CASE REPORT

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A novel missense variant of *SCN4A* co-segregates with congenital essential tremor in a consanguineous Kurdish family

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Abstract

Essential tremor (ET) is a neurological disorder characterized by bilateral and symmetric postural, isometric, and kinetic tremors of forelimbs produced during voluntary movements. To date, only a single *SCN4A* variant has been suggested to cause ET. In continuation of the previous report on the association between *SCN4A* and ET in a family from Spain, we validated the pathogenicity of a novel *SCN4A* variant and its involvement in ET in a second family affected by this disease. We recruited a Kurdish family with four affected members manifesting congenital tremor. Using whole-exome sequencing, we identified a novel missense variant in *SCN4A*, NM_000334.4: c.4679C>T; p.(Pro1560Leu), thus corroborating *SCN4A's* role in ET. The residue is highly conserved across vertebrates and the substitution is predicted to be pathogenic by various in silico tools. Western blotting and immunocytochemistry performed in cells derived from one of the patients showed reduced immunoreactivity of SCN4A as compared to control cells. The study provides supportive evidence for the role of *SCN4A* in the etiology of ET and expands the phenotypic spectrum of channelopathies to this neurological disorder.

1 | INTRODUCTION

Essential tremor (ET [MIM 190300]) is characterized by postural, isometric, and kinetic tremors that are visible and persistent and are restricted to hands and forearms (Deuschl et al., 1998). It is rarely accompanied with tremor of legs, but the tremor of the head develops in as many as 30–60% of the ET patients (Louis, 2013). While ET is not a fatal condition, it can be a major cause of social stigma, primarily affecting activities of daily living such as writing, drinking, and eating (Louis, 2005).

The clinical presentation of ET overlaps with multiple disorders such as Parkinson's disease (PD) and Dystonia and so on, and differential diagnosis is challenging to establish, especially at the earlier stages. The clinical recommendations are periodically provided by the Tremor Investigation Group (TRIG), the Movement Disorder Society (MDS), and Washington Heights-Inwood Genetic Study of Essential Tremor (WHIGET) (Bain et al., 2000; Deuschl et al., 1998; Louis et al., 1997). The diagnosis is based on excluding other tremor types. These concordantly exclude tremor produced due to dystonia, Parkinson disease, hyperthyroidism, use of certain medicine or alcohol abuse as ET (Deuschl et al., 1998). Resting tremor is traditionally associated with Parkinson disease but action tremor is restricted to ET, however, resting tremor can also be noted in severe forms of ET (Koller & Rubino, 1985).

The pathomechanism of ET is not completely understood. It has been hypothesized to be either a neurodegenerative disorder of the cerebellum or the manifestation of dynamic oscillatory disturbances of neurologic origin (Deuschl & Elble, 2009).

ET is one of the most prevalent neurological disorder; a meta-analysis of 28 different populations estimates 0.9% of pooled prevalence of all ages which increased to 4.9% in the age group ≥65 years (Louis & Ferreira, 2010). The majority (50–70%) of ET cases is hereditary (defined as two immediate family members diagnosed before 65 years of age) and familial cases presumably follow an autosomal dominant mode of inheritance (Deuschl & Elble, 2009). OMIM catalogued six ET loci termed ETM1–ETM6 (checked on September 14, 2021), but only five susceptibility genes—*DRD3*, *HS1BP3*, *FUS*, *TENM4*, and *NOTCH2NLC*—have been characterized so far. These were identified in ET families from diverse ethnic groups (Higgins et al., 2005; Hor et al., 2015; Lucotte et al., 2006; Merner et al., 2012; Sun et al., 2020). Among these, the latest report is the identification of a tri-nucleotide (GGC) repeat expansion in the five prime untranslated region (5′-UTR) of *NOTCH2NLC* in two unrelated Chinese families manifesting essential tremor (Sun et al., 2020). Notably, molecular genetic defects of *DRD3* and *HS1BP3* could not be replicated (Kuhlenbaumer et al., 2014; Zimprich, 2011). Furthermore, several other genes (*HTRA2*, *DNAJC13*, *SORT1*, *NOS3*, *KCNS2*, *HAPLN4*, and *USP46*) had been proposed to cause familial ET but their candidacy was also not replicated (Liu et al., 2016; Rajput et al., 2015; Sanchez et al., 2015; Unal Gulsuner et al., 2014). Recently, a missense variant of *SCN4A* was reported to cause ET in five affected members of a Spanish family. However, a second report supporting the link between *SCN4A* and ET has been missing (Bergareche et al., 2015).

