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A Novel Mutation in *KCNQ1* Associated with a Potent Dominant Negative Effect as the Basis for the LQT1 Form of the Long QT Syndrome

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Abstract

Introduction—Long QT Syndrome (LQTS) is an inherited disorder characterized by prolonged QT intervals and life-threatening polymorphic ventricular tachyarrhythmias. LQT1 caused by *KCNQ1* mutations is the most common form of LQTS.

Methods and Results—Patients diagnosed with LQTS were screened for disease-associated mutations in *KCNQ1*, *KCNH2*, *KCNE1*, *KCNE2*, *KCNJ2* and *SCN5A*. A novel mutation was identified in *KCNQ1* caused by a 3 base deletion at the position 824–826, predicting a deletion of phenylalanine at codon 275 in segment 5 of *KCNQ1* ($\Delta F275$). Wild-type (WT) and $\Delta F275$ -*KCNQ1* constructs were generated and transiently transfected together with a *KCNE1* construct in CHO-K1 cells to characterize the properties of the slowly activating delayed rectifier current (I_{Ks}) using conventional whole-cell patch-clamp techniques. Cells transfected with WT-*KCNQ1* and *KCNE1* (1:1.3 molar ratio) produced slowly activating outward current with the characteristics of I_{Ks} . Tail current density measured at -40 mV following a 2 sec step to $+60$ mV was 381.3 ± 62.6 pA/pF ($n=11$). Cells transfected with $\Delta F275$ -*KCNQ1* and *KCNE1* exhibited essentially no current. (Tail current density: 0.8 ± 2.1 pA/pF, $n=11$, $p=0.00001$ vs WT). Co-transfection of WT- and $\Delta F275$ -*KCNQ1* (50/50) along with *KCNE1* produced little to no current (tail current density: 10.3 ± 3.5 pA/pF, $n=11$, $p=0.00001$ vs WT alone), suggesting a potent dominant negative effect. Immunohistochemistry showed normal membrane trafficking of $\Delta F275$ -*KCNQ1*.

Conclusion—Our data suggest that a $\Delta F275$ mutation in *KCNQ1* is associated with a very potent dominant negative effect leading to an almost complete loss of function of I_{Ks} and that this defect underlies a LQT1 form of LQTS.

Keywords

Ion channel; Mutation; Inherited syndrome; Electrophysiology; Torsade de Pointes

INTRODUCTION

Long QT syndrome (LQTS) is characterized by QT prolongation in the surface ECG, syncope, and sudden cardiac death secondary to an atypical polymorphic ventricular tachycardia known as Torsade de Pointes (TdP). Mutations in ten different genes causing inherited LQTS have been identified. Mutations in the *KCNQ1* (KvLQT1) gene can cause both the autosomal dominant Romano-Ward syndrome and the autosomal recessive Jervell and Lange-Nielsen syndrome.¹ *KCNQ1* and *KCNE1* (*minK*) form the slowly activating component of the delayed rectifier K⁺ current (I_{Ks}), which contributes to cardiac repolarization. Functional expression of mutant *KCNQ1* channel proteins in heterologous expression system has revealed a loss of channel function in most cases.¹ Over one hundred mutations have been identified and shown to be associated with a variety of ion channel dysfunction mechanisms.² The objective of this study was to evaluate the functional consequences of a novel single amino acid deletion mutant, Δ F275, in a mammalian heterologous expression system.

METHODS

Clinical case presentation

A 14-year-old girl was referred for genetic analysis. Her ECG at annual check-up showed a prolonged QT interval of 520–560 ms. She had syncopal attack at 10 years of age, but no family history of sudden cardiac death and normal hearing. The surface ECG at rest showed broad-based tall T wave typical LQT1^{3,4} and a QTc interval of 515 ms (Figure 1A). Holter recording and treadmill exercise stress test did not reveal a clinically significant arrhythmia. However, her QTc prolonged from a baseline of 475 to 555 ms during a treadmill exercise stress test. Infusion of epinephrine (0.3mg/kg/min) during electrophysiological study prolonged QTc from a baseline of 470 ms to 620ms and induced a polymorphic VT (Figure 1B). Programmed electrical stimulation in the absence of epinephrine did not induce ventricular tachycardia (VT) or fibrillation (VF). Both parents displayed a normal ECG and genetic screening was declined.

Genetic analysis

The protocol of this study was approved by the respective IRBs of the institutions involved in the study. After obtaining written informed consent, genomic DNA was isolated from peripheral blood lymphocytes by conventional methods.⁵ The genomic DNA was amplified on GeneAmp® PCR System 9700 thermal cycler by standard polymerase chain reaction (PCR) technique using the primers as described previously.^{6,7} For genetic screening, we used single-strand conformation polymorphism (SSCP) followed by DNA sequencing or PCR-sequencing method for *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2* and *KCNJ2* genes. Abnormal conformers were sequenced with ABI377XL genetic analyzer (Applied Biosystems, Foster City, CA). Electropherograms were compared with the *KCNQ1* wild-type sequence (GenBank accession number AJ006343) using the DNASIS Ver. 3.7 software (HITACHI, Japan).

