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Original Article

L51P, a novel mutation in the PAS domain of hERG channel, confers long QT syndrome by impairing channel activation

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Abstract: The *human ether-à-go-go-related gene* (hERG) potassium channel mediates the repolarization of ventricular action potentials. Mutations in the *KCNH2* cause long QT syndrome (LQTS) and are associated with cardiac arrhythmias and sudden death. Here, we functionally analyzed a mutation of hERG potassium channel (p.L51P), gaining novel insights into clinical genotype-phenotype relationships. Potassium currents were recorded by whole-cell patch clamping in HEK293 cells transiently transfected with wild-type and/or mutant hERG potassium channel. Immunofluorescence assay and confocal imaging were undertaken to study the effects of L51P mutation on channel trafficking. The models of the protein structure of hERG and its mutations are predicted by Amber16 software. Molecular dynamics (MD) of individual protein were performed with Particle Mesh Ewald (PME). The production of MD simulations of hERG-WT and hERG-Mut at constant pressure and temperature were carried out with SHAKE. L51 was a conservative amino acid, located in the Per-Arnt-Sim (PAS) domain of the amino terminus. L51P caused loss of function via impairing channel activation. L51P was predicted to destroy hydrophobic structure in the PAS domain, thus causing the failure of channel opening. In summary, the present study identifies L51P as a novel mutation of hERG potassium channel. L51P mutation mechanistically impairs channel activation, reducing channel functionality.

Keywords: L51P, long QT syndrome, hERG, PAS, channel activation

Introduction

Congenital long QT syndrome (LQTS) is characterized by an abnormal prolongation of the QT interval in the 12-lead electrocardiogram (ECG) and the occurrence of life-threatening ventricular tachyarrhythmias, particularly *torsade de pointes*. It causes recurrent syncope and sudden cardiac death (SCD) [1]. More than 600 mutations have been identified in 16 LQTS genes. Causative variants in three genes, *KCNQ1*, *KCNH2* and *SCN5A*, are detected in the vast majority of individuals with clinical diagnosis of LQTS [2, 3]. Mutations in *KCNH2,* which encodes *human ether-à-go-go-related gene* (hERG) potassium channel, result in type 2 LQTS (LQT2).

The hERG channel generates the rapidly-activating delayed rectifier current (I_{Kr}) and is responsible for ventricular repolarization in humans [4]. The channel switches between closed, open or inactivated states. Like other voltage-gated channels, activation gates of hERG channels are tightly packed in the closed state at resting potential, blocking the entrance of the pore. Upon depolarization, the channel is poised to be opened. However, the inactivation of hERG channels exhibits several distinctive yet unique features. Once the activation gates are open, inactivation occurs rapidly. Given that the inactivation is much faster than the activation, no currents could be mediated by hERG channels during depolarization. This property of hERG channel critically determines the duration of the plateau phase of action potential in myocytes [5]. Although hERG channels do not mediate current at this phase, activation gates continue to open slowly and proceed with depolar-

Table 1. Primer sequences for hERG point mutations

Mutation	Primer Pairs (5'-3')
L51P	GCTTCTGCGAGCCGTGCGGCTACTC
	GAGTAGCCGCACGGCTCGCAGAAGC
L51G	GCTTCTGCGAGGGGTGCGGCTACTC
	GAGTAGCCGCACCCCTCGCAGAAGC

ization. During repolarization, activation gates of hERG channels return to the closed state, referred to as deactivation. The speed of deactivation, however, is very slow. In addition, hERG channels recover from inactivation with a velocity much more rapid than that of the deactivation, thereby allowing potassium efflux and facilitating repolarization [5]. Thus, loss-of-function mutants of hERG channels with altered structures in crucial functional domains frequently lead to LQTS.

The hERG channel consists of a 6-helix transmembrane core-region flanked by two cytosolic domains: an N-terminal Per-Arnt-Sim (PAS) domain and a C-terminal cyclic-nucleotide homology binding domain (cNBD). The PAS domain contains an N-terminal Cap (PAS-Cap, residues 1-25), the PAS core (residues 26-75), a connector helix (residues 76-87), and a scaffold region (residues 88-135) [6]. Residues 26-135 are also known as the globular region with a capability of interacting with cNBD [7]. The PAS domain, a hot spot for clinical mutation studies, has enabled the interrogation of novel clinical genotype-phenotype relationships. The vast majority of missense mutations in PAS domain have been documented to accelerate channel deactivation, reduce protein stability and/or impair channel trafficking [8-14]. Here we describe a previously unrecognized missense *KCNH2* mutation (c.152T>C, p.Leu51Pro) residing in the PAS core domain. Unlike most *KCNH2* mutations, p.Leu51Pro causes long QT syndrome by impairing channel activation.

Materials and methods

Clinical evaluation and genetic analysis

Patients were clinically evaluated by the Second Xiangya Hospital of Central South University. The study was approved by the Medical Ethics Committee of the hospital and conforms to the principles outlined in the Declaration of Helsinki. Informed consent for genetic analysis was given prior to the collection of blood samples for DNA extraction.

