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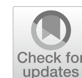

Two rare autosomal recessive neurological disorders identified by combined genetic approaches in a single consanguineous family with multiple offspring

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Abstract

Introduction Neurodevelopmental disorders (NDDs) refer to a broad range of diseases including developmental delay, intellectual disability, epilepsy, autism spectrum disorders, and attention-deficit/hyperactivity disorder caused by dysfunctions in tightly controlled brain development. The genetic backgrounds of NDDs are quite heterogeneous; to date, recessive or dominant variations in numerous genes have been implicated. Herein, we present a large consanguineous family from Turkiye, who has been suffering from NDDs with two distinct clinical presentations.

Methods and results Combined in-depth genetic approaches led us to identify a homozygous frameshift variant in *NALCN* related to NDD and expansion of dodecamer repeat in *CSTB* related to Unverricht-Lundborg disease (ULD). Additionally, we sought to functionally analyze the *NALCN* variant in terms of mRNA expression level and current alteration. We have both detected a decrease in the level of premature stop codon-bearing mRNA possibly through nonsense-mediated mRNA decay mechanism and also an increased current in patch-clamp recordings for the expressed truncated protein.

Conclusion In conclusion, increased consanguinity may lead to the revealing of distinct rare neurogenetic diseases in a single family. Exome sequencing is generally considered the first-tier diagnostic test in individuals with NDD. Yet we underline the fact that customized approaches other than exome sequencing may be used as in the case of ULD to aid diagnosis and better genetic counseling.

Keywords Neurodevelopmental disorders · *NALCN* · *CSTB* · Long PCR · Exome sequencing · Linkage analysis

Seda Susgun and Emrah Yucesan contributed equally to this work.

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Introduction

Neurodevelopmental disorders (NDDs) including autism spectrum disorder and intellectual disability/developmental delay (ID/DD) are characterized by defects in brain development and function that may affect cognition, behavior, and motor skills [1]. The phenotypic heterogeneity of NDDs has been attributed both to the variable expressivity of the phenotype within the distinct NDD category and also to the comorbid features, including epilepsy, motor deficits, behavioral and psychiatric issues, and other congenital malformations [2].

NDDs are also heterogenous on the genetic level. To date, various genes and loci have been implicated in NDDs based on copy number variation analyses and next-generation sequencing approaches, particularly exome sequencing [3–5]. Indeed, in 2019 exome sequencing was recommended as a first-tier clinical diagnostic test for individuals with NDDs [6].

It has been reported that clinically and molecularly diagnosed patients may have multilocus pathogenic variations (MPV) almost approaching 5% [7, 8]. Among these MPVs, the significant proportion comprises *de novo* variants in autosomal dominant (AD) or X-linked disease genes. Observation of MPVs in double autosomal recessive (AR) disease genes is much lower with a percentage of around 10% [7, 8]. As is known, in populations with a high rate of parental consanguinity, there is the increased contribution of AR private pathogenic variations to disease phenotypes [5]. Accordingly, increased consanguinity may result in observing two AR disorders in a single family [9]. Taken together, combined genetic analyses in such families can aid in dissecting the identity of the associated genes and provide genetic counseling.

Herein, we have set out to analyze the genetic defects in a family from Turkiye who has multiple siblings with an initial clinical diagnosis of Unverricht-Lundborg disease (ULD) and an undiagnosed form of NDD. ULD is an AR form of progressive myoclonus epilepsy that is associated with the cystatin B (*CSTB*) gene (ULD MIM: # 254800, Gene MIM: *601145) [10]. Expansion of the dodecamer repeat in the putative promoter region of *CSTB* is responsible for the majority of the ULD cases [11]. Therefore, we have used an in-depth genetic approach first to sequester homozygous regions associated both with ULD and NDD in this family and then to identify the associated variation with long PCR or whole exome sequencing (WES). Biallelic pathogenic variation in the *NALCN* gene (NM_052867.2:c.3056dupT, MIM: *611549) identified for the undiagnosed NDD phenotype has also been functionally analyzed. Our findings demonstrate the value of next-generation sequencing (NGS) in improving exact molecular diagnosis for Mendelian diseases and providing better genetic counseling for families.

