A novel *de novo* heterozygous variant of the KCNQ2 gene: Contribution to early-onset epileptic encephalopathy in a female infant

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Abstract. Early-onset epileptic encephalopathy (EOEE) represents one of the most severe epilepsies, characterized by recurrent seizures during early infancy, electroencephalogram (EEG) abnormalities and varying degrees of neurodevelopmental delay. The KCNQ2 gene has been reported to have a major role in EOEE. In the present study, a 3-month-old female infant from the Chinese Lisu minority with EOEE was analyzed. Detailed clinical evaluations and next-generation sequencing were performed to investigate the clinical and genetic characteristics of this patient, respectively. Furthermore, the three-dimensional structure of the mutant protein was predicted by SWISS-Model and the expression of KCNQ2 protein in the patient was assessed by flow cytometry. It was observed that the patient presented with typical clinical features of EOEE, including repeated non-febrile seizures and significant EEG abnormalities. A novel heterozygous missense variant c.431G>C (p.R144P) in KCNQ2 was identified in the patient and the genotyping of KCNQ2 in the patient's parents suggested that this variant was *de novo*. Subsequently, the breakage of hydrogen bonds between certain amino acids was predicted by structural analysis of the mutant protein. Flow cytometric analysis detected a significant reduction buts not complete loss of native KCNQ2 protein expression in the patient (25.1%). In conclusion, a novel variant in KCNQ2 was confirmed as the genetic cause for EOEE in this patient. The present study expanded the pathogenic mutation spectrum of KCNQ2, enhanced the understanding of the molecular pathogenesis of EOEE and provided novel clues for research on the genotype-phenotype correlation in this disease.

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Abbreviations: ARHGEF9, Cdc42 guanine nucleotide exchange factor 9; ASD, atrial septal defect; CDKL5, cyclin-dependent kinase-like 5; EEG, electroencephalogram; EOEE, early-onset epileptic encephalopathy; KCH, Kunming Children's Hospital; KCNT1, potassium sodium-activated channel subfamily T member 1; NGS, next-generation sequencing; PBMCs, peripheral blood mononuclear cells; PNKP, polynucleotide kinase 3'-phosphatase; REVEL, Rare Exome Variant Ensemble Learner; SCN8A, sodium voltage-gated channel α subunit 8; SLC2A1, solute carrier family 2 member 1; SOAP, Short Oligonucleotide Analysis Package; STXBP1, syntaxin-binding protein 1; VSD, voltage-sensing domain

Introduction

Early-onset epileptic encephalopathy (EOEE) is a group of severe epilepsies characterized by refractory seizures with progressive brain dysfunction in the early infantile period, accompanied by complex etiologies (1,2). In addition to perinatal brain injury, metabolic diseases and structural brain malformations, genetic defects have also been indicated to contribute to the pathogenesis of EOEE through participating in processes such as the generation and pruning of synapses and the differentiation and migration of neurons (3). With the development of high-throughput sequencing technology, opportunities have been provided to investigate the underlying molecular-genetic basis of epilepsy. More than 100 genes have been identified to be related to EOEE, such as Cdc42 guanine nucleotide exchange factor 9 [ARHGEF9; Online Mendelian Inheritance in Man (OMIM) #300607], cyclin-dependent kinase-like 5 (CDKL5; OMIM #300203), potassium sodium-activated channel subfamily T member 1 (KCNT1; OMIM #614959), sodium voltage-gated channel α subunit 8 (SCN8A; OMIM #614558), solute carrier family 2 member 1 (SLC2A1; OMIM #614847) syntaxin-binding protein 1 (STXBP1; OMIM #602926), polynucleotide kinase 3'-phosphatase (PNKP; OMIM #605610) and potassium voltage-gated channel subfamily Q member 2 (KCNQ2; OMIM #602235) (4-14). Among these, KCNQ2 is the causative gene for 7-10% of cases of EOEE (15-17).

The KCNQ2 gene, a member of the KCNQ family, maps to chromosome 20q13.33, consists of 19 exons and encodes a voltage-gated potassium-channel subunit $K_V7.2$ (also known as KCNQ2 protein), which is widely distributed in the central nervous system (18,19). The KCNQ2 protein is composed of 872 amino acids and contains an N-terminal cytoplasmic tail, a long C-terminal region (including four α-helical regions A-D) and six transmembrane segments (S1-S6) that form four voltage-sensing domains (VSD) (S1-S4), as well as a pore domain (S5, S6 and the loop between them) (Fig. 1). The KCNQ2 protein and its homologous KCNQ3 protein constitute homo- and hetero-tetrameric ion channels considered as the molecular basis of neuronal M-channels, which induce an M-current (a slowly deactivating, voltage-dependent and non-inactivating potassium current) and have a major role in controlling neuronal excitability through limiting repetitive firing of action potentials (20,21). A previous functional study by Jentsch (22) suggested that a 25% down-regulation of M-currents may be sufficient to cause the onset of epilepsy in infants. Echoing this finding, numerous studies confirmed that mutations in the KCNQ2 gene usually resulted in over-excitability of the neurons by affecting the generation of M-currents, thus causing the occurrence of seizures (23,24). Consequently, haploinsufficiency and a dominant-negative effect, resulting from the loss of KCNQ2 protein function caused by KCNQ2 mutations (such as c.740C>T, c.853C>A, c.860C>T, etc.) (25,26), are currently recognized as the primary drivers of KCNQ2-related EOEE (27,28). Furthermore, the severity of the clinical phenotype is strongly related to the degree of protein dysfunction or deficiency determined by various KCNQ2 mutations (29). However, the genotype-phenotype correlation in this disease has remained to be fully elucidated.

