Heterozygous KCNJ10 variants affecting Kir4.1 channel cause paroxysmal kinesigenic dyskinesia

Xiaojun Huang
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University

Xin Fu
collaborative innovation center for brain science, Shanghai children 's medical center

Jingying Wu
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University

Xiaoqi Hong
songjiang Hospital and songjiang research institute, Shanghai Jiaotong University School of Medicine

Ziyi Li
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University

Lan Zheng
Fudan University Minhang Hospital

Qing Liu
Peking Union Medical College Hospital

Shendi Chen
Shanghai Jiao Tong University Medical School Affiliated Ruijin Hospital

Beisha Tang
Xiangya Hospital Central South University Department of Neurology

Yuwu Zhao
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University

Xiaorong Liu
institute of neuroscience of the second affiliated hospital of Guangzhou Medical University

Xunhua Li
Sun Yat-sen University First Affiliated Hospital

Xiaoli Liu
Shanghai Fengxian Central Hospital

Zaiwei Zhou
Shanghai xunyin biotechnology Co.,Ltd

Li Wu
Shanghai Jiao Tong University School of Medicine Affiliated Ninth People's Hospital
Kan Fang  
Shanghai General Hospital

Ping Zhong  
Suzhou Hospital of Anhui Medical University

Mei Zhang  
First Affiliated Hospital of Anhui Medical University

Xinghua Luan  
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University

Wotu Tian  
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University

Xiaoping Tong  
Department of anatomy and physiology, Shanghai Jiaotong University School of Medicine

Li Cao ( caoli2000@yeah.net )  
Shanghai Sixth People's Hospital: Shanghai 6th Peoples Hospital Affiliated to Shanghai Jiao Tong University  https://orcid.org/0000-0003-1742-9877

---

Research Article

**Keywords:** Paroxysmal kinesigenic dyskinesia, KCNJ10, Kir4.1, cerebellum

**Posted Date:** September 1st, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-3292429/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background

Paroxysmal kinesigenic dyskinesia is the representative form of paroxysmal dyskinesia, and its mechanism is unclear. Although paroxysmal kinesigenic dyskinesia is mostly attributed to genetic factors, more than 60% of paroxysmal kinesigenic dyskinesia cases are of uncertain mutations. We searched for novel genetic causes of paroxysmal kinesigenic dyskinesia and explored the corresponding pathophysiology.

Methods

A cohort of 476 probands with primary paroxysmal kinesigenic dyskinesia of uncertain genetic causes were enrolled for whole exome sequencing. Gene Ranking, Identification and Prediction Tool, a method of case-control analysis, was applied to identify the candidate genes. Another 46 probands were subsequently screened with Sanger sequencing. Whole-cell patch-clamp recording was applied to verify the electrophysiological impact of the identified variants. A mouse model with cerebellar heterozygous knockout of the candidate gene was generated via adeno-associated virus injection, and dyskinesia-like phenotype inducement and rotarod tests were performed. In vivo multiunit electrical recording was applied to investigate the change in neural excitability in knockout mice.

Results

Heterozygous variants of potassium channel inwardly rectifying subfamily J member 10 (KCNJ10) mainly clustered in patients with paroxysmal kinesigenic dyskinesia compared with the control groups. Fifteen variants were detected in 16 out of 522 probands (frequency = 3.07%). Patients with KCNJ10 variants tended to have a later onset age and shorter duration of attacks than patients with proline-rich transmembrane protein 2 mutations. Inwardly rectifying potassium channel 4.1 (Kir4.1) is highly expressed in the cerebellum of mice, and its expression pattern is consistent with the natural course of paroxysmal kinesigenic dyskinesia. Further electrophysiological recordings revealed that all the variants identified in patients led to different degrees of reduction in Kir4.1 currents, and mice with heterozygous conditional knockout of Kcnj10 in the cerebellum presented dystonic posture with epidural KCl stimulation in cerebellum, as well as poor motor coordination and motor learning ability in rotarod tests. The firing rate of deep cerebellar nuclei was significantly elevated in Kcnj10-cKO mice, indicating abnormal hyperexcitability in the Kir4.1-deficient mouse model.

Conclusion

We identified heterozygous mutations of KCNJ10 as a novel genetic cause of paroxysmal kinesigenic dyskinesia. Based on the findings in the present study, we suppose that the impaired function of Kir4.1 might lead to defective homeostatic maintenance of extracellular potassium and glutamate levels and thus cause abnormal neuronal excitability. The findings elucidated the pathogenesis of paroxysmal kinesigenic dyskinesia, though additional efforts are needed to reveal the role of Kir4.1 in movement disorders.
Introduction

Paroxysmal kinesigenic dyskinesia (PKD), the most common type of paroxysmal dyskinesia, is characterized by transient and recurrent dystonic or choreoathetoid attacks precipitated by sudden voluntary movements.\(^1,2\) Primary PKD is attributed to genetic factors, and approximately one-third of PKDs are caused by mutations in proline-rich transmembrane protein 2 (\textit{PRRT2}), identified in 2011 as the first causative gene for PKD.\(^3,4\) In addition, \textit{PRRT2} is not only the cause of PKD but also the reason for several other paroxysmal neurological diseases, indicating the shared mechanism between PKD and other episodic disorders.\(^5\)\textsuperscript{–}\textsuperscript{10}\) Moreover, multiple genes related to episodic neurologic disorders have also been reported to cause PKD attacks, including \textit{PNKD}, \textit{SLC2A1}, \textit{KCNMA1}, \textit{DEPDC5}, \textit{KCNA1}, \textit{SCN8A}, and \textit{CHRNA4}.\(^11\)\textsuperscript{–}\textsuperscript{15}\) Although previous findings support the hypothesis that PKD is probably related to the disturbance or imbalance in neural excitability,\(^16\)\textsuperscript{–}\textsuperscript{18}\) the underlying mechanisms remain unclear. It has been well accepted that the basal ganglia-thalamo-cortical circuit is highly associated with PKD due to its clinical manifestation.\(^19\) However, increasing evidence from both clinical and mechanistic studies has highlighted the role of the cerebellum in PKD.\(^17\)\textsuperscript{–}\textsuperscript{21}\)

No genetic cause is found in more than 60% of PKD individuals,\(^1,5\)\) unceasing efforts have been made to seek causative genes for PKD. Using the GRIPT (Gene Ranking, Identification and Prediction Tool) tool, we identified the \textit{TMEM151A} gene as the second major gene responsible for PKD, accounting for approximately 4.80\% of \textit{PRRT2}-negative patients.\(^22\) Here, we apply a similar strategy of case-control analysis on whole-exome sequencing (WES) data in large-scale samples of PKD from the China Paroxysmal Dyskinesia Collaborative Group (CPDCG) to map a novel candidate gene for PKD.