2 | MATERIALS AND METHODS

2.1 | Subjects

We recruited a consanguineous Kurdish family with four members of the fourth and fifth generation diagnosed with ET (Figure 1a). The study was carried out according to the rules described in the Declaration of Helsinki. Prior to molecular analyses, we collected written informed consent and got this study approved by the Ethics Commission of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

2.2 | Whole-exome sequencing

We used whole-blood to extract DNA using FlexiGene DNA kit (Qiagen, 51206) from four members (IV-6, IV-7, V-1, and V2) of the family. In case of probands, IV-5 and V-3, we extracted DNA from primary fibroblasts and buccal smear using Quick Extract™ DNA Extraction Solution (Lucigen, QE0905T) and Buccal-Prep Plus DNA Kit (Bio-Budget Technologies GmbH, BPP-50), respectively. Whole-exome sequencing (WES) was conducted on DNA samples of the mother (IV-6) and her son (V-2) using NimbleGen SeqCap EZ Human Exome Library v2.0 enrichment kit. Samples were run on an Illumina HiSeq 2000 sequencing system (paired-end reads, 2 x 100 bp) and data were analyzed as described before (Hussain et al., 2012). For variant interpretation, we used VARBANK, our in-house database and analysis platform (http://varbank.ccg.uni-koeln.de).

2.3 | Co-segregation analysis and variant interpretation

The co-segregation analysis was performed by Sanger sequencing of the targeted genomic region of *SCN4A*. Several in silico prediction tools were used to predict the pathogenicity of *SCN4A* variant which are given in Appendix S1 (Supplementary method section). For analyzing the conservation status of proline at position 1560, sequences retrieved from UniProtKB or NCBI were aligned by Clustal W.