The present study reported on a female infant from the Chinese Lisu minority suffering from EOEE caused by a novel *de novo* KCNQ2 variant and the clinical and genetic characteristics of this patient were determined.

Materials and methods

Subjects and clinical evaluation. A 3-month-old female patient and her parents were recruited from the Department of Respiratory and Critical Care Medicine, Kunming Children's Hospital (KCH; Kunming, China). This patient was hospitalized in September 2021 due to recurrent non-febrile convulsions. There was no consanguinity between the parents. Written informed consent was obtained from the patient's parents prior to performing clinical evaluations and collecting blood samples from any subject (October 2021 and March 2022). The present study was performed according to the Declaration of Helsinki (2013 version) as well as relevant laws of China and approved by the Ethics Committee of KCH (September 18, 2021).

Next-generation sequencing (NGS)
Sample collection and DNA extraction. Blood, urine and oral mucosa swab samples were obtained from the proband. The genomic DNA was extracted from these samples using a DNA Isolation Kit (BioTeke Corporation) according to the manufacturer's protocol. Subsequently, the DNA concentration was measured by the Qubit dsDNA HS Assay Kit on a Qubit fluorometer (both from Invitrogen; Thermo Fisher Scientific, Inc.) and the DNA quality was detected by electrophoresis on 1% agarose gels.

Construction of DNA library. The preparation of DNA libraries was performed by a KAPA Library Preparation Kit (Kapa Biosystems; Roche Diagnostics) following the manufacturer's instructions. Specifically, the genomic DNA was randomly fragmented into ~200 bp pieces and these fragments were then subjected to purification, end repair, poly-A tailing reaction and adapter ligation. Finally, the prepared DNA libraries were amplified through PCR (the compositions of the PCR mixture included 10 µl Library DNA, 12.5 µl 2X KAPA HiFi HotStart ReadyMix, 1 µl PCR Primer Premix and 1.5 µl water) with the following thermocycling conditions: initial denaturation at 98°C for 2 min, 8 cycles of denaturation at 98°C for 30 sec, annealing at 65°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 4 min.

Targeted gene capture. The capture of targeted genes was performed by hybridization of the capture probes (cat. no. 5190-9494; Agilent Technologies, Inc.) to the prepared DNA libraries and the removal of non-hybridized library molecules. Dynabeads® MyOne™ Streptavidin T1 (Invitrogen; Thermo Fisher Scientific, Inc.) in binding buffer were added to the probe-library hybridization mixture to absorb the probes with target fragments. For purification and elution, the pooled magnetic beads were washed with washing buffer 1 (25°C, 10 min), 3 (65°C, 15 min) and elution buffer. Next, the captured DNA was amplified using PCR the following PCR mixture: 21 µl 2X KAPA HiFi HotStart ReadyMix, 1 µl of 5 µM primer and 20 µl captured library beads suspension. The PCR amplification program was 98°C for 2 min; followed by 98°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec, for 13 cycles; and finally 72°C for 4 min. The products were subsequently purified by Agencourt AMPure XP beads (Beckman Coulter, Inc.). The quality inspection of the final product was performed with a Qubit dsDNA HS Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions.

Sequencing. The resulting libraries were loaded onto the Illumina HiSeq2500 platform (Illumina, Inc.) and then subjected to NGS following the manufacturer's specifications to generate paired-end 200-bp reads.

