Seizure 39 (2016) 34–43

Contents lists available at ScienceDirect

Seizure

journal homepage: www.elsevier.com/locate/yseiz

SCN1A gene sequencing in 46 Turkish epilepsy patients disclosed 12 novel mutations

Sunay Usluer [a], Seda Salar [a,2], Mutluay Arslan [b], Uluç Yiş [c], Bülent Kara [d], Pınar Tektürk [e], Betül Baykan [e,f], Cihan Meral [g], Dilşad Türkdoğan [h], Nerses Bebek [e], Özlem Yalçın Çapan [a,1], Aslı Gündoğdu Eken [a], S. Hande Çağlayan [a,*]

[a] Department of Molecular Biology and Genetics, Boğaziçi University, İstanbul, Turkey
[b] Department of Child Neurology, Gülhane Military Medical School, Ankara, Turkey
[c] Department of Pediatrics, Division of Child Neurology, Dokuz Eylül University School of Medicine, İzmir, Turkey
[d] Department of Pediatrics, Kocaeli University Medical Faculty, Kocaeli, Turkey
[e] Department of Neurology, Clinical Neurophysiology and Child Neurology Units, Istanbul University, Istanbul Faculty of Medicine, Istanbul Turkey
[f] Department of Neurology, Istanbul University Epilepsy Center, Istanbul Turkey
[g] Department of Child Neurology, Gülhane Military Medical School, İstanbul, Turkey
[h] Department of Child Neurology, Marmara University, Medical Faculty, Istanbul

ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form 11 May 2016
Accepted 12 May 2016

Keywords:
Dravet syndrome
Epileptic encephalopathy
GEFS+
SCN1A mutation

ABSTRACT

Purpose: The SCN1A gene is one of the most commonly mutated human epilepsy genes associated with a spectrum of phenotypes with variable degrees of severity. Despite over 1200 distinct mutations reported, it is still hard to draw clear genotype–phenotype relationships, since genetic and environmental modifiers contribute to the development of a particular disease caused by an SCN1A mutation. We aimed to initiate mutational screening of the SCN1A gene in Turkey and advance further our understanding of the relationship between the SCN1A sequence alterations and disease phenotypes such as GEFS+, DS and related epileptic encephalopathies.
Methods: Mutational analysis of the SCN1A gene was carried out in 46 patients with DS, late-onset DS, GEFS+ and unspecified EE using either direct Sanger sequencing of the coding regions and exon/intron boundaries or massively parallel sequencing.
Results: Nineteen point mutations, 12 of which were novel were identified, confirming the clinical diagnosis of the patients. Patients with a mutation (either truncating or missense) on linker regions had significantly later disease onset than patients with mutations in homology regions. The presence of SCN1A mutations in two clinically unclassified patients supported the association of SCN1A mutations with a wide range of phenotypes.
Conclusion: The conventional Sanger sequencing method was successfully initiated for the detection of SCN1A point mutations in Turkey in epilepsy patients. Furthermore, a modified strategy of massively parallel pyro-sequencing was also established as a rapid and effective mutation detection method for large genes as SCN1A.

1. Introduction

The *SCN1A* gene, located on 2q24.3, encodes the voltage-gated sodium channel alpha1 subunit ($Na_v1.1$, $\alpha1$). $Na_v1.1$ channels are responsible for the neuronal excitability and expressed in the central and peripheral nervous systems and in cardiac myocytes. They are located particularly in the soma and dendrites and in the axonal initial segment in a subset of inhibitory interneurons [1]. The mutations found in the *SCN1A* gene are the most common genetic cause of early epileptic encephalopathies (EE) and have been associated with a spectrum of phenotypes including Dravet Syndrome (DS), Generalized Epilepsy with Febrile Seizure Plus (GEFS+), Borderline Severe Myoclonic Epilepsy of Infancy (SMEB), Doose syndrome (MAE), infantile spasms and some other infantile epileptic disorders [2–4]. Mutations vary from point mutations to

* Corresponding author. Tel.: +90 212 359 6881; fax: +90 212 287 2468.
E-mail address: hande@boun.edu.tr (S.H. Çağlayan).
[1] Current address: Department of Molecular Biology and Genetics, Arel University, İstanbul, Turkey.
[2] Current address: Institute of Neurophysiology, Chraite University Medicine, Berlin, Germany.

http://dx.doi.org/10.1016/j.seizure.2016.05.008
1059-1311/

microdeletions, translocations and intra/intergenic duplications [2,5–7]. The *SCN1A* gene variants are listed on three webpages [8–10]. While gain-of-function mutations lead to increased neuronal excitability, loss-of-function mutations reduce the inhibitory action of GABAergic interneurons [11]. DS is a common form of EE and about 80% of DS patients have an *SCN1A* mutation approximately 90% of which arising *de novo*. DS is characterized by infantile onset, fever-sensitive, treatment resistant seizures and intellectual disability [12]. Seizure onset is in the first year of life, generally at around 6 months. At onset, seizures can be generalized or unilateral, clonic and tonic-clonic. During the course of the disease afebrile, myoclonic or focal seizures or atypical absences can also occur. Until seizure onset, development is normal. However, developmental delay and other neurological defects begin during the second year of life [1].

Despite the identification of many mutations in the *SCN1A* gene, the genotype–phenotype correlation is still not clear suggesting the involvement of either genetic or environmental modifiers in the development and progression of various diseases [13,14]. Mutations causing truncation of the channel protein and missense mutations affecting voltage sensitivity and/or pore regions are more frequent in DS patients and missense mutations affecting channel function less severely are more frequent in GEFS+ [15].