To predict the consequences of the variant on the protein structure, we retrieved an X-ray structure of SCN4A protein (pdb-code 6agf, 3.2 A resolution) from the Protein Data Bank (www.rcsb.org). We used PyMOL 2.3 (www.pymol.org; Schrödinger, LLC) for
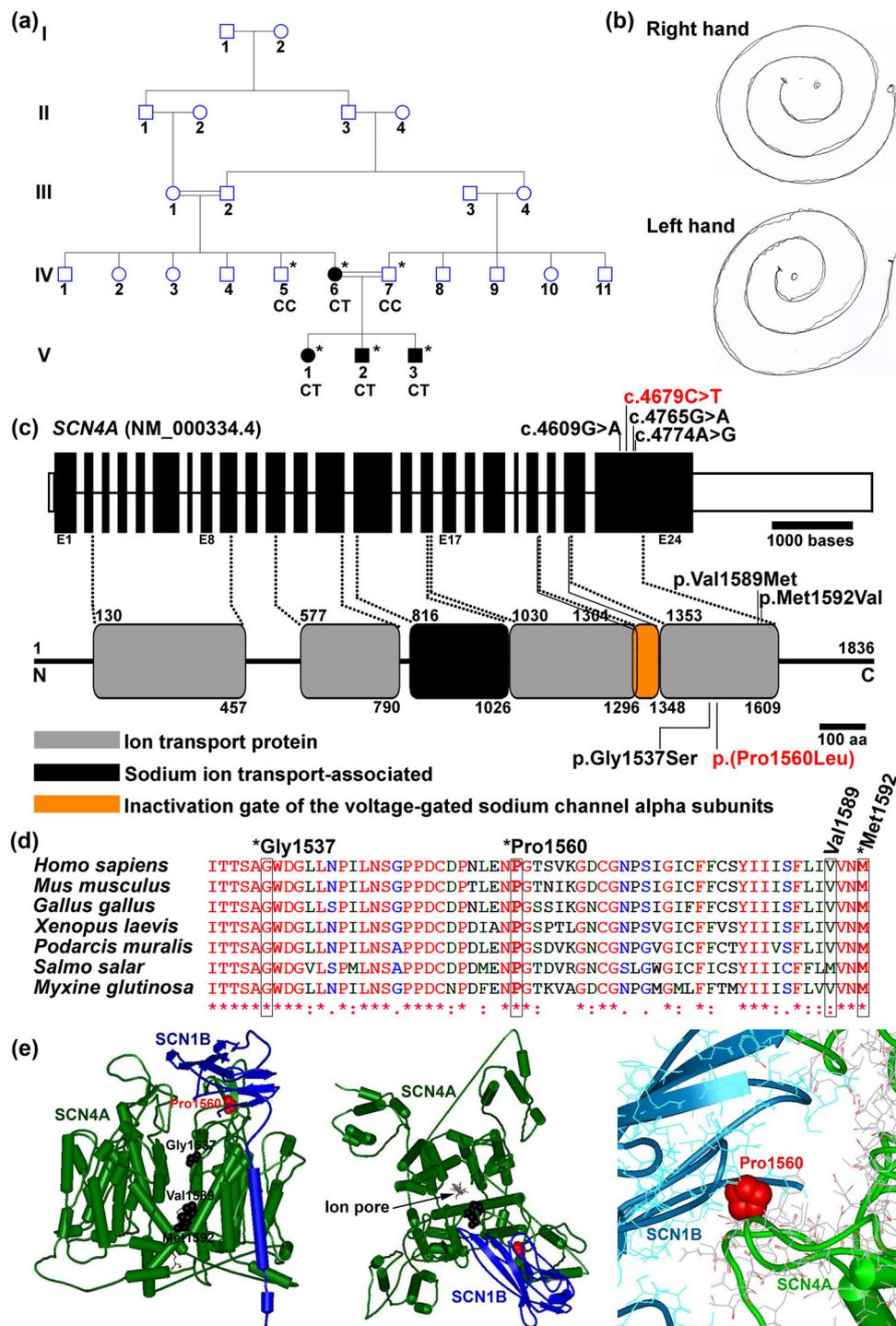
FIGURE 1 Clinical and molecular findings of ET family. (a) Five-generation pedigree of Kurdish family manifesting congenital essential tremor. Asterisks shown on the top of each symbol mark individuals participating in this study. The genotype of *SCN4A* for each individual is shown below each symbol. (b) Representation of the Archimedes spirals showing the magnitude of tremor of both hands in affected member (IV-6). (c) Upper panel: schematics of genomic structure of *SCN4A* showing 24 exons, shown by vertical bars—filled for coding and unfilled for noncoding exons. Black horizontal line shows intron drawn without scale. Scale bar for exon is indicated. Variant in red was identified in this study and in black are previously reported ones. Lower panel: Schematic of sodium channel protein type 4 subunit alpha (SCN4A) drawn according to the indicated scale. Variants identified for ET in this study and previously reported in Spanish family are shown by red and black colors, respectively, placed below the image whereas those causing paramyotonia congenita of von Eulenburg and normokalemic and hyperkalemic periodic paralysis are shown on the top of the structure. (d) Alignment of stretches of the SCN4A protein showing the conservation of mutated residues Pro1560, Gly1537, Val1589, and Met1592 (boxed regions) in several species of vertebrates. (e) Three-dimensional structure of SCN4A (green) and subunit β-1 (blue) complex (pdb-code 6agf). Left panel; schematic representation showing side view of the complex, middle panel; top view. Pro1560 is shown in red whereas previously described variants are in black situated near the ion pore. Right panel; zoomed region of the complex showing Pro1560 in space filling representation (red)

structural analysis and WebLab viewerPro (Molecular Simulations Inc.) for data visualization.