Constructs for electrophysiological and confocal studies

The wild-type (WT) *KCNQ1* and *KCNE1* cDNAs were generated as described previously.⁸ The deletion of 3 nucleotides, TCT, corresponding to phenylalanine at position 275 was introduced to WT-*KCNQ1* cDNA by site-directed mutagenesis with primers of 5'-CGGCTTCCTGGGCCTCATCTCCTCGTACTT-3' and 5'-AGTACGAGGAGATGAGGCCAGGAAGCCG-3', and the Δ F275-*KCNQ1* clone was confirmed by sequencing.

Cell culture and transient transfection

CHO-K1 (Chinese hamster ovary) cells were obtained from American Type Cell Collection and cultured in Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO₂ incubator at 37°C. Cultured cells were seeded in 35-mm dishes 1 day before transfection and transiently transfected with various plasmids using FuGENE6 lipid based transfection reagent (Roche Diagnostics Co., Indianapolis, IN). In the electrophysiologic experiments, 0.75, 0.5 or 0.375 µg of WT-*KCNQ1* and/or 0.75, 0.375 or 0.25 µg of Δ F275-*KCNQ1* together with 0.75 µg of WT-*KCNE1* were transfected into CHO-K1 cells. pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA) was co-transfected to allow for identification of the transfected cells. Cells displaying green fluorescence 48–72h after transfection were studied electrophysiologically.

Electrophysiological recordings

To investigate the effects of the Δ F275-*KCNQ1* mutation on I_{Ks}, we performed whole-cell patch-clamp experiments on CHO-K1 cells transfected with WT- and/or Δ F275-*KCNQ1* channel. Briefly, cells were placed in a perfusion chamber (PDMI-2, Medical Systems Corp.) mounted on the stage of an inverted microscope (TE2000, Nikon, USA). Cells were superfused with normal external solution containing (in mmol/L) 132 NaCl, 4.8 KCl, 2 CaCl₂, 1.2 MgCl₂, 5 Glucose and 10 HEPES-Na (pH=7.4 adjusted with HCL). Patch pipettes were fabricated from borosilicate glass capillaries (1.5 mm O.D., Fisher Scientific, Pittsburgh, PA). Pipettes were pulled using a gravity puller: PP-83 (Narishige, Japan) and filled with pipette solution of the following composition (mmol/L) 110 Aspartic acid, 5 ATP-K₂, 11 EGTA, 10 HEPES and 1 MgCl₂ (pH=7.35 adjusted with KOH). The pipette resistance ranged from 2–5 MΩ when filled with the internal solution. All recordings were made at room temperature.

After forming a gigaseal, the cell membrane was ruptured by applying negative pressure. Current signals were recorded using an Axopatch 200A amplifier (Axon Instruments Inc., Foster City, CA) and series resistance errors were reduced by 60–70% with electronic compensation. All signals were acquired at 500 Hz to 5 kHz (Digidata 1322, Axon Instruments, Foster City, CA) with a personal computer running Clampex 9 software (Axon Instruments, Foster City, CA) and filtered at 5kHz with a 4 pole Bessel low pass filter. Membrane currents were analyzed with Clampfit 9 software (Axon Instruments, Foster City, CA).

Immunohistochemistry

Immunohistochemistry was performed to assess protein localization of WT- and Δ F275-*KCNQ1* channels. Briefly, CHO-K1 cells transfected with WT- and/or Δ F275-*KCNQ1* were fixed with an ethanol-acetone mixture and permeabilized with 0.2% Triton X-100. Cells were incubated overnight at 4°C with 1:100/200 dilutions of primary goat polyclonal antibodies against the *KCNQ1* channel protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) 48 hours after transfection. Cells were incubated with a 1:1,000 dilution of anti-goat alexa-488 conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for 2 hours at room temperature. Cells were mounted using Pro-Long antifade mounting media (Molecular Probes).

Immunofluorescence stained CHO-K1 cells were visualized by confocal microscopy (Olympus Fluoview FV300). Cells were excited at 488nm using an argon laser and emission was collected via 525 nm band pass filter and photomultiplier tube (40X oil immersion lens).

Statistics

Results are expressed as mean \pm S.E.M. Difference between groups were tested by one-way ANOVA followed by Scheffe's modified F-test for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

RESULTS

We initially evaluated DNA from the patient for mutations in the *KCNQ1* gene using single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction product. An abnormal pattern of bands was detected in exon 6. Further examination by direct sequencing of the abnormal fragment showed a deletion of TCT at position 824–826 in *KCNQ1* (Fig. 2A). This abnormal sequence causes a single amino acid deletion of phenylalanine at position 275 in the S5 segment of *KCNQ1* channel. The phenylalanine at position 275 is highly conserved among different species. (Fig. 2B) Predicted topology of WT- and mutant-*KCNQ1* channel is shown in Fig. 2C. This mutation was not detected in more than 200 unrelated ethnically-matched healthy individuals. No mutations were identified in any of the other long QT-related genes screened (*KCNH2*, *KCNE1*, *KCNE2*, *SCN5A*).

