Association of a synonymous *SCN1B* variant affecting splicing efficiency with Benign Familial Infantile Epilepsy (BFIE)

Sunay Usluer[1], Melek Aslı Kayserili[1*], Aslı Gündoğdu Eken[2], Uluc Yiş[3], Costin Leu[4**], Janine Altmüller[4], Holger Thiele[4], Peter Nuernberg[4], Thomas Sander[4], S. Hande Çağlayan[2]

[1] Formerly affiliated with the Department of Molecular Biology and Genetics, Boğaziçi University, Istanbul, Turkey

[2] Department of Molecular Biology and Genetics, Bogazici University, Istanbul, Turkey

[3]Department of Pediatrics, Division of Child Neurology, Dokuz Eylül University School of Medicine, İzmir, Turkey

[4] Cologne Center for Genomics, University of Cologne, Cologne, Germany

*Present address: Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

** Present address: NIHR University College London Hospitals Biomedical Research Centre, Department of Clinical and Experimental Epilepsy, UCL Institute of Neurology, London, United Kingdom

Corresponding author: Server Hande Çağlayan, Ph.D.,

Department of Molecular Biology and Genetics,

Boğaziçi University, İstanbul, Turkey

Tel: +90 -212 359 6881

Fax: +90 -212 287 2468

Email: hande@boun.edu.tr

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Abbreviations:

BIS: Benign Infantile Seizures

BFIC/BFIS: Benign Familial Infantile Convulsions/ Seizures

BFIE: Benign Familial Infantile Epilepsy

WES: Whole Exome Sequencing

AD: Autosomal Dominant

SCN1B: Voltage Gated Sodium Channel Beta 1

ABSTRACT

Benign familial infantile epilepsy (BFIE) is clinically characterized by clusters of brief partial seizures progressing to secondarily generalized seizures with onset at the age of 3-7 months and with favorable outcome. *PRRT2* mutations are the most common cause of BFIE, and found in about 80% of BFIE families. In this study, we analyzed a large multiplex BFIE family by linkage and whole exome sequencing (WES) analyses. Genome-wide linkage analysis revealed significant evidence for linkage in the chromosomal region 19p12-q13 (LOD score 3.48). Mutation screening of positional candidate genes identified a synonymous *SCN1B* variant (c.492T>C, p.Tyr164Tyr) affecting splicing by the removal of a splicing silencer sequence, shown by *in silico* analysis, as the most likely causative mutation. In addition, the *PRRT2* frameshift mutation (c.649dupC/p.Arg217Profs*8) was observed, showing incomplete, but high segregation with the phenotype. *In vitro* splicing assay of *SCN1B* expression confirmed the *in silico* findings showing a splicing imbalance between wild type and mutant exons. Herein, the involvement of the *SCN1B* gene in the etiology of BFIE, contributing to the disease phenotype as a modifier or part of an oligogenic predisposition, is shown for the first time.

HIGHLIGHTS

- A large family of four generations showing BFIE phenotype including 13 affected and 15 healthy sampled individuals, was analyzed.
- Whole genome linkage analysis showed significant linkage to chromosome 19p12-q13 with a LOD score of 3.48.
- Sequence analysis of the most plausible candidate, *SCN1B*, identified a synonymous variant c.492T>C/p.Tyr164Tyr segregating with the disease phenotype and affecting splicing efficiency as shown by *in silico* analysis and minigene *in vitro* assay system.
- WES analysis outside of the linkage region identified a common *PRRT2* c.649dupC/Arg217Profs*8 mutation, known to be associated with BFIE phenotype, showing incomplete penetrance with the disease in the family.
- Synonymous *SCN1B* variant most probably contributes to the BFIE phenotype as part of an oligogenic predisposition or as a modifier.
- Signifies the importance of evaluation of potentially pathogenic effects of synonymous variants.

1. INTRODUCTION

Benign Infantile Seizures (BIS) is one of the three benign epilepsy syndromes others being BFIS (Benign Familial Neonatal Seizures) and BFNIS (Benign Familial Neonatal Infantile Seizures) that are characterized by partial seizures occurring in the first two years of life, however with different ages of onset. BIS was described by Fukuyama in 1963 as clusters of partial seizures with subacute onset between 3 to 20 months of age comprising motor arrest, impairment of consciousness, staring, and convulsive movements[1]. In 1992 Vigevano described the familial form of the disease and named it as Benign Familial Infantile Convulsions/Seizures (BFIC or BFIS). The seizures are age dependent and patients have normal motor development before and after the disease duration[2] and is known to have an autosomal dominant mode of inheritance[3]. Herein, Benign Familial Infantile Epilepsy (BFIE) will be used instead of BFIS as suggested by the ILAE Commission on Classification and Terminology in 2010[4].