Materials and methods

Identification of ULD and NDD-associated variants

Patients and clinical assessments

A family from Turkiye with ten children had been recruited for this study. The parents (I-1 and I-2) were first-degree cousins, two sibs (II-1 and II-2) had an initial clinical diagnosis of ULD, and two sibs were born with an undiagnosed NDD (II-7 and II-9) (Fig. 1A). Physical, neurological, and electroencephalography (EEG) examinations together with consultation on family history were performed. DNA was extracted from peripheral blood samples of all family members using the QIAamp DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany).

Genotyping

Two different SNP genotyping platforms were used throughout the study. II-3, II-4, II-5, and II-9 were genotyped using Illumina Human HumanCytoSNP-12 BeadChip kit (300K), while II-1, II-7, and II-9 (replicated as a batch control) were genotyped using Illumina HumanOmniExpress BeadChip (700K) kit (Fig. 1A).

Analysis of *CSTB* to dissect the ULD phenotype in the family

SNP array–based homozygosity of the region encompassing the *CSTB* gene in individual II-1 was consistent with the diagnosis of ULD. In order to detect the presence of *CSTB* dodecamer expansion, we have PCR amplified the *CSTB* promoter region encompassing the GC rich dodecamer (CCCCGCCCCGCG)n using primers 5-CCCGGAAAG ACGATACCAG-3 (forward) and 5-CGGCTTCTTTCG CTCCAG-3 (reverse). The PCR refractive individuals for this region (II-1, II-2) were further analyzed with long PCR through the service provided by Ulm University, Institute of Human Genetics (Fig. 1B).

Dissecting the genetic cause underlying the undiagnosed NDD phenotype in the family

LOD score analysis in the pedigree was performed selecting II-7 and II-9 as the only affected individuals in the family and using matching SNP data from 300K and 700K chips. This analysis was run using ALLEGRO version 1.2c software under the software package easyLINKAGE plus version 5.08 assuming recessive inheritance with full penetrance [12]. Haplotypes in regions with positive linkage peaks were manually inspected for identical by descent (IBD) inheritance of the shared haplotypes in the affected children. WES was performed in two affected