Conventional whole-cell patch-clamp experiments were conducted on cells transfected with WT- and/or mutant-*KCNQ1*. Representative current traces of each transfection protocols are shown in Fig. 3A–E. Cells transfected with 0.75 μg of WT-*KCNQ1* together with 0.75 μg of *KCNE1* exhibited slowly activating outward current compatible with I_{Ks} recorded from native cardiac myocytes (Fig. 3A), whereas cells transfected with same amount of $\Delta\text{F275-KCNQ1}$ + *KCNE1* produced little to no current (Fig. 3E). Cells transfected with 0.375 μg of WT-*KCNQ1* (+ *KCNE1*) expressed slightly less I_{Ks} compared to those transfected with 0.75 μg of WT-*KCNQ1*, although the differences were not statistically significant (Fig. 3B). Co-expression of 0.375 μg of WT-*KCNQ1* with the same amount of $\Delta\text{F275-KCNQ1}$ (+ *KCNE1*) showed little to no current (Fig. 3D). Increasing the ratio of WT to ΔF275 from 1:1 to 2:1, resulted in a slight recovery of I_{Ks} current (Fig. 3C). These observations indicate a potent dominant negative effect of $\Delta\text{F275-KCNQ1}$.

Summary data of current-voltage relationships for peak current recorded and tail currents recorded upon repolarization to -40mV for the various transfection protocols are shown graphically in Fig. 4(A, B). Current-voltage relations were not significantly different between 0.75 and 0.375 μg of WT. I-V relation for $\Delta\text{F275-KCNQ1}$ channels was nearly flat and significantly different from WT ($p=0.00001$). Co-transfection of WT- and $\Delta\text{F275-KCNQ1}$ (1:1) along with *KCNE1* also produced little to no current ($p=0.00001$ vs WT). Increasing WT to $\Delta\text{F275-KCNQ1}$ ratio from 1:1 to 2:1 significantly increased developing current ($p=0.00251$). Summary data for current intensity recorded during depolarizations to $+60\text{mV}$ and tail currents recorded upon repolarization to -40mV shown in Fig. 4(C, D). These results once again indicate that the $\Delta\text{F275-KCNQ1}$ mutant exerts a very potent dominant-negative effect on channel function or expression.

To determine whether the changes observed on I_{Ks} is due to impaired trafficking of the protein to the plasma membrane, we determined the localization of WT and mutant *KCNQ1* channel proteins using immunohistochemical techniques.

CHO-K1 transfected with WT-*KCNQ1* and/or $\Delta\text{F275-KCNQ1}$ cells were incubated with a goat polyclonal antibody against the *KCNQ1* channel protein. Localization of the immunolabeled protein was assessed by confocal imaging, following incubation of the cells with an Alexa-488 conjugated secondary anti-goat antibody. Positive staining was observed in all transfected CHO-K1 cells, but not in non-transfected controls (Fig. 5A). Cells transfected with WT (Fig. 5B), $\Delta\text{F275-KCNQ1}$ (Fig. 5C) or WT+ $\Delta\text{F275-KCNQ1}$ (Fig. 5D) all displayed peripheral

staining. Similar results were obtained in 15 cells transfected with WT (4 transfections), 14 cells transfected with $\Delta F275$ -*KCNQ1* (4 transfections) and 5 cells transfected with WT + $\Delta F275$ -*KCNQ1* (2 transfections).

These findings suggest that the loss of function observed with the $\Delta F275$ mutant is not due to a defect in trafficking of mature *KCNQ1* channels from the ER/Golgi complex to the cell membrane.

DISCUSSION

LQT1 is caused by gene mutations in *KCNQ1* and it is the most common form of congenital LQTS, responsible for approximately 43% of LQTS-linked mutations. *KCNQ1* encodes the pore-forming α -subunit of the I_{Ks} potassium channel. *KCNE1* encodes the β -subunit. Over one hundred mutations in *KCNQ1* have been reported to cause a loss of function and thus predispose to the development of LQTS. Reduced levels of I_{Ks} lead to prolongation of action potential duration (APD) and thus to prolongation of the QT interval and the development of TdP. The present study identifies a novel *KCNQ1* mutation (F275del or $\Delta F275$) caused by a 3 nucleotide deletion, resulting in deletion of a phenylalanine in the S5 segment of *KCNQ1*. Functional analysis of the mutated channel protein revealed a major loss of function consistent with the LQT1 phenotype of the patient.

The $\Delta F275$ -*KCNQ1* channel failed to produce any current when co-expressed with *KCNE1*, indicating that the mutant was unable to form functional homo-multimeric channels, or could not be expressed on the surface membrane due to a trafficking defect. Subcellular localization of the channel proteins revealed normal trafficking of the mutant protein, thus pointing to a functional dysfunction of the channel as the cause for the loss of current. Co-expression of WT- and $\Delta F275$ -*KCNQ1* channel produced little to no I_{Ks} , suggesting that the $\Delta F275$ -*KCNQ1* suppresses the function of the WT channel in a potent dominant-negative manner. Thus, the heteromultimeric channel containing mutant subunits is largely non-functional. The strong dominant negative suppression is consistent with the clinical phenotype observed in our patient with QTc intervals at rest as long as 560 msec, which prolonged by 150 ms in response to epinephrine infusion, leading to induction of TdP. The Δ QTc induced by epinephrine is longer than that observed in LQT1 patients with the Romano-Ward syndrome.^{9,10} These characteristics approach those described for patients with Jervell and Lange-Nielsen syndrome, the homozygous recessive form of LQT1.¹¹ Although several dominant-negative *KCNQ1* mutants have been reported,^{8,12–15} the $\Delta F275$ -*KCNQ1* mutation herein described appears to result in the most prominent dominant-negative suppression of I_{Ks} .