We found that rare variants of potassium inwardly rectifying channel subfamily J member 10 (\textit{KCNJ10}) clustered in both \textit{PRRT2}- and \textit{TMEM151A}-negative PKD patients, while in the control group, variants in \textit{KCNJ10} seldom occurred, suggesting that mutations in the \textit{KCNJ10} gene are potentially associated with PKD. Subsequently, we further investigated the electrophysiological changes in \textit{KCNJ10} variants and the relationship between Kir4.1 and PKD in \textit{Kcnj10}-deficient mice.

Materials and methods

Participants, data collection, and patient consent

In total, 522 PKD probands without \textit{PRRT2} or \textit{TMEM151A} mutations were enrolled in our study. All probands were from the dataset of the China Paroxysmal Dyskinesia Collaborative Group (CPDCG). Whole-exome sequencing (WES) was applied to 476 probands. In addition, the WES database containing 1600 samples of non-paroxysmal disorders (WESctrl\_1600) was used to compare the frequency difference of candidate genes between PKD and non-paroxysmal diseases. Another 46 \textit{PRRT2}- and \textit{TMEM151A}-negative PKD probands and unaffected individuals (n = 500) serving as matched healthy controls were subsequently examined for variants in candidate genes by Sanger sequencing.
The PKD patients’ clinical features were evaluated by at least two neurologists, and the clinical diagnostic criteria are detailed as follows:\textsuperscript{1}: First, patients should meet both core symptoms: 1) Kinesigenic triggers and attacks presenting as dystonia, chorea, ballism, or a combination; 2) No loss of consciousness during attacks. In addition, supportive evidence such as the presence of aura, an attack duration < 1 min, a positive result of a high-knee exercise test or a good response to low-dose voltage-gated sodium channel blockers could further confirm the diagnosis, while PKD patients with secondary aetiologies were excluded. Detailed clinical information on demographics, family history, features of attacks and response to treatment were collected via in-person interviews and clinical questionnaires.

This study was approved by the ethics committee of the Sixth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China and was registered at [http://www.chictr.org.cn](http://www.chictr.org.cn) (registration number: ChiCTR2100050834). All participants or their legal guardians signed written informed consent.

**Whole-exome sequencing and bioinformatics**

Genomic DNA was extracted using a standard phenol/chloroform extraction protocol. WES was performed on 476 both \textit{PRRT2}- and \textit{TMEM151A}-negative PKD probands using SureSelect v6 reagents for capturing exons and the HiSeq X Ten platform for sequencing. Alignment to the human genome assembly hg19 was carried out, followed by recalibration and variant calling. Then, we built a large allele frequency database containing 5000 virtual samples based on the gnomAD database (https://gnomad.broadinstitute.org/).

Referring to the method (GRIPT) described by Wang \textit{et al.} in 2018,\textsuperscript{23} we used this collection of data as a control group to filter the common human genome variants. The minor allele frequency (MAF) was set at < 0.01%. The Rare Exome Variant Ensemble Learner (REVEL) scores were applied to annotate the variants, which was considered an ensemble method for predicting the pathogenicity of missense variants by integrating all the analysis tools. To filter benign variants as much as possible, we set the cut-off of REVEL at 0.3. It should be noted that REVEL could not evaluate the pathogenesis of loss-of-function (LOF) variants; therefore, we set a default value of 0.9 for these variants. Among the genes filtered through GRIPT, we compared the frequency of rare variants in these genes between 476 PKD patients and WESctrl\textsubscript{1600}. Genes with much higher frequency in PKD patients were selected, and we further focused on those whose expression is predominantly in the central nervous system, especially motor-related regions, including the cortex, basal ganglia, thalamus and cerebellum.

**Sanger sequencing of the candidate gene**

Sanger sequencing was performed to determine the presence of the variants filtered out by GRIPT analysis. Furthermore, we sequenced all the exons and flanking introns of the candidate genes with direct polymerase chain reaction using customized primers in 46 other \textit{PRRT2}- and \textit{TMEM151A}-negative PKD probands as well as 500 neurologically normal controls. Afterwards, the frequency and predicted pathogenesis of all the detected variants detected via Sanger sequencing were evaluated with various population databases and software, including REVEL, the 1000 Genomes Project (http://www.internationalgenome.org), the Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/projects/SNP), the gnomAD database, the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), MutationTaster (http://www.mutationtaster.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), and SIFT
Among these tools, the 1000 Genomes Project, the Single Nucleotide Polymorphism Database and the gnomAD database are used to evaluate the frequency of the variants. SIFT and PolyPhen-2 are applied aiming to predict the pathogenicity of missense variants. The algorithm of SIFT is based on the evolutionary conservatism of sequence while PolyPhen-2 is based on mixed features of sequence and structure. REVEL is a tool of comprehensive evaluation of missense variants. As SIFT, PolyPhen-2 and REVEL are not applicable of assessing nonsense or frameshift variants, we additionally use MutationTaster to evaluate the pathogenicity of these types of variants. The primers for Sanger sequencing of the exons in KCNJ10 are listed in Supplementary Table 1b.

Construction of plasmids

Fusion plasmid constructs of human cDNA encoding full-length KCNJ10 (NM_002241) with GFP at the N-ter were generated by Genecreate Technology (Wuhan, China). The wild-type (WT) KCNJ10 gene was synthesized by overlapping PCR, during which the fragment was synthesized with overlapping primers. Then, the target fragment was cloned into the pcDNA3.1 vector by Gibson assembly. Finally, the exact sequence was validated through direct Sanger sequencing. The mutations c.436C > T, c.539G > A, c.889C > T, c.883C > T, c.910_912del, c.724A > G, c.776T > C, c.1028G > A, c.554C > A, c.321_322del, c.811C > T, c.422delC, c.191G > A, c.1042C > T and c.1105G > A were introduced into the WT constructs by site-directed mutagenesis.

Cell culture transfection

HEK 293T cells were obtained from the Cell Bank of Chinese Academy of Sciences (www.cellbank.org.cn) and maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37°C in a humidified incubator with 5% CO2. One day before transfection, cells were plated at 150,000 cells per well in 6-well culture dishes. The next day, cells were transfected with 2.5 µg of GFP control plasmid DNA or KCNJ10-GFP wild-type (KCNJ10-WT) or mutant plasmid DNA using Lipofectamine 3000 transfection reagent (Invitrogen).

Immunofluorescence

HEK 293T cells transfected with the respective expression constructs were washed in PBS and fixed using 4% paraformaldehyde (PFA) for immunofluorescence analysis. Cells were blocked with 10% normal donkey serum (NDS) and 0.3% Triton X-100 in 0.01 M PBS (PBST) for 60 min, incubated with primary antibodies against chicken anti-GFP (1:1000, ab13970, Abcam) and rabbit anti-Kir4.1 (Kcnj10) (APC-035, Alomone Labs) in blocking solution at 4°C overnight and incubated with Alexa Fluor 488 or 594 secondary antibodies (1:1000, Life). DAPI (40,6-diami-dino-2-phenylindole) (1:10,000, Life) was used for nucleic acid staining. Images were taken with a Zeiss 710 confocal microscope.