In order to initiate mutational screening of the *SCN1A* gene in Turkey and advance further our understanding of the relationship between the *SCN1A* sequence alterations and disease phenotypes such as GEFS+, DS and related epileptic encephalopathies, the conventional Sanger sequencing method and a modified strategy of massively parallel pyro-sequencing for rapid detection of mutations were established in this study.

2. Materials and methods

2.1. Subjects

Forty-six Turkish patients suspected to have DS, late-onset DS (LO-DS), unspecific EE (UEE) and GEFS+ were included in this study with the informed consent of their families. The study was conducted with the approval of the Institutional Review Board for Research with Human Subjects.

The study cohort consisted of DS ($N = 30$), LO-DS ($N = 10$), GEFS+ ($N = 4$) and UEE ($N = 2$) patients. DS was characterized by febrile or afebrile tonic or tonic-clonic seizures with onset within the first year of life and yielding developmental stagnation and regression onwards [16]. Patients with LO-DS had the same clinic with DS patients but disease onset was beyond the first year of age. GEFS+ was characterized with febrile seizures starting early in life that continued beyond the age of six, generally with a positive family history [17]. Two patients, due to the lack of complete clinical information did not fit into definitions of DS, LO-DS and GEFS+ but were also included since they had UEE characterized by absence seizures at onset and a degree of mental retardation later.

2.2. SCN1A gene amplification

Genomic DNA was extracted from K'EDTA-treated peripheral blood samples using MagNa Pure Compact Nucleic Acid Isolation Kit Large Volume (Roche Diagnostics, Mannheim, Germany).

All 26 exons including exon/intron boundaries of the *SCN1A* gene were amplified by polymerase chain reaction (PCR) in 36 separate reactions using previously reported primer pairs [18]. For massively parallel sequencing, all amplicons were fluorometrically quantified using Quant-iT-PicoGreen dsDNA assay Kit (Invitrogen, ABD) on Light Cycler 480 (LC480II, Roche Diagnostics, Mannheim, Germany). Amplification conditions are available upon request.

2.3. Sanger sequencing

Purified PCR products were Sanger sequenced at Macrogen, Seoul, Korea. All mutations were confirmed by repeated PCR and re-sequencing. Nucleotides were numbered according to reference sequences NM_001165964.1 and NP_001159436.1 where A of the ATG start codon corresponded to nucleotide number one. Bioinformatics tools namely, Mutation Taster [19], Polyphen [20] and Provean [21] were used to evaluate the putative effects of novel mutations.

2.4. Massively parallel amplicon sequencing (MPAS)

SCN1A amplicons of each patient were pooled at equal molecule amounts and purified by MinElute DNA purification kit (Qiagen, ABD). Ten nucleotides long MID sequences specifying each patient were ligated to amplicons in each pool using GS Rapid Library Preperation Kit Lib-L (Roche, Germany) as described in the GS Junior Rapid Library Preparation Manual. Concentration of pooled amplicons was measured both by Quant-iT-PicoGreen dsDNA assay Kit (Invitrogen, ABD) and also by qPCR using KAPA NGS quantification kit (KAPA systems, ABD) on LightCycler 480II. Dilutions were made to have single fragment per bead and the sequencing library was prepared for emulsion PCR (emPCR) using GS Junior Titanium emPCR Kit Lib-L (Roche, Germany) as described in GS Junior emPCR Lib-L manual. DNA attached beads were picked up magnetically and pyrosequenced using GS Junior sequencing kit by following the instructions in the GS Junior sequencing method manual. Amplicon sequences were analyzed by Amplicon Variant Analyzer (AVA) program (Roche, Gernmany) using the *SCN1A* reference sequence, PCR primer and MID sequence information. In the result of AVA analysis, a variant list was obtained for each patient. The variants were filtered for known SNPs and unique variants were validated by Sanger sequencing.

2.5. Statistical analyses

Statistical analysis was conducted using SPSS software (Version 22) nonparametric tests module for Independent Samples Kruskal–Wallis Test with 95% confidence interval.

3. Results

A total of 46 patients with DS, LO-DS, EE and GEFS+ phenotypes were analyzed for *SCN1A* sequence variations by Sanger sequencing (30 patients) and Massively Parallel Amplicon Sequencing (16 patients) methods. Out of 46 patients, 19 (41.3%) had a sequence variation in the *SCN1A* gene, 12 of which were novel. Genetic variations and clinical information of patients are given in Tables 1 and 2, respectively. Among 30 patients with DS phenotype 12 had a mutation in the *SCN1A* gene (40%), on the other hand, only three out of 10 LO-DS patients were positive for *SCN1A* mutations (30%). Two of the four GEFS+ patients had a maternally inherited *SCN1A* mutation. Two UEE patients had a novel *de novo* frameshift mutation (2DS4) and a previously reported and paternally inherited variant (11DS23). This variant was present in the ExAC database with low frequency (0.0015).

3.1. Clinical summary of patients with SCN1A mutations

All patients except 11DS23, 29DS57 and 57DS121 had FS at disease onset (84%) and three patients had their first seizure after vaccination. All patients except 29DS57 had variable degrees of mental retardation with additional autistic features, speech delay and motor delay. Among DS patients, only 6DS12, 14DS26 and 15DS27 were pharmacoresistant (Table 1).

Table 1
Clinical information.