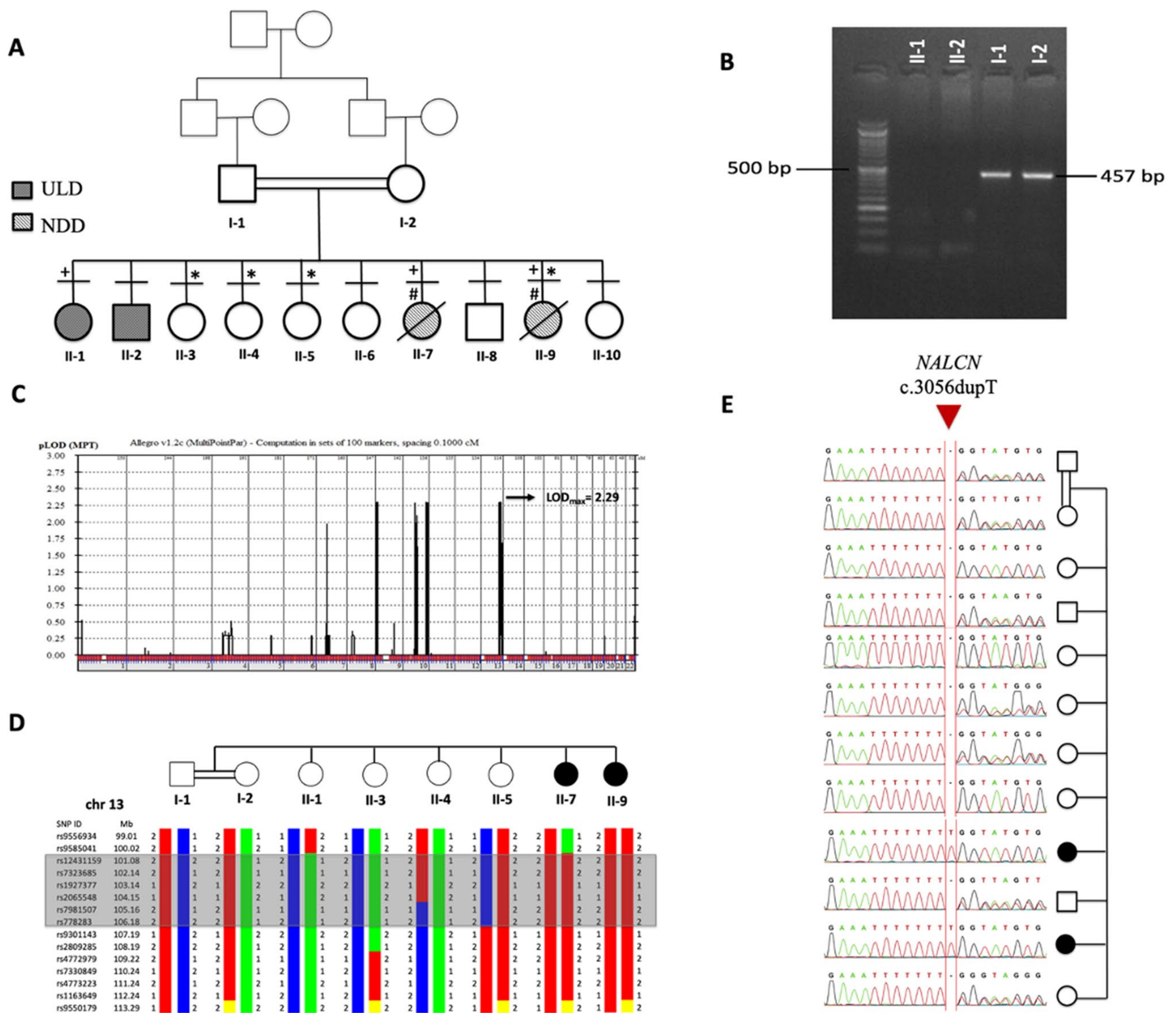

Fig. 1 Genetic findings in the family. **A** Pedigree of the family. "*" indicates individuals were 300K SNP genotyped, "+" indicates individuals were 700K SNP genotyped, and "#" indicates individuals have WES data. **B** Conventional PCR results of *CSTB* expansion region. The PCR refractive individuals (II-1, II-2) were further analyzed with long PCR. **C** Multipoint LOD scores were obtained by Allegro in the family along the autosomes. **D** SNP-derived haplotypes around the linkage region including *NALCN* on chromosome 13. The gray square indicates linkage region sharing in two affected siblings. **E** Segregation of *NALCN* variant within the family

sibs (II-7 and II-9) as previously reported [13]. The candidate variants residing in LOD score-positive regions were validated by Sanger sequencing.

Functional characterization of the truncating NALCN variant associated with NDD

Upon identification of a frameshift variant in *NALCN*, we have first investigated the possible involvement of the nonsense-mediated mRNA decay (NMD) mechanism that may cause a null effect. For this purpose, HEK293T cells were transfected with wild-type *NALCN* ($NALCN^{WT}$) and mutant-type *NALCN* ($NALCN^{Mut}$) encoded plasmids and treated with 0.1 mM cycloheximide (CHX) for 4 h. Afterward, the cells were subsequently harvested, and RNA expression levels were assessed through a real-time quantitative PCR method (detailed in supplementary data).

Then, we sought to analyze the role of the truncated protein on the electrophysiological properties of cells due to potential escape from the NMD mechanism. Therefore, MCF7 cells were transfected with $NALCN^{WT}$ and expressing truncated NALCN protein ($NALCN^{Trun}$) plasmids followed by whole-cell patch-clamp technique to evaluate possible current alteration in a truncated protein (detailed in supplementary data).

Statistical analysis

In the present study, whether the data were distributed normally was tested with the Shapiro-Wilk test. In comparing the data with normal distribution between two independent groups, a *t*-test was used. The non-normal distribution of the data between two independent groups was evaluated with the Mann-Whitney *U* test. Statistical analyses were performed at GraphPad Prism 8.0 program (GraphPad Software, Inc., CA, USA) with a significance level of 0.05 and 95% confidence level.