A number of mutations in *KCNQ1* have been shown to cause trafficking defects, leading to haploinsufficiency (T587M and $\Delta S276$) or a dominant-negative effect (A178fs/105). These variations are often missense mutations in the N-terminus, S5, pore region or C-terminus.^{8,16,17} Recent studies indicate that specific regions of *KCNQ1* (N-terminal juxtamembranous domain or C-terminus amino acids 610–620) are critical for channel surface expression.^{18,19} However, mutations outside these domains can lead to trafficking problems of varying degrees.²⁰ In the case of the *KCNH2* channel, the C-terminus region, including the cyclic nucleotide binding domain, is thought to be essential for channel trafficking, because it contains crucial sequence linked to endoplasmic reticulum retention and the interaction site with GM130 required for channel protein trafficking.²¹ In the present study, the mutation affected the mid-portion of the S5 segment and no trafficking defect was detected.

The I_{Ks} channel is comprised of 4 *KCNQ1* subunits. In the case of a heterozygous mutation in *KCNQ1*, as in the present case, mutant and WT subunits may combine to form a heteromultimeric channel. When the mutant *KCNQ1* subunits interfere with the function of

the wild-type subunits, I_{Ks} will be reduced by greater than 50%. This greater than expected loss of function is referred to as a dominant-negative effect. Mutations in the pore region, as in the case of $\Delta F275\text{-KCNQ1}$, are more likely to produce a dominant negative effect.^{12,22} In contrast to $\Delta F275\text{-KCNQ1}$, the molecular phenotype of $\Delta S276\text{-KCNQ1}$ has a very weak dominant negative effect leading to a recessive form of LQTS.¹⁷

In summary, we have identified a novel single amino acid deletion in domain 5 of *KCNQ1* in a patient with LQTS. Functional analysis revealed normal trafficking nonfunctioning channel with very strong dominant-negative suppression of I_{Ks} , consistent with a pronounced LQTS phenotype and sensitivity to epinephrine.