ID	Sex	Onset	Seizure Type		Dev. Symptoms	EEG	MRI	AEDs	Response to AED	Family History	DG	*SCN1A* Mutation & Inheritance	Additional Info
			Onset	Cont.									
1DS1	M	10d	FS	FS, GTCS	MD, MR	NA	NA	VPA, T, C	yes	Yes	DS	c.655 A > G* de novo	
2DS4	F		FS, Absence	NA	MR, Autistic	N	NA		yes	Yes	UEE	c.3341-3342delAA* de novo	
6DS12	M	5mo	FS, Eye Deviations, Convulsions	AFS	MR			VPA,PHB	YES	No	DS	c.239-244 delTGGACC* de novo	
								VPA, O	YES				
								VPA, T, L	YES				
10DS22	M	4MO	FS, GTCS (AV)	FS + AFS	SD, PR, Sp.Ed.	G SW	N	VPA,LT, T, Z, E, C, CBZ	No	No	DS	c.602 + 1 G > A de novo	
								VPA, L, CL	No				
								S	Yes				
11DS23	F	5Y	Abs	Abs, Atonic	SD, PR, Sp.Ed.	Focal SW (TP)	N	VPA, LT, CL	yes	No	UEE	c.1625 G > A Pat.	Cleft Palate Operation (6MO)
14DS26	M	5MO	FS, Eye Deviations, GTCS	AFS, GTCS	SD, PR, Sp.Ed.	GSW	N	PHB	No	No, Cons. Parents	DS	c.4402-4406 delCAAGA* de novo	
								VPA, CBZ, T, L, LT	No				
								S	Agg.				
								VPA, CL	No				
15DS27	M	3,5MO	FS Tonic-Clonic	GTCS MC, Drop Attacks	Severe MR MD	GSW	N	L, CL, VPA, T, PHB, S	Yes	NO	DS	c. 4883T > G N/A	
20DS46	F	9MO	FS, Clonic	GTCS, FS, AFS	SD, PR, Sp.Ed.	GSW	BFCA	PHB	No	Yes, Cons. Parents	DS	c.2887delC* de novo	
								PHB, O	No				
								PHB,O, VPA	No				
								VPA, O, CL	Reduction				
24DS50	M	6Y	FS Tonic	Complex -Partial Sz.	Severe SD,Fine MD	GS	N	L, VPA, O, PHB, C		No	LO-DS	c.1837C > T N/A	
26DS52	F	11MO	FS Clonic	GTCS	PR	GSW	N	PHB, CL, L, VPA, S	No	NO	DS	c.530G > A N/A	
29DS57	M	4MO	Tonic (AV)	FS	Normal	GSW	N			Yes	DS	c.301C > T de novo	
34DS68	M	4Y	FS Tonic-Clonic	Eyelid Myoclonia	Moderate SD Fine MD	GSW	BFCA	VPA, PHB, LT, KLZ, L, TPM, S		No	LO-DS	c.4498-1G > T* N/A	
36DS73	M	6MO	FS, Unilateral & Secondary GTCS Fr. SE	Unilateral& Secondary GTCS, MC	Severe MMR Severe Autistic Features	Multi-focal SW	N	VPA, LCP, STP, PRM	Fair	Yes	DS	c.241G > A* de novo	
48DS104	M	8MO	FS	GTCS, ASE MC, Atypical Abs	Moderate MR	GSW FSW	N	LT, L, T, CBZ, PHB, VPA, S,Z	No	No	DS	c.1696C > T * N/A	(SUDEP)
54DS112	F	6MO	FS (long duration)	AFS Focal, MC, Atypical Abs, Tonic (During Sleep)	Mild MMR	Focal or GSW (rare)	N	VPA, C	yes	No	DS	c.2567G > A* de novo	
								T	No				
55DS119	F	2Y	FS	Abs. Tonic-Clonic	Mild MR	GSW	N	L, VPA,	yes	Yes	GEFS+	c.5726A > G* Mat.	
56DS120	M	3Y	FS	GTCS Myoclonic	Moderate MR Polyneuropathy	GSW PS	N	PH, Z, VPA	No	No	GEFS+	c.1811G > A Mat.	
57DS121	F	3Y	Tonic	AFS CPS SE	Severe MMR	FSW	Right MTS	LT,CBZ, VPA	no	No	LO-DS	c.5141A > T* N/A	