A Trio-WES strategy was applied to identify the causal variants from all the patients. The pathogenicity of the variants was predicted according to the current recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology [15]. The identified mutations among all family members were validated by Sanger sequencing.

Site-directed mutagenesis

The wild-type hERG (hERG-WT) plasmid was generously donated by Prof. Robert Kass and Dr. Lei Chen (Columbia University). Mutant constructs of L51P and L51G (hERG-Mut) were prepared with Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instruction. Primers were shown in **Table 1**. Mutation constructs were verified by Sanger sequencing.

Transient transfection of WT and mutant constructs in HEK293 cells

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (Gibco) under 5% CO_2 atmosphere. hERG-WT or hERG-Mut was transfected respectively with accessory subunit-MiRP1 using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. In current-recording experiments, hERG-WT or hERG-Mut was transfected respectively with GFP at a ratio of 10:1. GFP was used as an indicator of hERG-positive cells.

Immunofluorescence assay and confocal imaging

HEK293 cells were transfected with hERG-WT or hERG-Mut respectively and seeded in 35 mm Cover glass-bottom Confocal dish (SPL, 200350). Briefly, cells were fixed with 4% buffered paraformaldehyde for 30 min and permeabilized with Triton X-100 (0.1%) for 10 min. The cells were blocked with 5% BSA for 1 hour at room temperature to shield nonspecific binding sites. Cells were then incubated with anti-hERG (1:1000, Sigma) at room temperature for 2

hours and at 4°C overnight. Excessive primary antibody was washed off with PBST three times, 10 min per time. Secondary antibody (1:1000, Invitrogen) was incubated for 1 hour at room temperature and then incubated with Hoechst 33258 (1 μg/mL) for 5 min to stain nuclei. Imaging was performed with a laser scanning microscope (Olympus, Japan).

Whole cell current recordings

Whole cell patch clamp recordings were performed on transiently transfected HEK293 cells at room temperature with an Axopatch-200B amplifier (Axon Instruments). Pipettes were pulled with P2000 puller (Sutter) with resistance between 1.5 to 3.5 megohms. The cells were perfused with bath solution containing (mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4 with NaOH). The pipette solution contained (mM): 130 KCl, 1 $MgCl_2$, 5 EGTA, 5 MgATP, 10 HEPES (pH 7.2 with KOH). The voltage protocols were shown in the figure legends. Current amplitude was normalized versus cell capacitance as current density (pA/pF), and was used for I-V curve analysis.

Bioinformatics analysis

The impact of point mutations on the structure and function of the protein were initially assessed with PolyPhen-2 [16], SIFT [17] and I-Mutant2.0 [18]. The protein molecules of hERG-WT full length (FL) was then retrieved from Protein Data Bank (PDB ID: 5VA1) with high resolution of 1.65 Å. The mutated structure of FL hERG-Mut (L51P) was manually analyzed by software Pymol, and the retrieved hERG-Mut protein structure was then energy minimized up-to 1000 cycles with the assistance of Amber99ffsb force field in Amber16 software.

To evaluate the conformational changes between full length of hERG-WT and hERG-Mut with each subunit, we executed 10 ns full-scale atomistic molecular dynamics simulation using the AMBER general atom force field for the ligands and the AMBER FF12SB for the proteins.

Molecular dynamics (MD) of each protein was performed under periodic boundary conditions (PBC) with 10 Å non-bonded cut-off and using Particle Mesh Ewald (PME) for the long-range electrostatics. The initial structure of each protein has been firstly energy-minimized to 0K using a total of 1000 minimization steps (500 steepest descent followed by 500 steps with the conjugate gradient method). This minimization procedure was followed by two heating cycles consisting of 5000 MD steps. The first cycle raised the system temperature to 100 K. Finally the system was equilibrated to the simulation temperature of 300 K with the second cycles. The production of MD simulations of hERG-WT and hERG-Mut at constant pressure and temperature were carried out for 10ns each with a 2 fs integration step size with SHAKE.

Based on the final 6ns MDs trajectory, 6000 snapshots for hERG-WT and hERG-Mut structures were extracted from the last 6ns trajectory for the final average structure of protein complex.

Data analysis

All data are presented as mean ± S.E.M. (standard error of mean). N value denotes the number of cells. Statistical difference was determined by an unpaired two-tailed Student's *t*-test or one-way ANOVA with the Bonferroni *post hoc* test. *P*-value of <0.05 was considered as statistically significant.

Results

Screening of long QT genes

The proband (II:1; **Figure 1A**) was a 28-year-old female, who experienced recurrent syncope or seizure when awakened by an alarm clock or loud noise. Upon one of her syncopic episodes in the local hospital, *torsade de pointes* was recorded with ECG (**Figure 1B**). Her ECG revealed a wide T wave at rest, albeit a 54 bpm sinus rhythm with normal PR (148 ms) and QRS (84 ms) interval (**Figure 1C**). The corrected QT interval (QTc) was 475 ms. Echocardiography, exercise ECG test, and 24-hour Holter were completely normal. Evaluation of the family history identified the patient's mother (I:2; **Figure 1A**) died suddenly at an age of 24.