Acknowledgements

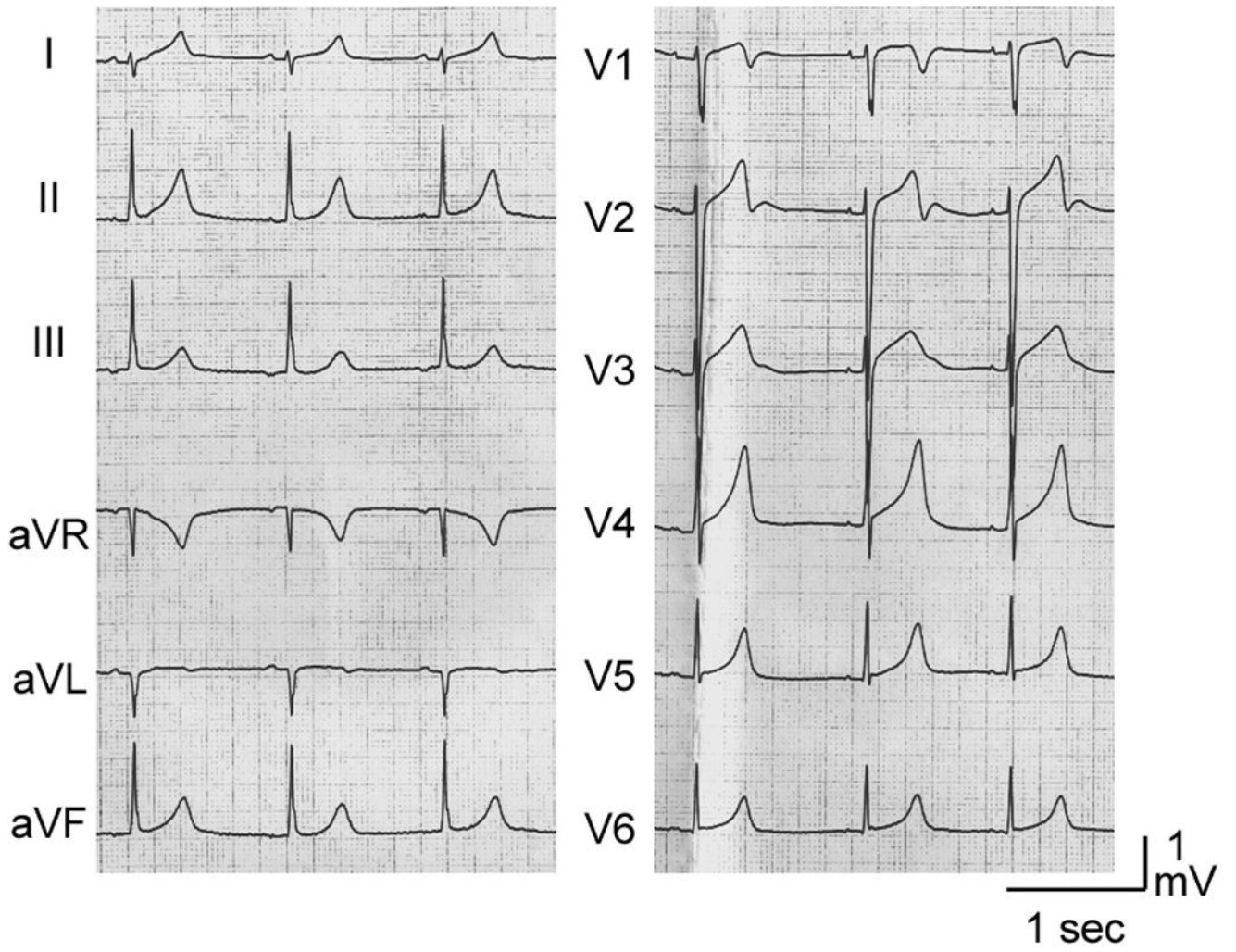
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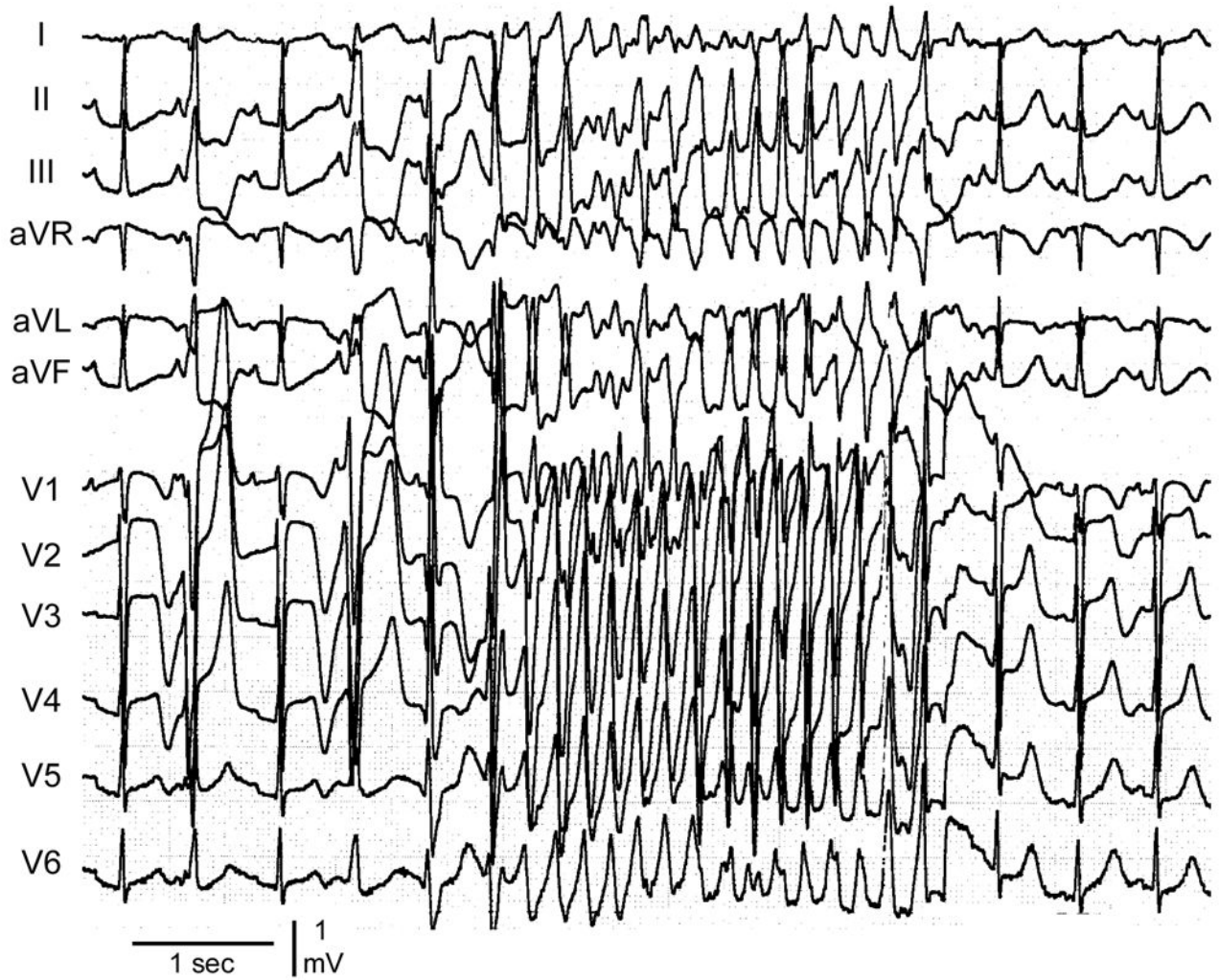


Figure 1. Electrocardiograms of the patient

A. 12-Lead ECG of the patient at rest. Broad-based tall T waves are observed. QTc interval is 515 ms at rest. **B.** Torsade de Pointes arrhythmias induced following administration of epinephrine (0.3mg/kg/min). Her QTc interval prolonged to 620 ms.