27DS53	F	11MO	Eye Dev., tonic Sz. upper extremities (AV)	GTC	GDD, SD	GSW (onset-during)	N	CL,T	Still seizures 4-5/day	Yes	DS	c.1294 G > T* N/A	Seizures fr. during arousal
3DS7	F	2y	FS, Myoclonus						yes	No	GEFS+		
7DS15	M	3MO	FS (AV) Head deviation, Long duration	Focal Sz., MC, Clonic leg jerks, Persisting conscious-ness	Walking and speaking after 5Y, Learning Disability, Sp.Ed.		N	VPA + T	yes	Yes	DS		
								VPA +C	yes				
8DS20	F	12MO	FS (After infection, for 2 days, 20 times), MC, Tonic	FS, myoclonic, tonic, rare atonic	Mild MMD, SD	SW in bilateral central regions	N	VPA +C	yes	Yes	DS		
12DS24	F	4Y	Eye Deviation,GTC	Tonic mouth dev. Hyper-salivation, GTC (Before Falling Asleep or During Arousal)	Minimal SD	GSW	Suspected cortical dysplasia	VPA + L	yes	no	LO-DS		
								VPA	No				
								VPA + O	Agg.				
17DS29	F	1.5Y	GTCS	GTCS, Hemiclonic,Atonic	MMD	GSW	Left TO atrophy	VPA + L+ CL	yes	No	LO-DS		Obesity
18DS30	M	3.5Y	FS SE	Fr. Complex partial AFS	PD, SD Sp.Ed.	GSW	N	VPA + L+ CL	no	Yes	LO-DS		
22DS48	M	1.5Y	FS (AV), long duration, GTCS, SE	GTCS	Dev. delay before onset, Severe PD, Sp.Ed.	GSW	N	Multi-AED	no	No	LO-DS		Corpus callosotomy operation at age of 4, Ataxia
								4 AED together	still seizures 5-6/day				
23DS49	M	5Y	SE lasting for 20 days, GTCS	GTCS, Atypic abs, Complex partial seizures	Moderate, PD, Sp.Ed.	GSW (onset), GS (During)	N	Multi-AED	No	No	LO-DS		Right frontal partial lobectomy
								4 AED together	still seizures 2/week				
25DS51	M	6Y	GTCS, FS, AFS Sec. Generalized Abs. Sz.	GTCS	Mild MR	GSW (onset-during)	N	VPA + CL	no	No	LO-DS		Photo-sensitivity
30DS60	F	3MO	Afebrile GTCS (AV)	FS, GTCS, Fr. SE	Mild MR, MD (before onset of epilepsy)	GSW (onset-during)	N	PHE + LT+ Ketogenic diet	yes	No	DS		
31DS61	F	6MO	Febrile SE	Partial Sz, Atypical abs.	GDD, Moderate MR	N (at onset)	N	VPA+ T	Seizure free since 4 years of age	No	DS		Micro-cephaly, Ataxia, Spasticity/ Hypertonia, apraxia in hand
32DS66	M	3MO	FS, Tonic (5 min)	GTCS, Afebrile GTC after age of 6, Abs. after age of 7	Mild MR, Special Education	GSW (onset-during)	N	Multi-AED	Ongoing abs. Sz., several/day	No	DS		Vomiting-Diarrhea attacks (Febrile, until 4Y)
33DS67	M	4MO	FS GTCS (lasting 2 months)	FS (AV, 12 MO), MC at sleep (after 1Y), FS GTC (until 4Y)	Mild MR, Special Ed.	N (at onset), left frontal ED	N	PHE	No	Yes	DS		
								V + O	No				
								VPA + O	Still seizure a few times per month				
39DS85	F	6MO	FS, SE	Arrest of Activity (2Y), atonic astatic (3Y), Hemiclonic/tonic (bet.1-2Y)	GDD, autistic features	N (at onset), Multifocal (ongoing)	N			Yes; Cons. Parents	DS		
41DS90	M	4MO	Atonic	GTCS-Clonic, MC, Aonic	GDD, Severe MR	GSW (onset-during)	Cerebral atrophy			no	DS		meconium aspiration, axial& pyramidal hypotonia, spasticity, Pyramidal signs, atopic facies
42DS93	F	7MO	GTCS	GTCS+ head drops	GDD, Severe MR	GSW+ Fast Rhythyms	N	VPA, CLZ, ZNS, Lev/	No	FS, MR, ID, (Sister)	DS		Pyramidal signs, Dyspraxia, ADHD, Hypothelorism
								TOP,LEV now	Yes				

Table 1 (*Continued*)

ID	Sex	Onset	Seizure Type		Dev. Symptoms	EEG	MRI	AEDs	Response to AED	Family History	DG	*SCN1A* Mutation & Inheritance	Additional Info
			Onset	Cont.									
43DS94	M	4days	MC and epileptic spasms,	MC and epileptic spasms, GTC, SE	GDD, Moderate ID, Severe MR	GS, FSWS, MED	nonspecific (asymmetric ventricular dilatation at 6 years)	VPA, PHB, T,CL, PRG, L	No	no	DS		abnormal neonatal period, pyramidal signs, dyspraxia, attention deficit, aggressive behavior
44DS96	M	7.5MO	febrile convulsive status epilepticus (30 min) (AV)	Febrile, afebrile, GTK/ GTN (4Y), focal motor (6Y)	GDD (after SE at age of 4Y, improved after 8months)	Right TP (6Y)	NA	VPA, PHB	No		DS		
45DS99	F		Febrile GTK/GTN		regression	Polyspike Waves	Abnormal			Yes	DS		hypotonia
47DS103	F	5MO	GTCS	GTCS	Normal	N	N	Diazepam		Yes	DS		
49DS105	M	2MO	GTCS	GTCS	Normal	N	N	PHB		No	DS		
50DS106	M	2MO	Epileptic Spasm (AV)	Epileptic Spasms	GGD, no developmental milestones, severe ID,	N	at 4 month old: corpus callosum agenesis, brain stem and cerebellar hypoplasia, generalized sulcal asymmetry at 16 month old: plus optic nerves and optic chiasm hypoplasia			Cons. Parents	DS		Axial hypotonia, spasticity, pyramidal signs, bilateral optic atrophy, large ears, inverted V-shaped mouth, bilateral clinodactyly of fifth fingers of feet, ASD
51DS107	M	14MO	FS in cluster	FS, atonic posture/ drop attack after screaming until age of 4, GTCS after age of 4,5	SD, ADHD, learning disability	at onset: backround rthym irregularity and right anterior epileptic discharges, last EEG: generalized paroxsysms	N	VPA + L + PRD	yes	No	LO-DS		
58DS122	M	5MO	FS, Left-sided focal clonic Sz.	GTCS (4Y)	SD, MR, mild ID, Visuospatial functions, attention and memory deficient	N	N	multiple AED	no	2 times FS in sister	DS		pyramidal signs
59DS125	M	46 days	MC and infantile spasms	FS and AFS, GTCS, MC, focal and hemi-convulsive Sz.	Fine MD, SD, mild ID, learning disability,	at onset: GS, FSWS, MED, last EEG: fast rhythmic activity (16Y)	cerebellar atrophy	PRD, VPA, CLZ, L and antipsychotics	No	Cons. Parents hearing deficiency in family	DS		cyanosis during birth, ataxia, horizontal and rotatory nystagmus, bilateral dysmetria and dysdiadochokinesia, aggressive behavior, cerebellar signs (ataxia)
60DS126	F	6MO	Febrile GTCS	Afebrile GTCS (5Y)	Mild ID and MR, executive functions, attention and memory deficient,	GSW	N	VPA + L + PHE	yes	NO	DS		
62DS131	M	18 MO	MC	Hemi-convulsion tonic	SD, MD, moderate ID	MED	N			Yes	GEFS+		Pyramidal signs