Results

Clinical presentation

Patients with ULD

In the last examination, the eldest sibling was at the age of 29 (II-1). She was reported to have no symptoms till the age of 10. After this age, she has begun to experience myoclonic seizures, typically triggered by auditory stimuli. She developed severe impairments in motor and cognitive functions. EEG revealed generalized epileptiform activity and magnetic resonance imaging (MRI) showed prominent cerebellar folia along with cerebral and cerebellar atrophy. Unfortunately, her epilepsy was refractory. Her younger brother (II-2) had a similar disease history with refractory epilepsy and motor-mental delay.

Patients with NDD

Two affected sisters (II-7 and II-9) had severe congenital motor-mental developmental delay and progressive epilepsy. Myoclonus progressive seizures had begun from 3 months old for patient II-9. Unfortunately, both patients (II-7 and II-9) were lost at the ages of 13 and 9, respectively.

Long PCR results for ULD phenotype

Dodecamer expansion in the promoter of the *CSTB* gene was determined with 60±2 repeats in two siblings with ULD (II-1 and II-2, Fig. 1B).

Genetic and functional analyses for NDD phenotype

Whole genome genotyping and Linkage analysis

Linkage analysis using a combination of 300K and 700K arrays data through PLINK in an autosomal recessive model revealed three linkage peaks on chromosomes 9, 10, and 13 with maximum LOD scores of 2.29 each (Fig. 1C).

WES and segregation analyses

WES data were filtered for linkage interval and candidate variants that were shared between affected individuals were validated by Sanger sequencing and segregation analysis. The linkage interval of chromosome 13, which includes the strongest candidate variant, is compiled in Fig. 1D. This collective effort has led us to identify a frameshift variant in *NALCN* (NM_052867.2:c.3056dupT, p.(Leu1019Phefs*30), rs772394714) in the homozygous state as the most likely gene associated with the NDD phenotype in the family. Segregation of the variant within all family members was consistent with recessive inheritance pattern (Fig. 1E). The variant was classified as pathogenic with evidence codes of PVS1, PP1, PM2, and PS3 [14].

RNA expression analyses

The mRNA level of $NALCN^{Mut}$ was significantly lower compared to $NALCN^{WT}$ at t=0 time point most probably due to nonsense-mediated mRNA decay (p=0.0379). However, there was an expression of $NALCN^{Mut}$, which could be due to the low-level escape of $NALCN^{Mut}$ mRNAs from NMD. After treatment with CHX for 4 h, mRNA levels of $NALCN^{Mut}$ were statistically significantly increased compared to the initial expression level through NMD blocking (t=0 $NALCN^{Mut}$ vs CHX $NALCN^{Mut}$, p=0.0051) (Fig. 2A).

Electrophysiological findings

$NALCN^{WT}$ or $NALCN^{Trun}$ plasmid transfected MCF7 cells were used for current recording, and these two groups were compared to assess alteration. The Patch-clamp recording revealed that the current of $NALCN^{Trun}$ expressed cells was increased compared to $NALCN^{WT}$ (p=0.0007) (Fig. 2B). This finding supports that truncated NALCN protein might have the gain of protein function.

Discussion

In populations with high parental consanguinity, genomic studies have especially uncovered recessive disease-causing alleles [15, 16]. Moreover, there is a substantial increase that the offspring are born with two different autosomal recessive disorders due to identical descent inheritance of independent pathogenic alleles [9]. It is not surprising as we are all heterozygous for potentially pathogenic yet silent recessive variations, which may result in severe diseases in the subsequent generations due to parental consanguinity [17]. In this instance, next-generation sequencing combined with linkage analysis provides an important opportunity for diagnosis and genetic counseling [15, 16, 18]. Herein, we