Next-generation sequencing of all currently known long QT genes demonstrated that the proband and her sister II:3 carried a heterozygous missense mutation in the *KCNH2* gene (NM_000238.3:c.152T>C) encoding for p.L51P

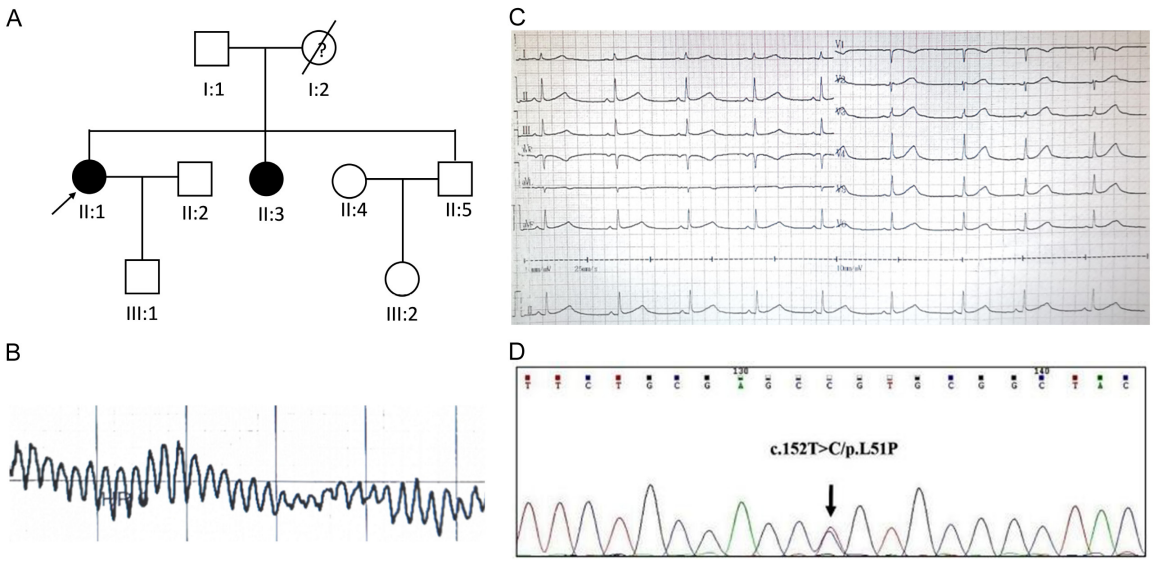

Figure 1. Clinical data. A. Pedigree of the studied family. Circles and squares represent females and males, respectively. Black symbols, clinically affected subjects with LQTS, the arrow indicates the proband. B. The electrocardiogram record of torsade de pointes. C. Twelve-lead electrocardiogram of the proband (paper speed 25 mm/s). D. DNA sequence chromatograms depicting the heterozygous c.453dupC and the c.152T>C change of the KCNH2 gene.

hERG (**Figure 1D**). To the best of our knowledge, this mutant has not been annotated in any available public database. The novel mutation in exon 2 of the *KCNH2* gene was further verified by Sanger sequencing. This missense mutation changes T to C at the 152 base pair of hERG gene. It encodes a mutant channel with the leucine-to-proline (L51P) mutation located in the N-terminal conservative PAS domain (**Figure 2**).

Since the mid-1970s, β blockers have been the mainstay of therapy for asymptomatic and/or symptomatic LQTS individuals with prolonged QT intervals. The patient was thus placed on a regime of propranolol and oral K^+ supplements. In the meantime, implantation of implantable cardioverter defibrillator (ICD) was recommended to but rejected by the patient. During the follow-up, she kept taking oral K^+ supplements but stopped the propranolol therapy due to significant sinus bradycardia.

Function of WT and mutant channels

Loss of channel function owing to mutations in hERG is the most common pathogenic mechanism underlying LQTS. Initially, the impact of L51P mutation on the structure and function of hERG was assessed with PolyPhen-2, SIFT and I-Mutant2.0. Polyphen-2 and SIFT categorize the point mutation as "possibly damaged"; it was predicted by I-Mutant as a mutated variant with markedly compromised structural stability. Subsequently, whole-cell patch clamp recordings were performed to verify the function of L51P mutation (**Figure 3A**). An initial "hook" could be observed at the beginning of deactivation traces in hERG-WT channels (**Figure 3B**). The slow activation and deactivation, along with rapid kinetics of inactivation and recovery of inactivation, is characteristic of hERG currents [5]. A fraction of hERG channels inactivate rapidly during depolarization. This inactivation state is removed promptly by repolarization, accounting for the ascending part of the hooked tail currents. By contrast, the descending part of hooked tail currents results from channel deactivation. As in **Figure 3**, representative traces for WT and L51P channels were shown (**Figure 3B-D**). I_{peak} of WT-I_{Kr} was plotted versus the testing voltage, with the curve being classically "bell-shaped" (**Figure 3E**). No currents could be elicited from L51P channels (**Figure 3C**). Given the heterozygous genotype of the patient, WT-hERG and mutated-hERG were co-transfected at a ratio of 1:1. I_{peak} and I_{tail} were significantly decreased (**Figure 3D-F**).