Agg.: Aggravation, ED: Epileptic Discharges; GTCS: Generalized Tonic-Clonic Seizures; GTCS: Generalized Tonic-Clonic Seizure; L: Levetiracetam; M: Male; mo: Months; N: Normal; N/A: Not Available; NA: Not Applied; Psy.: Psychiatric; S:Stiripentol; ADHD:Attention-Deficient Hyperactivity Disorder; ADS: Atypical Dravet Syndrome; AED: Anti-Epileptic Drug,; AFS: Afebrile Seizure, ASD:Autism Spectrum Disorder; AV: After Vaccination; BFCA: Bilateral Frontoparietal Cerebral Atrophy, CBZ: Carbamazepine, CD: Cortical Dysplasia, CL:Clobazam, Cons.: Consanguineous; Cont: Continuing; Dev.:Developmental, DG::Diagnosis; E:Ethosuximide, ED:Epileptic Discharges; UEE:Unspecified Epileptic Encephalopathy; EEG: Electroencephalograph, F: Female, FCA: Frontoparietal Cerebral Atrophy; FP:Frontoparietal, Fr.: Frequent, FS: Febrile Seizures, FSWS:Focal Sharp Waves/Spikes; GDD:Global Developmental Delay; GS:Generalized Slowing; GSW: Generalized Spike Wave; GTC: Generalized Tonic Convulsion; ID:Intelligence Deficiency; LGS: Lennox-Gestaut Syndrome, LOC:Loss Of Consciousness;LT: Lamotrigine, Mat.:Maternally İnherited; MC: Myoclonic; MD:Motor Delay; MED:Multifocal Epileptiform Discharges; MMD:Motor And Mental Delay; MMR:Motor And Mental Retardation; MR: Mental Retardation; MRI: Magnetic Resonance Imaging; MTS: Mesial Temporal Sclerosis, O: Oxcarbazepine, Pat.:Paternally İnherited; PH:Phenytoin, PHB: Phenobarbital, PME:Progressive Myoclonic Epilepsy; PR:Psychomotor Retardation; PRD:Primidone; SD:Speech Delay; SE: Status Epilepticus, Sp.Ed.:Special Education; SW: Spike Wave, Sz.:Seizure; T: Topiramate, TO: Temporooccipital; TP:Temporoparietal, V:Vigabatrin; VPA: Valproic Acid, Y:Years, Z:Zonisamide.

Table 2
SCN1A genotypes of the patients (reference sequences NM_001165964.1 from cds start and NP_001159436.1, * denotes novel mutations that are not present in *SCN1A* database [10]).

Patient	GRch37(hg19)	Exon	cDNA	Protein	Mutation	Protein Domain	Sequencing Method
1DS1	166909401	E5	c.655 A > G*	p.R219G	Missense	DIS4	Sanger
2DS4	166892561-166892562	E16	c.3341-3342delAA*	p.K1142RfsX5	Frame Shift	DII–DIII	Sanger
6DS12	166929888-166929893	E1	c.239-244 delTGGACC*	p.L80-D81del	In frame deletion	N-terminal	Sanger
10DS22	166911147	IVS4	c.602 + 1 G > A	-	Splice site	DIS3	Sanger
11DS23	166901590	E10	c.1625 G > A	p.R542Q	Missense	DI–DII	Sanger
14DS26	166852614-166852618	E24	c.4402-4406 delCAAGA*	p.Q1468RfsX14	Frameshift	DIII–DIV	Sanger
15DS27	166848818	E26	c. 4883T > G*	p.I1628S	Missense	DIVS4	Sanger
20DS46	166893016	E16	c.2887delC*	p.L963FfsX1	Frameshift	DIIS6	Sanger
24DS50	166900385	E11	c.1837C > T	p.R613X	Nonsense	DI-DII	Sanger
26DS52	166911220	E4	c.530G > A	p.G177E	Missense	DIS2-S3	Sanger
29DS57	166915162	E2	c.301C > T	p.R101 W	Missense	N-terminal	Sanger
34DS68	166850927	IVS24	c.4498-1G > T	-	Splice acceptor	DIII-DIV	Sanger
36DS73	166929891	E1	c.241G > A*	p.D81 N	Missense	N-terminal	MPAS
48DS104	166900526	E11	c.1696C > T *	p.P566S	Missense	DI-DII	MPAS
54DS112	166894581	E15	c.2567G > A*	p.G856D	Missense	DIIS3-S4	MPAS
55DS119	166847975	E26	c.5726A > G*	p.Q1909R	Missense	C-terminal	MPAS
56DS120	166900411	E11	c.1811G > A	p.R604H	Missense	DI-DII	MPAS
57DS121	166848560	E26	c.5141A > T*	p.D1714V	Missense	DIVS5-S6	MPAS
27DS53	166903363	E9	c.1294 G > T*	p.A432S	Missense	DI–DII	MPAS