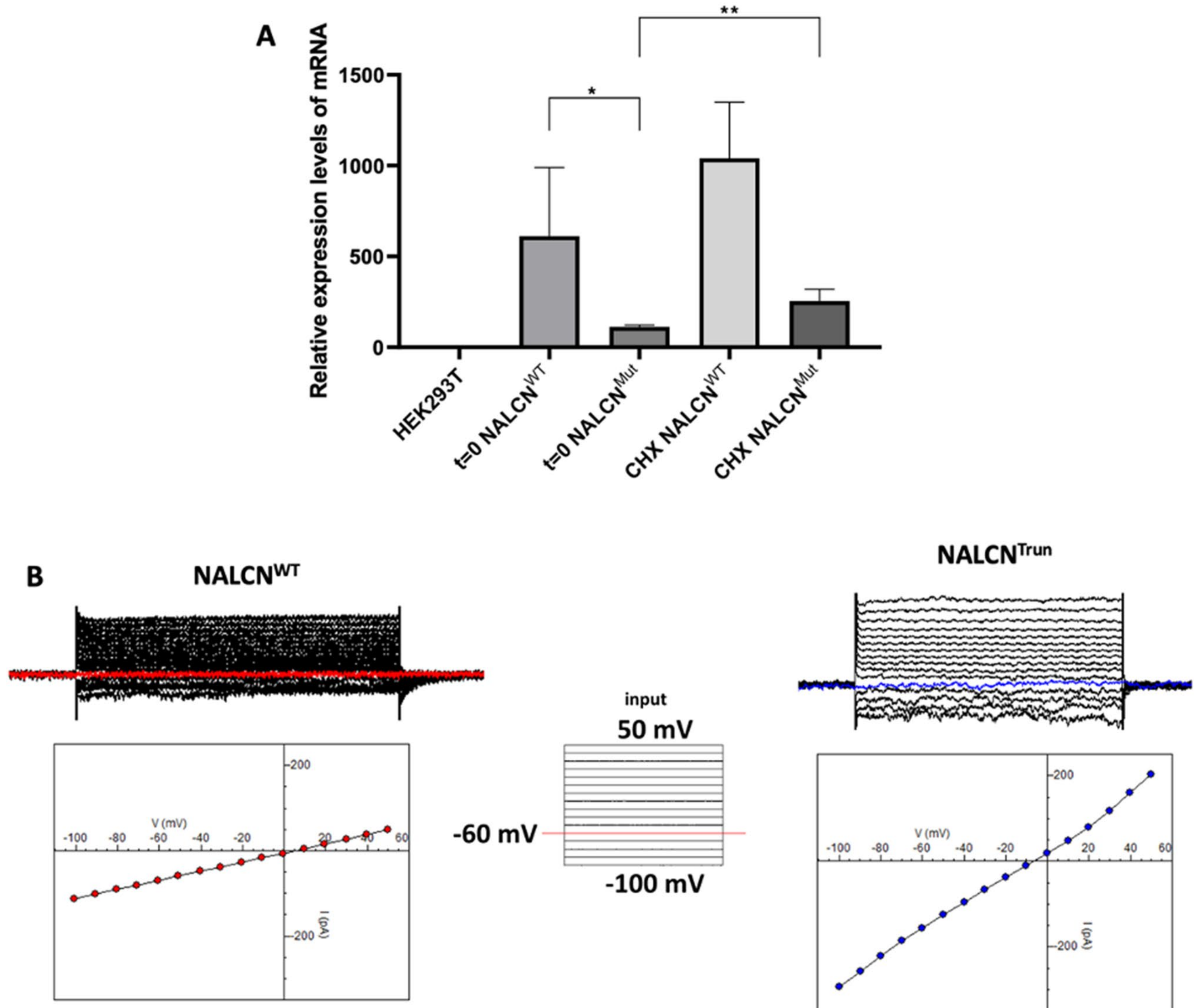

Fig. 2 Functional experiment results for the *NALCN* variant. **A** Relative mRNA expression levels of $NALCN^{WT}$ and $NALCN^{Mut}$ before and after cycloheximide (CHX) treatments. **B** Patch-clamp recording findings of cells transfected with $NALCN^{WT}$ and $NALCN^{Trun}$ plasmids

present a large consanguineous family suffering from two rare recessive neurological disorders (ULD and NDD) with multiple children born to first-degree cousin parents.