Bioinformatics analysis of NGS data. Raw image files from the Illumina HiSeq2500 platform were preprocessed using Bcl2Fastq software (version 2.20; http://support.illumina.com) for BCL-to-FASTQ conversion and demultiplexing. The generated raw reads were cleaned and filtered to remove low-quality data using Cutadapt (version 1.2.1; https://cutadapt.readthedocs.io/en/stable/) and then aligned to the human reference genome using the Short Oligonucleotide Analysis Package (SOAP) aligner tool (version 2.21; soap.genomics.org.cn/soapsnp.html). Genome Analysis Toolkit (version 3.7; https://www.broadinstitute.org/gatk/) was utilized to remove the redundant PCR duplicates and recalibrate the base quality score. The obtained insertions and deletions as well as single nucleotide polymorphisms were detected by SAMTOOLS (version 0.1.19; http://samtools.sourceforge.net/). Variant annotation was performed with ANNOVAR (http://www.openbioinformatics.org/annovar/).
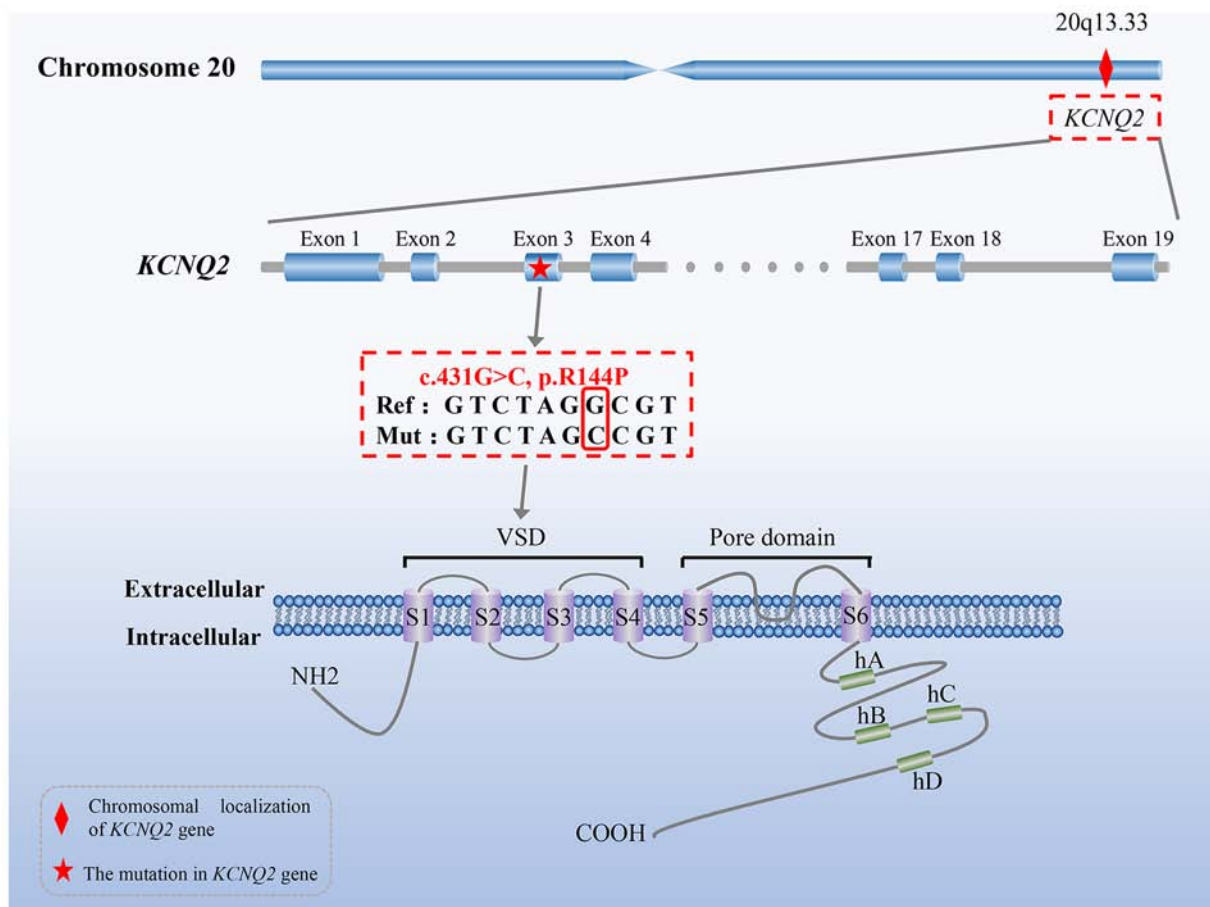
Figure 1. Schematic representation of the KCNQ2 gene and its encoded protein. The KCNQ2 gene is located on chromosome 20q13.33, including 19 exons, and the novel mutation identified in the present study had occurred on exon 3. The KCNQ2 protein consists of an intracellular N- and C-terminal region (containing helixes A, B, C and D) and 6 transmembrane domains (S1-S6), which constitute 4 VSDs (S1-S4) and a pore domain (S5, S6 and the middle P-loop). Mut, mutation; Ref, reference; VSD, voltage-sensing domain.

Pathogenicity prediction of the identified variant. The potentially damaging effect of the identified variant on protein function was predicted using Rare Exome Variant Ensemble Learner (REVEL; https://sites.google.com/site/revelgenomics/downloads), which is an ensemble pathogenicity analysis tool based on the Random Forest algorithm.

