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Seznam příloh:

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Detection rate of causal variants in severe childhood epilepsy is highest in patients with seizure onset within the first four weeks of life

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Abstract

Background: Epilepsy is a heterogeneous disease with a broad phenotypic spectrum and diverse genotypes. A significant proportion of epilepsies has a genetic aetiology.
In our study, a custom designed gene panel with 112 genes known to be associated with epilepsies was used. In total, one hundred and fifty-one patients were tested (86 males / 65 females).

Results: In our cohort, the highest probability for the identification of the cause of the disease was for patients with a seizure onset within the first four weeks of life (61.9% clarification rate) – about two times more than other groups. The level of statistical significance was determined using a chi-square analysis.
From 112 genes included in the panel, suspicious and rare variants were found in 53 genes (47.3%).
Among the 151 probands included in the study we identified pathogenic variants in 39 patients (25.8%), likely pathogenic variants in three patients (2%), variants of uncertain significance in 40 patients (26.5%) and likely benign variants in 69 patients (45.7%).

Conclusion: Our report shows the utility of diagnostic genetic testing of severe childhood epilepsies in a large cohort of patients with a diagnostic rate of 25.8%. A gene panel can be considered as a method of choice for the detection of pathogenic variants within patients with unknown origin of early onset severe epilepsy.

Keywords: Epilepsy, Epileptic encephalopathy, Targeted gene panel testing, MPS, Phenotype, KCNQ2

Background

Epilepsy is a heterogeneous disease with a broad phenotypic spectrum and diverse genotypes. A significant proportion of epilepsies has a genetic aetiology [1].

Severe childhood epilepsies are a very heterogeneous group of diseases, both clinically and genetically. Epilepsies might be inherited in an autosomal dominant fashion with mutations being often de novo, yet a good proportion of patients exhibit an autosomal recessive inheritance [1].

New methods of gene panel sequencing such as Massively Parallel Sequencing (MPS) enable a feasible approach to finding the causal variant in these patients, however the interpretation of the variants is often challenging.

The objective of our study was to identify the genetic aetiology of epilepsy in patients with severe early onset epilepsies.

Methods

Severe epilepsy is considered to be an intractable epilepsy which usually begins in infancy and is associated with global developmental delay, cognitive dysfunction and ongoing epileptiform activity that causes further cognitive slowing and decline. Drug-resistant epilepsy is defined as a failure of two or more appropriately selected and adequately tried anticonvulsant medications to achieve seizure freedom [2, 3].

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Patients

One hundred and fifty-one unrelated patients with severe childhood epilepsy were included in the study (86 males / 65 females). In the majority of cases, the epilepsy occurred sporadically (138/152), while in the remaining 13 patients the occurrence was familial.

Probands were referred for genetic analysis over a period from March 2015 to December 2016. The patients' legal representatives all gave informed consent and the study was approved by the local ethics committee. Brain MR imaging revealed no structural abnormalities in any of the patients. DNA samples from both parents were collected for the interpretation of variants; in two exceptional cases this was not possible.

Optional: previously tested with array CGH – to exclude chromosomal aberrations as the cause of epileptic seizures.

Design

A custom gene panel design was created with SureDesign (SureDesign release 3.5.x, Agilent, California, USA) application. Genes were chosen according to these criteria:

1. "Known" epilepsy genes: At least two published reports describing a causal relationship between variants in the gene and epilepsy.

OR

2. At least one published report describing a causal relationship between variants in the gene and epilepsy in two or more unrelated patients.

At first, according to these criteria and a literature search, we included 97 relevant genes in the gene panel design (07/2015). Then, the second version (in 03/2016) was enriched by newly reported genes up to a final number of 112. Genes included in the designs are listed in the Additional file 1: part II.

Sequencing was performed on a MiSeq Desktop Sequencer from Illumina (Illumina, California, USA) with 2 × 150 bp sequencing kit (20 samples per run).

Data analysis

Data were analysed by two independent software tools - NextGene (NextGENe 2.41, Softgenetics, Pennsylvania, USA) and SureCall (SureCall 3.0.3.x, Agilent, California, USA).

NextGene analysis was performed with default settings.

SureCall analysis was performed on default settings except for "SNP Read depth filter". This value was set to value 10. The aim of this analysis was to increase the sensitivity of the whole process.

Afterwards, Alamut Batch (Alamut Batch 1.5.2, Interactive Biosoftware, Rouen, France) was used for annotating merged VCFs into a tabular file.

Variant evaluation

An Alamut Batch annotated file in tabular format was used as the input for evaluation. Variants were then filtered according to the following workflow:

1. Variants found in three or more patients from the same run were filtered out

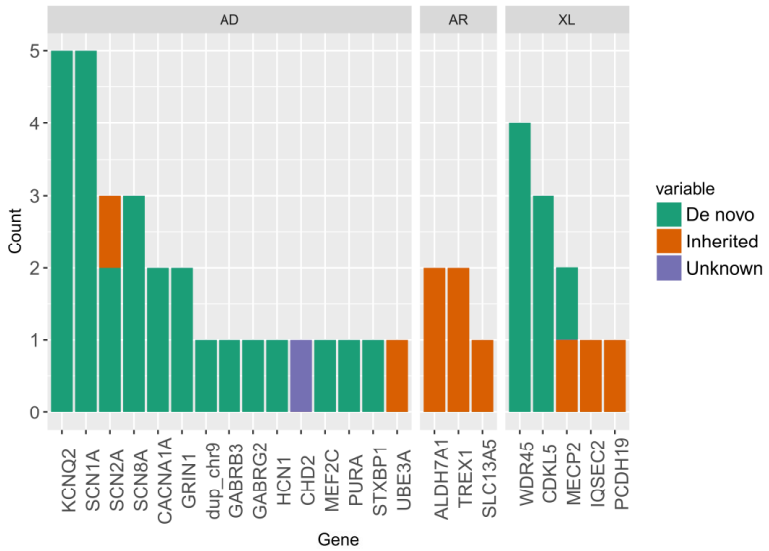


Fig. 1 The number of pathogenic variants in individual genes stratified by inheritance mode. AD = autosomal dominant, AR = autosomal recessive, XL = X-linked. Legend: X axis: all genes Y axis: Number of pathogenic variants

Table 1 List of variants found in cohort classified as Pathogenic or Likely Pathogenic

Gene	Ref Seq	DNA-level	Protein level	AD/AR	DN/INH	Prediction (SIFT, PolyPhen2,ClinVar)			ExAC all
Pathogenic AD variants									
CACNA1A	NM_001127221.1	c.13319826G > T		AD	DN				
CACNA1A	NM_001127221.1	c.2663A > T	p.Gln888Leu	AD	DN				0.0005
dup chr9	CNV			AD	DN				
GABRB3	NM_000814.5	c.841A > G	p.Thr281Ala	AD	DN	D	PD		
GABRG2	NM_000816.3	c.968G > A	p.Arg323Gln	AD	DN	D	PD		
GRIN1	NM_007327.3	c.2443G > A	p.Gly815Arg	AD	DN	D	PD	P	
GRIN1	NM_007327.3	c.1643G > A	p.Arg548Gln	AD	DN	T	PD		
HCN1	NM_021072.3	c.1189A > G	p.Ile397Leu	AD	DN	T	B		
KCNQ2	NM_172107.2	c.826A > C	p.Thr276Pro	AD	DN	D	B		
KCNQ2	NM_172107.2	c.1004C > G	p.Pro335Arg	AD	DN	D	PD		
KCNQ2	NM_172107.2	c.701C > T	p.Thr234Ile	AD	DN	D	PD		
KCNQ2	NM_172107.2	c.913_915delTTC	p.Phe305del	AD	DN				
KCNQ2	NM_172107.2	c.913_915delTTC	p.Phe305del	AD	DN				
MEF2C	NM_002397.4	c.766C > T	p.Arg256*	AD	DN				
PURA	NM_005859.4	c.812_814del	p.Phe271del	AD	DN				
SCN1A	NM_001202435.1	c.1244 T > A	p.Ile415Lys	AD	DN	D	PD		
SCN1A	NM_001165963.1	c.5384A > G	p.Glu1795Gly	AD	DN	D	PD		
SCN1A	NM_001165963.1	c.4384dup	p.Tyr1462Leufs*24	AD	DN				
SCN1A	NM_001165963.1	c.1178G > A	p.Arg393His	AD	DN	D	PD	P	
SCN1A	NM_001165963.1	c.1525C > T	p.Gln509*	AD	DN				
SCN2A	NM_001040142.1	c.2774 T > C	p.Met925Thr	AD	DN	D	PD		
SCN2A	NM_001040142.1	c.5009C > T	p.Thr1862Ile	AD	DN	T	PD		
SCN8A	NM_014191.3	c.4921C > G	p.Leu1641Val	AD	DN	D	PD		
SCN8A	NM_014191.3	c.2549G > A	p.Arg850Gln	AD	DN	D	PD	LP	
SCN8A	NM_014191.3	c.4850G > T	p.Arg1617Leu	AD	DN	D	PD		
STXBP1	NM_003165.3	c.1654 T > C	p.Cys552Arg	AD	DN	D	B		
UBE3A	NM_130838.1	c.1149G > C	p.Glu383Asp	AD	INH				
Pathogenic AR variants									
ALDH7A1	NM_001182.4	c.1318-1G > C		AR	INH				0.00041
ALDH7A1	NM_001182.4	c.518-14_518delinsCA		AR	INH				
SLC13A5	NM_177550.3	c.425C > T	p.Thr142Met	AR	INH	D	PD	P	0.00081
TREX1	NM_016381.3	c.10621072del	p.Leu354Phefs*22	AR	UNK				
TREX1	NM_016381.3	c.1072A > C	p.Thr358Pro	AR	INH	T		P	0.0016
Pathogenic X-linked variants									
CDKL5	NM_003159.2	c.2578C > T	p.Gln860*	XL	DN				
CDKL5	NM_003159.2	c.463 + 5G > A		XL	DN				
CDKL5	NM_003159.2	c.1247_1248del	p.Glu416Valfs*2	XL	DN			P	
IQSEC2	NM 001111125.2	c.3206G > C	p.Arg1069Pro	XL	INH	D	PD		
MECP2	NM_004992.3	c.1219_1229del	p.Asp407Glnfs*25	XL	DN				
WDR45	NM_007075.3	c.654del	p.Arg219Alafs*69	XL	DN				
WDR45	NM_007075.3	c.970_971del	p.Val324Hisfs*17	XL	DN				
WDR45	NM_007075.3	c.511C > T	p.Gln171*	XL	DN				

Table 1 List of variants found in cohort classified as Pathogenic or Likely Pathogenic *(Continued)*

Gene	Ref Seq	DNA-level	Protein level	AD/AR	DN/INH	Prediction (SIFT, PolyPhen2,ClinVar)			ExAC all
WDR45	NM_007075.3	c.344 + 4A > C		XL	DN				
Likely pathogenic variants									
CHD2	NM_001271.3	c.3782G > A	p.Trp1261*	AD	UNK				
MECP2	NM_004992.3	c.925C > T	p.Arg309Trp	XL	INH	D	PD	VUS	
PCDH19	NM_001184880.1	c.698A > G	p.Asp233Gly	XL	INH	D	PD		

Legend: Data were analysed by SureCall and NextGENe with parameters mentioned in the methods section
SIFT – D: deleterious, T: tolerated;
PolyPhen2 PD probably damaging, *B* benign, *PoD* possible damaging;
ClinVar – VUS Variant of uncertain significance, *P* pathogenic;
AR autosomal recessive, *AD* autosomal dominant, *XL* X-linked, *INH* inherited, *DN* de novo

2. Variants with a higher percentage (over 1%) in population databases (ExAC, 1000G) were deprioritized
3. Classification into four groups based on criteria in the annotated file (such as: ACMG classification [4], prediction programs – SIFT [5], Polyphen2 [6], Mutation taster [7], Clinvar [8], conservation, inheritance, X-linked disease). Groups were defined by pathogenicity of the variant: Pathogenic, likely pathogenic, variants of uncertain significance, benign and likely benign were grouped together.

CNV analysis

We used tools integrated into NextGENe for the detection of copy number variations. For the analysis, samples were compared with healthy control samples. NextGENe CNV tools perform a detection based on the Hidden Markov Model [9].

The resulting report shows INDELs in a tabular file. For precise analysis, this CNV Tool was performed against a different group of healthy controls, see Additional file 1: part III.

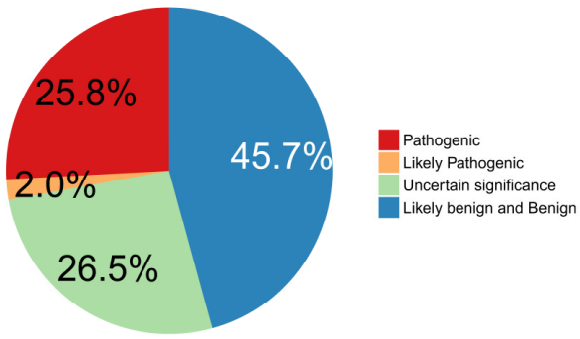


Fig. 2 Patients classified by detected variants There were 25.8% of probands with pathogenic variants (39/151), 2% probands with likely pathogenic variants (3/151), 26.5% with variants of uncertain significance (40/151), and 45.7% of probands without suspicious variants (69/151). If the proband had more than one variant, the "most pathogenic" variant was used to designate pathogenicity

Parental testing

For each pathogenic or likely pathogenic variant, Sanger sequencing and segregation analysis was performed and evaluated.

Results

After analysis of the whole dataset, we selected 99 SNVs and one CNV for further analysis. From 112 genes included in the panel, suspicious and rare variants were found in 53 genes (47.3%).

Most frequently, variants were found in *SCN1A* (eight occurrences) and *KCNQ2* (five occurrences). All variants found in the project were then stratified into these four classes:

- Pathogenic
- Likely pathogenic
- Variants of uncertain significance (VUS)
- Benign and likely benign

For the next step, Sanger sequencing of variants, classified as Pathogenic or Likely pathogenic, was used; the total was 42 SNVs in 22 genes out of 112 in the panel (19.6%). Furthermore we identified one CNV on chr 9. (Fig. 1 and Table 1).

The rest of the variants (VUS and Benign) are summarized in Additional file 1: part IV.

Among the 151 probands included in the study, we identified pathogenic variants in 39 patients (25.8%), likely pathogenic variants in three patients (2%), variants of uncertain significance in 40 patients (26.5%) and likely benign variants in 69 patients (45.7%) (Fig. 2). Two patients were carriers of two pathogenic variants – one was a compound heterozygote for two variants in trans in the *ALDH7A1*, the second was a compound heterozygote for two variants in trans in the *TREX1*.

Inheritance patterns

Our results showed that the majority of pathogenic or likely pathogenic variants were found in genes that follow autosomal dominant pattern of inheritance (27 SNVs in 14 genes), also CNV on chr9 was found to be acting with AD inheritance. Another 5% were found in genes associated with autosomal recessive inheritance (five SNVs in three genes), and finally 24% were found in genes with X-linked inheritance (11 SNVs in five genes).

For 40 patients, out of 42 classified with pathogenic or likely pathogenic variants, DNA samples from both parents were available for segregation analysis by Sanger sequencing.

In 34 patients, including 33 with SNVs and one with CNV, these variants arose de novo. These de novo variants were found in 15 genes and CNV was found in chromosome 9.

Nine variants in seven genes (detected in 7 patients) were inherited.

In two cases only one parental sample was available. In one of these cases the detected variant was inherited from the mother.

Information about maternal or paternal inheritance of inherited (and unknown) variants are available in Additional file 1: part V.

The distribution of the age at seizure onset and age at inclusion into the study

The distribution of the age at seizure onset and age at inclusion into the study is described in the Additional file 1: part I. The information was gathered from the patient's documentation. The median age at seizure onset of the whole group was 14.5 months; the first quartile was 4 months and the third quartile was 36 months. The median age at inclusion into study was 93 months; the first quartile was 49.5 months and the third quartile was 169 months.

The probability of finding the pathogenic variant in relation to age at seizure onset

The gene panel testing indicated that the highest probability for finding the cause of epilepsy was in the cohort of patients with the earliest onset of seizures, i.e. within the first four weeks of life – 61.9% clarification rate (13/21 patients).

In other age groups the clarification rate was lower: 35.8% (19/53) in the group of patients with first seizure between four weeks and 12 months of age; 11.1% (5/45) in the group with first seizure between 12 months and 36 months of age; and 15.6% in patients with first seizure after the age of 36 months (5/32) (Fig. 3).

The dataset was analysed using the Chi squared statistic and the results were statistically significant ($p = 0.000052$; the result is significant at $p < 0.05$). Moreover, we always compared two groups with the Fisher's exact test and significant relationships between the age at onset and clarification rate were observed for group 1 (<four weeks) and group 2 (4 weeks – 12 months) $p = 0.0673$; group 1 and group 3(12 months – 36 months) $p = 0.0001$; group 1 and group 4 (> 36 months) $p = 0.0009$. However, no

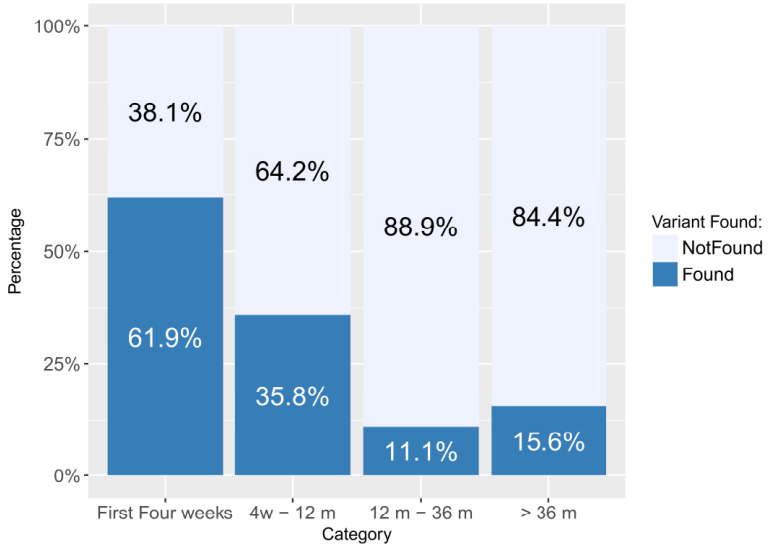
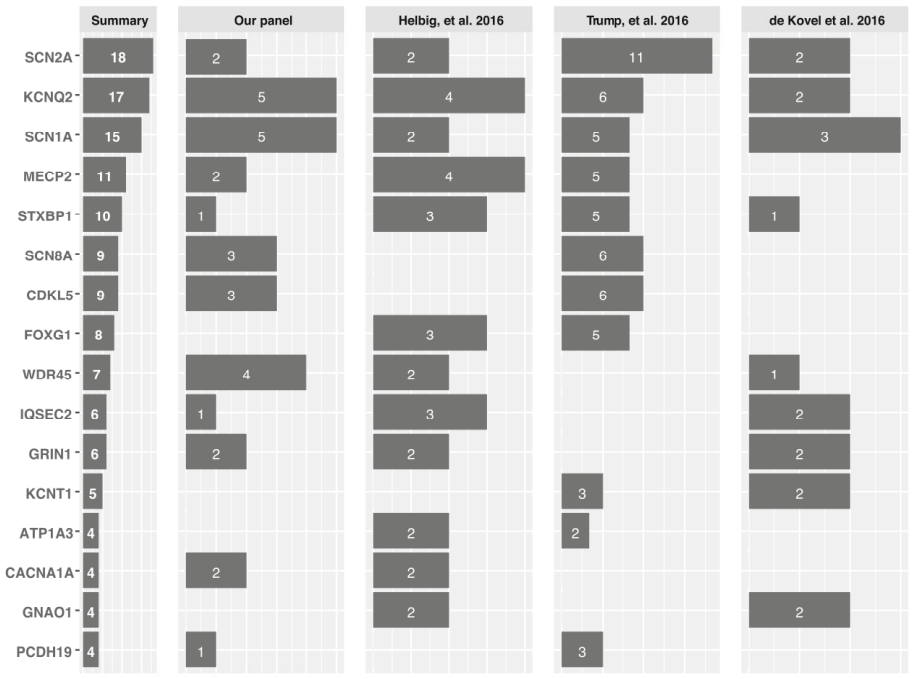


Fig. 3 Classification based on age at the onset of seizures: Legend: X axis: probands were divided into 4 groups by the age at the first seizure – the first four weeks of life, four weeks to 12 months of age, 12 months to 36 months years of age and older than 36 months., Y axis: Percentage of variants (not found) – pathogenic and likely pathogenic variants were marked as "variant found"



SUM genes	22	79	21	20
SUM variants	44	108	71	29

Most frequent	KCNQ2, SCN1A	KCNQ2,MECP2	SCN2A	SCN1A
Probands in cohort	152	314	400	360
Genes in panel	112	WES	46	351
Panel efficiency	26%	38%	18%	8%

Fig. 4 Results of our gene panel analysis were compared with three other published studies that focused on targeted sequencing in patients with epilepsy. For comparison, the following sources were used: [9–11]

significant difference was observed when comparing group 3 vs. group 4 (p = value 0.7330).