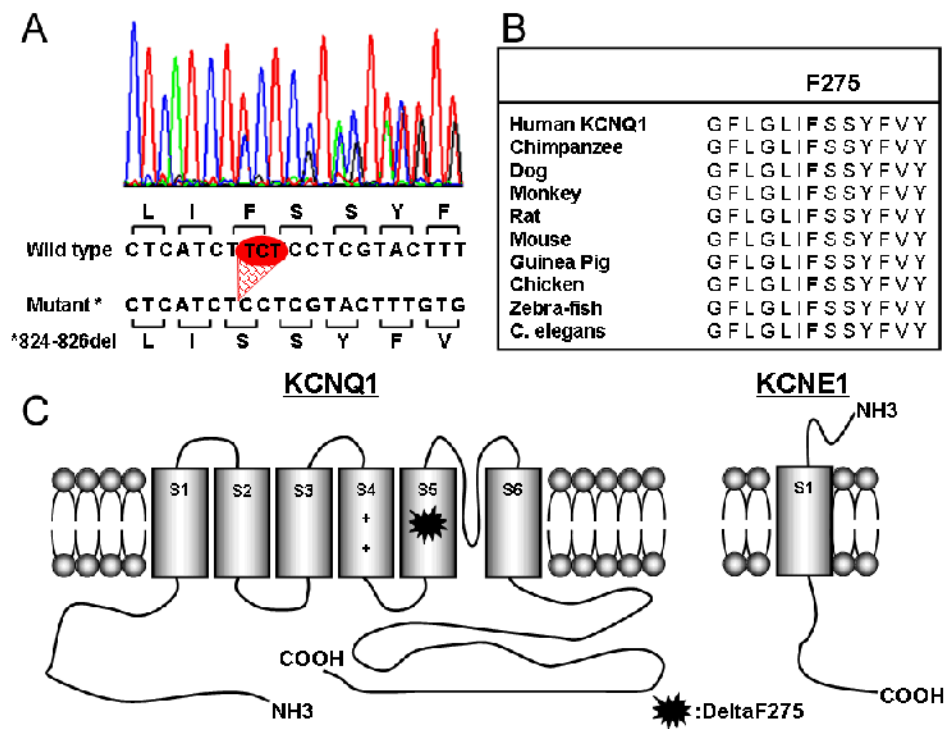


Figure 2. $\Delta 275$ F mutant at *KCNQ*

A. Chromatogram showing a heterozygous deletion at the position of 824–826.

B. Amino acid sequence alignment showing conservation of Phenylalanine 275 in multiple species

C. Schematic topology of *KCNQ1* and *KCNE1* proteins forming I_{Ks} . $\Delta 275$ F mutation was located at the S5 segment of *KCNQ1*.