Electrophysiological recordings

HEK-293T cells were transfected with mutant plasmid or control vector for 24 h. Glass pipettes with a resistance of 3–4 MΩ were filled with intracellular solution containing (in mM) 125 K-gluconate, 15 KCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 3 Na2-ATP, and 0.3 Na-GTP and pH set to 7.3. The external solution contained (in mM) 150 NaCl, 10 glucose, 10 HEPES, 2 CaCl2, 5 KCl, and 1 MgCl2, with the pH adjusted to 7.3 with Tris-
Base. BaCl₂ (100 µM) was used to block Kir4.1 channel currents in all experiments. All the cells were visualized with an upright epifluorescence microscope (BX51WI; Olympus, Tokyo, Japan) equipped with differential interference contrast optics and an infrared CCD camera (optiMOS, Q IMAGING; Olympus, Tokyo, Japan). Whole-cell recordings were made from every mutant and wild type with a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Signals were low-pass filtered at 2 kHz and sampled at 20 kHz using Digidata 1550A (Molecular Devices) in all experiments.

Mouse model

All animal experiments were conducted according to protocols approved by the Animal Care & Welfare Committee of Shanghai Jiao Tong University School of Medicine. Kir4.1<sup>lox/lox</sup> mice (Stock No: 026826) and Rosa26-mGFP mice (Stock No: 007676) were purchased from Jackson Laboratories, USA. C57BL/6J mice were purchased from Charles River (Beijing Vital River Laboratory Animal Technology). All animals were housed in the laboratory animal facility in the Department of Laboratory Animal Science at Shanghai Jiao Tong University School of Medicine.

Real-time quantitative PCR (RT-qPCR)

The whole brain, forebrain or cerebellum tissues were rapidly dissected on ice and homogenized in TRIzol Reagent (Tsingke) at 4°C. RNA was then extracted and dissolved in nuclease-free water to a final concentration of 1000 ng/µl. Total RNA was reverse transcribed using HiScript III RT SuperMix (R323-01, Vazyme), followed by RT-qPCR using the ArtiCanATM SYBR qPCR kit (TSE501, Tsingke) and an ABI StepOne PlusTM thermocycler (Applied Biosystems), according to the recommendations of the manufacturer. Dissociation curve analysis was carried out after PCR amplification to confirm the absence of nonspecific amplification products and primer dimers. The results of real-time PCR were normalized to control values. Information on the primers used is listed in Supplementary Table 1a.

Immunohistochemistry

Mice were euthanized by barbiturate overdose and perfused transcardially with 0.01 M PBS followed by 4% PFA. Brains were removed, post-fixed overnight and dehydrated in 30% sucrose. Samples were then embedded in optimal cutting temperature compound (OCT) and stored at -80°C before sectioning. Tissues with regions of interest were cut by a cryostat (CM1950, Leica) at a thickness of 30 µm. Sections were rinsed with 0.01 M PBS 3 times for 10 to 15 min, after which they were blocked with 4% NDS at room temperature (RT) for 2 hours. Then, the samples were incubated with primary antibodies with 1% NDS in PBST at 4°C overnight. After PBST rinsing for 3 changes, the samples were reacted with fluorescent dye-conjugated secondary antibodies with 1% NDS in PBST with DAPI (1:1000, D9542, Sigma–Aldrich) at RT for 2 hours and then rinsed three times before mounting with antifade mounting medium (0100-01, Sothern Biotech). Images were taken with an Olympus SLIDEVIEW™ VS200 Slide Scanner and Nikon C2 confocal microscope.

Primary antibodies included rabbit anti-Kir4.1 (1:800, APC-035, Alomone Labs), mouse anti-GFAP (1:500, MAB360, Sigma–Aldrich) and goat anti-GFP (1:500, ab190289, Abcam). Secondary antibodies conjugated to Alexa Fluor 488 (AF488), AF568 and AF647 were 1:1000 diluted, including AF488 donkey anti-goat (705-
545-003, Jackson), AF568 donkey anti-rabbit (A10042, Invitrogen) and AF647 donkey anti-mouse (715-605-151, Jackson).

**Adeno-associated virus (AAV) administration**

For local knockout of *Kcnj10* in the cerebellum by viral injection, either Kir4.1<sup>+/flo</sup<x>−::Rosa26-mGFP or WT mice were injected with AAV2/5-gfaABC1D-iCre-WPRE-pA (~ 10<sup>13</sup> gc/ml, S0611-5, Taitool Bioscience). In brief, 6-week-old mice were anaesthetized with isoflurane (3% for induction and 1.5% for maintenance) and then placed on a stereotaxic apparatus (51730D, Stoelting), with a heating pad used to maintain body temperature. After subcutaneous injection of 2% lidocaine for perioperative analgesia and cleaning with 10% povidone iodine and 70% ethanol, a midline incision was made, and the skull was exposed and cleaned with cotton swabs. The small holes (~ 0.6 mm in diameter) for entry of pipettes were drilled on the skull using an electric cranial drill (SD-300, Yuyan). One microlitre of AAV2/5-gfaABC1D-iCre-WPRE-pA (~ 10<sup>13</sup> gc/ml) was injected into the cerebellum vermis of mice at two sites (coordinates from bregma: 6.0 mm posterior, 0.0 mm lateral, 2.0 mm and 3.0 mm ventral) using a gastight syringe (7803-04, Hamilton) and glass pipettes at a slow rate (~ 100 nl/min) controlled by Quintessential Stereotaxic Injector (53311, Stoelting). Pipettes were left in place for at least 10 min. Animals were allowed to recover from anaesthesia on a heat pad after the injection was completed. Three weeks after the surgery, at least 3 mice were sacrificed for immunohistological analysis to verify the efficiency of virus transfection, and others were used for subsequent rotarod tests and *in vivo* multi-unit electrical recording.

**Epidural KCl stimulation**

Mice were anesthetized, placed on the stereotaxic apparatus as AAV administration. To induce dyskinetic postures in animals, KCl (1 M)-soaked cotton ball was applied on the local surface of the cerebellar cortex. Then behaviour was monitored by an experimenter and recorded by a camera. The dyskinesia-attacked mice were expected to exhibit abnormal postures (such as an extended and stiff limb or/and tail, flatten posture, exaggerated truncal flexion, and truncal twisting). If the dyskinetic movements or postures appeared after stimulation and lasted for more than 5 s, the dyskinesia attack was recorded. The average occurrence of dystonia was calculated in each group (WT/ *Kcnj10*-cKO).

**Rotarod test**

Mice were placed onto a rotating rod with auto acceleration from 4 to 40 rpm in 5 min and maintenance at 40 rpm for another 5 min (47650, UGO). A trial ended if the animal fell off the rods or gripped the device and spun around for 3 consecutive revolutions without attempting to walk on the rods. The latency and speed of the rod at the endpoint were recorded for each mouse in four repeated trials to evaluate motor coordination and motor learning ability.