Discussion

We were able to identify the cause of severe childhood epilepsy in 25.8% of patients from our cohort and this finding concurs with previously published reports [1, 2]. From the 112 genes in the panel, pathogenic or likely pathogenic variants occurred in 22 of them.

MPS gene panel testing enables the testing of a large number of genes in parallel with very high coverage and low costs. More supportive information is presented in Additional file 1: part VI.

Over 80% of the pathogenic variants arose de novo, as they were not present in the parental samples (confirmed by Sanger sequencing).

Special cases

The first case was a patient with a CHD2 variant, where the father's sample was not available. The second patient, with two heterozygous variants in gene TREX1, has one variant inherited from his/her mother (p.Thr358Pro) while the other variant is of unknown inheritance, as the father's sample was not available. A female proband with Angelman syndrome has a variant in gene UBE3A inherited from her healthy mother. This is caused by imprinting, when the proband's maternal allele is active and the paternal is not expressed. The mother inherited the variant from her father.

Comparison with previously published reports about epilepsy gene panels

In order to assess the sensitivity and specificity of our approach, we compared our data with previously published reports. The analysis is presented in Fig. 4.

In agreement with the study by Helbig, et al. [10], the diagnostic rate was approximately 30%. Moreover, the vast majority of the pathogenic variants in currently known genes are de novo. Our results are very similar to those shown by Trump, et al. [11] (94%). According the study by Kovel, et al. [12], in which the authors adopted a very different approach and designed a large panel consisting

of 351 genes, the diagnostic efficiency of their panel did not increase and was much lower than the expected 30%.

Designing a large panel can help to find some rare variants which other panels cannot reveal; but it also involves higher costs, lower coverage, lower read depth and more difficult interpretations of the more variants that are multiplied by each sample. Based on our experience, we would recommend the design of a panel with approximately 100 well selected genes.

Age at seizure onset

Our results show that the probability of finding the pathogenic variant is the highest in patients with the earliest age at seizure onset (results in Fig. 3). Among our patients, the detection rate for pathogenic or likely pathogenic variant found in a group with seizure onset during the first four weeks of life was 61.9%.

In the groups where the onset of seizure was later, the number of pathogenic or likely pathogenic variants was found to be significantly lower. In a group where the age of seizure onset was between four weeks to 12 months it was 35.8%; between 12 months and 36 months, 11.1%; and after 36 months, 21.7%.

In effect, the earlier the phenotype is manifest then the chances of finding a pathogenic variant are significantly higher. This is shown in Fig. 3.

This trend has also been described previously by Helbig, et al. [10].

CNV testing

In our cohort two CNVs were found. The first classified as Pathogenic (on chr 9) for epilepsy and the second classified as VUS. These were detected using NextGENe CNV comparison tool based on Hidden Markov Model Results and were further confirmed by an Array CGH.

Conclusion

Our study has proven that MPS gene panel is a powerful tool for the DNA diagnosis of severe MRI negative childhood epilepsies. Today, a gene panel is an optimal method for the identification of pathogenic variants in highly heterogeneous disorders such as the genetically determined disorders including severe childhood epilepsies. In a cohort of 151 patients, we were able to identify the cause of epilepsy in 27.8% of patients (39 patients with pathogenic variant and three with likely pathogenic variants).

Additional file

Additional file 1: Part I Age distribution among patients, first box plot is age of seizure onset, second age of inclusion into study. Part II List off all genes included in panel. Part III Process of CNV analysis. Part IV List of variants of uncertain significance or likely benign found in our cohort. Part V. Part VI Advantages of the gene panel testing. (DOCX 150 kb)

Abbreviations

AD: Autosomal dominant; AR: Autosomal recessive; CNV: Copy number variation; MPS: Massively parallel sequencing; SNV: Single nucleotide variant; VUS: Variant of uncertain significance; WES: Whole exome sequencing

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DS participated in the bioinformatics analyses and drafted the manuscript. PL carried out the molecular genetic studies and evaluated the data. MK and JN participated in the molecular genetic studies and carried out the Sanger sequencing. KS performed the clinical evaluation, neurological consultation and recruited the patients. MV performed genetic consultations; PS recruited the patients and designed the study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of University Hospital Motol.

Consent for publication

Informed consent was obtained from all patients or their legal representatives.

Competing interests

The authors whose names are listed in Authors name section certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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DATABASES

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Variant pathogenicity evaluation in the community-driven Inherited Neuropathy Variant Browser

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Abstract

Charcot-Marie-Tooth disease (CMT) is an umbrella term for inherited neuropathies affecting an estimated one in 2,500 people. Over 120 CMT and related genes have been identified and clinical gene panels often contain more than 100 genes. Such a large genomic space will invariantly yield variants of uncertain clinical significance (VUS) in nearly any person tested. This rise in number of VUS creates major challenges for genetic counseling. Additionally, fewer individual variants in known genes are being published as the academic merit is decreasing, and most testing now happens in clinical laboratories, which typically do not correlate their variants with clinical phenotypes. For CMT, we aim to encourage and facilitate the global capture of variant data to gain a large collection of alleles in CMT genes, ideally in conjunction with phenotypic information. The Inherited Neuropathy Variant Browser provides user-friendly open access to currently reported variation in CMT genes. Geneticists, physicians, and genetic counselors can enter variants detected by clinical tests or in research studies in addition to genetic variation gathered from published literature, which are then submitted to ClinVar biannually. Active participation of the broader CMT community will provide an advance over existing resources for interpretation of CMT genetic variation.

KEYWORDS

database, inherited neuropathy, variants of unknown significance, VUS

1 | INTRODUCTION

Charcot-Marie-Tooth disease (CMT) and related disorders represent inherited conditions affecting the peripheral nervous system (Reilly, Murphy, & Laurá, 2011; Saporta & Shy, 2013). Initially reported in the late 19th century by physicians Jean Martin Charcot, Pierre Marie, and Howard Henry Tooth, CMT is now considered amongst the most common inherited neurological disorders and affects an estimated one in 2,500 people (Reilly et al., 2011). Typical findings include high arches of the feet, as well as muscle weakness and atrophy, sensory loss, and deep tendon reflexes, all more pronounced in the distal extremities (Pagon et al. 1993). These findings result from the progressive, length-dependent, degeneration of motor and/or sensory axons.

CMT is clinically and genetically heterogeneous. The clinical onset, rate of progression, nerve conduction velocities, and involvement of motor and sensory axons differ among the subtypes (Reilly et al., 2011; Saporta & Shy, 2010). The motor nerve conduction velocities in the arms separate CMT in demyelinating (CMT1), axonal (CMT2),

and intermediate forms (Dyck & Lambert, 1968; Reilly et al., 2011; Saporta & Shy, 2010). Each major subtype is also classified based on mode of inheritance and genetic etiology. The advances in gene discovery enabled by next-generation sequencing (NGS) have revealed the extent of locus and allelic heterogeneity, with over 120 CMT and related genes identified (Timmerman, Strickland, & Zuchner, 2014). The "traditional" yield for definitive genetic CMT diagnosis is around 65%; however, diagnostic yields ranging from 19% to 53% have been observed from NGS approaches (Walsh et al., 2017). Because ~30% of CMT families remain without a genetic diagnosis (Fridman et al., 2015; Murphy et al., 2012; Saporta et al., 2011), and recently discovered genes typically affect a small proportion of patients, there are likely additional unidentified causes.

With so many CMT genes, variants of unknown significance (VUS) are frequently identified during genetic testing. Standards and guidelines have been developed to interpret pathogenicity of VUS including their frequency within a large control population, cosegregation with disease status, and informatics/experimental evidence (MacArthur et al., 2014; Richards et al., 2015). The task of categorizing individual alleles within these genes according to pathogenicity is daunting, yet highly clinically relevant. Data sharing and disease-variant databases are salient solutions to VUS interpretation. Efforts have been implemented to collect and store genetic variation from published literature and clinical laboratories, such as the Human Gene Mutation Database (HGMD), the Human Variome Project (HVP), and ClinVar (Bean & Hegde, 2016). Such initiatives are necessary for and will lead to improved variant interpretation; however, currently, erroneous pathogenicity annotations in clinical diagnostic laboratories and in the published literature have introduced false pathogenicity claims into disease-variant databases (MacArthur et al., 2014; Manrai et al., 2016; Xue et al., 2012). Discovering these errors and properly annotating novel variation will require cooperative curation efforts from experts within each disease community.

The Inherited Neuropathy Consortium (INC) is an international group of academic medical centers dedicated to clinical research of CMT. Among the INC's goals is the construction of Web-based resources for clinicians and researchers. Supported by the INC, we have created a unique, community-driven, variant sharing browser for the CMT clinical and research community. The Inherited Neuropathy Variant Browser (INVB) provides simple, user-friendly access to currently reported CMT variants, including patient-level genotypic and phenotypic information. INVB will act as a complementary satellite database, where all INVB data have been submitted to ClinVar—an open-access database of clinically observed variation (Rehm et al., 2015).

Despite current variant collection efforts, the INC recognizes that a potentially large proportion of CMT genetic variation remains uncaptured. Within the academic setting, additional observations of published variation are now rarely shared after the initial gene discovery report (Bean, Tinker, da Silva, & Hegde, 2013). Likewise, data sharing from clinical laboratories is limited owing to lack of funding and infrastructure, limited phenotype information, or company policies (Bean et al., 2013). Another goal of the INVB is to encourage submission of all observed genetic variation as well as obtaining reports from clinical laboratories, when possible. To minimize burden on users, the INVB accepts direct submission of genetic variation, which will be synced biannually with ClinVar. The INVB's interactive rating system and discussion platform will lead to improved interpretation of VUS across the Mendelian CMT genes.

2 | METHODS

2.1 | Data collection, standardization, and curation

The CMT genetic variation data were collected from multiple sources including the HGMD, the Inherited Peripheral Neuropathies Mutation Database, results published by the Inherited Neuropathies Consortium, the supplemental data published by Athena and Quest Diagnostics, and manual data entry from collaborators (Cooper, Ball, & Krawczak, 1998). All collected and submitted data conform to institutional review boards and ethical guidelines. The initial set of genes was selected based on the published literature; however, users have the ability to add new genes as they are described as causes of CMT and related disorders. For phenotypic data, the CMT neuropathy score was imported from the Inherited Neuropathies Consortium's natural history study when available (Fridman et al., 2015). Additionally, each variant was annotated with the minor allele frequency from the Exome Aggregation Consortium (Karczewski et al. 2016).

The coding and protein sequence variant annotation follows recommendations of the Human Genome Variation Society (Dunnen et al. 2016). HUGO Gene Nomenclature Committee's Multi-symbol checker tool was used to validate all gene symbols. Each variant's syntax is standardized automatically with Mutalyzer Syntax Check, and the protein sequence variation was populated with Mutation Taster (Schwarz, Rödelsperger, Schuelke, & Seelow, 2010; Wildeman, van Ophuizen, Dunnen den, & Taschner, 2008). The Variant Effect Predictor was used to fill in missing variant effect data (McLaren et al., 2016). All publications require a valid PubMed ID, and the title, author, and reference metadata is automatically retrieved and saved in the database.

Continuing the community-driven nature of this database, CMT researchers and clinicians have begun to adopt individual genes to curate. In the initial release, each adoptee verified the genetic variation data. For future curation, biannual reports of new submissions will be generated for manual review and curation.

2.2 | Database creation and user interface

The INVB is built using PHP scripts for the backend and a relational database (Microsoft SQL Server) to store the data. The curated data were imported into the corresponding tables within the database using SQL scripts. The database has audit capability to track user-initiated changes. The graphical user interface for the Web page was built using the AngularJS framework, Bootstrap stylesheet, and HTML.

2.3 | Data submission

The INVB is designed for simple and fast variation upload. User registration and log-in is required for any variant submissions. The submitter must complete all required submission fields including: variant in HGVS notation, gene (drop-down selection of current CMT genes), protein notation, variant type, genotype, and NCBI mRNA reference sequence ID. For compound heterozygous variants, the submitter should select the "Add Compounded Variant" button to submit the variants together.

For data curation purposes, the data source is also required. The possible data sources are: clinical report (including clinical lab name), published article (including Pubmed ID), or research finding (indicated by Sanger sequencing or NGS). A comment box allows for any additional information pertinent to the variant that the submitter wishes to include.

Each variant submission is assigned a randomly generated family ID to allow for genotypic data. This family ID is displayed in the public browser. The submitter has the option to input a private family ID during submission. The private ID, along with the matched public ID, will be available only to the submitter in his/her account page. The purpose of the optional private family ID is to assist submitters with data entry tracking to minimize duplicate entries.

3 | RESULTS

3.1 | Graphical user interface

The variant browser is accessible to users through an online graphical user interface (https://hihg.med.miami.edu/neuropathybrowser). The Website consists of four different main tabs: Home, Sign Up, Contact Us, and Log In (Figure 1). The Home tab is the homepage of the Website and contains most of the functionality. It contains a quick search option along with three other multiselect boxes in order to filter for a gene, variant type, or data source (Figure 1A and B). For the "gene" filter, users can select a single gene, multiple genes, or all genes. The "variant type" filter is grouped into three categories: loss-of-function (LoF), missense, and others. The "LoF" category includes frameshift insertions and deletions (INDELs), stop-gained, and splice altering variants; the "missense" category includes in-frame INDEL, stop-lost, and missense variant; the "others" category includes 3′ untranslated region (UTR), 5′ UTR, intergenic, noncoding exons, intronic, upstream of gene, and downstream of gene variants. Finally, the "data source" filter includes three options: clinical report, published article, or research finding.

Query results are displayed in graph and table format. For each gene, the graphic result contains three viewing options: Allele, CMTNS (CMT neuropathy score) (Murphy et al., 2011), or Exome Aggregation Consortium minor allele frequency (ExAC MAF) (Karczewski et al. 2016) (Figure 1C). Each plot uses the "Mutations Needle Plot" and displays the known protein sites and domains, retrieved from NCBI. The default '"allele" plot displays the protein location of each allele, count of observations, and variant type. The "CMTNS" and "ExAC" plots require available data for at least one variant within the gene in order to be displayed. The "CMTNS" plot shows CMTNS for each allele and the associated age at onset. The "ExAC" plot displays the ExAC MAF (release 0.3.1). Currently, not all genes have the full set of annotated information available.

The table result consists of seven sortable columns: gene, variant, protein notation, variant type, rating, links, and data source (Figure 2). The data are grouped by primary variant into a gray expandable row (Figure 2A). The expanded view shows detailed information about each variant (Figure 2B). In the expanded view, each variant is grouped by the public family ID (Figure 2C) and the observed genotype and zygosity (Figure 2D). For example, the variant in Figure 2E is shown twice to indicate its homozygous zygosity (HOM). Alternatively, Figure 2F displays the variant in the compound heterozygous state (HET). For easy submission, additional families with the same genotype can be added by clicking the button next to the zygosity status (Figure 2G). Each family's data source can be viewed, edited, or deleted in the expanded view (Figure 2H). Lastly, each individual variant can be rated through the variant rating system, described below (Figure 2I).

3.2 | Variant rating system

The variant pathogenicity rating system is based upon the standardized terminology of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015). The average rating of a variant is represented as a five-star system in the table of query result. Each star corresponds to the ACMG terminology: benign, likely benign, uncertain significance, likely pathogenic, pathogenic (starting from 1 star rating). Registered users can rate a variant within the result table and can provide additional comments about their rating (Figure 2I). A history of all ratings and comments will be maintained for each variant. Importantly, any individual rating allows for free-text commenting. This creates a track record of evidence in support of the specific rating.

3.3 | Database summary statistics

The INVB currently contains 3,809 unique variants within 82 genes. The genes currently contain a median of 16 variants, an interquartile range from 4.0 to 69.5, and a maximum of 720 variants in *GJB1* (Figure 3A). A total of 4,558 unrelated families exist with the following genotypes: 2,244 heterozygous, 528 homozygous, and 301 compound heterozygous (Figure 3B). Currently, 1,475 families were reported from clinical laboratories without genotypic information. All new submissions will require genotypic data. The currently available phenotypic data highlights the clinical variability of CMT with an age of onset range of 9–75 years and a CMT neuropathy score of 2–24 (Figure 3C) (Murphy et al., 2011). The majority of variants are coding (Figure 3D). However, we encourage the submission of noncoding variation to cover the full spectrum of the genetic architecture of CMT. As whole-genome sequencing usage increases we expect the amount of noncoding variation to expand. The variant rating system is becoming a popular feature with 179 ratings to date (Figure 3E).

FIGURE 1 Overview of Inherited Variant Browser homepage and search options. **A**: The quick search field displays an autocomplete dropdown option and can be queried by: gene name, variant coding position, variant protein position, publication title, publication author, variant type, and data source. **B**: The multiselect searches enable multiple selections across gene, variant type, and data source. These fields allow users to conduct complex queries, such as all missense and LoF variants reported in published articles in *MFN2*, *GJB1*, and *GDAP1*. **C**: For single gene queries, variants can be displayed in three plots: allele, CMTNS, and ExAC. These plots can be toggled or hidden by selecting the buttons above each plot

3.4 | Usage examples

In order to illustrate use cases for the variant browser, we provide two examples that highlight current and future advantages.

1. Locus and allelic frequency are determinants of expected maximum tolerated population allele frequencies and directly affect pathogenicity evaluation (Whiffin et al., 2017; Wiel et al. 2017). In addition, genic sub-regions, such as protein domains and exons, have been shown valuable in determining genetic tolerance and thus pathogenicity (Gussow, Petrovski, Wang, Allen, & Goldstein, 2016). For example, nonsynonymous variants are observed across nearly all of GJB1, while such variants are clustered at specific protein domains in DNM2 (Figure 4A). By cataloging the genetic variation (and phenotypic details) observed in CMT genes, CMT-specific genetic tolerance metrics can be developed. Genetic tolerance metrics support clinical VUS interpretation as well as reveal insights into the biological mechanisms of disease genes (Samocha et al. 2017).
2. The Variant Rating System is a low threshold tool that allows clinicians and researchers to share and record supporting evidence for variant interpretation. Figure 4B shows a real example of a commentary from a user regarding conflicting data: the frequency of a variant in a healthy population (indicates benign) and abnormal electrophysiological data (indicates pathogenic). For quick interpretation from the table view, the variant is rated as two-star "likely benign," whereas the detailed view shows users' comments. The intuitive user interface allows for simple and fast user interactions, which will greatly improve the community's ability to interpret VUS.