Mechanism underlying decreased function of L51P mutation

The decreased function of L51P is presumably attributable to: 1) impaired trafficking and decreased expression of hERG in the plasma

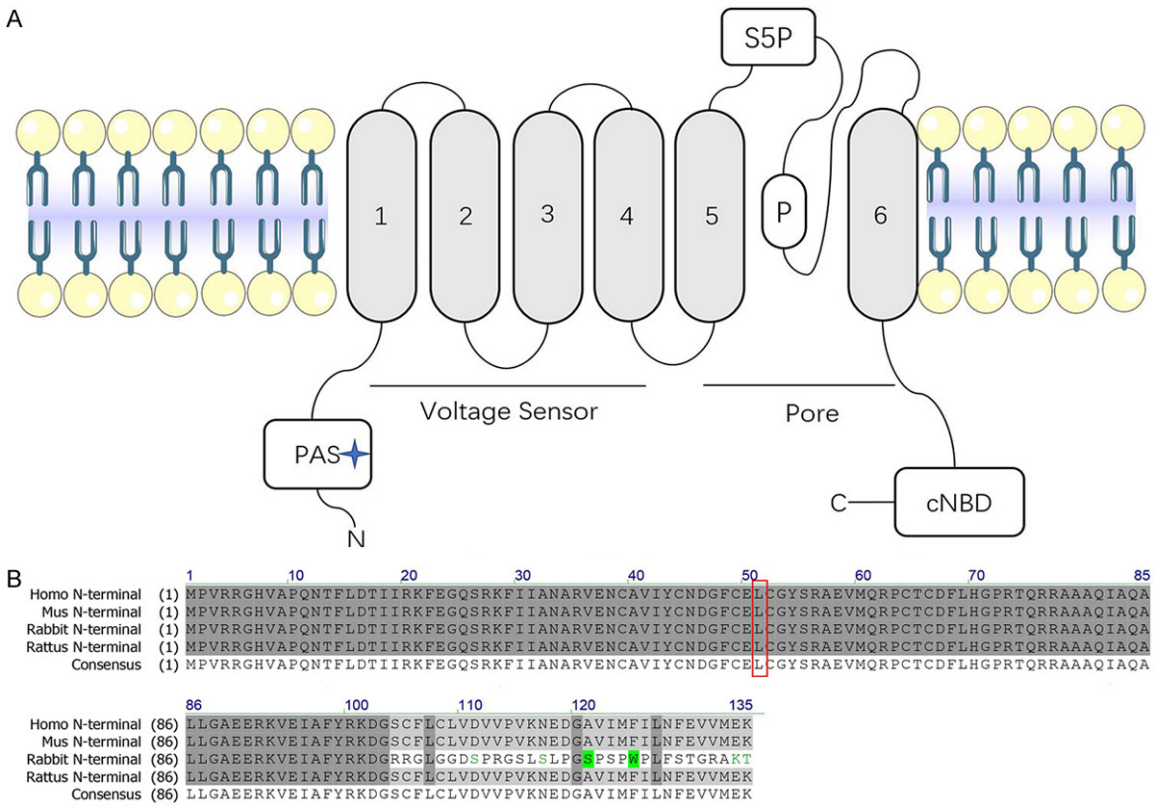

Figure 2. The cartoon of hERG α-subunit and alignment of PAS domain in different species. A. hERG α-subunit consists of six transmembrane domains (S1-S6). S1-S4 make up the voltage sensor domain (VSD), and S5-S6 together with the pore loop constitute the pore domain. The channel contains large and flexible cytoplasmic NH_2- and COOH-terminals. The novel mutation L51P identified in this study is located in the PAS domain and marked with a blue star. B. Amino acid sequences of different species are aligned. Dark-gray areas represent the identical residues and light-gray shaded areas indicate the conserved sequences among various species. The mutated residue discussed in this study (L51) is highlighted with a red rectangular box. L51 is highly conserved, implying an important role in maintaining channel function.