Several linkage analyses in large families identified responsible loci in the chromosomal regions 19q12-13[5], 16p12- q12[6], 2q248[7] and 1p36.12-p35.1[8], demonstrating genetic heterogeneity. However, it was lately shown that *PRRT2* mutations were found in about 80% of BFIE families in various population[9,10]. The *PRRT2* (Proline Rich Transmembrane Protein 2) gene is located on chromosome 16 in a previously identified locus for BFIE and Paroxysmal Dyskinesia[11,12]. Overall, 85 % of all reported *PRRT* mutations are truncating mutations occurring at the c.649 (c.649delC/p.Arg217Glufs*12 and c.649dupC/p.Arg217Profs*8), suggesting a mutation hotspot[13]. In their study Zara et al observed *KCNQ2* mutations in all three phenotypes but *PRRT2* mutations only in BFIE patients[14].

Herein, we report a large multiplex family with a BFIE phenotype co-segregating with a synonymous variant in exon 4 of the *SCN1B* gene most probably affecting splicing efficiency of the mRNA, and with the known c.649dupC/p.Arg217Profs*8 mutation in the *PRRT2* gene.

2. MATERIALS AND METHODS

2.1. DNA Samples

Patient recruitment and genetic analyses were conducted as recommended by the Boğaziçi University Ethical Committee and upon signed consent of the patients.

DNA was extracted from 10 ml peripheral blood of BFIE family members by the NaCI method[15] or from 2 ml peripheral blood by MagNa Pure DNA Isolation Kit-Large Volume on MagNA Pure Compact instrument (Roche Diagnostics, Mannheim, Germany) or by Qiagen DNAmp DNA extraction kit according to the manufacturers' instructions. For total RNA isolation blood was collected from index patient and parents in PAX tubes and RNA was isolated using MagNa Pure RNA Isolation Kit on MagNA Pure Compact instrument (Roche Diagnostics, Mannheim, Germany). For population screening of the *SCN1B* mutation, ethnicity matched population control samples in our laboratory were used. In addition, population screening of several other variants were carried out using whole exome data of 300 individuals (TUBITAK Advanced Genomics and Bioinformatics Research Center, AGBRC).

2.2.Candidate Gene Analysis

Coding sequences and exon/intron boundaries of four candidate genes (*SCN2A, SCN1B, KCNQ2,* and *PRRT2*) were amplified by PCR using specific primers (primer sequences and PCR conditions may be provided upon request). Two point linkage analysis of variants segregating with the disease phenotype was conducted using the Superlink[16] module the easyLINKAGE[17] assuming an autosomal dominant (AD) inheritance with 80% penetrance.

2.3.Whole Genome Linkage Analysis

Genome-wide genotyping SNPs was conducted at Cologne Center for Genomics using the Illumina Infinium HumanLinkage-24 Genotyping BeadChip for all 28 family members. Genome-wide linkage analysis was performed using the EasyLINKAGE[17] program package. The Allegro[18] and SimWalk[19] modules, with the assumption of autosomal dominant inheritance and 70% penetrance, were used for multipoint parametric linkage analysis.

2.4.Whole Exome Sequencing

Whole Exome Sequencing for the patients 4BF14 and 4BF17 was done at Cologne Center for Genomics, using Illumina sequence capture with Illumina sequencing on a HiSeq200 instrument. Variants in the linkage region, shared by the two patients, and not observed in the local database of Cologne Center for Genomics were selected for further analysis by Sanger sequencing, together with other exonic variants of epilepsy-related genes outside of the linkage region. Epilepsy genes were selected according to OMIM or Pubmed with epilepsy or seizure as keywords. Channel genes were selected as listed in the report of Klassen *et al.*[20].

2.4.1. Analysis of the *SCN1B* c.492T>C/p.Tyr164Tyr Variant with *Rsa* I Digestion

The *SCN1B* c.492T>C variant in exon 4 created a restriction site for *Rsa* I enzyme. For RE digestion analysis of BFIE family and population control samples exon 4 were amplified by PCR, 10 µl of PCR products were digested with *Rsa* I enzyme (Fermentas) at 37 °C overnight and digestion products were analyzed by agarose gel electrophoresis.

