Identification of Kv11.1 Isoform Switch as a Novel Pathogenic 
Mechanism of Long QT Syndrome

Running title: Gong et al.; Kv11.1 isoform switch causes long QT syndrome

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Abstract:

Background - The KCNH2 gene encodes the Kv11.1 potassium channel that conducts the rapidly activating delayed rectifier current in the heart. The relative expression of the full-length Kv11.1a isoform and the C-terminally truncated Kv11.1a-USO isoform play an important role in regulation of channel function. The formation of C-terminal isoforms is determined by competition between the splicing and alternative polyadenylation of KCNH2 intron 9. It is not known whether changes in the relative expression of Kv11.1a and Kv11.1a-USO can cause long QT syndrome.

Methods and Results - We identified a novel KCNH2 splice site mutation in a large family. The mutation, IVS9-2delA, is a deletion of the A in the AG dinucleotide of the 3' acceptor site of intron 9. We designed an intron-containing full-length KCNH2 gene construct to study the effects of the mutation on the relative expression of Kv11.1a and Kv11.1a-USO at the mRNA, protein and functional levels. We found that this mutation disrupted normal splicing and resulted in exclusive polyadenylation of intron 9, leading to a switch from the functional Kv11.1a to the non-functional Kv11.1a-USO isoform in HEK293 cells and HL-1 cardiomyocytes. We also showed that IVS9-2delA caused isoform switch in the mutant allele of mRNA isolated from patient lymphocytes.

Conclusions - Our findings indicate that the IVS9-2delA mutation causes a switch in the expression of the functional Kv11.1a isoform to the non-functional Kv11.1a-USO isoform. Kv11.1 isoform switch represents a novel mechanism in the pathogenesis of long QT syndrome.

Key words: arrhythmia (mechanisms), long QT syndrome, potassium channels, hERG, alternative polyadenylation
Introduction

*KCNH2* or *human ether-a-go-go-related gene 1 (hERG1)* encodes the Kv11.1 channel that conducts the rapidly activating delayed rectifier K⁺ current (Iₖᵣ) in the heart.¹⁻³ Iₖᵣ is one of the major ion channel currents contributing to the repolarization of the cardiac action potential. Mutations in *KCNH2* cause long QT syndrome type 2 (LQT2).⁴ Several Kv11.1 isoforms with different N- and C-termini have been identified.⁵ The Kv11.1a isoform represents the full-length channel consisting of 1159 amino acids. There have been two Kv11.1 N-terminal isoforms identified to date, Kv11.1b and Kv11.1-3.1.⁶⁻⁸ Kv11.1b lacks the first 376 amino acids of the full-length channel and has an alternate 36 amino acid N-terminus, while the first 102 amino acids of Kv11.1a are replaced by 6 unique amino acids in Kv11.1-3.1. The C-terminal isoforms, Kv11.1a-USO and Kv11.1b-USO, contain the truncated USO C-terminus, in which the last 359 amino acids of Kv11.1a and Kv11.1b are replaced by an alternate 88 residue C-terminal end.⁹,¹⁰ Functional studies have shown that the Kv11.1a, Kv11.1b and Kv11.1-3.1 isoforms form functional channels with distinct gating properties.⁶⁻⁸ However, the Kv11.1a-USO and Kv11.1b-USO isoforms fail to form functional channels when expressed in mammalian cells.⁹⁻¹² The relative expression of Kv11.1 C-terminal isoforms has been proposed to play a significant role in the expression and function of Kv11.1 channels.¹⁰,¹¹

*KCNH2* pre-mRNA undergoes alternative polyadenylation to generate the Kv11.1a and Kv11.1a-USO isoforms.¹¹ The full-length Kv11.1a isoform is produced by polyadenylation at a poly(A) signal in exon 15, while the C-terminally truncated Kv11.1a-USO isoform is generated by polyadenylation at a proximal poly(A) signal within intron 9. The formation of the two isoforms is determined by competition between the splicing and alternative polyadenylation of intron 9. We have previously shown that the relative abundance of Kv11.1 C-terminal isoforms...
varies in different tissues, indicating that alternative processing of \textit{KCNH2} pre-mRNA is regulated in a tissue-specific manner.\textsuperscript{11} Since Kv11.1a and Kv11.1a-USO are produced from alternative processing of a single pre-mRNA precursor, an increase in Kv11.1a-USO would limit the production of full-length Kv11.1a, whereas a decrease in Kv11.1a-USO would allow a given pre-mRNA transcript to be processed to the functional Kv11.1a isoform. Thus, the relative expression of Kv11.1a and Kv11.1a-USO plays an important role in regulating Kv11.1 channel function. Whether mutations within \textit{KCNH2} intron 9 can disrupt the alternative processing of \textit{KCNH2} pre-mRNA and cause LQT2 is unknown.

In this study, we identified a novel \textit{KCNH2} splice site mutation, IVS9-2delA, at 3' acceptor site of \textit{KCNH2} intron 9 in a large LQT2 family. We showed that IVS9-2delA disrupted normal splicing and resulted in exclusive polyadenylation of intron 9. The isoform switch from the Kv11.1a to the Kv11.1a-USO completely abolished Kv11.1 current. Our findings suggest that the IVS9-2delA mutation causes LQT2 by a Kv11.1 isoform switching mechanism.

\textbf{Methods}

\textbf{Phenotyping}

The study was approved by the institutional review board and carried out upon receipt of informed consent. The participants were blood-related members of a large family with long QT syndrome. The pedigree included 16 blood-related family members from three generations (Figure 1A). Two family members declined to participate in the study. Phenotyping was performed based on the history of cardiac events, the assessment of QT intervals and T-wave morphology.