**In vivo multi-unit electrical recording**

Mice were anaesthetized with 5% chloral hydrate. A small hole was drilled overlying the targeted brain regions (coordinates from bregma: 6.3 mm posterior, 1.5 mm lateral, 2.3 mm ventral from dura), and the dura was removed carefully. Neural signals were recorded from a multichannel data acquisition system.
(Zeus, Bio-Signal Technologies). Electrodes consisted of 16 individually insulated nichrome wires (35 µm inner diameter, impedance 300–900 Kohm; Stablohm 675, California fine wire). The implanted electrodes were secured with dental cement, and electrophysiological recording was initiated after recovery. Spikes were extracted with high-pass (300 Hz) filters and sampled at 30 kHz. Real-time spike sorting was performed using principal component analysis (PCA). Offline Sorter (Plexon) was used for spike sorting refinement before analysing data in NeuroExplorer (Nex Technologies).

**Statistical analysis**

The experimental data were analysed using GraphPad InStat3., Origin 8, and CorelDraw12. The data are illustrated as the mean ± standard error (mean ± s.e.m.). Unpaired Student’s t test was used for statistical analysis of the two groups of data with a normal distribution. For data not conforming to a normal distribution or with heterogeneity of variance, the Mann–Whitney test was used to test the statistical significance. Two-way ANOVA was used for data on a quantitative dependent variable at multiple levels of two categorical independent variables. In the electrophysiological experiment, “n” represents the number of cells recorded. $p < 0.05$ indicates a significant difference.

**Results**

**Heterozygous variants in the KCNJ10 gene are associated with PKD**

A total of 13 rare heterozygous variants in *KCNJ10* were detected in 14 probands through exome sequencing (Fig. 1A), including c.191G > A (p.Trp64X), c.321_322del (p.Val109Glyfs*15), c.422delC (p.Pro141Hisfs*57), c.436C > T (p.Leu146Phe), c.539G > A (p.Arg180His), c.554C > A (p.Ala185Glu), c.724A > G (p.Thr242Ala), c.776T > C (p.Val259Ala), c.811C > T (p.Arg271Cys), c.883C > T (p.Gln295X), c.889C > T (p.Arg297Cys), c.910_912del (p.Glu304del) and c.1028G > A (p.Arg343His). In addition, 2 out of 46 PRRT2- and TMEM151A-negative PKD probands were detected with *KCNJ10* variants {c.1042C > T (p.Arg348Cys) and c.1105G > A (p.Gly369Ser)} by Sanger sequencing (Fig. 1A). In contrast, only 9 variants with low frequency were detected in WESctrl_1600 (16/522 versus 9/1600, 3.07% versus 0.56%). No pathogenic variants in *KCNJ10* were found in any of the 500 healthy controls.

**Clinical and genetic findings of PKD patients with heterozygous KCNJ10 variants**

Sixteen probands with *KCNJ10* variants were from 14 sporadic cases and 2 PKD families (Fig. 1A). The brother (II:1) in Family 1 and the father (I:1) in Family 2 experienced PKD attacks in adolescence as well. *KCNJ10* variants were co-segregated with patients in these 2 families. Family 2 was a typical autosomal dominant family while the co-segregation analysis of Family 1 showed that the asymptotic father of the two affected brothers was the carrier of the variant c.883C > T. In addition, the co-segregation analysis in partial sporadic cases showed that c.436C > T and c.554C > A were inherited from the father of the proband.
while c.321_322del was maternally inherited. However, all the parents with variants were asymptomatic, which suggested that incomplete penetrance also existed in KCNJ10-related PKD.

The detailed clinical manifestations of KCNJ10-related PKD patients are listed in Supplementary Table 3. The proportions of the main characteristics are shown in Table 1, and we further compared the genotype-phenotype correlations among patients with KCNJ10, PRRT2 and TMEM151A mutations. The relevant parameters of PRRT2-positive (PRRT2+) or TMEM151A-positive (TMEM151A+) PKD patients were quoted from our previously published research.\(^5\),\(^22\) The mean onset age of all the patients with KCNJ10 variants was 13.56 ± 3.27 years, which is later than that of PRRT2+ patients (9.36 ± 4.42 years) but similar to that of TMEM151A+ patients (12.93 ± 3.15 years). Four patients reported spontaneous remission of the disease, with a mean age of remission of 19.83 ± 0.64 years. The precipitating factors in KCNJ10-positive (KCNJ10+) patients were the same as those in patients with other genotypes, including sudden movement (100.00%), speed change (62.50%), emotional stress (68.75%), intentional movements (37.50%) and fatigue (18.75%). The majority of the patients (87.50%) experienced an aura before the onset of involuntary movements. Dystonia was still the most frequent type of attack (93.75%), followed by chorea (31.25%) and ballism (6.25%). The duration of attacks in KCNJ10+ patients, which mostly lasted within 10 seconds, was shorter than that in PRRT2+ patients. Face involvement was reported by 81.25% of patients. Most patients with KCNJ10 variants had a pure phenotype (87.50%), and approximately 56.25% (9/16) of patients were prescribed carbamazepine or oxcarbazepine as the first medical choice. Overall, patients with KCNJ10 variants still responded well to carbamazepine or oxcarbazepine, as 66.67% of patients experienced complete remission, and 22.22% had partial remission.
Table 1
Comparison of clinical manifestations of paroxysmal kinesigenic dyskinesia (PKD) patients with different genotypes