PCR and Sanger sequencing. PCR and Sanger sequencing for the three subjects were utilized to validate the candidate mutation verified by NGS. In brief, genomic DNA from the parents of the patient was isolated from peripheral blood using the genomic DNA extraction kit (Qiagen China, Co., Ltd.) and the primers (forward, 5'-ACCACAGCCTCTGACTCCA-3'; and reverse, 5'-CAACCCTTCCTGCCCAGAG-3') for amplifying exon 3 of KCNQ2 were designed by online primer design software PRIMER3 (http://frodo.wi.mit.edu/primer3). PCR amplification (compositions of the PCR mixture: 10 μl target DNA, 12.5 μl 2X KAPA HiFi HotStart ReadyMix, 1 μl PCR Primer Premix and 1.5 μl water; amplification program: Initial denaturation at 95˚C for 5 min, followed by 32 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, extension at 72˚C for 30 sec, and then a final extension at 72˚C for 7 min), purification of PCR products and Sanger sequencing with an ABI 3730 Genetic Analyzer platform (Applied Biosystems; Thermo Fisher Scientific, Inc.) were

performed sequentially. Sites of mutation were confirmed through comparing the sequencing results with the human KCNQ2 reference sequence (NM_172107.2) retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/).

Molecular modeling of the mutant KCNQ2 protein. SWISS-Model, an automated web-based homology modeling server (http://swissmodel.expasy.org/workspace/), was used to calculate the three-dimensional (3D) structure models of the mutant (Mut) and wild-type (Wt) KCNQ2 protein. The original and mutated amino acid sequences of KCNQ2 were uploaded to the above SWISS-Model workspace and the crystal structure 7CR1 fetched from Protein Data Bank (http://www.rcsb.org/pdb/) was selected as the best-matched template with a sequence identity of 62% and a coverage of 73%. The modeled structures were visualized and analyzed with PyMOL (https://pymol.org/2/).

Flow cytometry. The expression of KCNQ2 in all subjects was assessed by flow cytometry. The isolation of peripheral blood mononuclear cells (PBMCs) from peripheral blood of all subjects was performed by Ficoll density gradient centrifugation (MilliporeSigma) using a ratio of PBMC isolation reagents: Peripheral blood of 3:1. The obtained PBMCs were washed three times with PBS and then treated with
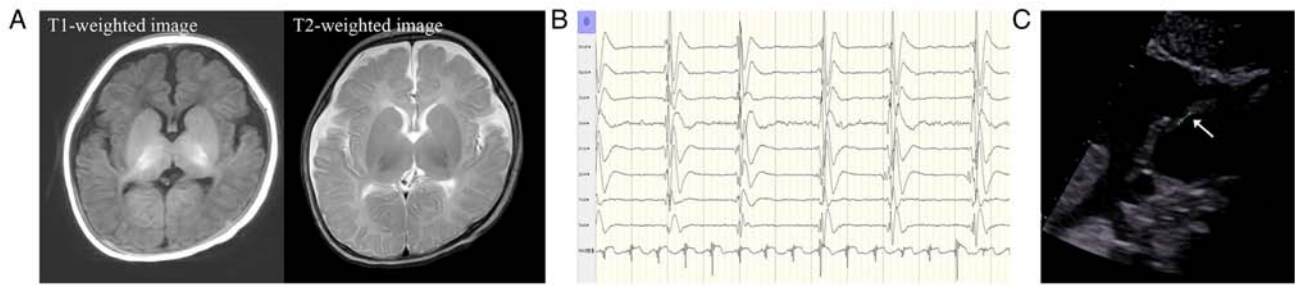
Figure 2. Imaging findings of the patient. (A) On brain MRI, no obvious abnormalities were observed. (B) Electroencephalogram exhibiting a burst suppression pattern during the recent follow-up. (C) On echocardiography, an atrial septal defect with a diameter of 2.5 mm was discovered (indicated by white arrow).

fixation/permeabilization buffer (eBioscience; Thermo Fisher Scientific, Inc.) for cell fixation and permeabilization. Rabbit anti-KCNQ2 (cat. no. ab22897; Abcam,) primary antibody was applied at 1:200 dilution overnight at 4˚C, and subsequently, samples were incubated with goat anti-rabbit IgG H&L (cat. no. ab150077; Abcam) secondary antibody diluted at 1:400 for 30 min at room temperature (protected from light). Measurement of fluorescence intensity and analysis of flow cytometric data were performed using a FACSCanto II flow cytometer (BD Bioscience; Thermo Fisher Scientific, Inc.) and FlowJo v8.8 software (FlowJo LLC), respectively.

Results

Clinical characteristics. The patient, born at full term via spontaneous vaginal delivery without asphyxia, was a three-month-old female infant from the Chinese Lisu minority, presenting with recurrent non-febrile convulsions. The infant suffered 7 episodes of unprovoked non-febrile seizures within 3 days prior to admission. Each onset lasted ~3-5 min and was characterized by clenched fists, upturned eyes, tonic spasm of limbs and unconsciousness. On admission to KCH, the patient underwent a physical examination, which indicated that the muscle strength, tension and reflexes of the extremities were normal. There was no family history of epilepsy for this patient.