4 | DISCUSSION

At a time when clinical multigene testing in highly heterogeneous Mendelian diseases is becoming a standard, the burden of VUS has risen to a point where it obstructs high quality diagnosis in individual patients. Neurologists and genetic counselors working with CMT patients are confronted by this issue daily. Although there are multiple potential solutions conceivable to address this problem, including future functional genomics platforms, a sensible and cost effective way forward is the comprehensive and public mapping of disease associated variation to disease-causing genes. We have collected CMT-specific genetic variation, along with genotypic and phenotypic data when available, from published literature, clinical lab reports, and our own in-house data. We then created the interactive, Web-based

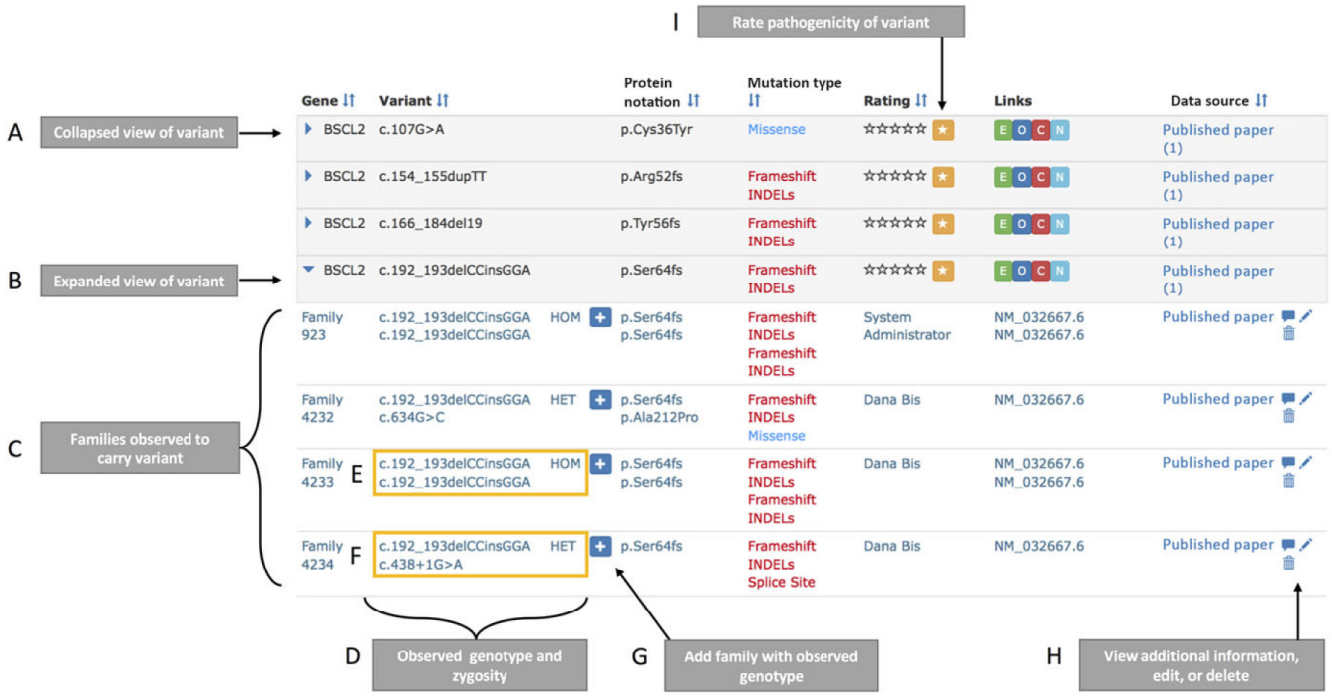

FIGURE 2 Overview of query result table. **A**: The collapsed view of each variant is returned by default. This view displays a high-level overview of the variant including gene symbol, cDNA sequence, protein notation, variant type, rating, link outs to ExAC (E), OMIM (O), ClinVar (C), and NCBI (N), and the data sources. **B**: The expanded row view shows additional information including: (**C**): the family-level information for each variant and (**D**) the observed variant zygosity and relevant genotype. **E**: Variants observed in the homozygous state is displayed twice and is marked as "HOM," whereas (**F**) variants observed in the compound heterozygous state are paired with the compounded variant and marked as "HET." **G**: The simple-add button allows a user to quickly add another family with the same observed variant(s). **H**: Buttons to view additional user comments, edit variant information, or delete a variant. **I**: In the collapsed view, the user can view the top-level variant rating. The star button allows the user to rate the variant and display the variant rating history

INVB accessible to, and relying on participation from, CMT researchers and clinicians to view the collected CMT variation. We have implemented an interactive rating system of genetic variation based on standardized terminology from the ACMG (Richards et al., 2015). The rating history for each variant, along with comments from raters, will be maintained to provide a rich perspective to users. The INC is encouraging its global membership to submit observed pathogenic variation, VUS and polymorphisms to the INVB. This platform enables a joint effort by the global CMT expert community to store, share, and discuss genetic variation to resolve VUS.

Although the frequency of a DNA variant within an unaffected population helps to categorize benign variation, only repeated observation of a variant in affected families will strengthen pathogenic classification. The continued aggregation of both disease-associated and benign variation will remain essential to further resolve VUS (Bean et al., 2013). Currently, 25%–30% of variants in databases can be clearly classified as benign or pathogenic (Bean & Hegde, 2016). We see an opportunity to create enthusiasm and attention in the CMT field to participate in the important task of collecting individual genetic test results from many clinics and different parts of the world. We were able to assemble an international group of experts committed to working together to classify variation and we hope the CMT community will serve as a positive example of joint enterprise within genomic medicine. The INC, the largest CMT-related consortium, has designated the interactive INVB as the CMT genetic tool of choice. The international character of the INC is attracting users from different geographic locations with diverse ancestries, such as the new Asia Oceanic Inherited Neuropathies Consortium. As minor allele frequencies can vary widely between ancestral populations, uniquely rare variants in one population might be less rare in another, thus excluding such an allele as pathogenic.

Similarly, large-scale population data, such as ExAC, a collection of over ~60,000 exomes from patients unaffected by severe pediatric diseases, has revealed previously reported pathogenic alleles as too common relative to the prevalence of CMT (Minikel et al., 2016). We therefore incorporated ExAC data into the INVB (Karczewski et al. 2016). Benign variants falsely assigned as pathogenic have been revealed and reported disease variant penetrance has been re-evaluated, such as p.Arg468His in *MFN2*. This variant has been implicated in variant screening studies at least four times; yet, the relatively high count of 265 heterozygous alleles out of 115,542 ExAC chromosomes excludes the variant as a causative, high penetrance allele in a dominant rare disease (Braathen, Sand, Lobato, Høyer, & Russell, 2010; Casasnovas et al. 2010; Engelfried et al., 2006; McCorquodale et al., 2011).

Though variant databases excel in providing overall allele frequencies, most capture genetic variants without listing individual patients and their genotypes; however, this information may contribute to variant interpretation (Lanthaler et al., 2014). Without knowing the heterozygous, homozygous, or compound heterozygous state, it may be difficult to determine causality in a recessive disorder. Furthermore,

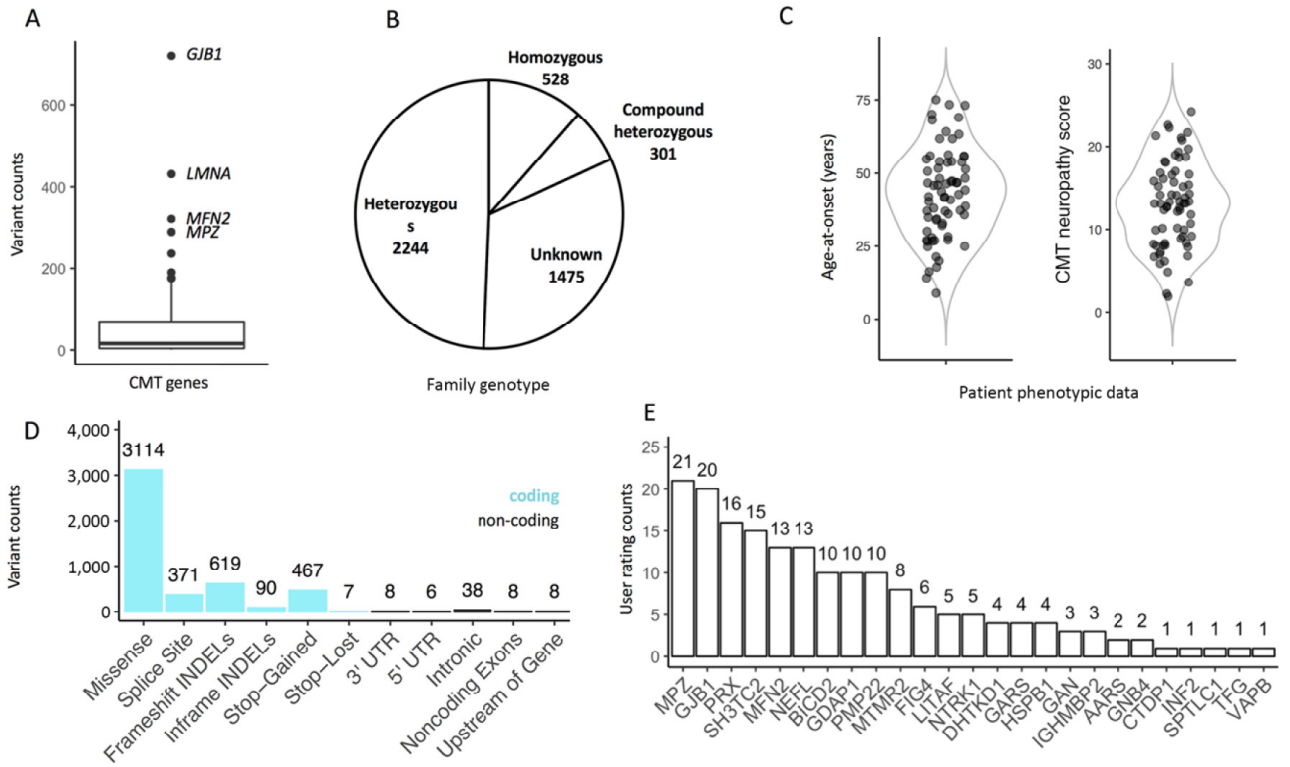

FIGURE 3 Inherited Neuropathy Browser descriptive statistics. **A**: Boxplot depicting the variant counts per CMT gene. **B**: Pie chart of variant zygosity counts. The unknown zygosity variants predominantly come from clinical lab reports that do not contain this information. **C**: Violin plots displaying available phenotypic data for a variant. **D**: Bar chart of variant functional consequences. Coding consequences are highlighted in blue. **E**: Bar chart of user ratings per gene

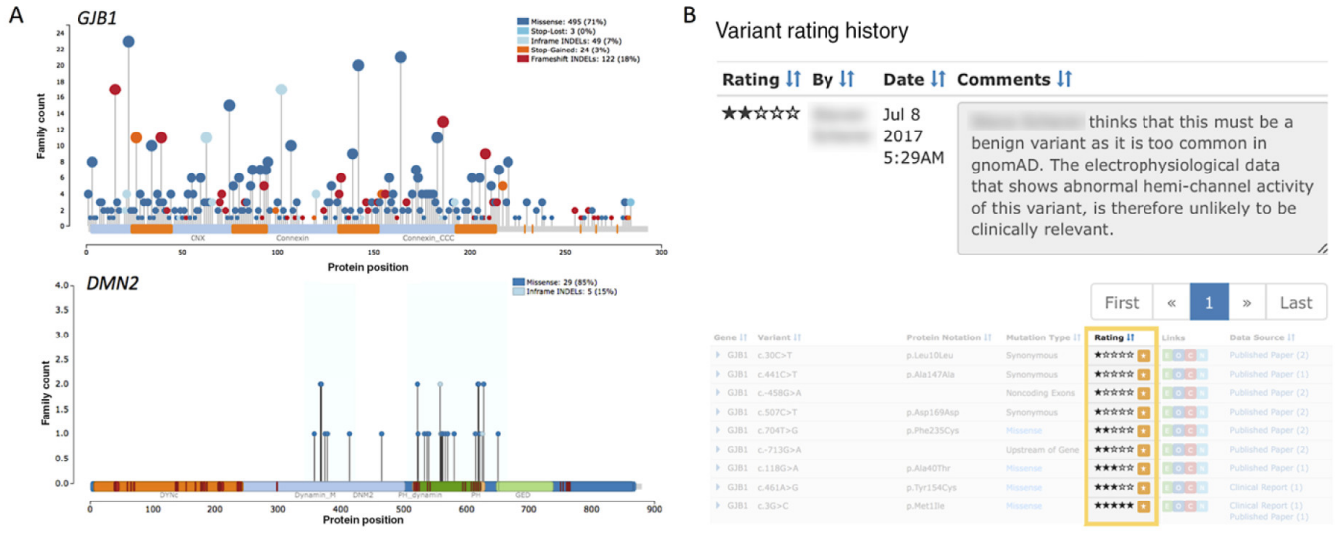

FIGURE 4 Inherited Neuropathy Browser usage examples. **A**: Allele plots displaying the missense and loss-of-function variants within *GJB1* and *DMN2*. The number of families with an observed variant is shown on the *y*-axis and is emphasized by the size of the needle head. The protein position and domain information is displayed on the *x*-axis. Blue regions in *DMN2* highlight the clustering of variation. **B**: Detailed variant rating history view displaying user comment and user ratings in collapsed table view

the pathogenicity of a compound heterozygous pair of alleles is directly influenced by each involved allele. For CMT specifically, genotypic information is particularly important because several genes, for example, *GDAP1* and *HSPB1*, can cause disease in dominant and recessive inheritance modes depending on the specific variant (Cassereau et al., 2011; Houlden et al., 2008; Sivera et al., 2010). Finally, genotypic information may well guide future oligogenic models of inheritance in individual patients and influence predictable phenotypic expression. For these reasons, we require both the observed zygosity and phenotypic information with each variant submission. Beyond variant interpretation, storage of these datasets will enable future genotype–phenotype correlation analyses.

In order to benefit the wider scientific community, all collected data have been submitted to ClinVar—a public archive of the interpreted clinical significance of variants for reported conditions at the National Center for Biotechnology Information (Landrum et al., 2016). ClinVar was developed to provide access to the interpretation of human variation and is an effort critical to genomic medicine (Landrum et al., 2014). As avid supporters of the ClinVar initiative, we recommend the entire community to submit variation data directly to the database. However, we have implemented direct submission to the INVB to collect data inappropriate for ClinVar (such as variants based solely on computational predictions) or from users who have chosen not to submit to ClinVar (such as CMT researchers outside of the United States). As we do not want to add to the issue of multiple fragmented data collection efforts with yet another database, we will perform two-way synchronization with ClinVar at least biannually. Users should look to ClinVar for the most up-to-date variant database and utilize the INVB as a tool to view and discuss this data. We hope the simple and easy-to-use interface of the INVB will facilitate communication about variant pathogenicity status among CMT researchers and clinicians and provide improved diagnostic abilities.

WEB RESOURCES

The Inherited Neuropathy Variant Browser (INVB): https://hihg.med.miami.edu/neuropathybrowser

Human Gene Mutation Database (HGMD): https://www.hgmd.cf.ac.uk/ac/index.php

The Human Variome Project (HVP): https://www.humanvariomeproject.org/

ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/

The Inherited Peripheral Neuropathies Mutation Database: https://www.molgen.vib-ua.be/CMTMutations/

HUGO Gene Nomenclature Committee's Multi-symbol checker tool: https://www.genenames.org/cgi-bin/symbol_checker

Mutations Needle Plot: https://www.npmjs.com/package/muts-needle-plot

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AUTHOR CONTRIBUTION

C.S. contributed with the database concept and creation, browser design and creation, variant curation, and manuscript preparation. D.M.B. contributed with the database concept, browser design, variant curation, variant annotation, and manuscript preparation. D.S. contributed with the variant annotation with protein domain information. A.S. contributed with the data collection. D.H. contributed with the database concept, browser design, and variant curation. M.M.R. contributed with the database concept, browser design, and variant curation. S.S.S. contributed with the database concept, browser design, and variant curation. M.S. contributed with the database concept, browser design, and variant curation. S.Z. contributed with the database concept, browser design, and variant curation.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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WILEY CLINICAL GENETICS

SHORT REPORT

Novel *SBF2* mutations and clinical spectrum of Charcot-Marie-Tooth neuropathy type 4B2

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Biallelic *SBF2* mutations cause Charcot-Marie-Tooth disease type 4B2 (CMT4B2), a sensorimotor neuropathy with autosomal recessive inheritance and association with glaucoma. Since the discovery of the gene mutation, only few additional patients have been reported. We identified seven CMT4B2 families with nine different *SBF2* mutations. Revisiting genetic and clinical data from our cohort and the literature, *SBF2* variants were private mutations, including exon-deletion and de novo variants. The neuropathy typically started in the first decade after normal early motor development, was predominantly motor and had a rather moderate course. Electrophysiology and nerve biopsies indicated demyelination and excess myelin outfoldings constituted a characteristic feature. While neuropathy was >90% penetrant at age 10 years, glaucoma was absent in ~40% of cases but sometimes developed with age. Consequently, *SBF2* mutation analysis should not be restricted to individuals with coincident neuropathy and glaucoma, and CMT4B2 patients without glaucoma should be followed for increased intraocular pressure. The presence of exon-deletion and de novo mutations demands comprehensive mutation scanning and family studies to ensure appropriate diagnostic approaches and genetic counseling.

KEYWORDS

autosomal recessive, Charcot-Marie-Tooth neuropathy, CMT4B2, de novo mutation, glaucoma, *SBF2*

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1 | INTRODUCTION

Hereditary peripheral neuropathies comprise a heterogeneous group of monogenic disorders of the peripheral nervous system. The most common form is Charcot-Marie-Tooth disease (CMT), which is characterized by distal limb muscle weakness and atrophy, foot deformities, gait problems, and sensory deficits.[1] CMT is divided into a demyelinating form with reduced nerve conduction velocities (NCVs) and Schwann cell and myelin abnormalities in nerve biopsies, and an axonal form with near normal NCV and histological evidence of axonal degeneration and regeneration.[1] CMT is associated with mutations in >80 genes and all types of inheritance.[2] Establishing genotype-phenotype correlations is meaningful for medical care and genetic counseling but often obstructed by the small number of individuals with mutations in a particular CMT gene. Mutations in the *SBF2* gene encoding SET-binding factor 2 (SBF2), also known as myotubularin-related protein 13 (MTMR13), cause autosomal recessive demyelinating CMT type 4B2 (CMT4B2).[3,4] Nerve biopsies show particular myelin abnormalities called focally folded myelin[5] and one of the initial studies[3] found an association with early-onset glaucoma. Since then, only few additional families and singleton cases have been reported (Table S1).

2 | MATERIALS AND METHODS

Investigations were performed in the course of routine clinical care. No new patient samples were required. Research was conducted according to the principles expressed in Declaration of Helsinki and was approved by the local Research Ethics committees. All participants or in case of minors and their legal representatives gave informed consent for the use of clinical data, collection of samples, and subsequent DNA analyses. Molecular genetic studies are described in Appendix S1. We searched publications in PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) as well as OMIM (Online Mendelian Inheritance in Man; https://www.omim.org/) and The Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php) entries for *SBF2* mutations. Cases for which at least a minimal set of clinical and neurophysiological data was available were considered further.