3.2. Summary of the genotype information

Out of 19 identified *SCN1A* mutations, 12 were missense, one was in-frame deletion and six were truncating mutations. Seven mutations were on the homology domains (one truncating and six missense), eight were on the linker domains (three truncating and five missense) and four were on either N- or C-Terminal domains (three missense and one in-frame deletion). Nine of the identified variants occurred *de novo* and three were inherited. Seven variants could not be tested for inheritance since parental samples were unavailable (Table 2 and Fig. 1). The effect of 12 novel variants on protein function was assessed by in silico tools, Mutation Taster [19], Polyphen [20], SIFT [21] and all were predicted to be pathological.

3.3. Genotype/phenotype correlation

DS is characterized by febrile or afebrile seizures starting in the first year of life. In our cohort disease onset for *SCN1A* positive patients ranged from 10 days to 5 years. Twelve patients had disease onset within the first year of life and six patients had unusual disease onset beyond 2 years of age. Because of this discrepancy median onset was 8.5 months but mean onset was 19.3 months. Patients with a mutation (either truncating or missense) on the linker regions had significantly later disease onset than patients with mutations on the homology regions (Independent samples Kruskal–Wallis test, $p < 0.05$). For instance, the DS patient 1DS1 with the earliest disease onset (10 days) had a missense mutation on the S4 transmembrane region (Voltage Sensor). In addition, while three DS patients with a mutation on the N-terminal cytoplasmic region had disease onset at around 5 months of age, two GEFS+ patients (55DS119 and 56DS120) with disease onset at 2 and 3 years of age, respectively, had a mutation on the C- terminal cytoplasmic region and on a linker region.

Regarding the inheritance of variants, three patients with remarkably late onset epilepsy (11DS23, 55DS119 and 56DS120) had an inherited mutation while the other three (24DS50, 34DS68 and 57DS121) could not be tested for inheritance due to unavailability of the parental samples.

It is known that, while DS patients more often have truncating mutations, patients with milder phenotypes like GEFS+ have missense mutations [10]. In our cohort, there were six truncating mutations in total. Out of these, three frameshift deletions and one splice acceptor mutation occurred in DS patients and one nonsense and one splice donor mutation were associated with LO-DS.

3.4. Clinical information of SCN1A negative patients

In our cohort of 27 *SCN1A* negative patients, 18 (67%) were diagnosed as DS, seven (26%) as LO- DS and two patients (7.4%) as GEFS+. Among the *SCN1A* negative patients, age of onset ranged from 1.5 to 72 months. While mean age of onset is 15 months, median is 6 months since 16/27 patients had disease onset within the first year of life. Though 13/27 patients had FS at seizure onset, ongoing seizures dominated by GTCS (10/30) in *SCN1A* negative patients. With regard to psychomotor functions, 92% (25/27) of *SCN1A* negative patients had a degree of psychomotor regression from learning disability to severe mental and motor retardation. Remarkably, two of the *SCN1A* negative patients had abnormal development before epilepsy. Six patients had ataxia or spasticity and one patient had gait problems (27DS53) that are comorbidities of DS.

3.5. Comparison of detection methods

In order to accelerate the mutational screening of the *SCN1A* gene, MPAS method was implemented. Gene regions and exon/intron boundaries of the *SCN1A* gene were amplified for 12 patients and sequenced in a single GS Junior sequencer run (Roche, Germany). To compare the two approaches, two Sanger negative and two Sanger positive (one missense and one deletion) samples were also included in MPAS. The deletion in patient 14DS26 (c.4402-4406 delCAAGA) was confirmed (Fig. 2a). However, the missense variation in patient 12DS24 was not observed by MPAS. Repeated Sanger sequencing showed that the initial result was a false positive. In the two Sanger sequencing negative patients, MPAS was also negative. In total, 16 patients were analyzed by MPAS, 6 variations in the *SCN1A* gene were detected and validated by Sanger sequencing without any false-positives. Fig. 2b shows MPAS and Sanger sequencing results of patient 54DS112.

4. Discussion

SCN1A is the most clinically relevant gene for a wide spectrum of epilepsy phenotypes and the search for a mutation in the *SCN1A* gene is the first widely accepted step in DNA diagnosis of patients