2.4 | Immunoblotting and immunofluorescence

To trace the amount and subcellular localization of SCN4A, we performed immunoblotting and immunofluorescence, respectively, on dermal primary fibroblasts derived from the affected mother (IV-6) and her elder healthy brother (IV-5) as control. The fibroblasts were propagated using established protocols described elsewhere (Hussain et al., 2012). For immunoblotting, we lysed the primary fibroblast cells in lysis buffer having the following components; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, with fresh amount of Proteinase Inhibitor Cocktail (PIC, Sigma). Following to the manual lysis of cells using 0.4×19 mm syringe having needle (needles 27G $\times$ 3/4″, Nr.20, BD Macrolane TM3), samples were incubated on ice for 15 min. After centrifugation, proteins in the supernatant were denatured in SDS sample buffer, and subjected for western blot analysis.

To visualize SCN4A on immunoblots, anti-rabbit IgG peroxidase coupled (Sigma A6154) secondary antibody (1:10,000 dilution) was used to detect rabbit anti-SCN4A antibody (Abcam, ab138079) with dilution of 1:200. As loading control, we used α-tubulin (rat monoclonal anti Y/L1/2) (Hussain et al., 2012) as a primary (1:20) and anti-mouse IgG peroxidase conjugated (Sigma, A4416) as secondary antibody (1:10,000 dilution). ImageJ was used to measure the band densities seen in western blot and were normalized with α-tubulin.

To investigate the effects of variant on subcellular localization of SCN4A, we performed immunofluorescence. For this purpose, we grew aforementioned primary fibroblasts overnight on 12 mm coverslips. The following day, cells were incubated shortly with phosphate-buffered saline (PBS). Immediately after removing PBS, the cells were fixed for 10 min with methanol (prechilled) at $-20°$C. Prior to incubation with primary antibody—rabbit anti-SCN4A (1:100, Abcam, ab138079)—overnight at 4°C, blocking was performed with 10% fetal bovine serum (FBS) for 2 h at room temperature (RT). The following day, cells were incubated thrice with PBS, each for 5 min, at RT. Secondary antibody, Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen, A21206), dilution 1:10,000, was incubated along with 4′,6-diamidino-2′-phenylindole (DAPI)—to stain DNA—for 30 min at room temperature in dark. After incubation three times with $1\times$ PBS, each for 5 min in dark, cells were mounted on glass slides with gelvatol. To visualize, confocal laser microscopy (Leica, LSM TCS SP5) was used. p value (Student's t test) was calculated using t-test Calculator of GraphPad (https://www.graphpad.com/quickcalcs/ttest1.cfm). Finally, Adobe Photoshop CS2 was used to prepare the composite images.

3 | RESULTS

3.1 | Clinical details of the patients

We present a family with congenital, nonprogressive essential tremor and sudden excessive sweating in four members (Figure 1a). All the affected members—age ranges from 9 months to 37 years—showed slightly delayed motor development but normal intellect, vision and hearing. None of the affected members had seizures. Our patients never exhibited paralysis or muscular hypotonia or atrophy. In addition, they showed no clinical signs of myotonia of the hand, face and tongue. Electromyography or electroencephalography was not performed in any of the family members. Clinical detail of each our patients is given below:

Case 1 (IV-6): Proband, 37-year-old, is fourth child of healthy consanguineous Kurdish parents. She had essential tremor since birth, accompanied by sudden excessive sweating. The sweat episodes were independent of whether the patient was at rest or in motion. She exhibited bilateral posture as well as kinetic tremor of the whole body and extremities increasing a slightly in arbitrary movements (Video S1). She had no voice tremor. Her writing was irregular but readable. In Archimedes Spiral Test (following a spiral shape with a pen), she faced more difficulty following with her left hand than with her right, which is her dominant hand (Figure 1b). The blood profile showed a normal potassium level. She did not show any other cerebellar, rigid-kinetic or dystonic symptoms, and was unremarkable for ectodermal abnormalities, seizures, or psychiatric disease.

Case 2 (V-1): The female patient is 8-year-old and the first child of IV-6. She had clinical presentation similar to her mother, such as nonprogressive essential tremor (Video S2) and sudden excessive sweating since birth. Skull sonography revealed normal cerebral structures and normal sized cerebral ventricles. A Doppler sonography of the anterior cerebral artery showed a normal flow profile without evidence of increased intracranial pressure. She learned to walk upright at the age of 17 months. She had average intellect and normal health otherwise.