2.5. *SCN1B* cDNA Analysis

Total RNA was isolated from the index patient and his parents using MagNa Pure nucleic acid extraction robot (Roche, Germany). All coding regions of the *SCN1B* gene were amplified using mRNA specific primers with the Long-Range Two-Step RT PCR Kit (Qiagen, USA), and analyzed by Sanger Sequencing (Macrogen, Korea).

2.6. Minigene Analysis of the *SCN1B* c.492T>C/p.Tyr164Tyr variant

Minigene method was described in detail by Kishore *et al.* and conducted according to their recommendations[21]. Briefly, pSpliceExpress Vector was purchased from the AddGene plasmid repository and amplified in *E. coli* cells. Isolated plasmid was sequenced by Macrogen, Korea using M13 Forward primer for confirmation. Exon 4 of the *SCN1B* gene was amplified from genomic DNA of sample 4BF14 with sequence specific primers and adaptors. The *SCN1B* variant was confirmed by Sanger sequencing, and cloned into pSE vector using the Gateway cloning method and the Gateway® BP Clonase™ II Enzyme Mix (Invitrogen, USA). Positive and negative clones for the *SCN1B* variant were selected with *Rsa* I digestion. Additionally, isolated plasmids were confirmed by Sanger sequencing.

Growth media of SH-SY5Y cells included 1:1 ratio of DMEM (no glucose, Invitrogen) and F12 (Invitrogen), 10% FBS, 1% penicillin/streptavidin, 1% non-essential amino acids (Invitrogen). Media was refreshed every two (or three) days and cells were splitted every eight to 10 days. For differentiation, SH-SY5Y cells were plated as 70-80% confluent in growth media. The growth media was changed the day after plating to differentiation media (DMEM low glucose, 1% penisilin/streptavidin, 1% N2 supplement, retinoic acid; final concentration 10 µM). Media was changed every 3 days and further differentiated for 9 days in culture. SH-SY5Y cells were transfected at the 5th day of differentiation according to manufecturer's protocol by using DNA: Lipo ratio of 0,2 µg DNA and 0,5 µl Lipofectamine 2000.

Two days after transfection total mRNA was isolated from differentiated SH-SY5Y cells using Zymo quick RNA miniprep kit as described by the manufacturer. Reverse transcription PCR was conducted using the Qiagen Long-Range 2-Step RT-PCR kit. The target region was amplified by a LongRange PCR step using rat insulin exons specific primers. Primers for cDNA production; *SCN1B* mRNA F: CTCCCGGGGACATTCTAAC and *SCN1B* mRNA R: TTCAAGGCTGGTGAGAGAGG. Targeted boost RT-PCR primers for all 3 transcripts were: *SCN1B* mRNA 2F: CTATTAATACCGGCGGCCC and *SCN1B* mRNA 4&5 R: ATTACGGCTGGCTCTTCCTT. Rat insulin exon 3 and exon 2 specific primers: pSE-RNEx3-F GCCCTGCCCAGGCTTTTGTCA and pSE-RNEx2-R GCAGAGGGGTGGACAGGGTAG.

3. RESULTS

3.1. BFIE Family

The index patient (4BF14) was diagnosed with BFIE at age of 5 months, had typical symptoms of BFIE, namely seizures with deviation of head and eyes to one side, cyanosis, apnea and general motor arrest. Several members of the family had similar symptoms and seizure type during infancy and remission about a year later. All were responsive to phenobarbital and seizure free after 2-3 years of age without medication. Only one patient (4BF26) needed medication (carbamazepine) in order to be seizure free in adulthood (≥45 years old) (Figure 4). 4BF17 deceased during the study. For other patients in generations III and IV clinical information were obtained during interviews with other family members especially with the member 4BF24. The pedigree was analyzed by Pedcheck[22] to

confirm family relationships. The index patient was negative for mutations in the candidate genes *SCN2A*, and *KCNQ2* by Sanger sequencing.

Genome-Wide Linkage Analysis

Whole genome multipoint linkage analysis with autosomal dominant inheritance and 70% penetrance identified a region with a significant LOD score of 3.48 on the chromosomal region 19p12-q13 (Figure 1A). Fine mapping defined the region of interest between markers rs959419 (chr19: 20826692, GRCh-37) and rs870379 (chr19: 35543756, GRCh-37) (Figure 1B). The haplotypes in the linkage region are shown in Figure 2. The linkage region included the *SCN1B* gene together with 76 other protein-coding genes.