<table>
<thead>
<tr>
<th></th>
<th>PRRT2+ N (%)</th>
<th>TMEM151A+ N (%)</th>
<th>KCNJ10+ N (%)</th>
<th>P Value (P1) (PRRT2 vs. KCNJ10)</th>
<th>P Value (P2) (TMEM151A vs. KCNJ10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>183</td>
<td>29</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probands</td>
<td>170</td>
<td>25</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (probands)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>123 (72.35)</td>
<td>18 (72.00)</td>
<td>16 (100)</td>
<td>0.037&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.056&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Female</td>
<td>47 (27.65)</td>
<td>7 (28.00)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Form</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure</td>
<td>124 (67.76)</td>
<td>25 (86.21)</td>
<td>14 (87.50)</td>
<td>0.174&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Complicated</td>
<td>59 (32.24)</td>
<td>4 (13.79)</td>
<td>2 (12.50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic</td>
<td>83 (48.82)</td>
<td>19 (76.00)</td>
<td>12 (85.71)</td>
<td>0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.686&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Familial</td>
<td>87 (51.18)</td>
<td>6 (24.00)</td>
<td>2 (14.29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at onset (y)</td>
<td>9.36 ± 4.42</td>
<td>12.93 ± 3.15</td>
<td>13.56 ± 3.27</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt; &lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.533&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Remission age (y)</td>
<td>21.01 ± 5.87</td>
<td>21.42 ± 2.33</td>
<td>19.83 ± 0.64</td>
<td>0.628&lt;sup&gt;d&lt;/sup&gt; &lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.157&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aura</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>135 (75.00)</td>
<td>27 (93.10)</td>
<td>14 (87.50)</td>
<td>0.414&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.932&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>45 (25.00)</td>
<td>2 (6.90)</td>
<td>2 (12.50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triggers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudden movement</td>
<td>183 (100.00)</td>
<td>29 (100.00)</td>
<td>16 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Speed change</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>123 (67.96)</td>
<td>25 (86.21)</td>
<td>10 (62.50)</td>
<td>0.655&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.145&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>58 (32.04)</td>
<td>4 (13.79)</td>
<td>6 (37.50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Move intention</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>70 (39.33)</td>
<td>15 (51.72)</td>
<td>6 (37.5)</td>
<td>0.886&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.360&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Differences among PRRT2+, TMEM151A+, KCNJ10+ patients compared by<sup>a</sup>χ² test, <sup>b</sup>Yates' corrected χ² test, <sup>c</sup>Fisher exact probability, and <sup>d</sup>two sample t-test. p value was set at 0.05.
<table>
<thead>
<tr>
<th></th>
<th><strong>PRRT2+</strong> N (%)</th>
<th><strong>TMEM151A+</strong> N (%)</th>
<th><strong>KCNJ10+</strong> N (%)</th>
<th><strong>P Value (P1)</strong> (PRRT2 vs. KCNJ10)</th>
<th><strong>P Value (P2)</strong> (TMEM151A vs. KCNJ10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>108 (60.67)</td>
<td>14 (48.28)</td>
<td>10 (62.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>97 (54.19)</td>
<td>19 (65.52)</td>
<td>11 (68.75)</td>
<td>0.262&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.826&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Fatigue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>82 (45.81)</td>
<td>10 (34.48)</td>
<td>5 (31.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>32 (17.98)</td>
<td>10 (34.48)</td>
<td>3 (18.75)</td>
<td>1.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.441&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Attack</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dystonia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>11 (6.01)</td>
<td>0 (0.00)</td>
<td>1 (6.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>172 (93.99)</td>
<td>29 (100.00)</td>
<td>15 (93.75)</td>
<td>1.000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.356&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Chorea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>94 (51.37)</td>
<td>21 (72.41)</td>
<td>11 (68.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>89 (48.63)</td>
<td>8 (27.59)</td>
<td>5 (31.25)</td>
<td>0.182&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ballism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>173 (94.54)</td>
<td>26 (89.66)</td>
<td>15 (93.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10 (5.46)</td>
<td>3 (10.34)</td>
<td>1 (6.25)</td>
<td>1.000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Duration (sec)</strong></td>
<td><strong>≤ 10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>65 (36.72)</td>
<td>17 (58.62)</td>
<td>13 (81.25)</td>
<td>0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.304&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10 to 30</td>
<td>87 (49.15)</td>
<td>11 (37.93)</td>
<td>3 (18.75)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>≥ 30</strong></td>
<td>25 (14.12)</td>
<td>1 (3.45)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><strong>Face involvement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>109 (59.89)</td>
<td>25 (86.21)</td>
<td>13 (81.25)</td>
<td>0.092&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.992&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>73 (40.11)</td>
<td>4 (13.79)</td>
<td>3 (18.75)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Differences among PRRT2+, TMEM151A+, KCNJ10+ patients compared by <sup>a</sup>χ² test, <sup>b</sup>Yates’ corrected χ² test, <sup>c</sup>Fisher exact probability, and <sup>d</sup>two sample t-test. p value was set at 0.05.
<table>
<thead>
<tr>
<th>Treatment effect</th>
<th>PRRT2+ N (%)</th>
<th>TMEM151A+ N (%)</th>
<th>KCNJ10+ N (%)</th>
<th>P Value (P1) (PRRT2 vs. KCNJ10)</th>
<th>P Value (P2) (TMEM151A vs. KCNJ10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Relief</td>
<td>91 (79.82)</td>
<td>14 (82.35)</td>
<td>6 (66.67)</td>
<td>0.191(^c)</td>
<td>0.550(^c)</td>
</tr>
<tr>
<td>Incomplete Relief</td>
<td>21 (18.42)</td>
<td>3 (17.65)</td>
<td>2 (22.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No response</td>
<td>2 (1.75)</td>
<td>0 (0.00)</td>
<td>1 (7.14)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Differences among PRRT2+, TMEM151A+, KCNJ10+ patients compared by \(^a\)\(\chi^2\) test, \(^b\)Yates’ corrected \(\chi^2\) test, \(^c\)Fisher exact probability, and \(^d\)two sample t-test. \(p\) value was set at 0.05.

Heterozygous KCNJ10 mutations lead to reduced Kir4.1 currents

All 15 mutants showed depolarized resting membrane potentials (RMPs) and increased membrane resistances (Rms) to different degrees compared with the wild type (Supplementary Table 2). Accordingly, apparent reductions in macroscopic K\(^+\) currents as well as Kir4.1-sensitive currents were observed in all the mutants (Fig. 2). Furthermore, according to the different degrees of decreased currents, the 15 mutants were categorized into 3 groups: complete blockade of Kir4.1 (bare currents were detected, including c.191G > A, c.321_322del, c.436C > T, c.554C > A, c.776T > C, c.883C > T, c.889C > T, c.910_912del), partial blockade of Kir4.1 (approximately 80% of the currents were detected, including c.539G > A, c.811C > T, c.1028G > A and c.1105G > A) and slight blockade of Kir4.1 (approximately 20% of the currents were blocked, including c.724A > G and c.1042C > T) (Fig. 2I).

The expression pattern of Kir4.1 in mice

To explore the expression pattern of Kir4.1 in the central nervous system, we measured the level of mouse Kcnj10 mRNA by real-time PCR. We found that in the whole brain, Kcnj10 was relatively low during the embryonic period, markedly increased during postnatal stages, peaked at postnatal day 30 (P30), and declined in adulthood (Fig. 3A). Similar expression trends were observed in the cerebellum (Fig. 3B) and forebrain (Fig. 3C). Immunofluorescence analyses in P30 mice confirmed that Kcnj10 mostly expressed in astrocytes (Fig. 3D) and was ubiquitously expressed in the central nervous system, with high levels in the cerebellar cortex, olfactory bulb, thalamus, hypothalamus, pons, cortex and hippocampus (Fig. 3E). Similar to mRNA level, the protein level of Kir4.1 also peaked at P30 during development, confirmed by immunofluorescence in the cerebellum and cortex of P7, P30, P56 and P84 mice (Fig. 3F-H).