Case 3 (V-2): The male proband is the second child of IV-6, he learned to walk upright at the age of 16 months. He also showed congenital tremor similar to his mother (IV-6).

Case 4 (V-3): She is 9 months old and had congenital tremor like her mother (IV-6), associated with sweat attacks. She was normally developed and no other health issues were recorded.

3.2 | Identification of the causal variant in *SCN4A*

In WES data of two affected individuals, we focused on heterozygous variants that were shared by both individuals. We preferentially searched for causative variants in genes implicated in the etiology of ET, and found a missense variant in *SCN4A*, NM_000334.4: c.4679C > T; p.(Pro1560Leu) (Figure 1c, upper panel). The gene was previously reported for ET association in a Spanish family (Bergareche et al., 2015). Sanger sequencing of all available family members showed co-segregation of the variant (c.4679C>T) with the disease, according to an autosomal dominant mode of inheritance (Figure 1a). The variant is listed in gnomAD (rs753838641) with two heterozygous alleles but it is absent in ClinVar and 2379 samples of our in-house dataset. In silico analyses using several tools predicted pathogenic effects of this variant on SCN4A (Table S1). It has a CADD score

of 24.4 thus placed in the category of pathogenic variations (Table S1). MuPro suggested decrease of SCN4A stability due to the predicted substitution p.(Pro1560Leu) (Table S1).

3.3 | In silico analyses revealed the pathogenic nature of the identified variant

SCN4A encodes sodium channel protein type 4 subunit alpha—a pore-forming subunit of the sodium channel present in skeletal muscle, which is a polypeptide of 1836 amino acids also known as voltage-gated sodium channel subunit alpha Nav1.4 (Pan et al., 2018). According to NCBI conserved domain databases, SCN4A has four ion transport protein domains, a sodium ion transport-associated domain situated within the middle of the protein and an inactivation gate of the voltage-gated sodium channel alpha subunits. Our variant residue, p.(Pro1560Leu), is located within the fourth ion transport protein domain (Figure 1c, lower panel), in an extracellular sequence stretch (pos. 1545-1574) situated between a pore-forming intramembrane part and transmembrane helix (Pan et al., 2018). The altered and two adjacent residues are strictly conserved (Figure 1d), depicting the functional significance of these specific residues. Proline in protein structures sits usually at positions where structural flexibility has to be restricted, especially in β-turns where it enables a correct refold of the polypeptide chain. In a cryo-EM structure of a complex consisting of shorter fragments of SCN4A and channel subunit β1—encoded by SCN1B—Pro1560 is located in a loop of SCN4A (Pan et al., 2018) which makes close contact with the channel subunit β1 (Figure 1e). A substitution by leucine keeps the hydrophobic character of the side chain, but may have local structural consequences, where an ion channel with its tight filtering for a special ion and subtle structural changes connected with the opening and closing of the channel may confer malfunction. These structural changes could also impair the interaction with subunit β1 (Figure 1e).

3.4 | Reduced expression of SCN4A

We next assessed the consequences of the SCN4A variant at the cellular level. Immunoblotting of cell lysate from patient-derived primary fibroblasts and those derived from a healthy individual indicated reduced immunoreactivity of SCN4A in the patient (Figure 2a). The comparison of band intensities and normalization with reference to α-tubulin showed reduction of 56% of SCN4A in mutant cells as compared to wild-type cells (Figure 2b). Immunofluorescence for SCN4A in primary fibroblasts showed the expected localization at the cell membrane (Pan et al., 2018) in both wild-type and mutant cells (Figure 2c, upper panel), but the latter showed a weaker signaling intensity (Figure 2c, lower panel). Thus, immunofluorescence analyses corroborated the findings of the western blots showing a reduced amount of the protein in mutant cells.

Taken together, we have provided compelling evidences, adding to the likelihood for the involvement of SCN4A in the etiology of ET by identifying a second variant in an unrelated family. Notably, we have also provided supporting evidence for the pathogenicity of the proposed variant at DNA and protein level.