The plausible candidate *SCN1B* gene in the linkage region was sequenced revealing a synonymous SNP (rs535042320, NM_001037.3, c.492T>C; NP_001028.1, p.Tyr164Tyr) in exon 4 (Figure 3A). The SNP is conserved in mammals, ultra-rare in the 282K samples of the Genome Aggregation Database (frequency $04.1\text{x}10^{-4}$, only present in South Asian population, http//:gnomad.broadinstitute.org), and was not observed in 461 unrelated controls of the same ethnicity as the family (Turkish). Analysis of the family members for the novel variant by *Rsa* I restriction digestion showed full co-segregation with the BFIE phenotype with a two-point LOD-score of 3.01 (AD inheritance, 70% penetrance). All of the 13 affected family members carried the mutation along with 3/15 healthy members of the family. ESE Finder[23] analysis showed that the mutation created an additional binding site for splicing regulator SRp55 (data not shown) and PESX[24] analysis showed that it abolished a silencer sequence (Figure 3B).

Genome-wide Exome Sequencing

The patients 4BF14 and 4BF17 were analyzed by whole exome sequencing considering two facts: (i) that the *SCN1B* variant may be co-segregating with the disease due to the basic haplotype in the linkage region masking other mutations and (ii) that there was no other plausible candidate in the region. Initially targeting the linkage region, filtering for unique variants shared by the two patients resulted in six variants in four genes, namely *ZNF98, SCN1B, ZNF792,* and *GRAMD1A*. Three other variants in two genes functionally similar to epilepsy related genes (*SLC7A10* and *KCTD15*) were observed in one

patient. All variations were validated by Sanger sequencing, except the variants in *ZNF98* and *SLC7A10*. The variants in *ZNF792*, a gene with no expression in the brain, were *in silico* predicted to be benign. The *GRAMD1A* gene has minimal expression in the brain, as reported by the EMBL-EBI Expression Atlas[25]. On the other hand, the *KCTD15* gene is known to be involved in the neural crest development[26].

Genome-wide unique variants shared by two patients outside the linkage region were also analyzed. When filtered for epilepsy and channel related genes that have expressions in the brain, 11 variants remained out of 6193 unique variants. While all 11 variants could be validated by Sanger sequencing, only two variants in *EFHC1* and *KCNJ10* were shared by other members of the family, but without a significant segregation with the phenotype or any evident modifying effect. However, a *PRRT2* mutation (c.649dupC/p.Arg217Profs*8)[13] known to be the main cause for BFIE was observed in one patient. The mutation was confirmed by Sanger sequencing and analyzed in all family members. Out of 13 affected individuals 11 carried the mutation together with 6 unaffected individuals. Supplementary Table 1 shows the two point LOD scores of all filtered variants based on the segregation analysis in the family. LOD scores were calculated using the SuperLink[17] program under the assumption of AD inheritance and 80% penetrance. The *PRRT2* mutation has the lowest LOD score and the variants on 3 genes (*SCN1B, ZNF792* and *GRAMD1A*) in the linkage region have the same LOD scores. *KCTD15* gene variant which is also in linkage region has relatively high LOD score, however, wild type allele with global allele frequency of 0.62 was segregating together with the disease in the pedigree.

SCN1B cDNA Analysis

To see the effect of the silent mutation on splicing, total mRNA was isolated from the blood sample of the index patient (4BF14) and his parents (4BF15 and 4BF16) and was analyzed by two-step RT-PCR resulting in no alteration between affected and healthy individuals. These samples were also analyzed by Sanger sequencing that showed silent *SCN1B* c.492 T>C/p.Tyr164Tyr variation in heterozygous state in the patient indicating that there is expression from both alleles and eliminated the possibility of NMD of the mutant allele. Overall evaluation of RT-PCR results of mRNA isolated from blood show that silent mutation did not have any effect on splicing and expression of the gene.

In Vitro Splicing Assay (Minigene)

In order to see the effect of the mutation in nerve cells an *in vitro* splicing assay was conducted. The target exon was cloned into an expression vector through Gateway cloning system. In this expression vector target exon was surrounded with rat insulin exons used as a positive control of transfection and expression.

Validation of the Minigene Assay With A Known GABRG2 Splicing Mutation

Prior to testing the effect of *SCN1B* c.492T>C/p.Tyr164Tyr variation on splicing, the efficacy of the minigene assay was shown using a splice site variant on the *GABRG2* gene (IVS6+2T>G). The mutation was reported by Kananura et al[27] and the splicing defect caused by the mutation was shown in both an *in vitro* system and also in a transgenic mouse by the retention of exon 6 into the intronic region[28].