Given the prominent role of the cerebellum in the pathogenesis of PKD, we generated Kcnj10-cKO mice with conditional heterozygous knockout of Kir4.1 in cerebellar astrocytes. Specifically, we crossed Kir4.1\(^{\text{flox/flox}}\) mice with Rosa26-mGFP mice. AAV2/5-gfaABC1D-iCre-WPRE-pA was then stereotaxically injected into the
cerebellar vermis of Kir4.1^+/floxed::Rosa26-mGFP (Kcnj10-cKO) and WT mice as controls (Fig. 4A). Three weeks after injection, to assess the transfection efficiency, immunohistochemical analyses were performed and revealed that 97.68 ± 0.67% (mean ± s.e.m., n = 3) of cerebellar astrocytes of Kcnj10-cKO coexpressed GFP (Fig. 4B, C) with ablation of Kir4.1 (Fig. 4D).

Because Kir4.1 channel in astrocyte are crucial for the maintenance of extracellular K^+ concentration and thus influence the neural excitability, we applied K^+ stimulation in the cerebellar cortex of Kcnj10-cKO mice to induce the dyskinetic postures in animals. The results showed that stiff lower limb and tail was induced in Kcnj10-cKO mice (occurrence%=66.7%, n = 6), while no abnormal dystonic posture was induced in WT mice (n = 6) (Fig. 4M, N). The induced dystonic phenotype suggested that impaired cerebellar Kir4.1 channel is associated with the abnormal motor function in mice.

The rotarod test was performed to assess the motor function of cerebellar Kcnj10-cKO mice, and the test was repeated four times for each mouse (Fig. 4E). Both the latency and the speed of the rod at the point of falling were significantly reduced in the Kcnj10-cKO group compared to the controls (Fig. 4F, G), indicating a deficiency of motor coordination in cerebellar Kir4.1-knockout mice. In addition, Kcnj10-cKO mice also showed poorer motor learning ability, as they failed to show improvement during repeated trials (Fig. 4H, I).

In the cerebellum, the deep cerebellar nuclei (DCN) play an essential role in motor coordination. Since poor balance was observed in Kcnj10-cKO mice, we subsequently investigated DCN neuronal firing in Kcnj10-cKO mice compared with WT mice (Fig. 4J), and the results showed that the average firing rate of the DCN neurons in Kcnj10-cKO mice was increased (WT: 41 recorded neurons from 4 mice ; Kcnj10-cKO: n = 62 recorded neurons from 4 mice) (Fig. 4K, L), indicating that hyperexcitability occurred in DCN neurons due to an elevated K^+ level in the extracellular space in Kir4.1-deficient mice.

**Discussion**

PKD is representative of paroxysmal dyskinesia, and it is widely accepted that primary PKD is attributed to genetic factors, but more than 60% of PKD patients are not identified with certain mutations. In our previous study, we identified TMEM151A as the second causative gene for PKD by means of GRIPT. Here, we applied the same strategy to analyse the WES data of both PRRT2- and TMEM151A-negative patients and eventually filtered out and focused on the KCNJ10 gene. Because PKD is a rare disease with incomplete genetic penetrance, the high frequency of KCNJ10 variants in PKD patients suggests its potential role in the pathogenesis of PKD.

KCNJ10 (NM_002241.5), located on chr 1q23.2, consists of 2 exons and encodes 379 amino acids. Its protein product is the inwardly rectifying potassium (Kir) 4.1 channel. The primary structure of the Kir4.1 subunit is composed of two transmembrane (TM) regions, an extracellular pore-forming region, which has a -G-Y-G- signature sequence acting as an ion filter, and intracellular N- and C-terminal domains (Fig. 1B). In this study, a total of 15 KCNJ10 variants (4 nonsense/frameshift and 11 missense) were identified, and most of them (11 mutations) cluster in the N-terminal cytoplasmic domain (Fig. 1B), while mutation c.436C>T (p.Leu146Phe) is in the transmembrane domain and two frameshift mutations (c.321_322del
(p.Val109Glyfs*15) and c.422delC (p.Pro141Hisfs*57)) are in the extra-membrane domain. It was initially known that homozygous and compound heterozygous \textit{KCNJ10} mutations are associated with sensorineural deafness, ataxia, impaired intellectual development, and electrolyte imbalance (SeSAME syndrome) and enlarged vestibular aqueducts.\textsuperscript{25} Frequent mutations of the \textit{KCNJ10} gene in patients with SeSAME syndrome can cause marked inhibition of Kir4.1 channels.\textsuperscript{24} To validate whether the variants in this study lead to the dysfunction of Kir4.1, we investigated the electrophysiological changes of the mutants, and the results illustrated that all the mutations caused the decreased Kir4.1 current to different degrees. Moreover, most mutations (13/15) profoundly reduced the Kir4.1 current ($\geq 80\%$ Kir4.1-sensitive currents blocked), indicating that the mutations identified in PKD patients significantly disturbed the function of Kir4.1. All the patients with \textit{KCNJ10} presented typical clinical features of PKD and it is hard to conclude the relationship between the degree of the Kir4.1 blockage and the severity of phenotype in this study. However, the clinical and genetic heterogeneity of PKD is widely accepted as one mutation could cause different phenotype varying from asymptomatic carriers to complicated form of PKD.\textsuperscript{1,5} The underlying reason for the heterogeneity is unclear but more collected data in the following study would assist to clarify the mechanism.

However, whether Kir4.1 defects are related to PKD needs further exploration. Therefore, we investigated the expression pattern of \textit{Kcnj10}, and the results showed that \textit{Kcnj10} mRNA was relatively low during the embryonic period, significantly increased during postnatal stages, peaked at adolescence (P30), and finally declined in adulthood. In 2015, Moroni et al. disclosed a gradual increment of Kir4.1 expression in the somatosensory cortex of rats during postnatal to P30.\textsuperscript{26} In the present study, we detected an increment before P30 but a progressive decrease during P30 ~ P84 of Kir4.1 expression in both cerebellum and cortex of mice by quantifying the immunoreactivity of Kir4.1 immuno-positive cells in cerebellum and cortex, which is accordance with the mRNA expression pattern during qPCR. The finding is consistent with the natural course of PKD, and the other two specific proteins related to PKD, \textit{Prrt2} and \textit{Tmem151a}, also share the same tendency of expression.