4 | DISCUSSION

We report a Kurdish family manifesting congenital essential tremor inherited in an autosomal dominant manner. Although, essential tremor is well established as a neurological disorder of adult onset, our family showed congenital tremor in all patients, that, to the best of our knowledge, has never been reported before. Even in the Spanish family, harboring the other reported SCN4A variant, c.4609G>A;p. Gly1537Ser (Figure 1c–e), affected members manifested tremor only at the age of 23 at the earliest (Bergareche et al., 2015). Early-onset tremor has been found to be associated with the familial form, while later-onset tremor is associated with rapid disease progression (Hopfner et al., 2016). Our study confirms the suggestion that younger age at onset of ET is mostly related to familial forms.

The patients were analyzed by WES of two patients from two different generations of this family. The variant of SCN4A, was selected for initial segregation analysis due to previous report of this gene linked with ET and epilepsy (Bergareche et al., 2015). Nevertheless, we also explored other shared variants between both affected members for their possible contribution to the pathogenesis of ET, but none of them qualified due to very high allele frequencies in gnomAD and/or in-house dataset. This result strengthened the association of SCN4A with ET diagnosed in the Kurdish family. Considering that ET is the most common movement disorder, it is not surprising that two alleles of the proposed SCN4A variant are found in gnomAD. This dataset also contains individuals with rare neuromuscular disorders. Furthermore, we could also speculate that these two individuals might have a nonpenetrant variant. Regarding the inheritance pattern of the SCN4A variant in affected member IV-6, we hypothesize that it is a de novo event, but this could not be assessed because IV-6's parents are not available. Nevertheless, they were reported to have no symptoms of essential tremor.

SCN4A has a well-established role in mediating the initiation and propagation of electrical potential in muscle cells (Cannon, 2015). Several gain-of-function variants of SCN4A have been known to underly increased enhanced muscle excitability and manifest five different allelic disorders—paramyotonia congenita, potassium-aggravated myotonia, hyperkalemic periodic paralysis, hypokalemic periodic paralysis, and congenital myasthenic syndrome (Xiuhai et al., 2008). Interestingly, this channel protein, Nav1.4, was initially attributed to skeletal muscle specific phenotypes, although its expression had been observed both in mouse brain and human cerebral cortex, highlighting its role in the brain (Bergareche et al., 2015). The congenital phenotype of ET in our cases and the previously reported Spanish cases reflects its significance and indispensable role in normal brain function.

Two of the previously reported substitutions of SCN4A are located closely to the variant reported here; one of them, p.(Val1589Met),
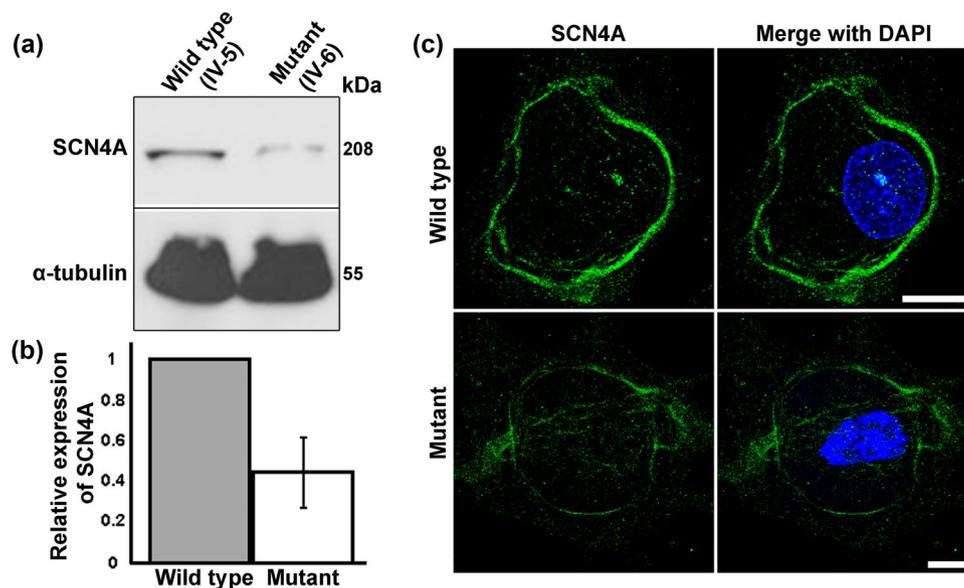
FIGURE 2 Effects of SCN4A variant at protein level. (a) A representative immunoblot, probed with SCN4A antibody, shows the reduced amount of protein in mutant cells compared to control. α-Tubulin (lower panel) is shown as internal control. (b) A bar graph comparing signal intensities from three independent immunoblots depicting a relative reduced amount of the protein in mutant as compared to the wild type, error bars represent SEM, p value = 0.033 (Student's t-test). (c) Confocal microscopy images of patient-derived primary fibroblasts (lower panel) and from wild type (upper panel) stained for SCN4A (green) and DAPI for DNA (blue). Scale bar is 10 μm