3 | RESULTS

We identified biallelic *SBF2* mutations in seven CMT families (Figure 1A). Genetic results but no further details of family G have been previously published.[6] We detected nine pathogenic *SBF2* variants, including three missense, three frameshift, one nonsense and two splice variants (Figure 1B, S1, Table S2). Except for c.134T>A,[6] mutations found in our patients had not been reported earlier and were not contained or extremely rare (frequency < 0.000005) in repositories representing "normal" variability of the human genome (Table S3). None of the families harbored convincing variants in any of the additional CMT loci and genes that were screened (Table S4). Missense mutations mapped to known protein domains (Figure 1B), affected residues that were conserved during evolution (Figure S2) and were predicted to be deleterious by bioinformatic tools (Table S3). The disease co-segregated with recessive inheritance of the *SBF2* mutations in all families but one: F-II:1 inherited the c.1601-2A>G variant from his father, while the c.5232-10_5244del variant was not detectable in parents' DNA (Figure 1A). Having excluded false maternity or sample mix-up (data not shown), analysis of patient derived mRNA and allele-specific reverse transcription polymerase chain reaction (RT-PCR) confirmed that the variants resided on different alleles, implicating de novo origin of c.5232-10_5244del in the maternal gamete (Figure S3).

Clinical presentations of 10 patients are summarized in Table 1. In five patients, glaucoma was diagnosed in the first year of life, while F-II:1 was diagnosed with marginally high intraocular pressure (IOP, 22 mm HG, normal ≤ 21) not before age 32 years. Four patients (aged 11-43) had no known vision problems. With regard to peripheral neuropathy, patients came to medical attention in the first or early second decade. Initial symptoms included foot deformities, muscle weakness, or gait problems. Three-year-old individual E-II:3 had no neurological deficits; however, glaucoma, slow NCV, and older siblings with CMT4B2 confirmed the diagnosis. C-II:1 had delayed early motor

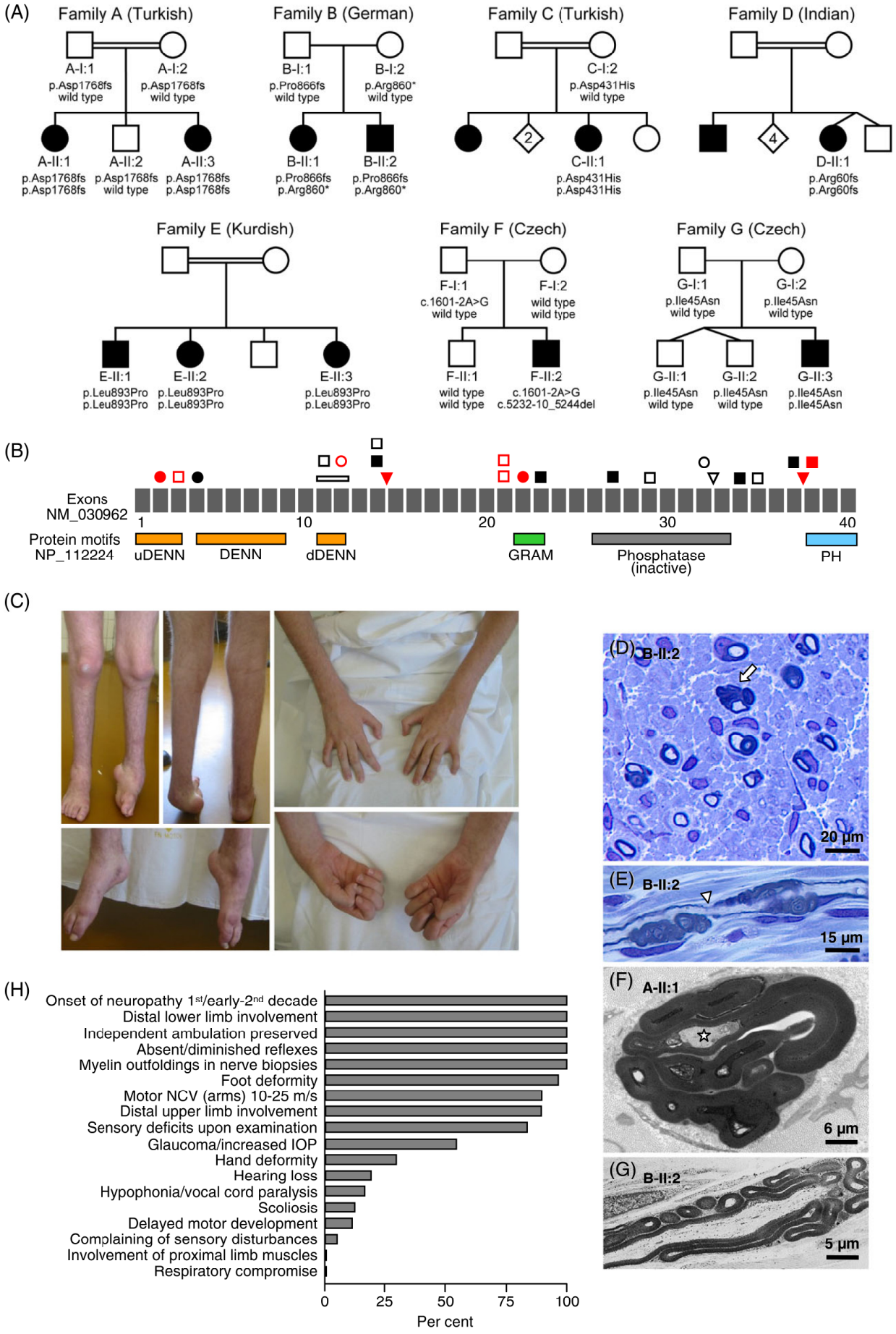

FIGURE 1 Legend on next page.

development (independent walking at age 2 years), while others reached early motor milestones normally. Disease progression was slow. At last examination (age range 3-35 years), all patients were able to ambulate independently, some utilizing assistive devices. All patients except E-II:3 had involvement of lower limb muscles, particularly foot and calf muscles (Figure 1C), and foot deformities requiring orthopedic surgery in five cases. Three individuals had normal function and bulk of distal upper limb muscles. In others, involvement of upper limb muscles was restricted to the hands except for F-II:1 with an additional involvement of forearms (Figure 1C). Claw-hand deformities were seen in three patients, two had scoliosis and one had hypophonia and a hoarse voice indicating vocal cord paresis. Function and bulk of proximal muscles were always normal. Deep tendon reflexes were absent in lower extremities and decreased or absent in upper extremities. None of the patients complained about sensory disturbances, hearing loss, or respiratory compromise. Upon examination, sensory deficits were missing in three cases and rather mild in others. Motor and sensory nerve evoked responses were either absent or revealed slow NCV (Table 1). Mean motor NCV was 23.6 m/s (range 13-36) in arm nerves and slower in leg nerves, indicating a demyelinating neuropathy. Nerve biopsies, taken for diagnostic purposes in the past, showed loss of large myelinated axons and signs of de- and remyelination (thin myelin and few onion bulb formations). Most striking findings were focal myelin protrusions emanating from the outer aspect of the sheaths. Electron microscopy confirmed that these protrusions consisted of irregular myelin outfoldings (Figure 1D-G).

Reviewing the available literature, we found 25 additional patients with *SBF2* mutations (Tables S1 and S2). Combining this data with information from our cohort, we determined the relative frequencies of signs and symptoms in CMT4B2 (Figure 1H).

4 | DISCUSSION

In this study, we report *SBF2* mutations and clinical findings in patients referred to our laboratories for genetic testing of CMT. Our data extend the spectrum of CMT4B2-causing variants, increasing the number of different mutations to 22 and bringing the number of published cases, for which at least a core set of clinical data elements was documented, to 35. As expected, CMT4B2 was rare in an unstratified CMT patient cohort (<0.5%) but may account for about 1% to 2% of cases with autosomal recessive CMT (this study and Zimon et al[7]).

SBF2 mutations are private recessive mutations, not present or very rare in databases representing "normal" human genetic variability. Most variants are micromutations, but an exon-deletion variant has been reported as well.[4] Genetically unresolved CMT patients could be heterozygous for two non-overlapping partial *SBF2* deletions which would be missed by Sanger or gene panel-based sequencing. One patient in our study had a de novo mutation on the maternal allele that had most probably arisen in the female gamete or early post-zygotically in the patient. While the contribution of de novo mutations to autosomal dominant diseases is obvious, they are not regularly considered in autosomal recessive conditions.[8] However, for accurate genetic counseling, this mode of inheritance should be taken into account as it reduces the recurrence risk in a family to a small remaining probability, although not to zero owing to possible parental germline mosaicism.

CMT4B2 displays a quite homogeneous neurological phenotype: Demyelinating sensorimotor neuropathy starting in distal limbs in the first decade, motor-predominant symptoms, foot deformities, slowly progressive course, and no major sensory deficits. Independent ambulation was preserved in nearly all cases, sometimes by the help of walking aids. Phenotypic homogeneity contrasts with the broad *SBF2* mutation spectrum with virtually every patient having a unique genotype. This is easily reconciled for loss-of-function variants that are all expected to result in no or truncated and unstable proteins. Similarly, destabilization leading to reduced protein levels is a common mechanism by which missense mutations cause disease,[9] but this has not yet been shown in CMT4B2. Alternatively, missense variants and in-frame deletions may render SBF2 functionless, for example, by disrupting interaction with partnering proteins that control intracellular transport[10], a known pathomechanism for demyelinating neuropathies.[11] Several *SBF2* in-frame deletion and missense mutations are in or near the domain DENN (differentially expressed in normal and neoplastic cells) which is critical for these interactions. Further evidence for disturbed intracellular trafficking in CMT4B2 comes from histological studies. Nerve biopsies from CMT4B2 patients show complex outfoldings of the myelin sheaths (focally folded myelin)[3,4] and in other types of CMT with focally folded myelin[12–15] myelin outfoldings have been shown[16] or implicated[17] to arise from disturbed membrane trafficking in diseased Schwann cells.

Early-onset glaucoma is a potential clue to *SBF2* mutations.[3] Glaucoma or increased IOP has been reported in ~50% of patients, and this proportion was similar in our cohort. Patients with normal ocular status during childhood and teens may develop glaucoma later in their lives, for example, F-II:1, diagnosed at age 32 years, and another patient, diagnosed at age 25 years (Tables 1, S1). Based on the initial reports,[3,4] glaucoma appeared to be related with

FIGURE 1 Genetic and clinical findings in CMT4B2. A, CMT families with affected individuals carrying biallelic *SBF2* variants. W, wildtype; M, mutation. B, *SBF2* mutations found in this and other studies. Square, nonsense and frameshift; triangle, splice-site; circle, missense; bar, in-frame exon-deletion mutation. Red symbols, mutations reported in this study; filled symbols, mutations causing neuropathy and glaucoma. Exons and protein domains are not drawn to scale. C, F-II:1 at age 23 years. Lower extremities: Wasting of distal leg muscles and severe left foot deformity (pictures taken after surgery of the right foot). Upper extremities: Atrophy of forearm muscles, claw hand deformity and thenar atrophy. D-G, Nerve biopsy findings. D, Loss of myelinated fibers, thin myelin, small onion bulb formations and abnormal proliferations of myelin sheaths (arrow). E, Myelin outfoldings associated with a node of Ranvier (arrowhead). F, Extended myelin outfoldings. Asterisk, axon. G, Longitudinal aspect of an axon with myelin folds. Semithin sections, toluidine blue stain (D-E), ultrathin sections, electron microscopy (F-G). H, Relative frequency of signs and symptoms based on data from our cohort and published cases (Tables 1 and S1) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Clinical, neurophysiological and nerve biopsy features in 10 individuals with biallelic *SBF2* variants

Family		A	B		C	D	E			F	G
Ethnicity/parental consanguinity		Turkish/first degree cousins	German/no		Turkish/first degree cousins	Indian/yes	Kurdish/first degree cousins			Czech/no	Czech/no
SBF2 mutations		p.[Asp1768fs];[Asp1768fs]	p.[Arg860*];[Pro866fs]		p.[Asp431His];[Asp431His]	p.[Arg60fs];[Arg60fs]	p.[Leu893Pro];[Leu893Pro]			p.[Val534fs];[Arg1744fs]	p.[Ile45Asn];[Ile45Asn]
Case/sex/AAE		A-II:1/F/22 years	B-II:1/F/15 years	B-II:2/M/11 years	C-II:1/F/12 years	D-II:1/F/43 years	E-II:1/M/18 years	E-II:2/F / 16 years	E-II:3/F/3 years	F-II:1/M/ 35 years	G-II:1/M/ 7 years
AAD ocular disease		6 months	n.a.	n.a.	n.a.	n.a.	Birth	Birth	2 months	32 years	Birth
Ocular pathology		Glaucoma	No	No	No	No	Glaucoma	Glaucoma	Glaucoma	Increased IOP	Glaucoma
AAD neuropathy/ first symptom		7 years/foot deformity	6.5 years/ steppage gait	6 years/waddling gait, hammer toes	7 years/distal weakness and muscle wasting	12 years/atrophy and weakness of leg muscles	Preschool age/gait problems, foot deformity	12 years/ hammer toes	Asymptomatic	Preschool age/foot drop, distal leg weakness	10 years/ foot deformity
Motor milestones		Normal	Normal	Normal	Walked at 2 years	Normal	Normal	Normal	Normal	Normal	Normal
Weakness	UL	No	n.r.	n.r.	Distal, mild	n.r.	No	No	No	Distal	n.r.
	LL	Distal	Distal	Distal	Distal, mild	Distal	Distal	Distal	No	Distal	Distal
Muscle atrophy	UL	Hands	Hands	Hands	Hands	Hands	No	No	No	Hands, forearms	Hands
	LL	Distal	Feet, calves	Feet, calves	n.r.	Distal	Distal	No	No	Feet, calves	Feet, calves
Sensory symptoms		No	No	No	No	No	No	No	No	No	No
Foot deformity/ surgery at age		Pes cavus, hammer toes	Pes cavus/ 15 years	Pes cavus, club foot/11 years	Pes equinus	Pes equinus	Pes cavus/ 11 years, hammer toes	Mild pes cavus, hammer toes	No	Pes cavovarus/ 23 years	Pes cavovarus/ 12 years
Hand deformity		Claw hands	No	No	Claw hands	n.r.	No	No	No	Claw hands	No
Deep tendon reflexes	UL	1+	n.r.	n.r.	1+	n.r.	0	0	0	1+	n.r.
	LL	0	0	0	0	n.r.	0	0	0	0	0
Abnormal sensory tests	UL	n.r.	No	No	Hands, mild	Hands, mild	No	No	No	Hands	n.r.
	LL	n.r.	No	No	Feet, mild	Feet, mild	Feet, lower legs	Feet, lower legs	No	Feet, lower legs	Distal
Walking		Walks long distances and climbs stairs	Walks independently, steppage gait	Walks independently, stumbles frequently	Walks independently, steppage gait	Walks independently but gait deteriorates	Walks almost normally, runs, climbs stairs	Walks almost normally, runs, climbs stairs	Normal	Walks independently but gait deteriorates	n.r.
Additional features		Scoliosis	Scoliosis	No	No	No	No	No	No	Hypophonia	No
Motor NCV/ CMAP	Median	A	15 m/s/0.2 mV	n.d.	n.r.	A	24 m/s/1.9 mV	n.d.	36 m/s/4.1 mV	A	22 m/s/n.r.
	Ulnar	18 m/s/3.0 mV	n.d.	n.d.	n.r.	A	24 m/s/4.3 mV	29 m/s/2.6 mV	32 m/s/3.5 mV	13 m/s/n.r.	n.d.
	Peroneal	A	12 m/s/n.r.	12 m/s/n.r.	n.r.	A	A	10 m/s/0.1 mV	26 m/s/1.2 mV	A	17 m/s/n.r.
	Tibial	n.d.	8 m/s/n.r.	n.d.	n.r.	A	17 m/s/n.r.	n.d.	n.d.	n.d.	n.d.
Sensory NCV/ SNAP	Median	A	n.d.	n.d.	n.r.	A	A	A	A	A	n.d.
	Sural	A	n.d.	n.d.	n.r.	A	A	A	n.d.	A	33 m/s/n.r.
Nerve biopsy		Severe fiber loss, myelin foldings	n.d.	Severe fiber loss, myelin foldings	Fiber loss, myelin foldings	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Abbreviations: A, absent response; AAD, age at diagnosis; AAE, age at examination; CMAP, compound muscle action potential; F, female; IOP, intraocular pressure; LL, lower limbs; M, male; NCV, nerve conduction velocity; n.d., not performed; n.r., not reported; SNAP, sensory nerve action potential; UL, upper limbs.

truncating mutations. Our and other studies challenge this view: Several individuals with missense mutations had glaucoma while cases with truncating mutations had no ocular abnormalities (Table 1, S1).

In essence, CMT4B2 patients and their families should be counseled on a rather moderate course of the neuropathy. Glaucoma is not a constant feature and genetic testing for demyelinating. CMT should include *SBF2*, irrespective of the presence or absence of glaucoma. At the same time, patients without ocular involvement appear at risk for developing glaucoma later in life and should be monitored for increased IOP. Copy number variants[4] and de novo mutations (this paper) do occur in the *SBF2* gene, which is relevant for molecular genetic testing and accurate reporting of recurrence risk.

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Conflict of interest

Authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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UBTF Mutation Causes Complex Phenotype of Neurodegeneration and Severe Epilepsy in Childhood

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Abstract

Introduction Neurodegenerative diseases of childhood present with progressive decline in cognitive, social, and motor function and are frequently associated with seizures in different stages of the disease. Here we report a patient with severe progressive neurodegeneration with drug-resistant epilepsy of unknown etiology from the age of 2 years.

Methods and Results Using whole exome sequencing, we found heterozygous missense de novo variant c.628G > A (p.Glu210Lys) in the *UBTF* gene. This variant was recently described as de novo in 11 patients with similar neurodegeneration characterized by developmental decline initially confined to motor development followed by language regression, appearance of an extrapyramidal movement disorder, and leading to severe intellectual disability. In 3 of the 11 patients described so far, seizures were also present.

Conclusions Our report expands the complex phenotype of neurodegeneration associated with the c.628G > A variant in the *UBTF* gene and helps to clarify the relation between this one single recurrent pathogenic variant described in this gene to date and its phenotype. The *UBTF* gene should be considered a novel candidate gene in neurodegeneration with or without epilepsy.

Keywords
- *UBTF*
- neurodegeneration
- epilepsy
- whole exome sequencing
- trio analysis

Introduction

Epilepsy is a group of chronic neurological disorders with recurrent seizures which can have both genetic and acquired causes. Several distinct childhood epilepsy syndromes have been linked to defects in particular genes. Changes in these genes typically cause dysfunction of neuronal ion channels, abnormal brain development, and disturbed energy metabolism leading to neurodegeneration.[1] Epilepsy phenotypes may include variable degrees of intellectual disability, developmental delay, autism spectrum disorders, other psychiatric disorders, and motor impairment.[2] Extensive discoveries in the last few years have been enabled by the rapid development

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of next-generation sequencing (NGS) technologies and established many associations between severe epilepsies and particular genes. In many patients, however, the underlying gene defect still remains to be discovered.

Recently, Edvardson et al[3] described a novel gain-of-function variant c.628G > A (p.Glu210Lys) in the upstream binding transcription factor (*UBTF*) gene in seven unrelated affected individuals, all suffering from similar developmental regression. A further four patients were described with very similar neurodegeneration also due to the same recurrent de novo variant c.628G > A.[4] This is the only known pathogenic mutation in this gene. Here we describe another patient who is older than most other reported patients suffering from severe progressive neurodegeneration and in whom we have identified the same unique heterozygous de novo variant by whole exome sequencing (WES).