Although the mechanism of PKD is not yet well elucidated, it has long been established that this disease is related to the disturbance or imbalance in neural excitability based on its clinical features and electrophysiological studies\textsuperscript{19}. Moreover, recent studies have highlighted that abnormal excitability in the cerebellum is involved in PKD.\textsuperscript{17,20,21} Because of the high expression of \textit{Kcnj10} in the cerebellum, we further explored the motor ability of cerebellar \textit{Kcnj10-cKO} mice. Poor balance and deficits in motor learning ability were obvious in \textit{Kcnj10-cKO} mice, revealing that the dysfunction of cerebellar Kir4.1 could disturb motor function in mice. Although Kir4.1 is mostly expressed in astrocytes in the nervous system, it plays a crucial role in maintaining the structural and functional integrity of the brain, including formation of the blood–brain barrier, regulation of synaptogenesis, and maintenance of metabolic, ionic, and neurotransmitter homeostasis.\textsuperscript{24} Among these functions, spatial potassium ($K^+$) buffering by astrocytes is an essential system for controlling extracellular $K^+$ concentration and neuronal excitability.\textsuperscript{27,28} It has been validated that the inhibition of Kir4.1 channels by gene mutations, expressional suppression, or pharmacological treatments could compromise the spatial buffering of $K^+$, resulting in increased extracellular $K^+$ and depolarized RMPs.\textsuperscript{24} In addition, Kir4.1 channel-mediated spatial $K^+$ buffering is
functionally linked to excitatory amino acid transporters (EAATs) and plays an important role in glutamate homeostasis in tripartite synapses. Kir4.1 channel blockade can cause a depolarization of astrocytes, which in turn inhibits astrocytic glutamate uptake via EAAT2. Elevated levels of extracellular K\(^+\) and glutamate are both key factors for the hyperexcitation of neurons. In accordance with the theory, we induced the dystonic posture in cerebellar Kcnj10-cKO mice by applying high K\(^+\) concentration on local surface of the cerebellar cortex. The inducement of the phenotype not only verified that defect Kir4.1 might be associated with the attack of PKD, but also indicated that the abnormal excitability is related with the impaired spatial K\(^+\) buffering by astrocyte. Further aberrant DCN neuronal firing was observed in cerebellar Kir4.1-deficient mice in the present study. In the cerebellum, the DCN plays an essential role in motor coordination, and its dysfunction has been implicated in animal models of dyskinesia. The increased rate of DCN firing in this study suggests hyperexcitability of the cerebellum, which is involved in the mechanism of PKD. In Prrt2 mutant mice, similar electrophysiological abnormalities in the DCN have also been observed. Therefore, we speculate that the dysfunction of Kir4.1 caused by heterozygous KCNJ10 variants might be associated with abnormal hyperexcitability in the cerebellum, which is involved in the pathogenesis of PKD. In other words, cerebellar Kir4.1 probably acts as a negative modulator of neural excitability in PKD. However, a more detailed mechanism of Kir4.1 in the regulation of cerebellar neurons in PKD remains to be discovered.

In this study, KCNJ10 variants accounted for approximately 3.07% (16/522) of PRRT2- and TMEM151A-negative PKD probands. Its frequency in overall PKD patients is lower than that of PRRT2 and TMEM151A mutations. Although DNA analysis was not conducted in all the parents of the probands, the current results of co-segregation analysis showed that incomplete penetrance is apparent in KCNJ10-related PKD, which is also common in PKD with PRRT2 and TMEM151A mutations. Due to the limited parents enrolled, the exact incomplete penetrance could not be obtained. However, PRRT2 mutations are identified in one-third of PKD patients and the prevalence of PRRT2 mutations in familial PKD is much higher than in sporadic cases, indicating that incomplete penetrance is more common in sporadic cases and other genotype except PRRT2. Following DNA analysis would be continued to figure out the accurate incomplete penetrance in PKD patients with KCNJ10 mutations and the underlying reason for the high frequency of incomplete penetrance in PKD. Regarding clinical manifestations, patients with KCNJ10 mutations are more similar to those carrying TMEM151A mutations than PRRT2+ patients. KCNJ10+ patients have predominantly sporadic cases, tending to have a later onset age and a short duration of attacks. Other phenotypic spectra of the KCNJ10+ patients, including triggers, attack form, occurrence of facial involvement and aura, were similar to the patients with PRRT2 and TMEM151A mutations in our previous studies. Nevertheless, as patients with KCNJ10 mutations are still limited, more subjects are needed to determine the definite phenotype-genotype correlation. Moreover, an increasing number of studies have reported the relationship between brain diseases and Kir4.1 dysfunction despite classical SeSAME, including depressive disorders, epileptic diseases, Huntington’s disease, autism spectrum disorders, Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, and neuropathic pain. In the present study, 3 mutations (c.889C > T/p.Arg297Cys; c.811C > T/p.Arg271Cys; c.1042C > T/p.Arg348Cys) were previously documented in other phenotypes (SeSAME syndrome; digenic non-syndromic hearing loss;
However, all the 3 documented variants were reported in cases with homozygous or compound heterozygous mutations, while heterozygous $KCNJ10$ mutations are the cause for PKD. In general, the symptoms in diseases with autosomal recessive inheritance are more severe than that in disorders with autosomal dominant inheritance. SeSAME syndrome is a complicated syndrome composed of several symptoms including seizures, sensorineural deafness, ataxia, impaired intellectual development, and electrolyte imbalance. In contrary, $KCNJ10$-related PKD is much less severe in manifestation compared with SeSAME. The different type of inheritance and phenotype indicate that impaired Kir4.1 in different degree could result in a wide range of clinical phenotypes and PKD would be a new spectrum and milder phenotype of Kir4.1-associated channelopathy.

**Conclusion**

Overall, we identified heterozygous $KCNJ10$ mutation as a novel genetic cause of PKD through the GRIPT method, broadening the known spectrum of PKD genotypes as well as Kir4.1-related channelopathy phenotypes. $KCNJ10$ mutations account for 3.06% of $PRRT2$- and $TMEM151A$-negative PKD, following the proportion of $PRRT2$ and $TMEM151A$ mutations in PKD patients. The phenotype of $KCNJ10$-related PKD is much more similar to that of patients carrying $TMEM151A$ variants, as $KCNJ10$ mutations are more common in sporadic cases and are prone to present a shorter duration of attacks and to have a later onset age. All the mutations identified in the present study led to reduced Kir4.1 currents to different degrees. Dystonic posture could be induced in heterozygous $Kcnj10$-cKO mice by high K$^+$ concentration in cerebellum and poor motor coordination and abnormal cerebellar electrophysiological changes were also observed. Based on the findings in the present study, we suppose that the dysfunction of cerebellar Kir4.1 might result in defective homeostatic maintenance of extracellular potassium and glutamate levels and thus lead to abnormal neuronal excitability, which is involved in the mechanism of PKD. The identification of the $KCNJ10$ gene for PKD elucidated the pathogenesis of PKD, although additional efforts are needed to further reveal the whole picture of the role of Kir4.1 in movement disorders.

**Abbreviations**

PKD
paroxysmal kinesigenic dyskinesia
$KCNJ10$
potassium channel inwardly rectifying subfamily J member 10
Kir4.1
inwardly rectifying potassium channel 4.1
SeSAME
seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance
$PRRT2$
proline-rich transmembrane protein 2
$TMEM151A$
transmembrane protein 151A
GRIPT
Gene Ranking, Identification and Prediction Tool.