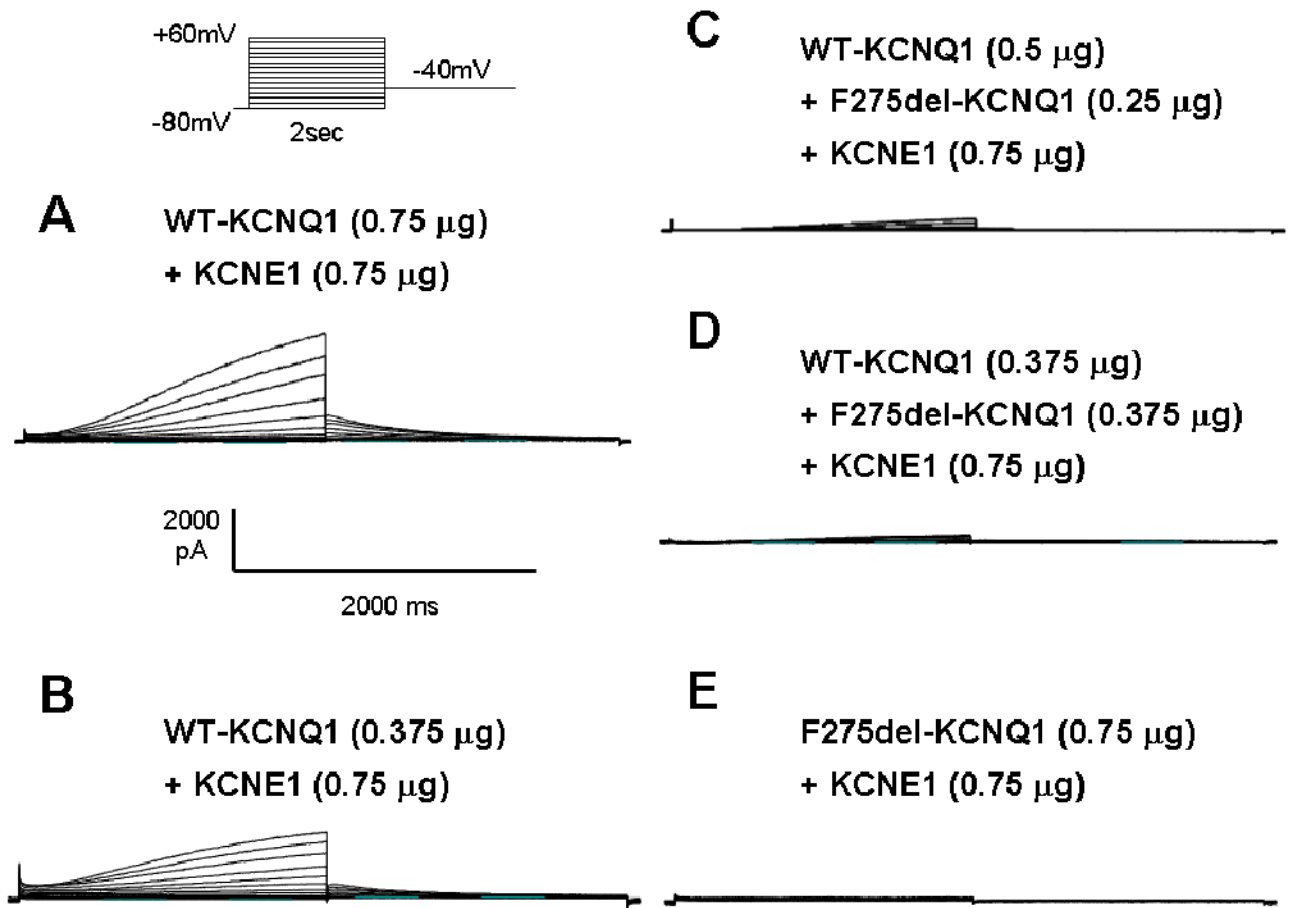


Figure 3. Representative current trace of WT- and/or $\Delta 275\text{F-KCNQ1}$ expressed in CHO-K1 cells

Cells of each panel were transfected as follows:

- A.** 0.75 μg of WT-KCNQ1 and 0.75 μg of KCNE1.
 - B.** 0.375 μg of WT-KCNQ1 and 0.75 μg of KCNE1.
 - C.** 0.5 μg of WT-KCNQ1, 0.25 μg of $\Delta 275\text{F-KCNQ1}$ and 0.75 μg of KCNE1.
 - D.** 0.375 μg of WT-KCNQ1, 0.375 μg of $\Delta 275\text{F-KCNQ1}$ and 0.75 μg of KCNE1.
 - E.** 0.75 μg of $\Delta 275\text{F-KCNQ1}$ and 0.75 μg of KCNE1.
- Pulse protocol is shown in the inset in the lower right.