The laboratory parameters revealed no significant abnormalities in 25-hydroxyvitamin D, thyroid function, blood glucose and bilirubin, thereby excluding tetany of vitamin D deficiency and convulsions caused by hypoglycemic or bilirubin encephalopathy. Neuroimaging tests and echocardiography were also completed after admission. Specifically, brain MRI of the patient indicated no pathological signs (Fig. 2A), while the electroencephalogram (EEG) revealed abnormal background activity at the time of admission (September 2021) and a burst suppression pattern at the most recent follow-up (March 2022) (Fig. 2B). In addition, an atrial septal defect (ASD) (diameter of 2.5 mm) was observed by echocardiography (Fig. 2C).

Identification of a novel de novo KCNQ2 variant. The proband and the proband's parents underwent genetic testing to investigate the potentially causative variant. According to the comparison of the sequencing result with the human reference genome, a novel heterozygous missense variant (c.431G>C) in exon 3 of the KCNQ2 gene (Fig. 3A) was identified in the proband (II-1). This single base substitution from G to C at nucleotide 431 led to an arginine (R)-to-proline (P) amino acid change at position 144 (p.R144P), thus possibly resulting in dysfunction of the encoded protein due to the fact that R144 in KCNQ2 is highly conserved among multiple species (Fig. 3B). In order to exclude the potential possibility of chimera, urine and oral samples from the proband were also used for DNA extraction and high-throughput sequencing. The KCNQ2 c.431G>C variant was also detected in the above two samples (Fig. 3A) and this finding corresponded with the sequencing result from the blood sample of the proband. To our knowledge, there have not been any previous reports about the c.431G>C variant in KCNQ2 up to now and this variant with an unknown frequency in the general population was predicted to be deleterious by REVEL. Neither previously reported pathogenic mutations for EOEE in KCNQ2 nor mutations in other EOEE-related genes (ARHGEF9, CDKL5, KCNT1, SCN8A, SCN2A, SLC2A1, STXBP1, etc.) were found in this patient. This novel missense variant was speculated to be the molecular pathological cause for the clinical phenotype of the proband. Neither of the proband's unaffected parents (the father, I-1; the mother, I-2) were carriers of this novel variant (Fig. 3C). Furthermore, the confirmation of the identity of the proband's biological parents was performed through paternity analysis (data not shown), thereby verifying that the KCNQ2 c.431G>C variant occurred *de novo* in the proband.

Structural analysis of KCNQ2 protein affected by the novel c.431G>C variant. To explore the possible role of the KCNQ2 c.431G>C variant in the protein structure and function, 3D structures of the Mut- and Wt-KCNQ2 protein were modeled (Fig. 3D). The comparative structural analysis of Mut- and Wt-KCNQ2 suggested that the identified variant (c.431G>C, p.R144P) disrupted the hydrogen bonds between the 140 and 144th amino acids and between the 147 and 144th amino acids. The breakage of hydrogen bonds may alter the local secondary structure and function of the protein.