**Declarations**

**Data availability**

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

**Author contributions**

Xiaojun Huang analyzed and interpreted the data, and was a major contributor in writing the manuscript. Xin Fu and Jingying Wu analyzed and interpreted the data, and also participated in writing the manuscript. Xiaoqi Hong assisted in the electrophysiological experiments. Ziyi Li interpreted the clinical data of enrolled patients. Lan Zheng, Qing Liu, Shendi Chen, Beisha Tang, Yuwu Zhao, Xiaorong Liu, Xunhua Li, Xiaoli Liu, Zaiwei Zhou, Li Wu, Kan Fang, Ping Zhong, Mei Zhang, Xinghua Luan participated in the clinical evaluation of recruited patients. Wotu Tian analyzed and interpreted the data, and revised the manuscript. Xiaoping Tong provided critical comments and suggestions in electrophysiological experiments, and further revised the manuscript. All authors read and approved the final version of the paper.

**Acknowledgements**

We appreciate the cooperation of all the patients and their families. We are indebted to all the members from the China Paroxysmal Dyskinesia Collaborative Group (CPDCG) for their contributions. In addition, we thank Dr Wu Jingchuan from the Department of Neurosurgery, Huashan Hospital, and the innovative research team of high-level local universities in Shanghai for their support (SHSMU-ZDCX20211901).

**Funding**

Prof. Cao received funds from the National Natural Science Foundation of China (No. 81870889) and the Training Program for Research Physicians of Innovative Translational Ability (SHDC2022CRD037).

Prof. Tong received funds from the Ministry of Science and Technology China Brain Initiative (2022ZD0204702), the National Natural Science Foundation of China (82271466, 31970904).

Dr. Wotu Tian received funds from the National Natural Science Foundation for Young Scholars of China (No. 82201398), the China Postdoctoral Science Foundation (No. 2022M712117), and the Shanghai Pujiang Program (22PJ052).

**Competing interests**

The authors report no competing interests.
Ethics approval and consent to participate

This study was approved by the Shanghai Jiao Tong University School of Medicine Affiliated Sixth People’s Hospital Foundation Ethical Committee. Written informed consent was obtained from all individual participants or their caregivers.

Consent for publication

Not applicable.

References


Figure 1

Heterozygous \textit{KCNJ10} variants in PKD patients and previously reported \textit{KCNJ10} variants. (A) The chromatograms and pedigrees of the \textit{KCNJ10} variants identified in PKD patients. All the mutations detected are located in the highly conserved region of proteins. I-1 (Father) in Family 1 was a carrier of mutation c.883C>T (p. Gln295X) without clinical manifestation, while both II-1 and II-2 presented as PKD. In Family 2, the mutation c.191G>A (p. Trp64X) was cosegregated in parents. (B) Schematic drawing of the protein domains and diagram of the 51 known \textit{KCNJ10} variants (NM_002241, green: previously reported, red:
discovered in this study). A total of 15 KCNJ10 variants (4 nonsense/frameshift and 11 missense) were identified in present study. 11 mutations cluster in the N terminus cytoplasmic domain, while mutation c.436C>T (p. Leu146Phe) locates in transmembrane domain and two frameshift mutations (c.321_322del (p. Val109Glyfs*15) and c.422delC (p. Pro141Hisfs*57)) are in extra-membranous domain.

Figure 2
Mechanistic studies of Kir4.1 mutants in HEK-293 cells display reduced macroscopic currents and Ba\(^{2+}\)-sensitive Kir4.1 currents. (A) Traces from HEK-293 cells that were transfected with Kir4.1-WT-EGFP, Val259Ala, Arg343His, and Arg348Cys. The macroscopic current waveforms were elicited by step depolarizations from −140 to +70 mV (in 10-mV steps). The mutants were three representatives of the three groups: mutants with current blocking by 99% (B), 80% (C), and 20% (D). (B-D) Average I/V plots for macroscopic currents for WT and mutant Kir4.1. (E) Traces from HEK-293 cells that were transfected with Kir4.1-WT-EGFP, Val259Ala, Arg343His, and Arg348Cys. The Kir4.1-sensitive currents were obtained by subtracting the currents with BaCl\(_2\) treatment (100 μM) from the macroscopic currents. The mutants were three representatives of the three groups: mutants with current blocking by 99% (F), 80% (G), and 20% (H). (F-H) Average I/V plots for Kir4.1-sensitive currents for HEK-293 cells expressing WT and mutant Kir4.1. (I) Statistical chart of Kir4.1-sensitive currents of WT and mutant Kir4.1 at -140 mV (pA). For the I/V plots, in some cases, the error bars (±s.e.m.) are smaller than the symbols used. *** p<0.001, * p<0.05.
Figure 3

**Temporal and spatial expression of Kir4.1.** (A-C) Relative expression level of *Kcnj10* mRNA in the whole brain (A), cerebellum (B) and forebrain (C) during development. The expression level in P30 was used as a calibration (mean ± s.e.m., n=3). (D, E) Representative immunostaining image of Kir4.1 in the P30 mouse brain. D, scale bar, 50 μm. E, Left panel, scale bar, 2 mm; right panel, scale bar, 500 μm. Ob, olfactory bulb; Ctx, cortex; Hp, hippocampus; Tha, thalamus; Hpt, hypothalamus; Cb, cerebellum. (F) Expression of Kir4.1 in
P7, P30, P56 and P84) mouse cerebellar and cortic astrocytes. Scale bar, 50 μm. (G, H) Quantification of Kir4.1 expression in the cerebellum (G) and cortex (H) (mean ± s.e.m., n=4, unpaired *t* test).

Figure 4

**Motor dysfunction and aberrant firing of deep cerebellar nuclei caused by cerebellar knockout of Kir4.1.** (A) Schematic diagram of conditional knockout of Kir4.1 in cerebellar astrocytes. (B-C) Representative image of AAV transfection of Kir4.1<sup>+/lox</sup>:Rosa26-mGFP mice. G, left panel, scale bar, 500 μm; right panel. Scale bar, 2 mm. H, scale bar, 50 μm. (D) Representative image of the ablation of Kir4.1 expression after AAV injection. Upper panel, WT group; lower panel, *Kcnj10-CKO* group. Scale bar, 50 μm. (E) Schematic diagram of the rotarod test. (F, G) Mean latency to fall (F) and maximum rotation speed of the rod (G) in WT and *Kcnj10-CKO* mice in the rotarod test. WT, n=9 mice; *Kcnj10-CKO*, n=8 mice; mean ± s.e.m., unpaired *t* test. (H, I) Latency to fall (F) and maximum rotation speed of the rod (G) of WT and *Kcnj10-CKO* mice in each trial of the rotarod test. WT, n=9 mice; *Kcnj10-CKO*, n=8 mice; mean ± s.e.m., two-way ANOVA. (J-K) Schematic
diagram (J) and representative traces of activity in the deep cerebellar nuclei of WT and $Kcnj10$-cKO mice (K). (L) Averaged firing rate in the deep cerebellar nuclei of WT and $Kcnj10$-cKO mice (WT: N=4 mice, n=41 neurons; $Kcnj10$-cKO: N=4 mice, n=62 neurons). (M, N) Representative image and occurrence of dyskinesia attack with epidural KCl stimulation in WT and $Kcnj10$-cKO mice (WT, n=6 mice; $Kcnj10$-cKO, n=6 mice).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable.docx