Methods

The study was approved by the Ethics Committee of 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol. The affected individual was selected by an experienced neurologist and epileptologist monitored by the University Hospital Motol. The patient's legal guardians signed informed consent.

WES was performed after NGS of a custom-designed gene panel (112 genes) for epilepsy and epileptic encephalopathy as described elsewhere.[5,6]

DNA (deoxyribonucleic acid) libraries were prepared from DNA samples from the proband and both parents and were sequenced in an outsourced laboratory (Macrogen Inc., South Korea) with SureSelect XT v.5 on Illumina's HiSeq4000. Data were analyzed from the provided FASTQ files in our laboratory with SureCall (Agilent Technologies, Santa Clara, CA, U.S.A.) and NextGENe (Soft-Genetics LLC, State College, PA, U.S.A.) software. To search for de novo variants, DeNovoGear algorithm was used.[7] Variants of interest were validated by Sanger sequencing. Correct parentity was tested using six microsatellite markers from three different chromosomes.

Afterwards, 112 individuals with unknown cause of nonlesional epilepsy were tested for the presence of c.628G > A (p.Glu210Lys) mutation in the *UBTF* gene by Sanger sequencing.

Results

Genetic counseling was performed as part of medical healthcare prior to genetic testing. The patient was first tested by NGS of a custom-designed gene panel with 112 genes.[6] Since no relevant causal variant was found by the gene panel sequencing, WES was performed in the family trio consisting of the patient and both parents. We performed Trio analysis and found a heterozygous variant c.628G > A (p.Glu210Lys) in the *UBTF* gene (NM_014233.3) not present in the parents and confirmed the variant by Sanger sequencing. It was the only de novo variant detected in the WES data in the patient. *UBTF* gene was not yet associated with any human disease at that time. This variant was not included in global/population databases such as the 1,000 Genome project (www.internationalgenome.org), Exome Aggregation Consortium (http://exac.broadinstitute.org), or Exome Variant Server (http://evs.gs.washington.edu/EVS) until recently reported in association with neurodegeneration[3,4] and eventually entered in the Human Gene Mutation Database (HGMD, Cardiff University, Cardiff, Wales, UK) Professional database (www.hgmd.cf.ac.uk).

The patient, a 13-year-old boy, was born from the first pregnancy to healthy parents. The perinatal period was uneventful. His early motor development was normal (he walked independently at 13 months), had mildly delayed speech development, saying first words at the age of 18 months. Before the onset of the deterioration, there were signs of fever followed by prolonged apathy (3 weeks) after immunization against morbilli, parotitis, and rubeola at the age of 15 months. His neurological problems started at the age of 2 years when the family noticed unsteady gait with frequent falls. Mental deterioration followed shortly after the onset of motor symptoms. The patient lost his verbal abilities, became apathetic, stopped playing with other children, and had difficulty with gross and fine motor skills. During the next 6 months there was progression of paleocerebellar syndrome with severe gait instability and upper motor neuron signs appeared–hypertonia, hyperreflexia, and positive pyramidal signs. The progression of all symptoms was rapid. At the age of 4 years, he was still able to walk independently but with severe ataxia, spasticity worsened, walking with support was possible until 4.5 years of age. Further on, stronger support for his gait was necessary. Mental deterioration continued and extrapyramidal signs, mostly dystonic attacks, appeared. Brain MRI (magnetic resonance imaging) performed at the age of 3 years that showed unspecific changes in white matter: hyperintensity on T2-weighted (T2W) and FLAIR images in the periventricular area and mild atrophy in the frontal and temporal lobe. T2 and FLAIR (fluid-attenuated inversion recovery) MRI images at both ages show bilateral symmetric hyperintense areas in ventromedial and dorsal thalamus. This is a nonspecific signal alteration. Representative MRI images at the age of 3 and 7 years can be seen in ►**Fig. 1**. At the age of 6 years he developed epileptic seizures. The seizures were tonic, tonic-clonic, and nonmotor (behavior arrests, autonomic, and emotional). The epilepsy was drug-resistant from the beginning. Electroencephalogram (EEG) was normal at the age of 3 years; atypical diffuse β activity appeared at the age of 5 years and frequently sharp waves at the age of 6 years. EEG finally showed continuous bilateral frontotemporal spikes and abnormal slow background activity. Ictal episodes show generalized paroxysmal fast activity. Treatment with diazepam, phenobarbital, topiramate, primidone, phenytoin, valproic acid, levetiracetam, and vigabatrine was only transient and partly successful or ineffective. The most effective was the combination of intravenous midazolam and levetiracetam. Final treatment was a combination of vigabatrin and primidone. He is still in electrical status epilepticus in this therapy and has seizure two to three times per hour. The seizures are without respiratory insufficiency.

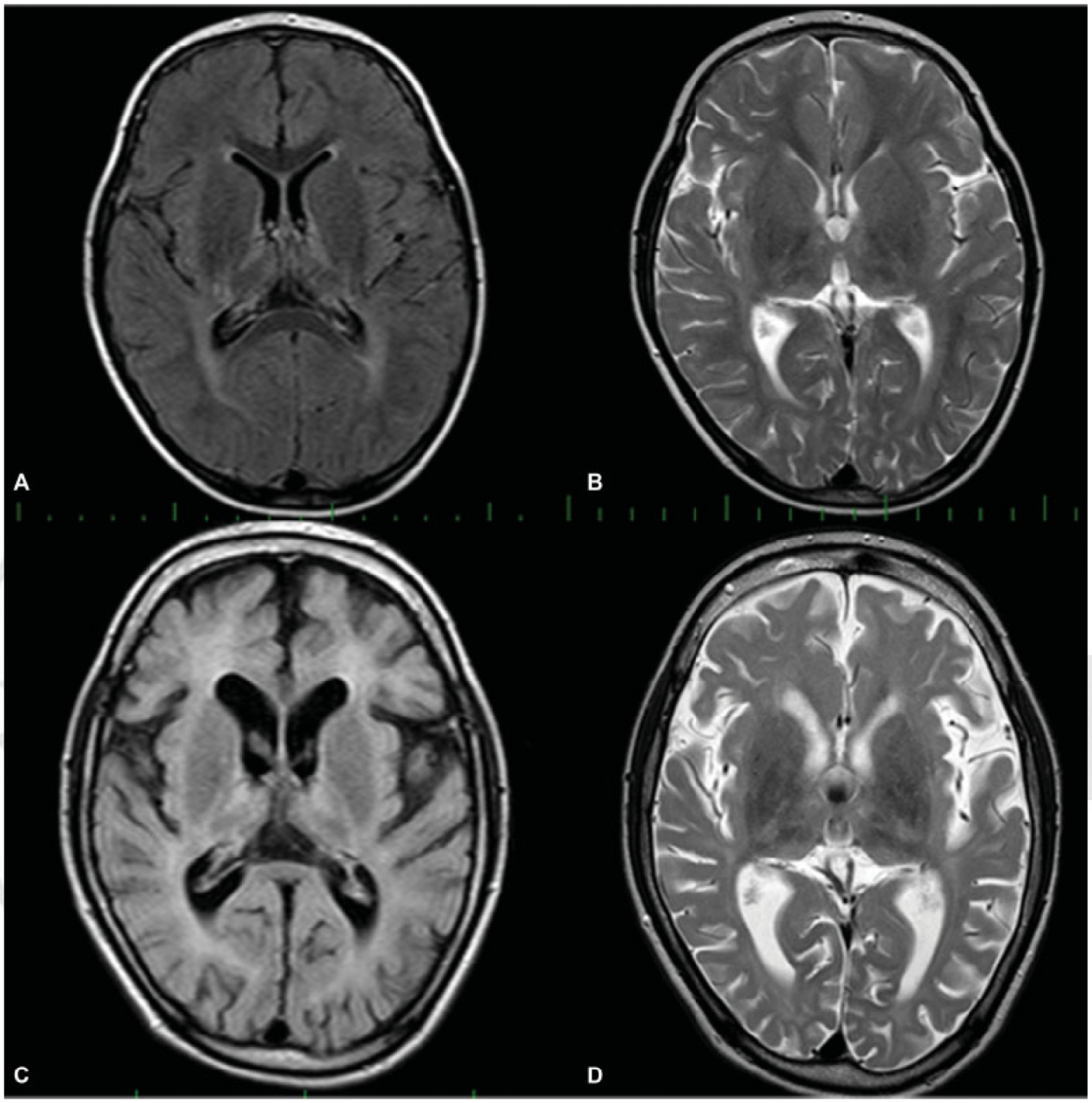

Fig. 1 Representative MRI slices from the patient. FLAIR **(A)** and axial T2-weighted **(B)** image at the age of 3 years–bilateral signal hyperintensity in the parieto-occipital white matter in FLAIR image. FLAIR **(C)** and axial T2-weighted **(D)** image at the age of 7 years–severe diffuse brain atrophy. T2 and FLAIR MRI images at both ages show bilateral symmetric hyperintense areas in ventromedial and dorsal thalamus. MRI, magnetic resonance imaging. FLAIR, fluid-attenuated inversion recovery.

The patient lost the ability of walking at the age of 6 years. Since the age of 11 years he is fed via gastrostomy. Currently he is fully immobilized, has a dystrophic habitus, and severe intellectual impairment. He cannot speak but he clearly reacts to the family members. He becomes calm in their presence. At the age of 13 years, there is severe central quadriparesis, extrapyramidal syndrome, and secondary scoliosis in his neurological status.

After the experience with this patient, we decided to search for this causal variant in the group of epilepsy patients with suspected genetic origin who were not previously resolved in our laboratory by any of the genetic tests. We sequenced the exon 7 of the *UBTF* gene in a cohort of 112 patients by Sanger sequencing; however, this c.628G > A (p. Glu210Lys) variant was not detected in any of them.

Discussion

We describe a Czech male patient with severe progressive neurodegeneration from the age of 2 years with diffuse progressive brain atrophy and suffering from drug-resistant epilepsy in the later stage of the disease. Using the Trio

analysis of WES data, which is known to be a powerful approach for finding de novo dominant causal mutations, we found a heterozygous missense variant c.628G > A (p.Glu210Lys) in *UBTF* gene in the patient but not in his parents. This was the only de novo variant detected in this patient but at the time of the first analysis, the *UBTF* gene was not yet associated with any human disease. We reanalyzed the data after 6 months and compared the results against currently available databases (Online Mendelian Inheritance in Man; OMIM). Literature (PubMed) search was also newly performed and we found that this variant was just recently reported in 11 patients with very similar neurodegeneration.[3,4] The phenotype of patients and the course of the disease reported in both publications is highly compatible with the clinical image of our patient. Moreover, a nonspecific signal alteration on thalamus mentioned above has not been described in published *UBTF* cases before. The common key feature present in our patient and in all the previously reported patients with the recurrent mutation (p.Glu210Lys) in *UBTF* is the motor and language regression. We believe that this c.628G > A de novo *UBTF* variant is the cause of the patient's neurodegeneration and subsequent psychomotor regression accompanied later by severe and drug resistant epilepsy.

It seems that in patients with *UBTF* mutations, phenotypic features show relatively high consistency among the affected 11 individuals described previously.[3,4] Our report confirms the previous findings and contributes to the identification of patients in later stages of the disease with apparently similar phenotype and course of disease and thus helps to expand the established genotype–phenotype correlation in complex diagnostics of rare disorders when even each individual case is important for establishing the clinical image. Our report together with both previous works strongly support inclusion of *UBTF* gene, or at least the critical exon with the recurrent mutation, among novel candidate genes for neurodegeneration disorder with or without epilepsy. The *UBTF* variant c.628G > A (p.Glu210Lys) is the only pathogenic variant known in the *UBTF* gene and is a hot spot for recurrent mutations. DNA testing of this gene should therefore begin with this prevalent mutation.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Conflicts of Interest

None of the authors has any conflicts of interest to disclose.

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Neonatal Onset of Epilepsy of Infancy with Migrating Focal Seizures Associated with a Novel *GABRB3* Variant in Monozygotic Twins

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Neuropediatrics

Abstract

Background Recently, a study providing insight into *GABRB3* mutational spectrum was published (Møller et al 2017). The authors report considerable pleiotropy even for single mutations and were not able to identify any genotype–phenotype correlations.
Methods The proband (twin B) was referred for massively parallel sequencing of epilepsy-related gene panel because of hypotonia and neonatal seizures. The revealed variant was confirmed with Sanger sequencing in the proband and the twin A, and both parents were tested for the presence of the variant.
Results We report a case of epilepsy of infancy with migrating focal seizures (EIMFS) of neonatal onset in monozygotic twins with a de novo novel *GABRB3* variant p.Thr281Ala. The variant has a uniform presentation on an identical genomic background. In addition, early seizure-onset epilepsy associated with *GABRB3* mutation has been until now described only for the p.Leu256Gln variant in the *GABRB3* (Møller et al 2017, Myers et al 2016) located in the transmembrane domain just as the p.Thr281Ala variant described here.
Conclusion De novo *GABRB3* mutations may cause neonatal-onset EIMFS with early-onset hypotonia, respiratory distress, and severe developmental delay.

Keywords
- GABRB3
- monozygotic twins
- epilepsy
- EIMFS – Epilepsy of Infancy with Migrating Focal Seizures

Introduction

Epileptic encephalopathies (EEs) are characterized by frequent and intractable seizures and poor developmental outcome.[1] The association of the *GABRB3* with EEs has been established, previously.[2] Patients with *GABRB3* pathogenic variants present with broad phenotypic spectrum, and genotype–phenotype correlations could not be demonstrated until now.[3] However, to date only one patient with an onset of seizures on day 1 of postnatal life and no association with epilepsy of infancy with migrating focal seizures (EIMFS) has been described.[3]

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The main clinical features of EIMFS are the onset of seizures in the first 6 months of life, nearly continuous electroencephalographic seizures, involving multiple independent areas originating in both hemispheres, and progressive deterioration of psychomotor development.[4,5]

We present monozygotic twins with severe EE with neonatal onset of EIMFS due to a de novo novel variant in the *GABRB3*.

Case Report

Patients and Methods

The study was approved by the ethics committee of University Hospital Motol. Parents provided informed consent according to the Declaration of Helsinki.

The twins were referred to our department at the age of 2 months because of serious hypotonia and neonatal-onset drug-resistant seizures.

A DNA sample from twin B was tested by massively parallel sequencing of a gene panel with the HaloPlex technology (Agilent, Santa Clara, California, United States). Ninety-seven genes known to be associated with severe childhood epilepsy were included in the panel. Data were analyzed using the SureCall software (Agilent) and the NextGENe software (Softgenetics, State College, Pennsylvania, United States). Variants were annotated with Alamut-Batch (Interactive Biosoftware, Rouen, France) and filtered according to the recommendations.[6]

Afterwards, the twin A and both parents were tested with Sanger sequencing for the presence of the variant.

Maternity and paternity was tested with a set of six highly polymorphic microsatellite markers from different chromosomes.

Clinical Examination

Female twins (monochoriati/biamniati) were born after spontaneous conception to healthy Caucasian unrelated parents. Family history was negative regarding epilepsy. Pregnancy was uneventful. Acute caesarean section was performed due to altered cardiotocography at gestational age of 37 weeks.

Birth weight and birth length of twin A and B were 1,980 g and 44 cm and 2,115 g and 46 cm, respectively (below the 3rd percentile). The APGAR score for both were 1 to 5 to 7. Both of them required reanimation after the birth. Both twins were very floppy from birth, but deep tendon reflexes were brisk. Spontaneous motor activity was minimal and their cry was very weak. Normal neonatal reflexes could not be elucidated. Their faces showed dysmorphic features such as remarkable dolichocephaly, tented upper lip vermilion, high-arched palate, and micrognathia. The head circumference of both children was 31 cm at the age of 7 days and 35 cm at 8 weeks of age (below the 3rd percentile).

Due to the absence of sucking reflex, they were fed by nasogastric tube. Oxygen was administered repeatedly because of episodes of hypoventilation. Some jerky movements of the limbs were already noted on the first day of life, but epileptic seizures were recognized only after performing electroencephalogram (EEG) during their second week of life. EEG showed frequent multifocal spikes and ictal recruitment of rhythmic activity above both hemispheres. Tonic posturing of limbs, trunk flexion, eye and head deviation, and bilateral alternating clonic movements of the limbs created the ictal semiology. The EEG at 2 months is shown in ►**Fig. 1**. The EEG recordings never showed any hypsarrhythmia.

Very detailed investigation of blood, urine, and cerebrospinal fluid (CSF) did not reveal signs of any metabolic disorder. Brain magnetic resonance imaging (MRI) was performed once (at 6 weeks of age) in both children and was without abnormalities. Karyotype was tested in both twins and was normal 46,XX. Microdeletion analysis using MLPA kit P-245 (MRC Holland, the Netherlands) was performed, and the results were without abnormalities (no deletion, no duplication detected). SurePrint G3 ISCA V2 CGH 4 × 180K (Agilent Genomics, United States) microarray was performed and no significant variants were found.

Seizures were drug-resistant in both children. Antiepileptic drugs were tried in various combinations. Phenobarbital, midazolam, clonazepam, topiramate, levetiracetam, vigabatrin, phenytoin, primidone, and their combinations were not able to completely control the seizures. Neither pyridoxine and pyridoxal-6 phosphate, nor ketogenic diet were effective.

A follow-up EEG remained abnormal with multifocal spikes and ictal build-up starting in various areas of the brain. In spite of this, the parents were only occasionally able to detect clinical ictal features in the second year of life.

At 17 months, the twins had a body length of 77 cm (10th percentile) and 75 cm (3rd percentile) in twin A and B, respectively. Head circumference was 41.7 cm in twin A and 41.2 cm in twin B (both below the 3rd percentile). Head circumference was evaluated with http://www.who.int/childgrowth/standards/second_set/chts_hcfa_girls_z/en/ (accessed November 20, 2017). The head circumference lines crossed the *Z*-score line: both twins tracked the –3 *Z*-score line from the beginning, but at the age of 17 months the growth started to fall away from the –3 *Z*-score line (and the growth line now tends away from the median). This indicates secondary progression of microcephaly. They have several facial dysmorphic features that became more prominent as they got older: microcephaly, hyperdolichocephaly, bitemporal narrowing, low set ears, hypotelorism, epicanthal folds, long eyelashes, sparse eyebrows, anteverted nares, long deep philtrum, everted upper lip vermilion, small chin, high-arched palate, and cone-like fingers. They have facial hypomimia, struggle with hypersalivation, and have to be fed with a nasogastric tube. They can swallow only a few milliliters of fluids administered by a syringe. They have no sucking reflex. Their muscle tone is extremely low, and on ventral suspension, they present as floppy babies. They have severe head lag while pulling up to sit and no head control except for a few seconds. Their spontaneous activity is minimal. They have severe central visual impairments and demonstrate no social contact.