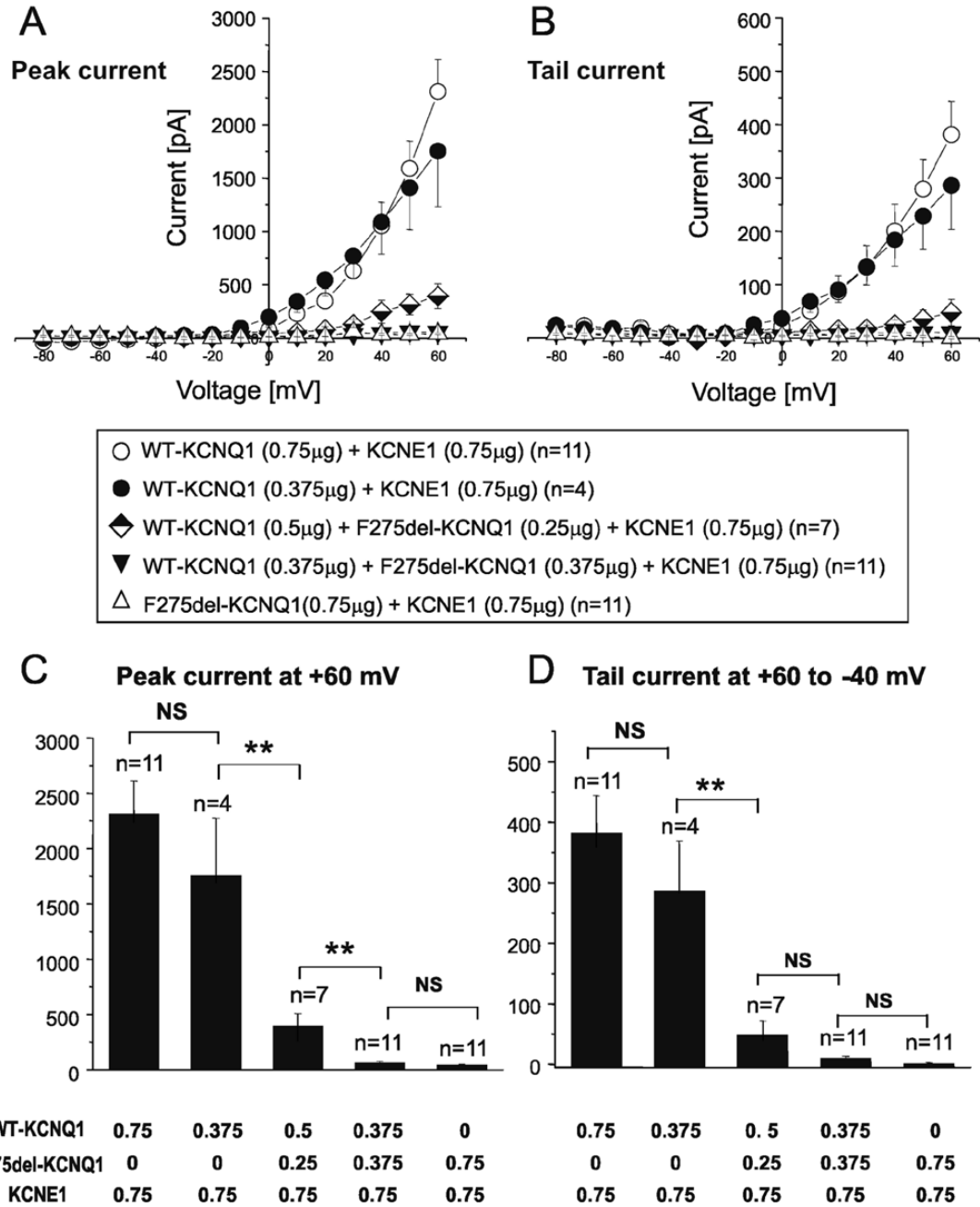


Figure 4. Current-voltage relationships of expressed currents

A. Current-Voltage relationship measured at the peak current during the test depolarization pulse.

B. Current-Voltage relationship measured of the tail current upon repolarization to -40mV following test depolarization.

C. Bar graphs showing current densities of developing (peak) recorded current at +60mV.

D. Bar graphs showing current densities of tail current recorded upon repolarization to -40mV from +60mV test depolarization.

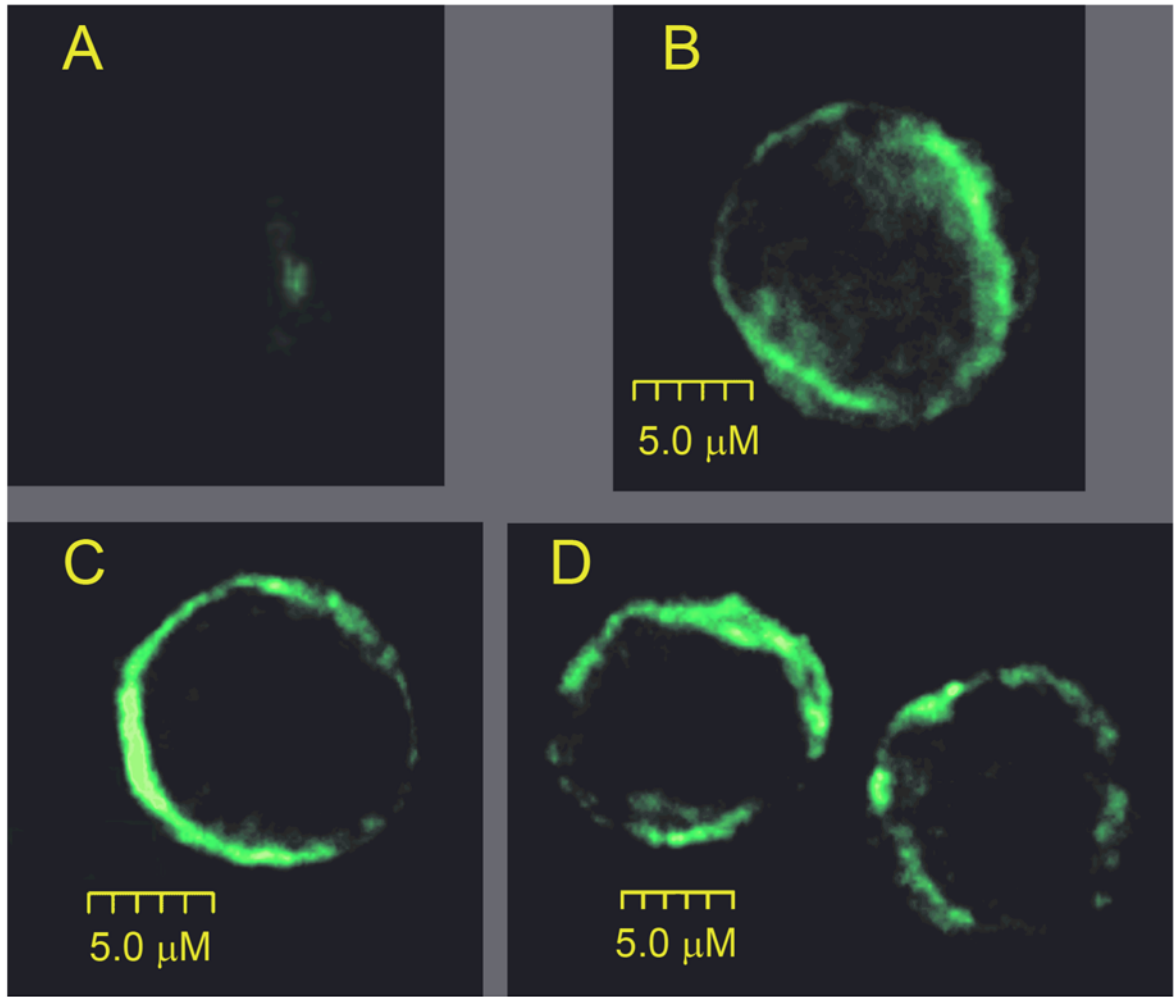


Figure 5. Protein localization of WT- and/or mutant-*KCNQ1* in CHO-K1 cells visualized by immunohistochemistry

Figure shows 0.15 μ m optical sections from the center of CHO-K1 cells transfected with WT-*KCNQ1* (B), Δ F275-*KCNQ1* (C) or Δ F275-*KCNQ1* + WT (D). A brighter fluorescence intensity near the plasma membrane reveals positive immunostaining of the KCNQ1 channel. Panel A shows the lack of any fluorescence signal from non transfected cells.