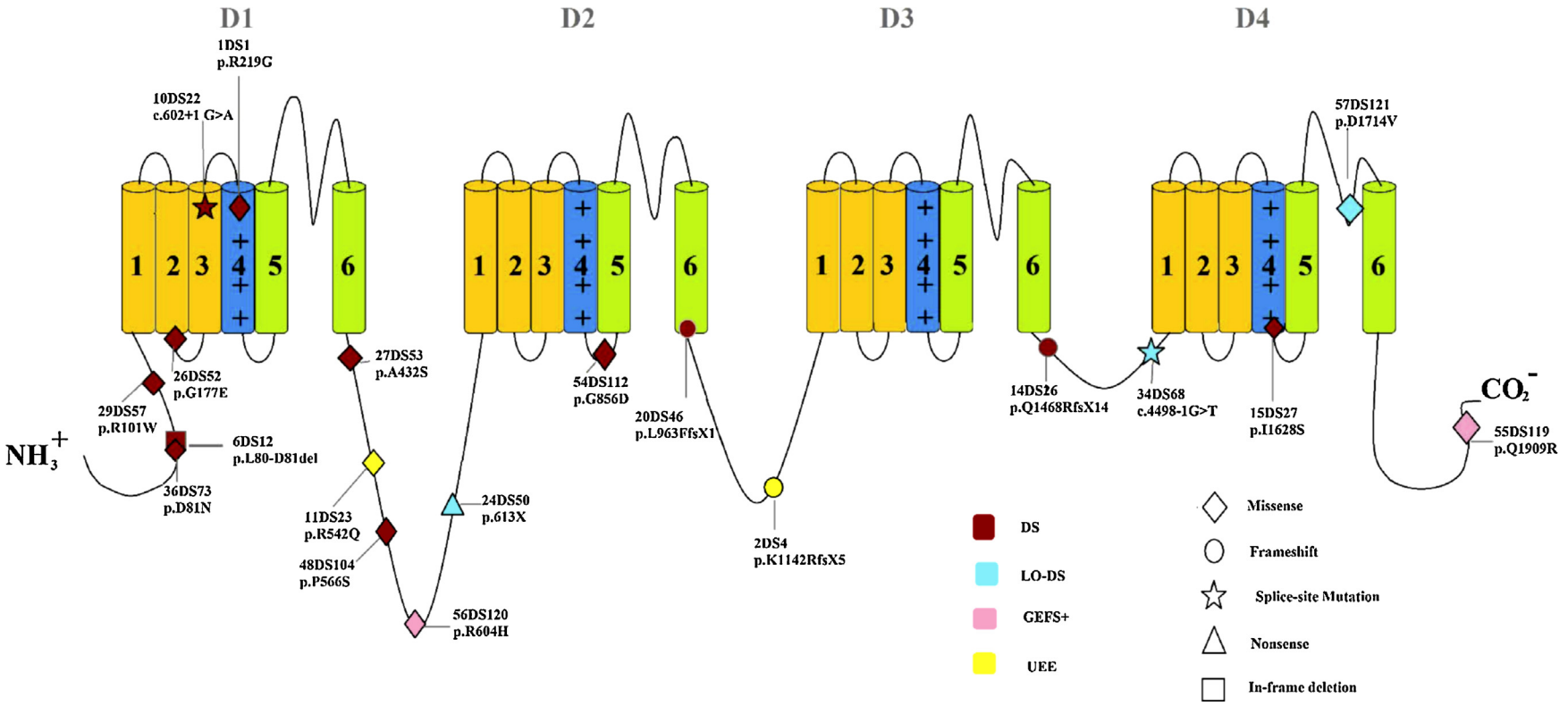

Fig. 1. Distribution of the identified mutations on $Na_v1.1$. α1 subunit. The voltage sensor domain (S4) is shown in blue and pore-forming units (S5 and S6) are shown in green.