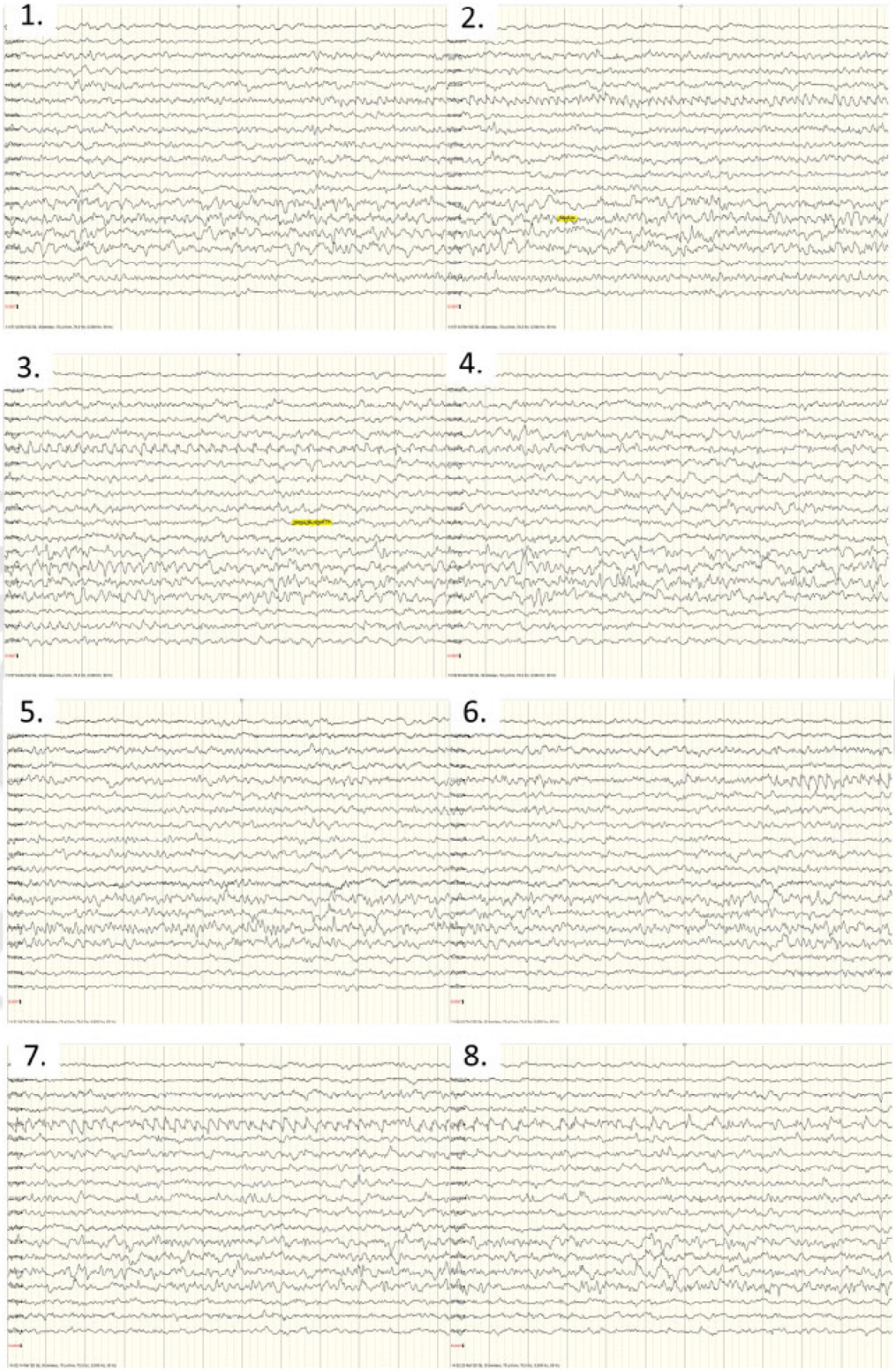

Fig. 1 Electroencephalogram (EEG)—twin A at the age of 2 months. Bilateral ictal built-up in independent areas is present.

The parents observe only sporadic seizures in twin A with wide mouth opening and upward eye rolling. Her EEG captured in sleep shows very frequent irregular spikes and sharp waves in both frontocentral regions. Physiological sleep transients are absent.

Twin B has more frequent generalized clonic seizures. While awake, EEG background activity is slow and frontocentrotemporal spikes and sharp waves are visible.

Seizures are more frequent during febrile illnesses and due to hyperthermia of other reasons.

Their current treatment is topiramate with phenytoin.

Results

We have detected the same p.Thr281Ala (c.841A > G) variant in the *GABRB3* gene (NM_000814.5) in heterozygous state in both twins, but not in the parents, showing the variant is de novo, because neither nonpaternity nor nonmaternity was observed.

This is a novel variant that has not been reported previously. The variant has no population frequencies in databases Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/, accessed January 15, 2017] or Exome variant server (EVS) (http://evs.gs.washington.edu/EVS/, accessed January 15, 2017], and is therefore extremely rare. Prediction algorithms (SIFT,[7] MutationTaster,[8] and PolyPhen2[9]) classify the variant as being deleterious. The variant occurs at a highly conserved residue. According to the American College of Medical Genetics (ACMG) recommendations,[6] the variant is classified as pathogenic (1 strong, 1 moderate, and 4 supporting criteria) and therefore we suggest it is causal for severe epilepsy in these twins.

Discussion

We present monozygotic twins with EIMFS and neonatal onset due to a novel pathogenic de novo variant in the *GABRB3* gene detected by massively parallel sequencing (MPS) gene panel testing.

EIMFS is a severe, early-onset EE presenting with bilateral partial seizures with migrating ictal EEG. Onset occurs typically between 40 days and 3 months (range, 1 day–6 months) after birth. Seizures are usually drug-resistant and the patients develop severe developmental delay and microcephaly.[10] Various pathogenic mutations have been described in this syndrome, *KCNT1* being the most frequent.[11] No *GABRB3* mutation was revealed as a cause of EIMFS up till now even though *GABRB3* is an established EE gene. Our report is also specific in describing monozygotic twins with the same causal mutation and almost identical phenotype.

Neurological status and head circumference are typically normal before the onset of seizures in EIMFS.[12] Most patients develop microcephaly and severe neurological impairment during the course of the disease.[13] Our cases were severely neurologically impaired from birth but epilepsy also started in the first week of life.

There are only a few cases of EIMFS starting in the neonatal period.[14]

Only one case of early onset of epilepsy (neonatal onset, day 1) associated with *GABRB3* mutation has been described before,[2] associated with the p.Leu256Gln variant. Our patients carry the p.Thr281Ala variant, and also present with neonatal onset. Both of these variants are in close proximity and are located in the α-helix (►**Fig. 2**). Their functional consequences may therefore be similar.

Since other tests (karyotype, Multiplex Ligation-dependent Probe Amplification [MLPA], array comparative genomic hybridization [CGH]) were not positive, we have no

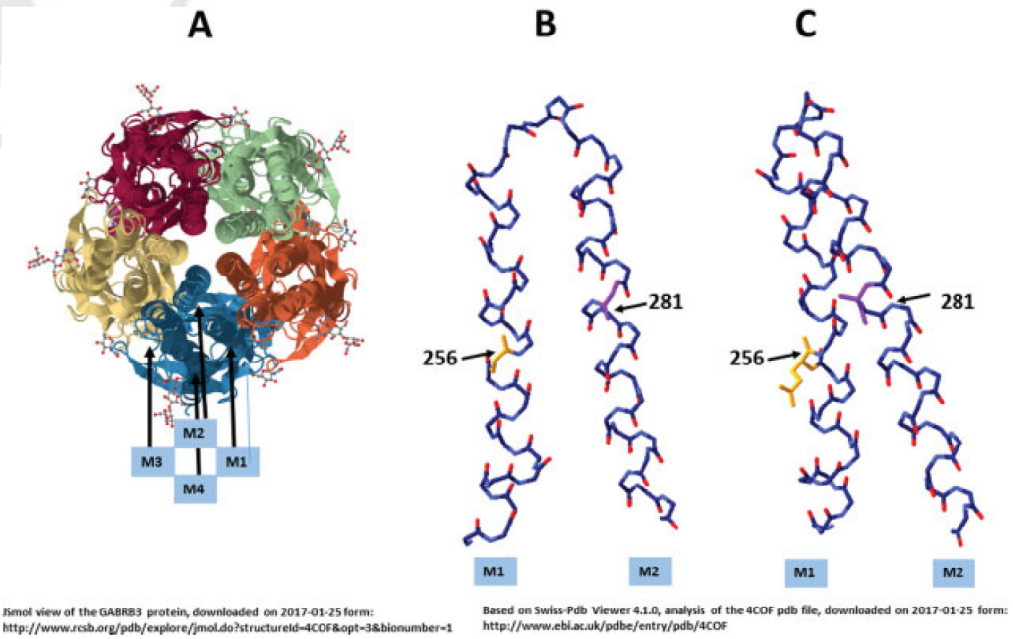

Fig. 2 *GABRB3*. (**A**) *GABRB3* is an ion channel, homopentamer. Each transmembrane unit consists of four α-helices, designated M1–M4. According to http://www.rcsb.org/pdb/explore/jmol.do?structureId=4COF&opt=3&bionumber=1 (accessed January 25, 2017). (**B**) Alpha-helix M1 and M2 are shown. Position p.Leu256 is highlighted in yellow, and position p.Thr281 is highlighted in violet. (**C**) Mutation p.Leu256Gln is highlighted in yellow, and mutation p.Thr281Ala is highlighted in violet. Both changes result in the formation of two new strong H-bonds. According to Swiss-Pdb Viewer 4.1.0,[15] from http://www.expasy.org/spdbv/, and analysis of the 4COF pdb file, downloaded from http://www.ebi.ac.uk/pdbe/entry/pdb/4COF (accessed January 25, 2017).

reliable explanation for the dysmorphic features in both twins. It may be associated with the *GABRB3* mutation, but there could also be other unknown explanation. Dysmorphic features have not been described previously in patients with *GABRB3* mutations, but there might be a relationship between the *GABRB3* mutations and dysmorphic features. Therefore, in future, we suggest that patients with *GABRB3* mutations should be also examined by an experienced clinical geneticist for the presence of dysmorphic features. To test this hypothesis, further studies are needed on a larger cohort of patients.

Conclusions

EIMFS can start at the newborn age with severe hypotonia, respiratory distress, and drug-resistant epilepsy. De novo pathogenic mutation in the *GABRB3* gene may cause severe EE with neonatal onset. In the monozygotic twins reported, the genotype–phenotype correlation was very high. Our cases broaden the spectrum of neonatal EEs of genetic etiology.

Conflict of Interest
None.

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ORIGINAL ARTICLE

Two Novel Variants Affecting *CDKL5* Transcript Associated with Epileptic Encephalopathy

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Background: Variants in the human X-linked cyclin-dependent kinase-like 5 (*CDKL5*) gene have been reported as being etiologically associated with early infantile epileptic encephalopathy type 2 (EIEE2). We report on two patients, a boy and a girl, with EIEE2 that present with early onset epilepsy, hypotonia, severe intellectual disability, and poor eye contact.
Methods: Massively parallel sequencing (MPS) of a custom-designed gene panel for epilepsy and epileptic encephalopathy containing 112 epilepsy-related genes was performed. Sanger sequencing was used to confirm the novel variants. For confirmation of the functional consequence of an intronic *CDKL5* variant in patient 2, an RNA study was done.
Results: DNA sequencing revealed *de novo* variants in CDKL5, a c.2578C>T (p. Gln860*) present in a hemizygous state in a 3-year-old boy, and a potential splice site variant c.463+5G>A in heterozygous state in a 5-year-old girl. Multiple *in silico* splicing algorithms predicted a highly reduced splice site score for c.463+5G>A. A subsequent mRNA study confirmed an aberrant shorter transcript lacking exon 7.
Conclusions: Our data confirmed that variants in the *CDKL5* are associated with EIEE2. There is credible evidence that the novel identified variants are pathogenic and, therefore, are likely the cause of the disease in the presented patients. In one of the patients a stop codon variant is predicted to produce a truncated protein, and in the other patient an intronic variant results in aberrant splicing.

Keywords: *CDKL5* gene, early onset seizures, infantile epileptic encephalopathy 2, massively parallel sequencing, splice site variant

Introduction

Cyclin-dependent kinase-like 5 (*CDKL5*) gene was first identified as a serine/threonine kinase 9 (*STK9*) with transcriptional mapping (Montini *et al.*, 1998). Severe X-linked infantile spasm (ISSX) caused by *STK9* disruption in two unrelated girls with infantile spasm syndrome was reported (Kalscheuer *et al.*, 2003). Later, neurodevelopmental disorder with infantile spasm, mental retardation, and phenotypic features of atypical Rett syndrome caused by variants in *CDKL5* was published (Tao *et al.*, 2004; Weaving *et al.*, 2004). Archer *et al.*, published in 2006 that *CDKL5* variants are the cause of infantile spasm, early epileptic seizures (later intractable) with onset in the first months of the life in females. Pathogenic variants in *CDKL5* were also noted, which are responsible for early infantile epileptic encephalopathy type 2 (EIEE2) characterized by early onset epilepsy, generalized tonic seizures, severe hypotonia, absent speech, and severe intellectual disability (Bahi-Buisson *et al.*, 2008; Fehr *et al.*, 2013).

The *CDKL5*-related encephalopathy is an X-linked dominant disorder, yet reported predominantly in females, only a few boys have been reported so far. The *CDKL5* gene is localized on chromosome Xp22.13 and contains 21 exons. *CDKL5* is an ubiquitously expressed protein with highest levels in the brain, thymus, and testes, involved in the brain maturation (Lin *et al.*, 2005; Rusconi *et al.*, 2008). The protein product shuttles in the cytoplasm and

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nucleus (Amendola *et al.*, 2014). In the nucleus, *CDKL5* also participates in the control of nuclear speckle morphology. Interestingly, the nuclear speckles in the interchromatin compartment are dynamic parts involved in the activation of a transcription factor by continuous activation of splicing factors. This mechanism affects pre-mRNA splicing (Ricciardi *et al.*, 2009). *CDKL5* and *MECP2* seem to share a common molecular pathway and symptoms from Rett syndrome and can also be found in some females with *CDKL5*-related encephalopathy. But findings from various studies suggest that *CDKL5* disorder is an independent entity with its own specific traits (Evans *et al.*, 2005; Fehr *et al.*, 2013). Variants in *CDKL5* occur mostly *de novo*, but germline mosaicism has also been published (Weaving *et al.*, 2004).

Three stages of *CDKL5*-related epilepsy have been described by Bahi-Buisson *et al.* (2008). At stage I, brief tonic seizures typically occur around the age of 4 weeks, interictal electroencephalography (EEG) is often normal at this period, but neurological examination reveals overall motor hypotonia and poor eye contact. Seizures usually respond to antiepileptic treatment. This "honeymoon" period may last several months, but EEG might already worsen at that time and motor and mental developments show no progression. Stage II presents with epileptic spasms or tonic seizures between the age of 6 months and 3 years. Infantile spasms and hypsarrhythmia are corticosteroid responsive at least in some cases, and some improvement of social contact and behavior is also reported. Severe mental and motor developmental delay, absent speech, and visual interaction are consistent with the diagnosis of epileptic encephalopathy. At stage III (after 3 years of age), patients have multiple types of seizures: epileptic spasms, tonic seizures, absences, and frequent myoclonic seizures. Some patients do not enter stage III and remain seizure free.

Previously, most of the *CDKL5*-related encephalopathies were shown to affect mainly females, probably because the studies included mostly females. Male patients with *CDKL5* variants have been reported later (Elia *et al.*, 2008; Fichou *et al.*, 2009; Sartori *et al.*, 2009). The clinical severity of the *CDKL5* disease for both genders has been described as being very similar (Liang *et al.*, 2011).

Materials and Methods

The study was approved by the Ethics Committee of 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol. Affected individuals were selected by an experienced epileptologist KŠ after referral to University Hospital Motol. We have a large cohort of patients with severe epilepsy of unknown etiology at our Centre for Epilepsies in University Hospital Motol. Patients were first selected by our epileptologist (K.Š.) and geneticist (M.V.) according to the criteria: negative brain magnetic resonance (MR) and availability of DNA samples from probands and both parents to verify variants. Previously, negative array comparative genome hybridization (CGH) testing is not a necessary condition. Patient's legal representatives signed informed consent.

DNA samples were isolated from peripheral blood using automated magnetic beads technique (iPrep™ PureLink™ gDNA Blood Kit; Invitrogen).

Massively parallel sequencing (MPS) of a custom-designed gene panel for epilepsy and epileptic encephalopathy with 112 related epilepsy genes was performed (HaloPlex; Agilent Technologies, Santa Clara, CA). The panel included *ADAR*, *ADCK3*, *ADSL*, *ALDH7A1*, *ALG13*, *AMT*, *AP4S1*, *ARHGEF9*, *ARX*, *ASAH1*, *ATP1A2*, *ATP1A3*, *BRAT1*, *C10ORF2*, *CACNA1A*, *CASK*, *CDKL5*, *CLCN4*, *CPT2*, *DCX*, *DEPDC5*, *DNM1*, *DOCK7*, *EEF1A2*, *FASN*, *FLNA*, *FOLR1*, *FOXG1*, *GABBR2*, *GABRA1*, *GABRB3*, *GABRG2*, *GAMT*, *GCSH*, *GLDC*, *GNAO1*, *GPHN*, *GRIN1*, *GRIN2A*, *GRIN2B*, *GRIN2D*, *HCN1*, *HDAC4*, *HNRNPU*, *CHD2*, *IQSEC2*, *KCNA2*, *KCNB1*, *KCNC1*, *KCNH5*, *KCNJ10*, *KCNQ2*, *KCNQ3*, *KCNT1*, *KCTD7*, *KIAA2022*, *MBD5*, *MECP2*, *MEF2C*, *MFSD8*, *MOCS1*, *MOCS2*, *MTOR*, *NEDD4 L*, *NRXN1*, *PANK2*, *PCDH19*, *PHF6*, *PIGA*, *PIGQ*, *PLCB1*, *PNKP*, *PNPO*, *POLG*, *PRIMA1*, *PRRT2*, *PURA*, *QARS*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *ROGDI*, *RYR3*, *SAMHD1*, *SCN1A*, *SCN1B*, *SCN2A*, *SCN8A*, *SLC12A5*, *SLC13A5*, *SLC19A3*, *SLC25A22*, *SLC2A1*, *SLC35A2*, *SLC6A1*, *SLC9A6*, *SPTAN1*, *ST3GAL3*, *STXBP1*, *SYN1*, *SYNGAP1*, *SYNJ1*, *SZT2*, *TBC1D24*, *TDP2*, *TPP1*, *TREX1*, *TSC1*, *TSC2*, *UBE3A*, *WDR45*, and *ZEB2*.

Our designed gene panel targeted coding regions of the genes and adjacent exon–intron boundaries (flanking introns ± 50 bp). Data from MPS were analyzed with SureCall software (Agilent Technologies) and NextGENe Software (SoftGenetics LLC, State College, PA). Average read length was 110 bp. 99.1% of the target region was covered at least 10×. Both methods are based on BWA-MEM alignment (Li and Durbin, 2010)

Sanger sequencing was performed with primers designed for exon 7 and 18 of the *CDKL5* gene to confirm the novel variants. Parentity was tested using six microsatellite markers.

For confirmation of the functional consequence of the intronic *CDKL5* variant in patient 2, a new blood sample for RNA study was taken. Total RNA was extracted from blood mononuclear cells using TRIzol reagent and transcribed into full length cDNA using Cloned AMV First-Strand cDNA Synthesis Kit according to manufacturer's instructions with Oligo (dT)20 as a primer (Thermo Fisher Scientific).

We designed primers for amplifications of cDNA of exons 4–8: forward ACATGAAATTGTGGCGATCA, reverse TACCATCTGGTGGCAACGTA. We expected aberrant splicing, therefore, we amplified exon 7 and adjacent exons to study the splicing changes. PCR products obtained from the cDNA were loaded on 2% agarose gel. The PCR products were sequenced using BigDye Terminator kit v3.1 (Life Technologies, CA). Capillary electrophoresis on the ABI 3130 Genetic Analyzer (Life Technologies) was performed.

Results

Patient 1

The patient is a 3-year-old boy, the first child of healthy, unrelated parents. He was born in the 38th gestational week through caesarean section because of cephalopelvic disproportion. Birth weight was 2750 g. Early postnatal cardiologic examination confirmed Fallot tetralogy. Postnatal adaptation was otherwise normal.