For the minigene assay *GABRG2* mutation was created using site directed mutagenesis and cloned into pSpliceExpress[21] vector with gateway cloning system. Mutant and wild type plasmids were used to transfect differentiating SH-SY5Y cells. After cDNA analysis RT-PCR products showed that the plasmid with *GABRG2* IVS6+2T>G mutation produced a longer DNA fragment. Sanger sequencing of RT-PCR products confirmed the observations by Tian and Macdonald[28] and the mutant plasmid showed the retention of the intron validating the efficacy of the splicing assay in SH-SY5Y cells (Supplementary Figure 1).

Minigene Assay of the SCN1B C.492 T>C/p.Tyr164Tyr Variation

Three plasmids, one with *SCN1B* exon 4 with c.492T>C/p.Tyr164Tyr mutation, one with wild type *SCN1B* exon 4 insert and empty vector were used to transfect differentiating SH-SY5Y Neuroblastoma cells. mRNA isolated from three separate transfections were used in cDNA analysis where two bands for mutant and wild type *SCN1B* vectors and only one band for empty vector were observed. The upper band (450 bp) represents *SCN1B* exon 4 surrounded by rat insulin exons and lower band (300 bp) represents rat insulin exons only (Figure 5). Right panel on Figure 5 illustrates the content of observed bands, confirmed also by Sanger sequencing.

Despite equal amount of mRNA templates used for all three reactions a much fainter band at 450 bp in wild type sample compared to the mutant was observed. Empty vector sample did not have any band at 450 bp. Overall the Minigene Assay indicated that the synonymous mutation c. 492 T>C/p.Tyr164Tyr in *SCN1B* promoted exon 4 splicing in differentiating neuroblastoma cells, confirming *in silico*-findings (Figure 3).

4. DISCUSSION

In this study, genome-wide linkage analysis of a large multiplex BFIE family with an index patient negative for mutations in *KCNQ2* and *SCN2A* genes resulted in a haplotype segregating with the disease with a significant LOD score of 3.48 in a 15 Mb region on the chromosomal segment 19p12-q13. This region has been previously reported as candidate linkage region for BFIE in five Italian families[5]. The mutational analysis of the most plausible positional candidate gene, the *SCN1B* gene, identified a synonymous nucleotide exchange (NM_001037.3 c.492T>C; NP_001028.1 p.Tyr164Tyr) fully segregating with the disease phenotype.

In addition to the synonymous *SCN1B* variant, WES identified in the linkage region *ZNF792*, and *GRAMD1A* variants shared by both patients and co-segregating with the risk haplotype. *ZNF792* is a zinc finger protein and may have a role in transcription regulation but not much is actually known about its function. According to the Expression Atlas database of EMBL-EBI[25] it is not expressed in brain and the effect of the variant was shown to be benign by *in silico* testing. The other segregating variant was a splice site variant in *GRAMD1A* that codes for GRAM domain-containing protein 1A, a seven pass transmembrane protein[29]. According to the Expression Atlas database of EMBL-EBI it has low expression in the brain and is a low frequency SNP in dbSNP (rs558298045). Both variants in *SCN1B* and *GRAMD1A* genes were not detected in at least 300 Turkish population controls. The frameshift mutation in *KCTD15* gene was not shared by the two patients, but it was included in the segregation analysis due to its function in neural crest formation. However, the wild type allele showed incomplete segregation with the disease phenotype (LOD score of 2.02) leaving *SCN1B* as the most plausible candidate gene.

Genome-wide analysis of all other genes did not reveal any other causative variant shared by the two patients, but WES data of patient 4BF14 showed a known variant, c.649dupC/p.Arg217Profs*8,

in the *PRRT2* gene at 16q11.2, which is associated with the BFIE phenotype[10]. However, the variant was present in only 11 out of 13 affected individuals and also in 6 unaffected individuals. Reported penetrance of *PRRT2* mutations is around 70%. Many reports of unaffected carriers of *PRRT2* c.649dupC/p.Arg217Profs*8 mutation is attributed to diagnostic difficulties[13], and that may also be the case in our family. However, affected individuals in this family were generally hospitalized due to long cluster of seizures and may not have escaped diagnosis.

Overall genome-wide linkage, WES and bioinformatic analysis emphasize the *SCN1B* synonymous c.492T>C/p.Tyr164Tyr variant and the *PRRT2* 649dupC frameshift mutation as the two strong candidates associated with the disease phenotype in the family.