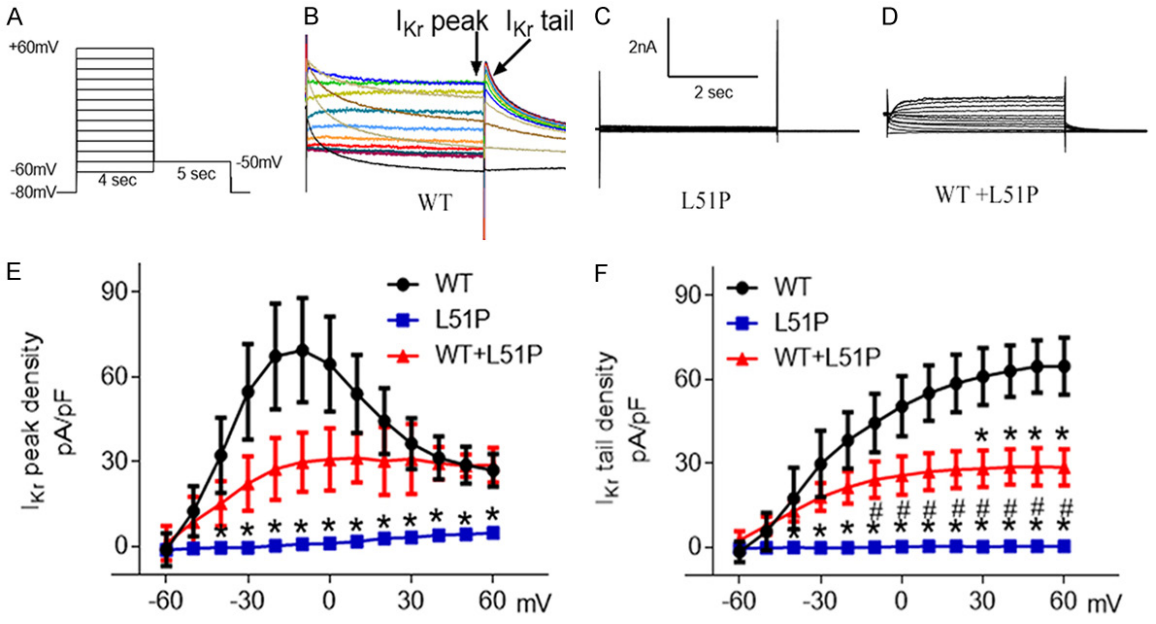

Figure 3. Whole-cell recordings of hERG-WT and hERG-L51P channels. (A) Voltage protocol. Cells were held at -80 mV, and then activated by 4-sec depolarizing steps from -60 to +60 mV in an increment of 10 mV. Cells were kept at -50 mV for 5 sec to elicit tail currents. (B-D) representative traces of hERG-WT (B), hERG-L51P (C) and WT-L51P (D) channels were shown respectively. (E) Typical "bell-shaped" I-V curves were presented. (F) The current density of tail current was plotted against corresponding potentials. (n=6-8; **P*<0.05 vs. HERG-WT; #*P*<0.05 vs. WT-L51P).