Analysis of KCNQ2 protein expression. The expression levels of native KCNQ2 protein in all subjects were assessed by flow cytometry to validate the pathogenicity of the novel *KCNQ2* c.431G>C mutation. The acquired data were processed by FlowJo v8.8 software and converted into histograms. As speculated, the flow cytometry results (Fig. 3E) indicated a significant reduction but not complete loss of native KCNQ2
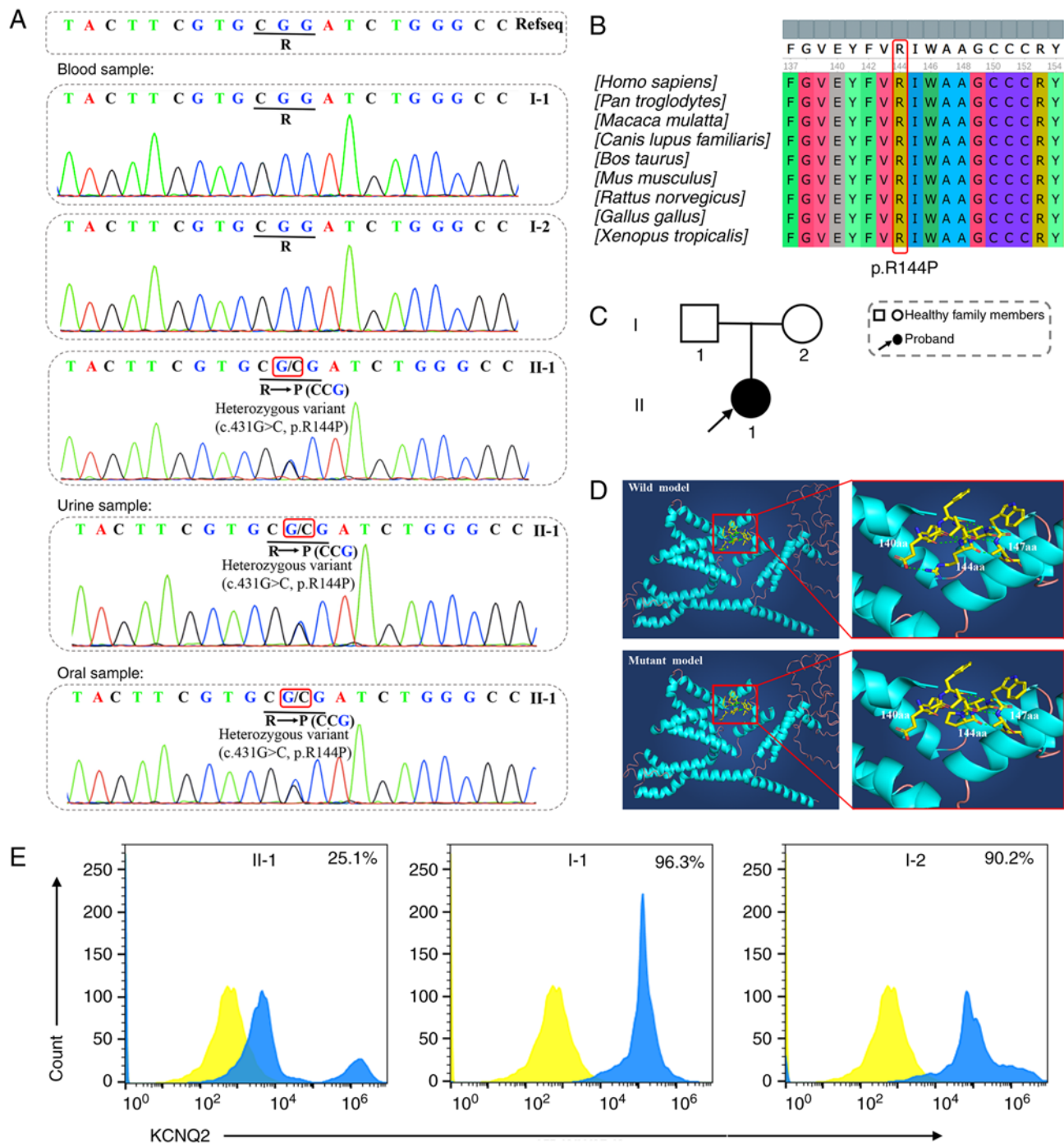
Figure 3. Mutational analysis of all participants and analysis of mutation pathogenicity. (A) Results of genetic testing. The proband had a novel *de novo* c.431G>C mutation in exon 3 of the KCNQ2 gene, while there was no mutation detected in the proband's parents. This novel *de novo* mutation was also detected in urine and oral samples from the proband through high-throughput sequencing. The identified mutation was marked with red boxes. (B) Evolutionary conservation analysis of amino acid 144 in KCNQ2. Conservation of the altered amino acid was revealed through sequence alignment. (C) The pedigree of the proband's family. II-1, the proband, is suffering from early-onset epileptic encephalopathy, while I-1 (proband's father) and I-2 (proband's mother) are healthy. (D) Structural model of the wild- and mutant-type (p.R144P) KCNQ2 protein. The novel mutation disrupted the hydrogen bonds between the 140 and 144th amino acids and between the 147 and 144th amino acids. (E) Flow cytometry results. A significant reduction but not complete loss of native KCNQ2 protein expression was identified in peripheral blood mononuclear cells from the proband (II-1, 25.1%). By contrast, no apparent downregulation of KCNQ2 expression was detected in the proband's father (I-1, 96.3%) and mother (I-2, 90.2%). In the image for II-1, two blue peaks were observed, which may be explained by the fact that a few PBMCs of the proband could express KCNQ2 normally. The peaks of the blank control were labeled in yellow and the peaks of the subjects were labeled in blue.

protein expression in PBMCs from the proband (II-1, 25.1%). By contrast, the values of the KCNQ2 protein expression in the proband's father (I-1) and mother (I-2) were 96.3 and 90.2%, respectively, and no prominent abnormalities were found.

Discussion

KCNQ2 variants, responsible for cell excitability control and maintenance of ion balance, were first discovered by

Table I. List of reported KCNQ2 variant sites in Chinese patients.