ULD, also named progressive myoclonic epilepsy 1 (PME1), is a rare neurodegenerative disorder and inherited autosomal recessively (MIM: #254800) [19]. ULD is the most common type among progressive myoclonic epilepsies and is characterized by seizures, myoclonus, ataxia, and cognitive decline [20]. The main cause of ULD has been defined as repeat expansion (>30 repeats) of a dodecamer sequence in the promoter region of the *CSTB* (cystatin B) gene which encodes inhibitor of lysosomal proteases [20, 21]. In the present study, we sought to find out repeat expansion regarding two siblings with suspected ULD; long-PCR revealed 60±2 repeats in affected siblings and was consistent with clinical findings.

NALCN is an orphan gene and encodes sodium ion leak channel which is mainly expressed in the nervous system. This channel plays a crucial role in modulating respiration, circadian rhythm, locomotion, and pain sensitivity by regulating the resting membrane potential (RMP) and excitability of neurons [22]. The importance of *NALCN* for RMP has been recapitulated in animal models that underline its indispensable function conserved across species. For instance, impaired expressions of *NALCN* resulted in hyperpolarization of hippocampal neurons' RPM and reduction in firing rate in mice. Additionally, bi-allelic knockout variants led to the premature death of mice within a day after birth due to disrupted respiratory rhythm [23].

Both dominant and recessive pathogenic variants in *NALCN* (MIM: * 611549) have been implicated in clinically

distinct NDDs. Heterozygous dominantly inherited variants lead to congenital contractures of the limbs and face, hypotonia, and developmental delay (CLIFAHDD, MIM: #616266), while recessively inherited variants cause infantile hypotonia with psychomotor retardation and characteristic faces 1 (IHPRF1, MIM: #615419) [24]. It is presumed that gain-of-function mutations are related to CLIFAHDD, whereas bi-allelic loss-of-function mutations are related to IHPRF1 [25]. As consistent, Bouasse and colleagues have shown missense mutations associated with CLIFAHDD present higher current in electrophysiological recordings compared to wild type [23].

We identified a bi-allelic *NALCN* frameshift variant in two siblings with NDD. As expected, we aimed to experimentally test whether mRNA bearing premature stop codon degrades through the NMD mechanism. Accordingly, the $NALCN^{Mut}$ mRNA level was low compared to $NALCN^{WT}$ mRNA, whereas after being treated with CHX increased compared to the initial level. This result was consistent with the NMD hypothesis resulting in null alleles. Nevertheless, we wanted to gain insight into what would happen if mRNA escaped from NMD and produced truncated protein under physiological conditions. Surprisingly, truncated protein has displayed a gain-of-function feature according to patch-clamp recording. This finding may be speculated as if the NMD mechanism could not work properly, heterozygous individuals would also show phenotypes like CLIFAHDD. Despite that, a 50% reduction in *NALCN* activity seems to be tolerable according to heterozygous loss-of-function variants in gnomAD [26]. Thus, it has been again underlined how an important control mechanism NMD is to protect organisms against incorrect protein production.

According to recent studies, the frequency of MPV should not be underestimated. Indeed, in a large NDD cohort from Turkiye, MPV's prevalence has been found to be 28.9% (in 51 of the 176) which mostly contained homozygous loci shared due to consanguinity [27]. Herein, the presented consanguineous family from Turkiye supports the aforementioned frequency.

Consequently, we have identified genetic background underlies two distinct rare neurologic diseases in a family thanks to the combination of plausible genetic approaches including linkage and haplotype analyses, targeted PCR approaches, and WES. Genetic identification of two separate disease genes in the same family has led us to conclude that the different neurological phenotypes in affected siblings are not solely due to the variable expressivity of the same disorder. Despite some limitations, WES is still quite a powerful and precise tool to elucidate disease-causing variants/genes in clinically rare and recessively inherited Mendelian diseases. Yet pitfalls of WES are well known [28]. Therefore, complementary and targeted genetic techniques chosen in the presence of proper clinical guidance may be required to resolve the additional pathogenicity as in the case of ULD.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10072-023-07211-y.

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Data availability Data are available from the authors on reasonable request.

Declarations

Ethics approval This study protocol was approved by the clinical ethics committee of the Istanbul University, Istanbul Faculty of Medicine. Written informed consents were obtained from/for all family members enrolled in the study according to the Helsinki Declaration.

Conflict of interest The authors declare no competing interests.

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