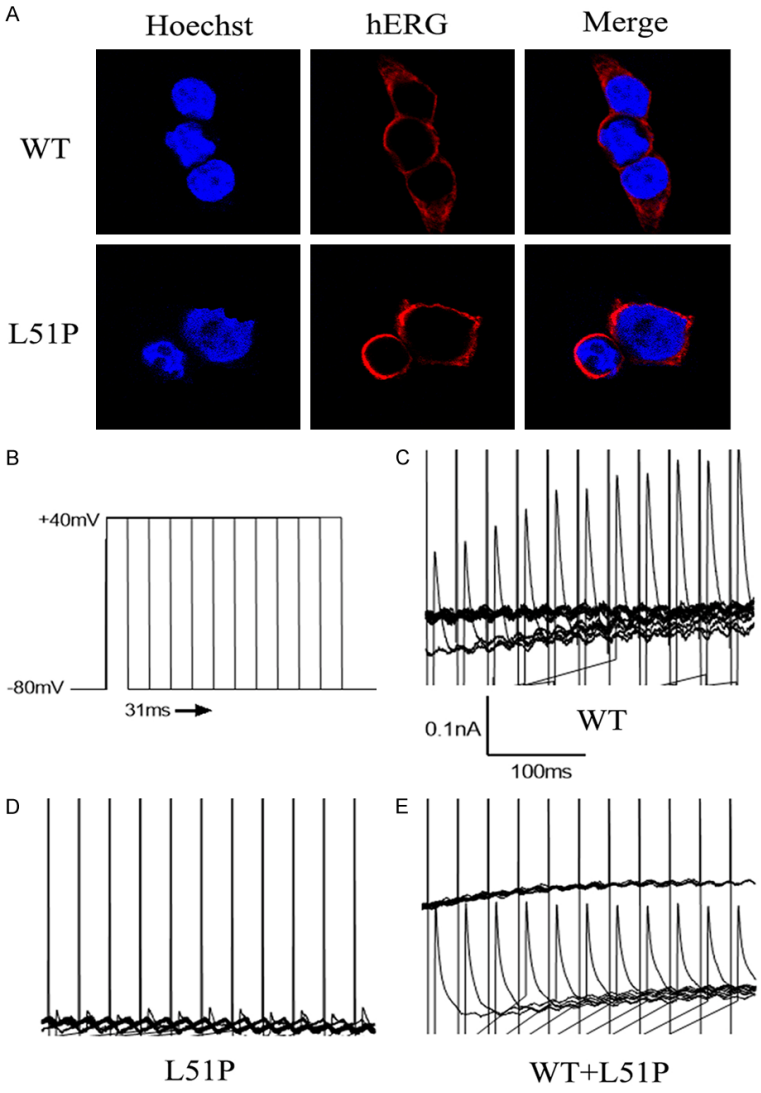

Figure 4. Mechanisms underlying hERG-L51P loss-of-function. (A) The expression level of hERG-L51P on cell surface was compared with hERG-WT using immunofluorescence staining (Blue-Hoechst 33258; Red-anti-hERG). (B) The activation of hERG channels was recorded with the envelope protocol. Cells were held at -80 mV before stepping to 40 mV for successively incremental durations. Then the voltage was repolarized to -80 mV and the peak tail currents were recorded. (C-E) Representative traces of hERG-WT (C), hERG-L51P (D) and WT-L51P (E) channels were shown respectively.

membrane; and/or 2) attenuated channel function. Hence, expression of WT-hERG and L51P-hERG was investigated with immunofluorescence assay. We observed that L51P mutation had no effects on channel trafficking (**Figure 4A**). The unusual overlapping gating kinetics of hERG channels rendered direct measurement of activation and inactivation technically difficult.

Taking this hurdle into account, a special "envelope protocol", as shown in **Figure 4B**, was employed to investigate channel activation of the L51P mutant. Cells were held at -80 mV before stepping to 40 mV for successively incremental durations; the voltage was then repolarized to -80 mV and the peak tail currents were recorded to reveal the proportion of channels activated during the preceding voltage (40 mV) (**Figure 4B**). With prolonged duration of depolarization, activatable hERG channels were increased in quantities, resulting in a larger initial hooked tail current during repolarization in cells expressing HERG-WT channels (**Figure 4C**). No currents could be elicited from L51P channels with this protocol (**Figure 4D**), implying deficient activation of the L51P mutant channel. In addition, L51P mutation also exerted suppressive effects on hERG-WT (**Figure 4E**).

The important role of L51 in stabilizing PAS structure and maintaining channel function

Based on bioinformatics analysis, L51, due to the hydrophobic environment and its long side chain, tends to form a stable and conservative hydrophobic domain (**Figure 5C**, **5E**). Polar amino acids K28 and R100 interact with surrounding negatively charged polar residues, thus forming

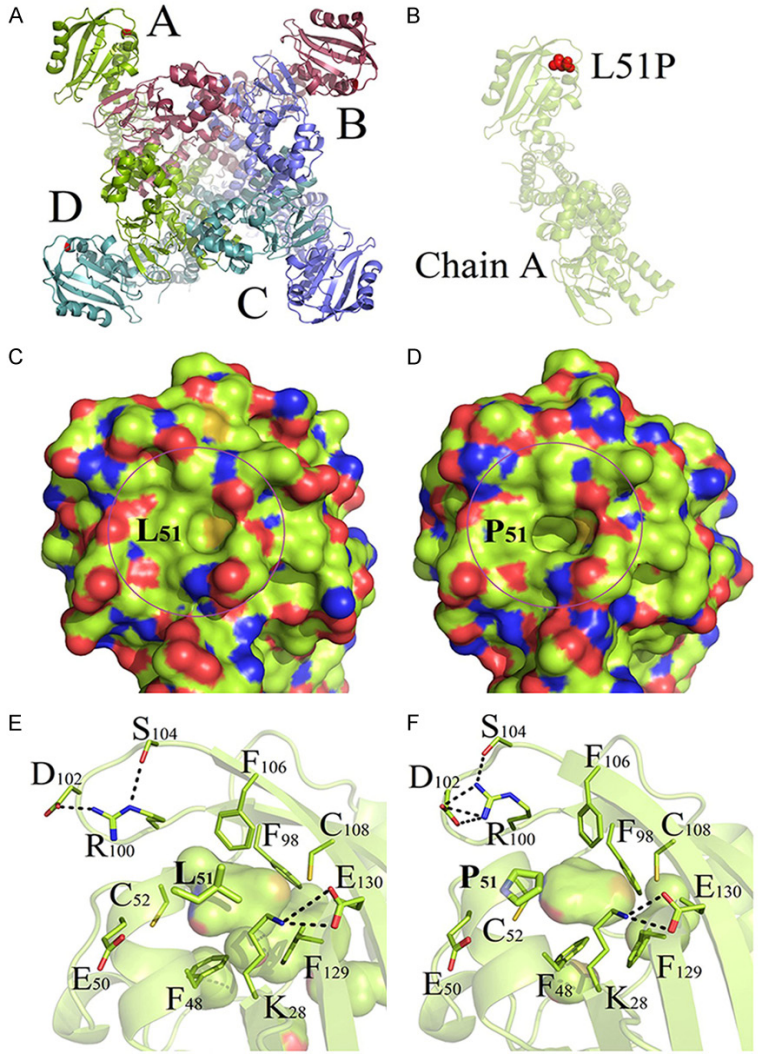

Figure 5. Structures of hERG-WT and hERG-L51P channels. (A, B) The crystal structures of hERG-WT (A) and hERG-L51P channel (B). (C, D) Surface residues and the electrostatic potential interface of PAS domain of hERG-WT (C) and hERG-L51P channel (D). (E, F) Structures of PAS domain of hERG-WT (E) and hERG-L51P channel (F). Bubbles reflect the internal space of PAS domain.