Nucleotide change	Amino acid change	Variant type	Variant class	(Refs.)
c.850T>C	p.Y284H	Missense	DM	(16)
c.871A>G	p.R291G	Missense	DM	(16)
c.710A>T	p.Y237F	Missense	DM	(16)
c.868G>A	p.G290S	Missense	DM	(16)
c.838T>C	p.Y280H	Missense	DM	(16)
c.1452G>C	p.E484D	Missense	DM	(16)
c.1284delG	p.Q429Rfs*5	Frameshift	DM	(16)
c.913T>C	p.F305L	Missense	DM	(16)
c.736G>C	p.A246P	Missense	DM	(16)
c.793G>A	p.A265T	Missense	DM	(16)
c.748G>T	p.V250L	Missense	DM	(16)
c.821C>T	p.T274M	Missense	DM	(16)
c.637C>T	p.R213W	Missense	DM	(16)
c.781T>A	p.F261I	Missense	DM	(30)
c.1742G>A	p.A518G	Missense	DM	(30)
c.1048A>C	p.N350H	Missense	DM	(31)
c.242T>C	p.L81P	Missense	DM	(31)
c.2506G>T	p.E836*	Nonsense	DM	(31)
c.958G>A	p.V320I	Missense	DM	(31)
c.998G>A	p.R333Q	Missense	DM	(31)
c.775G>A	p.D259N	Missense	DM	(31)
c.237T>G	p.N79K	Missense	DM	(31)
c.185C>T	p.A62V	Missense	DM?	(32)
c.839A>G	p.Y280C	Missense	DM?	(32)
c.2331delC	del 1 bp codon 777	Frameshift	DM	(32)
c.1948dupG	p.E650fs	Frameshift	DM	(33)
c.641G>A	p.R214Q	Missense	DM	(33)
c.916G>C	p.A306P	Missense	DM	(33)
c.1678C>T	p.R560W	Missense	DM	(33)
c.1019T>C	p.I340T	Missense	DM	(33)
c.766G>A	p.G256R	Missense	DM	(33)
c.365C>T	p.S122L	Missense	DM	(34)
c.956A>C	p.K319T	Missense	DM	(34)
c.830C>T	p.T277I	Missense	DM	(34)
c.1655A>C	p.K552T	Missense	DM	(34)
c.743dupT	ins 1 bp codon 248	Frameshift	DM	(35)
c.944G>A	p.G315E	Missense	DM	(36)
c.878T>C	p.L293P	Missense	DM	(37)
c.1057C>T	p.R353C	Missense	DM	(38)
c.1286G>A	p.C429Y	Missense	DM?	(39)
c.2015delG	del 1 bp codon 672	Frameshift	DM	(40)
c.2513_2514delAG	del 2 bp codon 838	Frameshift	DM	(41)

DM, pathogenic variant; DM?, likely pathogenic variant; del, deletion; ins, insertion.

Biervert *et al* (19) and Singh *et al* (21) in 1998 and were considered a major genetic cause of EOEE. The precise diagnosis and classification of EOEE rely on the combination of clinical data analysis and molecular genetic testing. Recently, through searching the Human Gene Mutation Database (HGMD; http://www.hgmd.org/), a total of 325 reported variant sites in the KCNQ2 gene were retrieved. Among these variants, 42 were verified in Chinese patients (16,30-41) and 83% (35/42) of them were missense variants, which were also primarily responsible for severe EOEE (Table I). In the present study, a novel *de novo* missense variant of the KCNQ2 gene (c.431G>C, p.R144P) was identified in a Chinese Lisu infant

with KCNQ2-related EOEE. It was noted that this patient with the KCNQ2 c.431G>C variant had a milder phenotype without significant psychomotor retardations in comparison to previously reported typical cases of KCNQ2-related EOEE (25,26).

The causative missense KCNQ2 variants associated with EOEE were mainly distributed in four hotspots, including the VSD (particularly in S4), the pore domain and two calmodulin (CaM)-binding α-helical regions (helix A and B) in the cytoplasmic C-terminal domain (42), and the function of the KCNQ2 protein was regulated by the above regions through different mechanisms (25). Specifically, the intracellular localization of KCNQ2 and the assembly as well as transport activity of the potassium channel were mediated by the interaction between the helix regions (A and B) with CaM. The P-loop (between S5 and S6) in the pore domain was found to have high potassium ion selectivity and permeability, which are critical for neuronal excitability. Of note, the voltage sensitivity of the $K_V7.2$ channel was conferred by the VSD. KCNQ2 mutations occurring in the VSD would reduce the voltage sensitivity by destabilizing the active VSD configuration (43). The voltage-sensing function of the VSD was endowed by charged residues, particularly the first four residues in S4, which was therefore regarded as the most crucial functional region in the VSD (44). The variants at the distal region of S4 disrupted the stability of the active VSD configuration but were not directly involved in voltage sensing. Conversely, the variants near S4 were able to squarely mediate the activation of gating pore currents. As Miceli *et al* (45) reported earlier, the effect of the KCNQ2 variants in the S1-S2 linker and S2 transmembrane segment on the voltage sensitivity and voltage-dependent activation of the $K_V7.2$ channel was smaller than that of KCNQ2 variants in S4 (43). Based on this, it may be speculated that the variants closer to S4 may result in more severe clinical phenotypes and this speculation may in part explain the phenomenon that the patient of the present study, who carried the c.431G>C variant located between S2 and S3, had a milder phenotype. Resonating with the above finding, the comparative structural analysis of Mut- and Wt-KCNQ2 protein in the present study suggested that the novel mutant (c.431G>C, p.R144P) led to the breaking of hydrogen bonds between several amino acids as compared to Wt-KCNQ2. Although this alteration would probably impact the local secondary protein structure of KCNQ2, no dramatic changes in the overall conformation of this protein were observed. This suggested that there may be a certain degree of KCNQ2 function damage, but this did not appear to be severe. Besides molecular modeling, flow cytometry was also performed to measure the expression of native KCNQ2 protein in the proband and the proband's parents. The result indicated that the novel missense mutation changed the normal structure of KCNQ2, resulting in the failure of protein encoded by the mutant KCNQ2 to bind to the normal anti-KCNQ2 antibody, which was reflected as the defect in expression of native KCNQ2. This evidence partially supported the pathogenicity of the novel mutation. However, the expression of native KCNQ2 protein in the proband (25.1%) was not completely absent, and this perhaps explained why the patient of the present study had a relatively mild phenotype compared to other reported KCNQ2-related EOEE cases. Another possible factor influencing the severity of the clinical phenotype is the type of mutation. In a previous study, the homozygous KCNQ2 variant