has been identified for Paramyotonia congenita of von Eulenburg (Matthews et al., 2008) and the other one, p.(Met1592Val), is known to be associated with both normokalemic and hyperkalemic periodic paralysis (Figure 1c–e) (Rojas et al., 1991; Xiuhai et al., 2008). Both of these mutations are described to influence the transmembrane helix located next—spanning from 1575 to 1597 amino acids (Pan et al., 2018)—to the one carrying our reported variant and thus explain the variable clinical spectrum. Analyzing the location of these variants, we found that p.Gly1537Ser, p.Val1589Met, and p.Met1592Val are located along the ion channel, whereas Pro1560Leu is slightly more distant (Figure 1e). This could potentially contribute to the different clinical manifestations of these variants. Interestingly, one of these variants, p.Gly1537Ser, has already been shown to affect ion flux (Bergareche et al., 2015).

The channel, Nav1.4 interacts with the β1 subunit (Pan et al., 2018). Subunit interactions usually stabilizes proteins against proteolytic degradation that might be enhanced if the subunit interaction is impaired. Furthermore, Pro in the loop position stabilizes the loop conformation; given more flexibility, expected when having Leu at that position, there is a greater possibility of increased proteolytic degradation. Considering these facts, we may speculate that marked reduction of SCN4A is most likely the consequence of proteolytic degradation rather than impaired protein synthesis. Previously, it was shown that impaired interaction of the alpha subunit of the voltage-gated sodium channel (VGSC) with SCN1B (β1 subunit) impedes its neuronal transport, where the β1 subunit was not translocated from the soma to the axonal initial segments and nodes of Ranvier (Kruger et al., 2016). These data suggest that neuronal transport of the β1 subunit is dependent on the formation of the α–β complex. We also

speculate that p.(Pro1560Leu) impairs the formation of the α–β complex, thereby affecting the neuronal transport of the β1 subunit.

Our study strongly suggests the involvement of *SCN4A* in the etiology of ET. We have shown that the identified variant of SCN4A reduces the amount of protein and thus, haploinsufficiency of *SCN4A* associated with a shortage of voltage-gated sodium channels may be responsible for the disease phenotype. Furthermore, our data expand the role of channelopathies to the etiology of ET.

CONFLICT OF INTEREST
No conflict of interest.

AUTHOR CONTRIBUTIONS
Muhammad Sajid Hussain wrote the manuscript. Peter Nürnberg, Shahid Mahmood Baig, Uzma Abdullah and Maria Asif, reviewed and edited the initial draft. Luitgard Graul-Neumann provided the clinical assessment. Maria Asif and Ionut Dragos Mocanu conducted all the functional experiments. Wolfgang Höhne generated three-dimensional structure of SCN4A and subunit β-1. Maria Asif, Janine Altmüller and Holger Thiele generated the whole-exome sequencing data. Maria Asif, Uzma Abdullah and Ehtisham Ul Haq Makhdoom analyzed the exome data and performed validation. Peter Nürnberg, Shahid Mahmood Baig and Muhammad Sajid Hussain provided

administrative oversight, supervision of experimental work and acquisition of funds.

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