solid salt bridges. The firm hydrophobic domain and strong salt bridges stabilize the crystal structure of PAS domain (**Figure 5A**). The smaller side chain of proline prominently shrinks the conservative hydrophobic domain (**Figure 5B**, **5D**, **5F**), indicating potentially remarkable effects on channel function. To substantiate the hypothesis, we further mutated L51 to G, an amino acid without side chain, and assayed its function (**Figure 6C**). As revealed, L51G bore resemblance to L51P in both models of structural collapse (**Figure 6A**, **6B**) and functional deficiency (**Figure 6D-F**). Our results suggest an irreplaceable role for L51 in regulating hERG channel function.

Discussion

The hERG channel mediates ventricular repolarization in humans by generating delayed rectifier potassium currents. Mutations of hERG channels, usually giving rise to loss of function, lead to LQT2 [19]. It has been documented that mutations in the PAS domain, located in the N-terminus, lead to loss-of-function through speeding deactivation (F29A, F29L, N33T, G53R, R56Q, C66G, H70R, A78P and L86R) [8] or impairing channel trafficking (T65P) [20]. Here, we have identified a novel mutation L51P residing in the PAS core domain of hERG channel. This newly undisclosed mutation impaired channel activation, causing sinus bradycardia with prolonged QT interval.

The most common mechanism by which hERG mutants cause LQTS is the loss of channel function and a dominant negative effect on hERG-WT when co-expressed [8]. Aberrant function or inefficient channel trafficking, collectively known as loss-of-function phenotype, is attributable to changes in critical structures of the channel. Wild-type hERG channel encompasses six transmembrane segments (S1-S6); S1-S4 constitute the voltage sensor domain (VSD), and the pore domain is composed of S5-S6 together with the pore loop. In addition to transmembrane regions, hERG channels contain long and flexible cytoplasmic NH_2- and COOH-terminus. The activation gate resides in S6 transmembrane helix and part of the COOH-terminal inner helices. Once the positively charged residues in the S4 sense membrane depolarization, the rearrangements of VSD are conveyed as a signal to the activation gate via S4S5 linkers. Slow VSD movement is a rate-limiting step for the slow activation of hERG channels. Inactivation mainly results from the rearrangement of S5P and pore

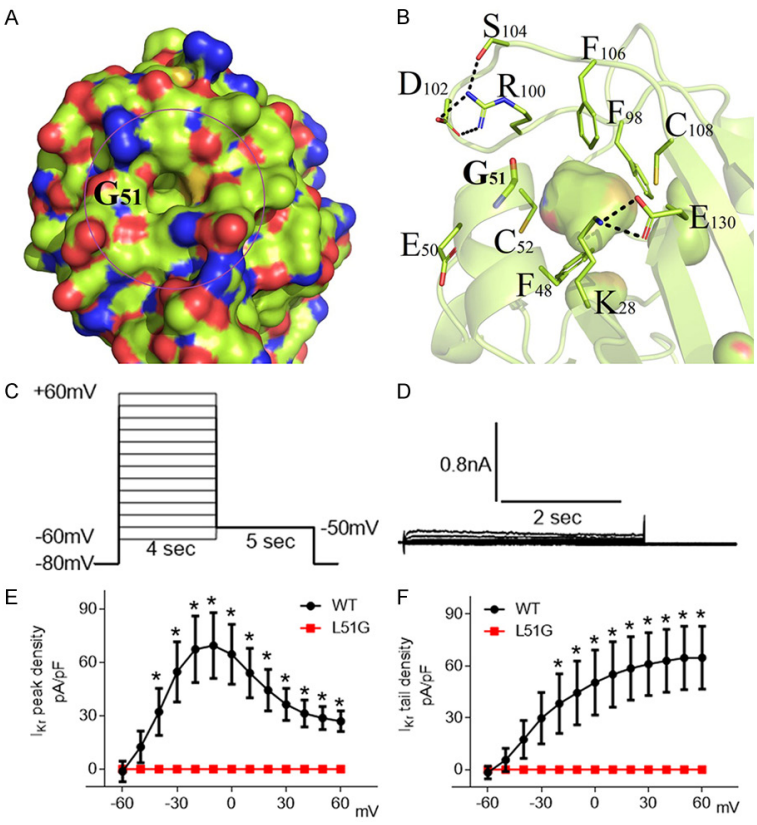

Figure 6. The effects of L51G on alpha-helix and PAS structure. A. Surface residues and the electrostatic potential interface of hERG-L51G channel. B. Structures of PAS domain of hERG-L51G channels. C. Voltage protocol. D. Representative traces of L51G were shown. E. Typical "bell-shaped" I-V curves were presented. F. The current density of tail current was plotted against corresponding potentials. (n=6-8; **P*<0.05 vs. hERG-WT; #*P*<0.05 vs. WT-L51P).