was lethal for mice; by contrast, no fatalities were observed in heterozygous mice despite the exhibition of neuronal hyperexcitation caused by reduced expression of KCNQ2 protein (46). Consistent with the previous study, the patient of the present study, who carried a heterozygous KCNQ2 variant only, presented with a mild EOEE phenotype without severe psychomotor retardations. According to the assessment of the patient's condition, the antiepileptic drug topiramate and certain conventional symptomatic (midazolam for sedation, etc.) and supportive therapies (levocarnitine for the facilitation of lipid metabolism, etc.) were eventually implemented for the patient. Certainly, poor neurocognitive development in EOEE is also a concern in spite of this abnormality not currently being observed in the patient. Follow-up surveillance for neurodevelopment in this patient will be maintained.

Apart from KCNQ2-related EOEE, ASD with a diameter of 2.5 mm was detected in this patient via echocardiography. ASD is one of the most common congenital heart diseases with an incidence of 1.6 per 1,000 live births and is anatomically characterized by absent tissue in the interatrial septum (47,48). Persistent left-to-right shunt via the defect may cause secondary pulmonary arterial hypertension with resultant fatal right heart failure and Eisenmenger syndrome (49,50). However, no related symptoms have been detected in the patient of the present study, and the ASD (only 2.5 mm in diameter) was considered to have a strong likelihood of healing spontaneously during childhood. Thus, no interventions for ASD were performed. However, high altitude may be a potential factor to take into account during the assessment for heart disease development. A higher risk of pulmonary hypertension and right heart failure was observed in the individuals living at high altitudes (51,52). Yunnan, where the patient resided, is a highland area located in the southwest of China with an average altitude of 2,000 m (53). Based on the aforementioned reasons, echocardiography will be performed regularly to monitor the patient's cardiac health.

In addition, the patient of the present study is from the Chinese Lisu minority in Yunnan, where >50% of Chinese ethnic minorities are settled (People's Government of Yunnan; http://www.yn.gov.cn). The rate of consanguineous marriage among the Lisu population is highest in China despite no consanguinity between the parents of the patient of the present study. The high incidence of genetic defects in ethnic minority groups remains a significant concern. Their unique cultural customs (particularly consanguineous marriage) and relatively isolated residential areas may predispose them to genetic disorders, which may perhaps also be a result of natural selection. Genome-wide association studies will be a valuable tool for the investigation of genetic variants in various populations.

Of note, there are three limitations to the present study. The first is that this novel mutation was not detected in any cohort of patients with EOEE. Furthermore, the study lacked an *in vitro* expression model of Mut- and Wt-KCNQ2. Finally, the study did not investigate the detailed relationship between the disruption of the hydrogen bonds (between the 140 and 144th amino acids) and damage to KCNQ2 function.

In conclusion, genetic analysis is crucial for diagnosing EOEE with high genetic and clinical heterogeneity. In the present study, a novel *de novo* KCNQ2 variant, which resulted in impaired function of the KCNQ2 protein, was identified in a Chinese

Lisu minority infant through NGS and Sanger sequencing. This variant was confirmed to underlie the EOEE in the patient of the present study. Overall, investigating and reporting novel causal KCNQ2 variants will expand the mutation spectrum of KCNQ2 and further contribute to genetic counseling and prenatal diagnosis. Future research should be dedicated to uncovering the exquisite molecular mechanisms of EOEE and clarifying the relationship between genotype and phenotype.

Availability of data and materials

The sequencing data that support the findings of the present study are available from the public DNA Data Bank of Japan (accession no. LC706559; http://getentry.ddbj.nig. ac.jp/getentry/na/LC706559/?format=flatfile&filetype=html& trace=true&show_suppressed=false&limit=10). The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HFL and HMF conceived and designed the study; TYY, JWY, FL and FW collected the clinical and genetic data; TYY, FL and FW performed flow cytometry; HFL, TYY and JWY analyzed the data; and HFL and HMF prepared the manuscript. All authors read and approved the final manuscript. HMF and HFL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Ethical approval for the present study was obtained from the Ethics Committee of KCH (Kunming, China).

Patient consent for publication

Informed consent was obtained from the parents of the patient.

Competing interests

The authors declare that they have no competing interests.

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