At the age of 4 weeks, he had a few staring spells with jerking of upper limbs and head deviation to the right. EEG was

normal. One month later, he presented with a series of short episodes of behavioral arrest and wide eye opening. Right-sided frontocentral spikes were seen on EEG at that time. At the age of 4 months, hypomotor seizures were frequent. When he was 1 year old, cardiosurgical correction of the Fallot tetralogy was performed. In the postoperative period, a series of epileptic spasms appeared with hypsarrhythmia on the EEG. Later he developed myoclonic seizures and tonic seizures.

Seizures have been intractable: valproic acid, topiramate, phenobarbital, vigabatrin, levetiracetam, phenytoin, clobazam, and their combinations were not permanently effective. Adrenocorticotropic hormone was partially effective. Ketogenic diet was administered from the age of 2 to 3 years with a reduction of seizure frequency, but only for a few months. At the age of 3.5 years, tonic seizures in series predominate but myoclonic seizures are present as well.

Psychomotor development was delayed since the first months. Overall hypotonia and poor social and visual contact were observed, as well. From the age of 3.5 years, relatively short lower limbs became apparent. He has no head control; he is not capable of independent sitting. Speech is absent; he performs only some unintelligible vocalizations and has central visual impairment.

Before performing epilepsy gene panel testing the patient underwent repeated brain MR imaging, showing only mild frontal atrophy. The metabolic screening and skin biopsy showed normal results. Targeted screening for DiGeorge syndrome was also negative. Moreover, array CGH was also done and no causal variants were found. The clinical phenotype is summarized in Table 1.

Patient 2

This female patient was born from the third, normal pregnancy of healthy, unrelated parents. Delivery and early postnatal period were uneventful. Bilateral tonic seizures of upper limbs followed by clonic jerking appeared at the age of 6 weeks. Initial EEG recordings did not show epileptiform activity, later bilateral frontocentral spikes were observed.

Seizures became more complex, usually starting with behavioral arrest ''freezing'' followed by tonic flexion or extension of the upper limbs and continued as asynchronous clonic jerking of the face, eyelids, and limbs and ended as a series of spasms. As she became older, the tonic phase was less prominent and the jerks were more asynchronous and irregular. Reactivity was disturbed during the seizures.

The seizures were drug resistant: phenobarbital and vigabatrin had partial effect; levetiracetam, clonazepam, valproic acid, topiramate, and phenytoin did not control the seizures, but seizures disappeared for 1 year at the age of 3 years. This happened without any intervention, there was no change in medication with vigabatrin, topiramate, and clobazam. During this period, she improved in walking and acquired some nonverbal communication skills. Seizures reoccurred at the age of 4 years. Therefore, ketogenic diet was implemented with good result: seizure freedom was achieved 3 months after initiation of the diet. The girl remains hypotonic, nonverbal, with severe intellectual disability and motor delay. Repeated brain MR and metabolic screening showed normal results. The clinical phenotype of the patient is summarized in Table 1.

Table 1. Clinical Phenotype of Patients

Variant	*Patient 1—male c.2578C>T (p.Gln860*)*	*Patient 2—female c.463+5G>A*
Age at seizure onset	4th week	6th week
Seizures	Multiple types	Multiple types in series
Drug-resistant seizures	Yes	Yes
Eye contact	Poor	Poor
Speech	Absent	Absent
Hand stereotypies	No	No
Intellectual disability	Profound	Severe
Hypotonia	+	+
Gait	Not achieved	Atactic gait
EEG	Multifocal discharges, hypsarrhythmia	Multifocal spikes
MR	Mild frontal atrophy	Normal

EEG, electroencephalography; MR, magnetic resonance.

A novel nonsense variant c.2578C>T (p. Gln860*) in hemizygous state in exon 18 of *CDKL5* according to the reference sequence NM_003159.2 was found in patient 1. The predicted reading frame is interrupted by a premature STOP codon, leading to a serious effect on the protein and probably nonsense-mediated mRNA decay. The variant was confirmed by Sanger sequencing of exon 18 of the *CDKL5* gene (Fig. 1) and was not found in the healthy parents, so we assume it occurred *de novo*. To our knowledge, this variant has not been previously described, is not listed in HGMD Professional (www.hgmd.cf.ac.uk/ac/index.php), and is absent from global/population databases such as the 1000 Genomes Project (www.1000genomes.org), Exome Aggregation Consortium (http://exac.broadinstitute.org), and Exome Variant Server (http://evs.gs.washington.edu/EVS). According to the ACMG criteria published in Richards *et al.* (2015), the variant has one very strong (PVS1: null variant in a gene wherein loss of function [LOF] is a known mechanism of disease), one strong (PS2: *de novo*: both maternity and paternity confirmed in the patient with the disease and no family history), one moderate (PM2: absent from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium), and ≥2 supporting criteria (PP3: multiple lines of computational evidence support a deleterious effect on the gene or gene product; PP4: the patient's phenotype or family history is highly specific for a disease with a single genetic etiology); therefore, we consider it as pathogenic and causal for EIEE2 in this patient.

In patient 2, MPS identified a heterozygous intronic variant close to the donor splice site of exon 7 of *CDKL5* gene (NM_003159.2). The variant occurs *de novo*, because it was not found in the healthy parents (parentity was tested). According to the ACMG rules, the variant has one strong and ≥2 supporting criteria. Therefore, the variant is evaluated as likely pathogenic.

Computer analysis using Alamut v.2.1. (Interactive Biosoftware, Rouen, France) based on the evaluation by a few

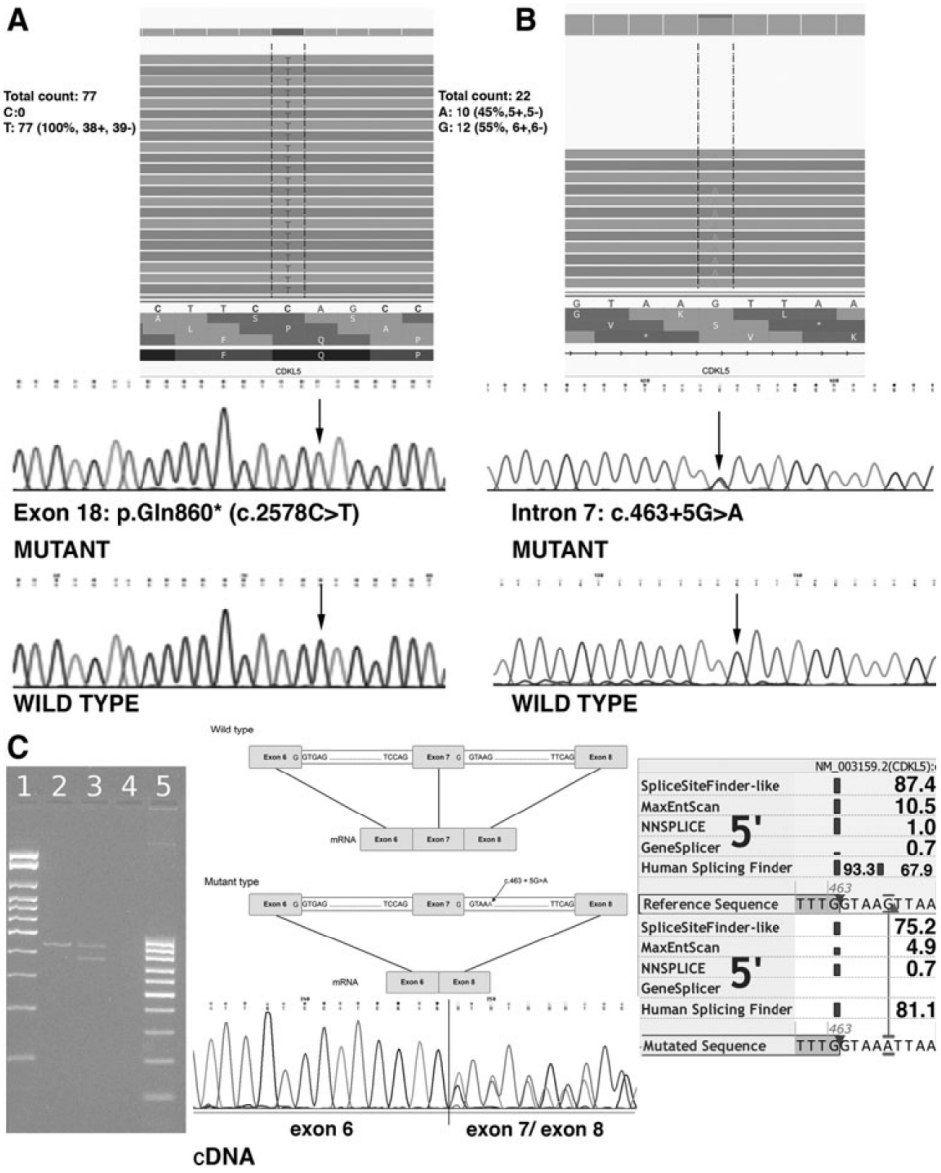

FIG. 1. Schematic view of two novel variants in the *CDKL5* gene and RNA study. **(A)** Variant p. Gln860* in the *CDKL5* gene in patient 1—visualized from IGV (http://software.broadinstitute.org/software/igv/), Sanger sequencing electrophoretogram of mutant and wild type allele. **(B)** Variant c.463+5G>A in the *CDKL5* gene in patient 2. **(C)** Two fragments formed due to the aberrant transcript visible on the 2% agarose gel, from the left side: 1 = LowRanger 100 bp DNA ladder (Norgen Biotek, CA, USA); 2 = control DNA sample; 3 = patient 2; 4 = negative control; 5 = 50 bp DNA ladder (Norgen Biotek, CA). Schematic presentation for mechanism and results of RNA splicing in wild and mutant type for c.463+5G>A variant. Predictions of splice sites in the wild type and mutated *CDKL5* sequences calculated using various predictor programs. A higher score indicates a strong splice site effect. *CDKL5*, cyclin-dependent kinase-like 5.

predictors SpliceSiteFinder-like (www.umd.be/HSF/), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSPLICE (www.fruitfly.org/seq_tools/splice.html), GeneSplicer (www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml), Human Splicing Finder (www.umd.be/HSF/) was performed. These different algorithms predicted high reduction of the splice site score (Fig. 1C). The difference between the sequences higher than 10%, such as in our case, predicts a strong effect in the splicing process (Shapiro and Senapathy, 1987).

PCR product from cDNA of patient 2 with splice site variant was loaded on 2% agarose gel. In comparison with the control sample, two fragments (differing in 60 nucleotides corresponding to exon skipping of exon 7) were observed due to the formation of an aberrant shorter transcript.

Sanger sequencing demonstrated the presence of exons 4, 5, 6, 7, and 8 on the wild type allele and exons 4, 5, 6, and 8 on the mutant allele.

Discussion

Most of the *CDKL5*-related encephalopathies were yet described in females and only rarely in males. This may be caused because variants in *CDKL5* were known as the cause for the so called atypical Rett syndrome. Therefore, *CDKL5* sequencing was frequently the next step after *MECP2* testing, which was performed predominantly in girls.

We present two novel variants in the *CDKL5* gene, one in a female and one in a male. The overall clinical manifestation of the male patient was similar to the phenotype reported in a few boys with rare point variants in *CDKL5* (Elia *et al.*, 2008; Wong and Kwong, 2015). Both children presented with different seizure types, two cases in particular showed very complex seizure types with features of tonic, clonic, and myoclonic convulsions as well as series of spasms. Seizures were drug resistant, but ketogenic diet was at least partially effective in patient 1 and granted

seizure freedom in patient 2. Global hypotonia and poor eye contact since early infantile age were present in both children. We did not observe gastrointestinal problems or scoliosis in our patients, but this can be due to their young age. Both patients had transient sleep problems due to myoclonic jerks; however, their epileptic origin has not been proven in either child. We have not observed any stereotyped hand movements typical for Rett syndrome patients. According to our knowledge, no co-occurrence of Fallot tetralogy or any other congenital cardiac anomaly with only *CDKL5* variants has been described. The only report describing a boy with severe encephalopathy, congenital cataracts, and tetralogy of Fallot attributes the phenotype to the interstitial deletion of Xp22 comprising also *CDKL5* (Van Esch *et al.*, 2007).

There is enough evidence that the variants are pathogenic and, therefore, are the cause of the disease in the presented patients. In patient 1, hemizygous variant p. Gln860* predicting premature stop codon, thus causing a serious effect on the protein, arose *de novo*. According to ACMG guidelines, we consider the variant as pathogenic, whereas in patient 2, the intronic variant c.463+5G>A affects the splicing and leads to the formation of aberrant transcript.

The splice donor site variant: NM_003159.2: c.463+5G>A in the intron 7 of the *CDKL5* gene may cause leaky splicing mechanism and leads to the formation of a 20 amino acids shorter protein without exon 7 (Fig. 1C). We showed that variant in intron 7 c.463+5G>A changes the 5′ donor splice site of intron 7. Aberrant splicing has been already described as a mechanism that could lead to EIEE2 (Weaving *et al.*, 2004; Archer *et al.*, 2006).

It has been suggested that splice site variants effect on the phenotype correlates with the proportion of functional CDKL5 due to mutated alleles (Bahi-Buisson *et al.*, 2008). The precise effect on the severity of the phenotype is not known. It is expected that a milder phenotype correlates with less severe mental retardation and the ability to walk even though gait is atactic. The clinical presentation in patient 2 is milder than in patient 1. This may be caused by leaky splicing. Also, variability of X inactivation in the different brain regions could lead to various phenotype manifestation (Weaving *et al.*, 2004). The other hypothesis is that exon 7 skipping (deletion of 60 nucleotides) causes in-frame deletion, protein is shortened only by 20 amino acids, and this may cause milder phenotype in the patient. Similar clinical phenotype with splice site variant IVS6-1G>T that also results in exon 7 skipping has been reported as pathogenic (Archer *et al.*, 2006). Our patient, similarly to the patient in Archer *et al.* (2006), had severe mental retardation, hypotonia, epilepsy seizure starting in the first months, and "honeymoon periods" with subsequent return of seizures.

Conclusions

We showed enough evidence that the variants are pathogenic and, therefore, are the cause of the disease in the presented patients. In one patient, stop codon variant causes a serious effect on the protein and the intronic variant in the second patient affects the splicing. Using RNA study, we were able to verify the formation of aberrant transcript in patient 2.

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Author Disclosure Statement

No competing financial interests exist.

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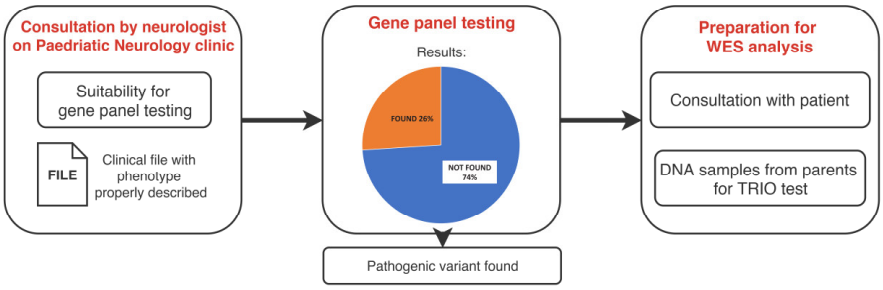
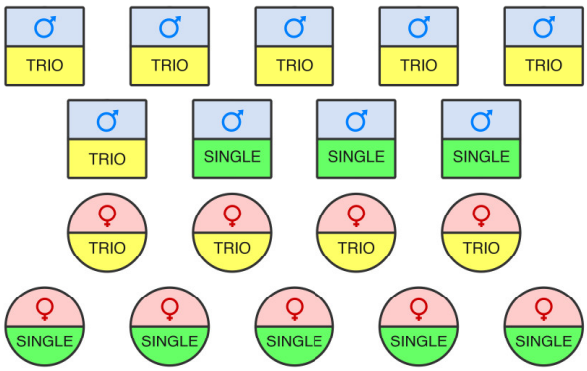
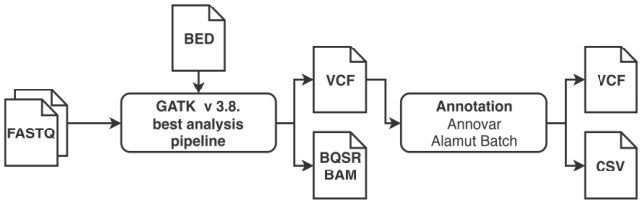




Methods

Bioinformatic analysis



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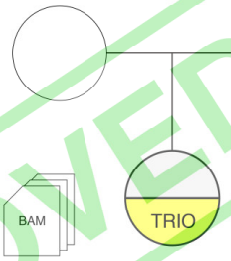

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n ~ 50 000 SNPs

- Population databases >0.01 (gnomAD, ExAC, 1000G)
- Predictions (ClinVar, PolyPhen, Hgmd...)
- Ref.gene.func (missense, synonymous...)
- Variant coverage, quality (DP ratio)
- OMIM and UniProt data
- In-house DB (WES)

n ~ 100 SNPs

prioritization by Exomiser based on HPO terms

weighted SNPs

TOP variants for manual check

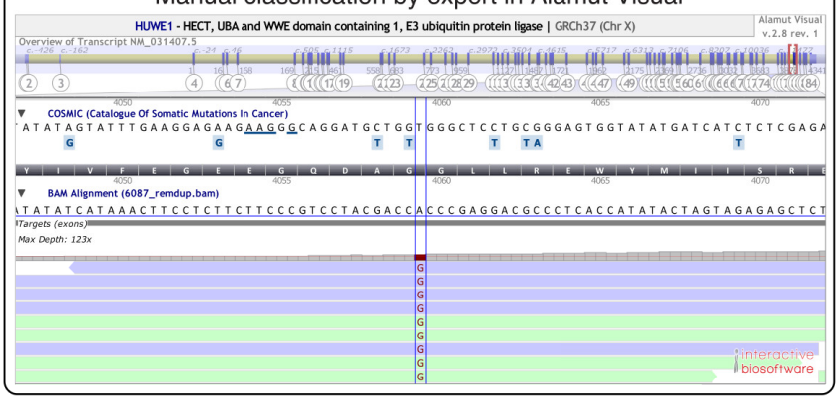
n ~ 10 SNPs

denovo prioritization by DenovoGear

weighted SNPs

TOP variants for manual check

Manual classification by expert in Alamut Visual



Results

SINGLE	*PPP2R5D* c.1267_1270delCTCT (p.Leu423fs)	SINGLE	*HUWE1* c.12195 G>C (p.Trp4065Cys)	SINGLE	*NARS2* c.83_84del (p.Leu28GInfs*17)	TRIO	*SETBP1* c.2601 C>G (p.Ser867Arg)	TRIO	*UBTF* c.628 G>A (p.Glu210Lys)

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Over 70% of pathogenic variants in Czech epilepsy cohort is propagated into protein domains

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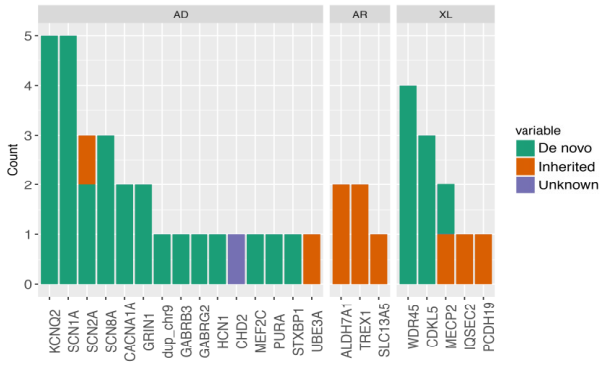
Introduction

Many studies that variation in conserved protein domains are less tolerated and thus are more likely connected to a clinical phenotype. These potentially significant consequences of DNA variation on domain structure and function makes them a valuable target for genomic studies.
In previous project we created database of protein domains published in PubMed, mapped to chromosomal location.
Our lab has long-time experience with NGS analysis in patients with neurodegenerative disorders. We are continuously implementing new approaches to optimize analysis workflow for NGS data.