The association of mutations in the voltage gated sodium channel beta 1 gene (*SCN1B)*, which modulates channel surface expression and channel conduction properties, with epilepsy has been controversially discussed[30]. *SCN1B* gene resides on chromosome 19, in the frequently reported BFIE linkage region. There are reports of mutations in *SCN1B* for temporal lobe epilepsy (TLE), genetic epilepsy with febrile seizures plus (GEFS+)[31] and Dravet syndrome[32]. It is also reported that *scn1b* null mice have retarded growth, ataxia, spontaneous seizures and die in postnatal day 21[33].The most striking feature of BFIE and the two other similar syndromes is their time dependent occurrence, which may be explained with temporal regulation of *SCN1B* splicing during mouse and human development. In human frontal lobe, the *SCN1B* isoform β1B (which is not a transmembrane protein and C terminus of the protein ends in intron 3 thus lacks exon 4) is expressed 10-fold higher than β1 variant at gestational week 22, and their levels become equal at week 36. However, after birth β1 level is 18-fold higher than β1B level[34,35]. It might be speculated that tissue specific factors may be shifting the balance of splicing regulators so that β1 is expressed more than β1B.

The *in-vitro* effect of the *SCN1B* synonymous β1 variant (NM_001037.3, c.492T>C; NP_001028.1, p.Tyr164Tyr) was shown using the system developed by Kishore et al.[21] by constructing a splicing reporter assay with mutant and wild type *SCN1B* exon 4 and pSpliceExpress vector in neuroblastoma cells. The total mRNA produced by the reporter vector when amplified by two step RT-PCR shows proper splicing with both the wild type and variant exon 4, but inhibition of splicing of exon 4 in the variant seems to be removed resulting in an altered β1:β1B ratio. *In vitro* splicing assay results additionally supported *in silico* findings that the variant was affecting splicing efficiency of the

SCN1B β1 variant. Technically the effectiveness of the minigene system testing for splicing defects was shown by assessing the function of a well-established *GABRG2* splice site variant. This *in vitro* system is promising for assessment of developmental regulation of *SCN1B* gene splicing.

Though WES analysis method usually filters out synonymous variants, recent studies show that this type of SNPs also have contribution to disease etiology either by affecting splicing efficiency and mRNA stability or altering binding sites for regulatory elements[36]. For example, in a recent report a novel synonymous mutation in *MPZ* gene was shown to cause Charcot-Marie-Tooth disease by shifting differential expression of alternative splice variants[37]. Taking these facts into account, though widely ignored, accumulating research on synonymous variants would eventually enlighten disease etiology in many episodic disorders like BFIE.

The large amounts of data generated by current technologies may actually have significant contributions to the understanding of basic genetic concepts such as low penetrance and modifier effects, however, the pedigree in Figure 4 showing the segregation of variants in *SCN1B* and *PRRT2* genes do not give an obvious clue as to the modifier effects of variations in either gene. One homozygous affected member for the *SCN1B* variation did not have a more severe phenotype (4BF17). The healthy phenotype of family members 4BF33 and 4BF85 (mother and son) who carry the variation in heterozygous and homozygous states respectively, have been confirmed by successive interviews with the family members. The observation of an unaffected individual homozygous for the *SCN1B* variant indicates an incomplete penetrance of the gene, in accordance with previous studies[38,39,40].

The results presented suggest that the *PRRT2* c.649dupC/p.Arg217Profs*8 frameshift mutation may not be sufficient to cause disease alone as it is present in several unaffected individuals. It may be concluded that the *SCN1B* c.492T>C/p.Tyr164Tyr splice site variant is also implicated in the etiology of BFIE in the family along with the known *PRRT2* c.649dupC/p.Arg217Profs*8 frameshift mutation and that both variants may be required to trigger disease, or that the *SCN1B* c.492T>C / p.Tyr164Tyr splice site variant acts as a modifier or as part of an oligogenic etiology. The role of the *SCN1B* variant in infantile epilepsy is not established and this report provides the first genetic evidence of *SCN1B* mutation causing the BFIE phenotype. Larger BFIE family cohorts should be investigated for *SCN1B* mutations and the synergistic effects of mutations in more than one gene. Contribution of *SCN1B* variants to BFIE phenotype may be underestimated when synonymous mutations may have been

ignored in other BFIE families. Moreover, all of the reported pathologic *SCN1B* variants associated with other epilepsy syndromes were observed on the same gene region shared by the two splice variants β1 and β1B[41].

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Disclosure of Conflicts of Interest

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

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