domain. S5P may serve as voltage sensing domain for fast channel inactivation [5]. The NH_2 terminus contains a PAS domain that renders hERG distinct from other voltage-gated potassium channels. PAS domain plays an important role in controlling slow deactivation via interaction with S4S5 linker [5, 21] or cNBD domain [5, 7, 22, 23].

Given the functionality of PAS domain, the L51P mutation of the patient in the present study would conceivably exhibit a loss-of-function phenotype via accelerated deactivation. To our surprise, no currents could be evoked from L51P hERG channels with either steady-state activation protocol or envelope protocol (**Figures 3A**, **4B**). On the basis of the available information on the residue position-channel function relationship, deficient activation of hERG largely results from mutations in VSD, S4S4 linker and S6 [5]. It is well known that PAS domain contains 5 anti-parallel β-sheets (βA-βE) and 3 α-helices (αA-αC) [24]. Residues 86-94 (between αC and βC) of PAS domain reportedly interact transiently and directly with S4S5 linker and stabilize hERG channels in the open state, thus leading to slow deactivation [5, 24]. Another putative model holds that PAS domain regulates deactivation by binding with the "cAMP binding pocket" of the cNBD domain [5, 7, 22]. Therefore, disruption of PAS motif represses the domain-domain interaction and accelerates deactivation [5, 21, 23], causing LQT2. Unexpectedly, hERG-L51P, a novel PAS domain-located mutation, resulted in loss-of-function by impairing channel activation.

Native I_{Kr} is composed of hERG1a and hERG1b heterotetramers. hERG1b lacks the first 373 amino acids of the hERG1a isoform but contains unique 36 amino acids at its N-terminus [5]. Although it has been demonstrated that hERG1b suppresses channel function by slowing channel activation [25], this effect could not be attributed to PAS domain alone. The region spanning from PAS domain to S1 may also contribute to regulate hERG channel activation in native I_{Kr}. Thus far, the majority of evidence suggests that PAS domain gates hERG channel principally by regulating channel deactivation. Recently, it has been found that the globular PAS domain-recognizing antibody accelerates the rate of activation [26]. This implied that PAS domain may participate in channel activation. Importantly, our data gleaned from a clinical patient with respect to a novel mutant of hERG channel uncovered a previously unappreciated pathogenic mechanism of hERG mutation. Specifically, the L51P mutation impaired hERG activation. Our results are the first line of evidence, to the best of our knowledge, demonstrating that PAS domain is important for hERG activation beyond channel deactivation. Physiologically,

wild-type PAS domain interacts with S4S5 linker and cNBD domain, and governs slow deactivation [5, 7, 22, 23]. Based on our results, it is inferable that PAS domain may interact with channel activation-related regions in closed state; freeze of the channel in closed state caused a deficiency in channel opening. It has been known that both S4S5 linker and cNBD domain contribute to channel activation. However, PAS domain only temporarily binds to S4S5 linker after the activation gate opens, while it remains interacted with the cNBD domain in both closed and open states [5]. We speculate that hERG-L51P may impair channel opening via interaction with cNBD domain. But further clues from bioinformatic analysis could not be gained due to a lack of the exact crystal structure of hERG cNBD domain. Previous studies have exploited the crystal structure of mouse EAG (homology of hERG) in simulation [23]. Unfortunately, mouse EAG did not share substantial similarity with hERG. Further investigations are needed in the future.

Our reported case of patient manifested prominent sinus bradycardia as well as prolonged QT intervals. However, sinus bradycardia in LQT2 seems rare, being sporadic as occasional cases in the afflicted families [27]. The presently known bradycardia-related mutations (R534C, A561V, K638del) are located in or around pore domain, and result in lowered current density, defective trafficking as well as altered channel gating [27]. Our case is the first example of bradycardia-associated, PAS domain-located and channel activation-suppressing hERG mutation. Intriguingly, inhibition of hERG with E-4031 (a classic inhibitor of hERG) showed similar effects to L51P mutation, decreasing heart rate [28, 29]. However, the exact role of hERG in pacemaker and bradycardia remains to be further explored.

In summary, L51P mutation has been demonstrated here to be a novel mutant of hERG channel, displaying a loss-of-function phenotype due to impaired channel activation. Our results offer novel insights into the function of PAS domain, underscoring its importance in controlling hERG opening. Our data suggest that targeted rescue of the function of PAS domain may be exploited for hERG channelopathies-related LQT2.

Acknowledgements

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Disclosure of conflict of interest

None.

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