Methods

We performed analysis on 60 variants, which were evaluated as pathogenic or likely pathogenic according to ACMG criteria, gathered from cohort of patients with epileptic encephalopathies.
Included variants were used from previous project published in paper:
https://doi.org/10.1186/s13023-018-0812-8



Variants were annotated by our custom database that provides information about all protein domains/features published in PubMed mapped on its chromosomal location. With this kind of information, we are able to determine whether is variant translated into specific domain and how change in this domain can affect function of protein. (Manuscript in preparation - schema in supplementary information)

Results

Out of 60 variants, 71% (43) was propagated into protein domains. Most common were Ion channel domains (14 variants), transmembrane regions (6 variants), and ion-channel transmembrane regions (5 variants). These regions are very conserved and changes in these structures can be harming for the whole organism. Many of the variants were annotated into multiple domains (60 variants into 93 domains). In the table below are listed annotated variants and their longest domain. (full table in suppl. file)

Variant	Chr	Position	Protein ID	Annotated domain
Ion transport prctein:				
HCN1_NM_021072.3 c.1189A>Gp.Ile397Leu	chr5	45396635	NP_066550.2	ion transport protein
KCNQ2_NM_172107.2 c.701C>Tp.Thr234Ile	chr20	62073874	NP_004509.2	ion transport protein
KCNQ2_NM_172107.2 c.826A>Cp.Thr276Pro	chr20	62071052	NP_004509.2	ion transport protein
KCNQ2_NM_172107.2 c.913_915delTTCp.Phe305del	chr20	62070962	NP_004509.2	ion transport protein
SCN1A_AB093548.1:c.542A>G p.Glu181Gly	chr2	166911208	NP_001159435.1	ion transport protein
SCN1A_NM_001165963.1 c.5384A>Gp.Glu1795Gly	chr2	166848401	NP_001159435.1	ion transport protein
SCN1A_NM_001165963.1c.1178G>Ap.Arg393His	chr2	166903479	NP_001159435.1	ion transport protein
SCN1A_NM_001165963.1c.4384dupp.Tyr1462Leufs*24	chr2	166854639	NP_001159435.1	ion transport protein
SCN1A_NM_001202435.1c.1244T>Ap.Ile415Lys	chr2	166903413	NP_001159435.1	ion transport protein
SCN2A_NM_001040142.1 c.2774T>Cp.Met925Thr	chr2	166201276	NP_001035232.1	ion transport protein
SCN2A_NM_021007.2:c.4756C>T p.Arg1586Cys	chr2	166243460	NP_001035232.1	ion transport protein
SCN8A_NM_014191.3 c.2549G>Ap.Arg850Gln	chr12	52159459	NP_001317189.1	ion transport protein
SCN8A_NM_014191.3 c.4921C>Gp.Leu1641Val	chr12	52200191	NP_001317189.1	ion transport protein
SCN8A_NM_014191.3c.4850G>Tp.Arg1617Leu	chr12	52200120	NP_001317189.1	ion transport protein
Transmembrane region:				
GABRB3_NM_000814.5 c.841A>Gp.Thr281Ala	chr15	26806318	NP_000805.1	transmembrane region
GABRB3_NM_000814.5:c.863C>A p.Thr288Asn	chr15	26806296	NP_000805.1	transmembrane region
GRIN1_NM_007327.3c.1643G>Ap.Arg548Gln	chr9	140058120	NP_000823.4	transmembrane region
KCNQ2_NM_172107.2 c.701C>Tp.Thr234Ile	chr20	62073874	NP_742105.1	transmembrane region
KCNQ2_NM_172107.2 c.913_915delTTCp.Phe305del	chr20	62070962	NP_742105.1	transmembrane region
SLC13A5_NM_177550.3c.425C>Tp.Thr142Met	chr17	6607319	NP_808218.1	transmembrane region
Ion channel:				
GABRB3_NM_000814.5 c.841A>Gp.Thr281Ala	chr15	26806318	NP_000805.1	ion-channel transmembrane region
GABRB3_NM_000814.5:c.863C>A p.Thr288Asn	chr15	26806296	NP_000805.1	ion-channel transmembrane region
GABRG2_NM_000816.3 c.968G>Ap.Arg323Gln	chr5	161576159	NP_000807.2	ion-channel transmembrane region
KCNQ2_NM_172107.2 c.826A>Cp.Thr276Pro	chr20	62071052	NP_004509.2	ion-channel transmembrane region
KCNQ2_NM_172107.2 c.913_915delTTCp.Phe305del	chr20	62070962	NP_004509.2	ion-channel transmembrane region
Cation transporter:				
GABRB3_NM_000814.5 c.841A>Gp.Thr281Ala	chr15	26806318	NP_000805.1	cation transporter family protein
GABRB3_NM_000814.5:c.863C>A p.Thr288Asn	chr15	26806296	NP_000805.1	cation transporter family protein
GABRG2_NM_000816.3 c.968G>Ap.Arg323Gln	chr5	161576159	NP_000807.2	cation transporter family protein
Others:				
SLC25A22_NM_001191060.1:c.347G>A p.Cys116Tyr	chr11	792935	NP_001177989.1	mitochondrial carrier protein
SLC25A22_NM_001191060.1:c.524G>T p.Arg175Leu	chr11	792616	NP_001177989.1	mitochondrial carrier protein
GRIN1_NM_007327.3c.2443G>Ap.Gly815Arg	chr9	140056634	NP_000823.4	ligand-binding domain
GRIN1_NM_007327.3c.1643G>Ap.Arg548Gln	chr9	140058120	NP_000823.4	ligand-gated ion channel
GRIN2A_NM_000833.4:c.593G>A p.Trp198*	chr16	10032230	NP_000824.1	LIVBP like domain
MEF2C_NM_002397.4 c.766C>Tp.Arg256*	chr5	88027590	NP_001180277.1	MADS box-containing tr.factors
MECP2_NM_001110792.1:c.433C>T p.Arg145Cys	chrX	153296882	NP_001104262.1	Methyl-CpG binding domain
ALDH7A1_NM_001182.4c.518-14_518delinsCA	chr5	125912902	NP_001173.2	NAD+-dependent dehydrogenase
MECP2_NM_004992.3 c.925C>Tp.Arg309Trp	chrX	153296354	NP_004983.1	NCOR2 interaction
PCDH19_NM_001184880.1c.698A>Gp.Asp233Gly	chrX	99662898	NP_001098713.1	nitrosylation - ion binding
ALDH7A1_NM_001182.4c.518-14_518delinsCA	chr5	125912902	NP_001173.2	nitrosylation - polypeptide binding
IQSEC2NM 001111125.2 c.3206G>Cp.Arg1069Pro	chrX	53267398	NP_001104595.1	PH domain
KCNJ10_NM_002241.4:c.313C>T p.His105Tyr	chr1	160012010	NP_002232.2	potassium channel - inward rectifier
PPP2R5D:c.1267_1270delCTCT (p.Leu423fs)	chr6	42977075	NP_001257405.1	protein phosphatase
PURA_NM_005859.4 c.812_814delp.Phe271del	chr5	139494575	NP_005850.1	PurA ssDNA and RNA-binding protein
STXBP1_NM_003165.3c.1654T>Cp.Cys552Arg	chr9	130444791	NP_001027392.1	Sec1 family
SLC25A22_NM_001191060.1:c.347G>A p.Cys116Tyr	chr11	792935	NP_001177989.1	Solcar2
SLC25A22_NM_001191060.1:c.524G>T p.Arg175Leu	chr11	792616	NP_001177989.1	Solcar2
PCDH19_NM_001184880.1c.698A>Gp.Asp233Gly	chrX	99662898	NP_001098713.1	tandem repeat domain
SLC13A5_NM_177550.3c.425C>Tp.Thr142Met	chr17	6607319	NP_001137310.1	Di- and tricarboxylate transporter
MECP2_NM_001110792.1:c.433C>T p.Arg145Cys	chrX	153296882	NP_001104262.1	DNA binding
PURA_NM_005859.4 c.812_814delp.Phe271del	chr5	139494575	NP_005850.1	DNA/RNA binding repeat
TREX1_NM_016381.3c.1062107delp.Leu354Phefs*22	chr3	48508951	NP_057465.1	ER localization
TREX1_NM_016381.3c.1072A>Cp.Thr358Pro	chr3	48508961	NP_057465.1	ER localization
FOLR1_NM_000802.3:c.439C>T p.Arg147Cys	chr11	71906737	NP_000793.1	folate receptor
HUWE1:c.12195 G>C (p.Trp4065Cys)	chrX	53563571	NP_113584.3	HECT domain
HUWE1:c.12195 G>C (p.Trp4065Cys)	chrX	53563571	NP_113584.3	HECT domain
UBTF:c.628 G>A (p.Glu210Lys)	chr17	42290219	NP_055048.1	HMGB-UBF_HMG-box
WDR45_NM_007075.3 c.511C>Tp.Gln171*	chrX	48933533	NP_001025067.1	WD40 repeat domain
WDR45_NM_007075.3 c.654delp.Arg219Alafs*69	chrX	48933277	NP_001025067.1	WD40 repeat domain
WDR45_NM_007075.3 c.970_971delp.Val324Hisfs*17	chrX	48932799	NP_001025067.1	WD7 repeat domain
SLC25A22_NM_001191060.1:c.347G>A p.Cys116Tyr	chr11	792935	NP_001177989.1	ADP/ATP transporter
SLC25A22_NM_001191060.1:c.524G>T p.Arg175Leu	chr11	792616	NP_001177989.1	ADP/ATP transporter
SLC13A5_NM_177550.3c.425C>Tp.Thr142Met	chr17	6607319	NP_001137310.1	Anion permease ArsB/NhaD.
NARS2:c.83_84del (p.Leu28Glnfs*17)	chr11	78285450	NP_078954.4	asparaginyl-tRNA synthetase

Conclusion

Our result shown, that most of the pathogenic variants are translated into protein domains. This information about protein domain can help experts in evaluation of NGS. On the other hand, we can see that many of these variants are not connected to any protein domain. Based on this fact we cannot decide pathogenicity on variant only based on these criteria.

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Gen	Počet asociovaných HPO termínů	HPO termíny
TRIOBP	3	Severe sensorineural hearing impairment HP:0008625; Autosomal recessive inheritance HP:0000007; Infantile onset HP:0003593;
CHCHD10	83	Ragged-red muscle fibers HP:0003200; Abnormal mitochondrial morphology HP:0008322; Skeletal muscle atrophy HP:0003202; Bulbar palsy HP:0001283; Areflexia HP:0001284; Neurodegeneration HP:0002180; Facial palsy HP:0010628; Autosomal dominant inheritance HP:0000006; Apraxia HP:0002186; Dyscalculia HP:0002442; Degeneration of the lateral corticospinal tracts HP:0002314; Neck flexor weakness HP:0003722; Paralysis HP:0003470; Perseveration HP:0030223; Parkinsonism HP:0001300; Calf muscle hypertrophy HP:0008981; Neuronal loss in the cerebral cortex HP:0007190; Sensorineural hearing impairment HP:0000407; Progressive cerebellar ataxia HP:0002073; Distal muscle weakness HP:0002460; Babinski sign HP:0003487; Proximal muscle weakness in lower limbs HP:0008994; Elevated serum creatine kinase HP:0003236; Proximal muscle weakness in upper limbs HP:0008997; Bilateral sensorineural hearing impairment HP:0008619; Restrictive ventilatory defect HP:0002091; Muscle weakness HP:0001324; Dyspnea HP:0002094; Difficulty walking HP:0002355; Tremor HP:0001337; Amyotrophic lateral sclerosis HP:0007354; Abnormal lower motor neuron morphology HP:0002366; Respiratory failure HP:0002878; Muscle spasm HP:0003394; Cerebral cortical atrophy HP:0002120; Emotional lability HP:0000712; Agitation HP:0000713; Fasciculations HP:0002380; Depressivity HP:0000716; Increased mitochondrial number HP:0040014; Abnormal upper motor neuron morphology HP:0002127; Increased intramyocellular lipid droplets HP:0012240; Paraparesis HP:0002385; Laryngospasm HP:0025425; Frontal lobe dementia HP:0000727; Xerostomia HP:0000217; Fatigue HP:0012378; Exercise intolerance HP:0003546; Progressive HP:0003676; Supranuclear gaze palsy HP:0000605; Slow progression HP:0003677; Distal lower limb muscle weakness HP:0009053; Disinhibition HP:0000734; Dysphagia HP:0002015; Frontotemporal dementia HP:0002145; Tetraparesis HP:0002273; Nausea and vomiting HP:0002017; Pes cavus HP:0001761; Hallucinations HP:0000738; Short stature HP:0004322; Ataxia HP:0001251; Anxiety HP:0000739; Pes planus HP:0001763; Apathy HP:0000741; Spinal muscular atrophy HP:0007269; Hammertoe HP:0001765; Increased serum lactate HP:0002151; Spasticity HP:0001257; Global brain atrophy HP:0002283; Dysarthria HP:0001260; Weakness of facial musculature HP:0030319; Hyporeflexia HP:0001265; Fatigable weakness of swallowing muscles HP:0030195; Pain HP:0012531; Generalized amyotrophy HP:0003700; Fatigable weakness of respiratory muscles HP:0030196; Mildly elevated creatine kinase HP:0008180; Proximal muscle weakness HP:0003701; Distal sensory impairment HP:0002936; Gliosis HP:0002171; Ptosis HP:0000508; Mutism HP:0002300; Generalized muscle weakness HP:0003324;
PRSS1	21	Hypoproteinemia HP:0003075; Failure to thrive HP:0001508; Pancreatitis HP:0001733; Autosomal dominant inheritance HP:0000006; Anal atresia HP:0002023; Autosomal recessive inheritance HP:0000007; Splanchnic vein thrombosis HP:0030247; Exocrine pancreatic insufficiency HP:0001738; Steatorrhea HP:0002570; Abdominal pain HP:0002027; Diabetes mellitus HP:0000819; Leukocytosis HP:0001974; Pancreatic pseudocyst HP:0005206; Jaundice HP:0000952; Fever HP:0001945; Abnormal thrombosis HP:0001977; Recurrent pancreatitis HP:0100027; Pleural effusion HP:0002202; Elevated C-reactive protein level HP:0011227; Abnormal enzyme/coenzyme activity HP:0012379; Pancreatic calcification HP:0005213;
HLA-DQB1	22	Weight loss HP:0001824; Urticaria HP:0001025; Abnormal blistering of the skin HP:0008066; Hallucinations HP:0000738; Eczema HP:0000964; Transient global amnesia HP:0010534; Slurred speech HP:0001350; Obesity HP:0001513; Achalasia HP:0002571; Excessive daytime sleepiness HP:0002189; Excessive daytime somnolence HP:0001262; Autoimmunity HP:0002960; Insomnia HP:0100785; Diabetes mellitus HP:0000819; Psoriasiform dermatitis HP:0003765; Abnormality of vision HP:0000504; Cataplexy HP:0002524; Macule HP:0012733; Abnormal rapid eye movement sleep HP:0002494; Erythema HP:0010783; Recurrent infections HP:0002719; Syncope HP:0001279;
HBB	89	Persistence of hemoglobin F HP:0011904; Hypoxemia HP:0012418; Reduced beta/alpha synthesis ratio HP:0011906; Abnormal bone structure HP:0003330; Reticulocytosis HP:0001923; Reduced alpha/beta synthesis ratio HP:0011907; Avascular necrosis HP:0010885; Autosomal dominant inheritance HP:0000006; Cataract HP:0000518; Chronic hemolytic anemia HP:0004870; Autosomal recessive inheritance HP:0000007; Heinz body anemia HP:0005511; Nonspherocytic hemolytic anemia HP:0001930; Chest pain HP:0100749; Arthralgia HP:0002829; Increased mean corpuscular volume HP:0005518; Microcytic anemia HP:0001935; Cardiorespiratory arrest HP:0006543; Stroke HP:0001297; Abnormality of metabolism/homeostasis HP:0001939; Hematuria HP:0000790; Nyctalopia HP:0000662; Fever HP:0001945; Increased red cell sickling tendency HP:0008346; Arrhythmia HP:0011675; Abnormality of the skeletal system HP:0000924; Recurrent bacterial infections HP:0002718; Recurrent infections HP:0002719; Depressed nasal bridge HP:0005280; Immunodeficiency HP:0002721; Abnormality of the dentition HP:0000164; Genu valgum HP:0002857; Osteoporosis HP:0000939; Pulmonary arterial hypertension HP:0002092; Muscle weakness HP:0001324; Dyspnea HP:0002094; Hypersplenism HP:0001971; Diabetes mellitus HP:0000819; Hypothyroidism HP:0000821; Hypertension HP:0000822; Leukocytosis HP:0001974; Delayed puberty HP:0000823; Jaundice HP:0000952; Cholelithiasis HP:0001081; Elevated serum creatinine HP:0003259; Hypoparathyroidism HP:0000829; Hepatomegaly HP:0002240; Feeding difficulties HP:0011968; Osteomyelitis HP:0002754; Abnormality of the nervous system HP:0000707; Upslanted palpebral fissure HP:0000582; Venous thrombosis HP:0004936; Paresthesia HP:0003401; Depressivity HP:0000716; Pigment gallstones HP:0011981; Adrenal insufficiency HP:0000846; Abnormality of the spleen HP:0001743; Splenomegaly HP:0001744; Neoplasm of the liver HP:0002896; Increased serum ferritin HP:0003281; Renal insufficiency HP:0000083; Neutropenia HP:0001875; Priapism HP:0200023; Pallor HP:0000980; Hemolytic anemia HP:0001878; Unconjugated hyperbilirubinemia HP:0008282; Increased lactate dehydrogenase activity HP:0025435; Skeletal dysplasia HP:0002652; Elevated hepatic transaminase HP:0002910; Abnormality of the hypothalamus-pituitary axis HP:0000864; Anxiety HP:0000739; Iron deficiency anemia HP:0001891; Cardiomyopathy HP:0001638; Thrombocytosis HP:0001894; Cardiomegaly HP:0001640; Retinopathy HP:0000488; Hypochromic microcytic anemia HP:0004840; Malabsorption HP:0002024; Skin ulcer HP:0200042; Abdominal pain HP:0002027; Hearing impairment HP:0000365; Anemia HP:0001903; Cirrhosis HP:0001394; Cholestasis HP:0001396; Visual impairment HP:0000505; Monochromacy HP:0007803; Malar prominence HP:0010620; Abnormality of the thorax HP:0000765; Abnormal hemoglobin HP:0011902;
PSPH	24	Feeding difficulties HP:0011968; Intellectual disability HP:0001249; Postnatal growth retardation HP:0008897; Seizures HP:0001250; Gastroesophageal reflux HP:0002020; Full cheeks HP:0000293; Intellectual disability, moderate HP:0002342; Intrauterinegrowth retardation HP:0001511; Autosomal recessive inheritance HP:0000007; Infantile onset HP:0003593; Cerebral atrophy HP:0002059; Global developmental delay HP:0001263; Hypospadias HP:0000047; Abnormal facial shape HP:0001999; Broad forehead HP:0000337; Narrow forehead HP:0000341; Generalized tonic-clonic seizures HP:0002069; Hyposerinemia HP:0012279; Esophagitis HP:0100633; Wide mouth HP:0000154; Micrognathia HP:0000347; Microcephaly HP:0000252; Hypertonia HP:0001276; Palpebral edema HP:0100540;

Ing. et Ing. David Staněk

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h-index:	4
Souhrnný IF:	19,48