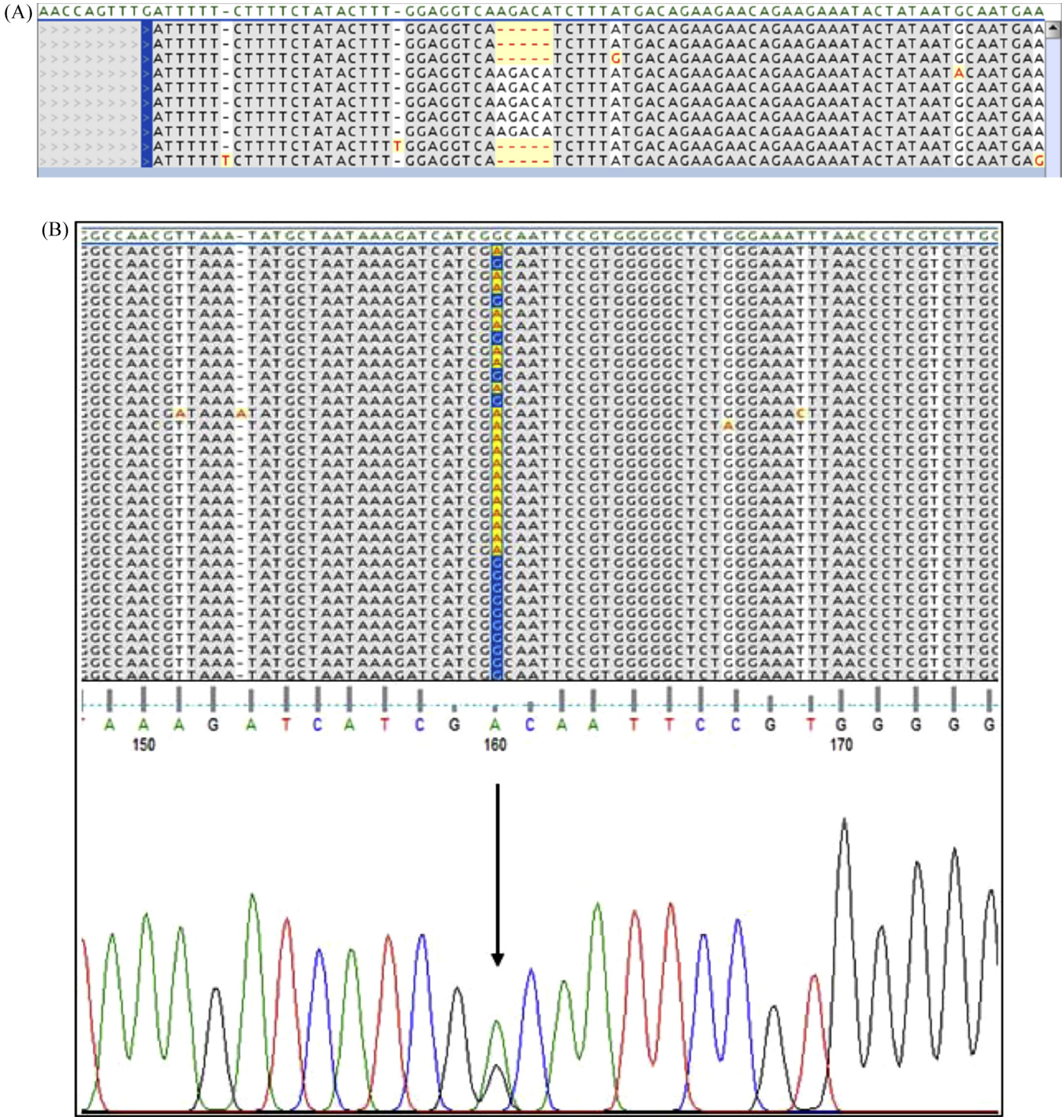

Fig. 2. (a): c.4402-4406delAGACA deletion in patient 14DS26 confirmed by pyrosequencing. (b) MPAS result of 54DS112 heterozygous for c.2567G > A (upper panel) and Sanger validation of the variant (lower panel).

suspected to have DS, GEFS+ and EE syndromes. It is reported that 80% of DS patients have an *SCN1A* mutation [12], and there are several reports of *SCN1A* mutations in patients with different epilepsy syndromes, also in patients with migraine and autism [22]. This study also demonstrated that in a clinically heterogeneous cohort, although biased toward DS phenotype, 46% of the patients had epilepsy caused by *SCN1A* mutations and the mutation ratio among DS patients were 41.3% which is significantly lower than the reported ratio. This fact may stem from our less stringent inclusion criteria or unavailability of data on DNA aberrations like indels and microdeletion/duplications. Furthermore, the DS phenotype in *SCN1A* negative patients may have resulted from mutations in other genes such as *PCDH19* [11] and *CHD2* [23] that were not analyzed in these patients.

In order to attribute a diagnostic value to *SCN1A* mutation screening, several researchers tried to make a prognosis based on *SCN1A* mutations. For instance, in a recent study by Zuberi et al. [15], a statistical analysis on 819 *SCN1A* mutations with respect to the mutation type (missense or truncating), effect of missense mutation on protein structure (Graham Score, GS) and also location

of the mutation on the protein was conducted. It was shown that truncating mutations resulted in earlier disease onset, same as high GS score missense mutations [15]. On the other hand, missense mutations on voltage (S4) and ion-pore regions were associated with DS phenotype rather than GEFS+ [10]. Although our cohort was not large, nevertheless, we observed that patients with a mutation (either truncating or missense) on linker regions had significantly later disease onset than patients with mutations in homology regions and inherited variants yielded to later disease onset and milder phenotype, in agreement with the above analysis.

Of all reported *SCN1A* mutations, 81.8% were novel [10]. However, in our cohort, this ratio was 63%. One splice site (c.602 + 1 G > A) and one missense mutation (C.530 G > A) mutation was reported in several DS patients phenotypically similar to patients 10DS22 and 26DS52, respectively. On the other hand, one nonsense variant (c.1837 C > T) and splice site mutation (c.4498-1 G > T) were reported in several DS patients but our patients had significantly later disease onset (6y and 4y, respectively). c.301 C > T variant was reported in DS patients with similar phenotype with patient 29DS57, though one previously reported patient [4] had later disease onset. Finally, c.1881 G > A variant was reported to be associated with DS or EE phenotypes, however, patient 56DS120 had a phenotype compatible with GEFS+ [10]. None of the variants identified in this study were present in public variant databases except the c.1625 G > A variant, which is present in ExAC database with a low frequency (0.0015). This previously reported variant was associated with JME [3], ASD [24], GEFS+ [25,26] and Infantile Epilepsy [27] with variable penetrance. The mutation disrupts the predicted tyrosine kinase site in the cytoplasmic loop 1, thus rather than demolishing channel function, it may have a regulatory effect on channel activity yielding a milder phenotype.

SUDEP is one of the most important causes of mortality in patients with epilepsy [28,29] and is more frequently reported in patients diagnosed with DS [30]. Patient 48DS1104 with a novel mutation (c.1696C > T) was deceased with definite sudden unexplained death in epilepsy (SUDEP) at the age of 20 following last of his habitual generalized tonic clonic seizures. SUDEP in this patient was supported with autopsy findings. The underlying mechanisms of SUDEP are still largely unknown and debated [31]. Therefore, reporting DS patients with SUDEP may help to predict which patients are at risk and to find relevant mechanisms for prevention [32,33].

Although *SCN1A* screening is important for epilepsy diagnosis, the large size of the gene renders Sanger sequencing cumbersome. The comparative analysis of Sanger and MPAS sequencing showed that MPAS, which is more rapid and cost-effective, could effectively replace conventional *SCN1A* screening methods.

The accumulating data on *SCN1A* mutations will provide better prognosis of epilepsy patients and will ultimately lead to personalized medicine. However, further analysis of parents and long-term follow up of patients are of great importance since they may shed light on the effect of mutations, on the onset and prognosis of the particular disease, elucidating genotype–phenotype relationships and providing better treatment options for patients.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

We would like to express our sincere appreciation to all patients and families for their contribution. We thank Yasemin Şen for her technical help in massively parallel sequencing. This study was supported by Boğaziçi University Research Fund Projects 07HB101 and 09S106 and TUBİTAK 110S518.

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