\textbf{Genotyping}

DNA was extracted from blood samples using the Puregene Blood Core kit (Qiagen,
Valencia, CA). The coding exons of four K+ channel subunits, KCNH2, KCNQ1, KCNE1 and KCNE2, associated with long QT syndrome, were amplified from genomic DNA by PCR using previously published primers.13 The primers for KCNH2 exon 10 were redesigned to obtain optimal sequence readings at the junction of intron 9 and exon 10 (E10-F: 5’-AGATTGCTTCCCGGTGT-3’ and E10-R: 5’-TGGGACTTTTGTA GGCTGCT-3’). The amplicons were sequenced using the Applied Biosystems 3730xL (Foster City, California). The Genomic DNA of 200 normal subjects were obtained from the Kaiser Permanente Northwest Biobank and used as a control.

**Cloning of the full-length KCNH2 gene and expression constructs**

The full-length KCNH2 gene was cloned from a human BAC clone consisting of the entire KCNH2 gene (RP11-166D23) using a lambda prophage red-mediated homologous recombination method.14 The cloned full-length KCNH2 gene is about 33 kb, including all exons and introns spanning from the start codon to the poly(A) signal. An abbreviated full-length KCNH2 gene construct, in which the two longest introns, intron 2 (14974 bp) and intron 5 (4437 bp), were shortened to 600 bp, was also generated. The abbreviated full-length KCNH2 gene is referred to as the short KCNH2 gene. The full-length and the short KCNH2 genes are driven by a CMV promoter. The constructs also contain the hygromycin resistance gene with a Flp recombination target site embedded in the 5’ coding region which is obtained from the pcDNA5 vector. Overlap extension PCR was used to generate a hemagglutinin (HA)-tagged short KCNH2 gene. The HA epitope tag along with short linkers TNSEHYPYDVPDYAVTFE (HA epitope underlined) was inserted between residues T443 and E444 in the extracellular S1-S2 loop of the Kv11.1 channel.

To generate the IVS9-2delA mutant short KCNH2 gene construct, we first PCR amplified
the genomic DNA fragment containing the IVS9-2delA mutation from the proband using the primers E10-F and E10-R (see above). The PCR amplicon was ligated into pCRII vector using TA cloning kit (Invitrogen, Carlsbad, CA) and the clone containing IVS9-2delA was identified by sequencing. A 90 bp fragment containing the IVS9-2delA mutation was inserted into the NcoI/Apal sites of the short KCNH2 gene by subcloning.

**Stable and transient transfection in HEK293 and HL-1 cardiomyocytes**

The full-length and short KCNH2 gene constructs were stably expressed in Flp-In HEK293 by co-transfection of the constructs (0.1 µg) with the Flp recombinase expression vector pOG44 (0.9 µg) using the Effectene method (Qiagen) and selected with 100 µg/ml hygromycin. The Flp-In HEK293 cells contain the FRT site at a single genomic locus, allowing stable integration of a single copy of the KCNH2 gene via Flp recombinase-mediated DNA recombination at a specific genomic location in all cell clones. HA epitope-tagged short KCNH2 gene constructs were transiently transfected into HEK293 cells or HL-1 cardiomyocytes by Lipofectamine 2000 (Invitrogen). Flp-In HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. HL-1 murine cardiomyocytes were cultured in Claycomb medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and 0.1 mmol/L norepinephrine (Sigma-Aldrich).15

**RNase protection assay and RT-PCR analysis**

RNA was isolated from HEK293 cells and HL-1 cardiomyocytes expressing short KCNH2 gene constructs with the RNeasy Kit (Qiagen). RNase protection assay (RPA) was performed using the RPAII and BrightStart BioDetect kits (Ambion, Austin, Texas) as previously described.16 Briefly, antisense RNA riboprobes were transcribed in vitro in the presence of biotin-14-CTP. The probe for detection of Kv11.1 mRNA levels contained 319 nt sequence from exon 9 to
intron 9. The total length of the probe was 460 nt and contained sequences from the pCRII vector at both ends. The probe for the hygromycin B resistance gene contained 158 nt of the gene and 70 nt from the pGEM vector. Yeast RNA was used as a control for the complete digestion of the probes by RNase. The relative intensity of each band was quantified using ImageJ and adjusted for the number of biotin-labeled cytidines in each protected fragment. The expression level of the hygromycin B resistance gene was used as an internal control for normalization. For RT-PCR analysis, the SuperScript III First-Strand DNA Synthesis kit (Invitrogen) was used to generate cDNA and PCR was performed using a forward primer in exon 9 (5'-GTGCTGAAGGGCTGAGCTGAG-3') and a reverse primer in exon 11 (5'-CCGACTGAAGCCACCCTCTTAAC-3'). The PCR products were visualized by electrophoresis on agarose gels and specific bands were isolated for sequence analysis.

Immunoblot analysis

Cells were washed with PBS and lysed with lysis buffer (50 mmol/L Tris-HCl, pH 7.4 containing 150 mmol/L NaCl, 1% Triton X-100) plus a protease inhibitor cocktail (100 μmol/L phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml of leupeptin, 2 μl/ml of aprotinin). The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred onto nitrocellulose membranes. After transfer, the membranes were blocked with 5% nonfat dry milk and 0.2% Tween 20 in PBS for 1 h. The membranes were probed with a 1:600 dilution of an anti-Kv11.1 antibody directed against the N-terminus of the protein (H175, Santa Cruz Biotechnology Inc., Santa Cruz, CA) or a 1:1000 dilution of an anti-HA antibody (HA.11, Convance, Berkeley, CA), and visualized with horseradish peroxidase-conjugated second antibody and ECL detection kit. The expression level of hygromycin B phosphotransferase encoded by the hygromycin B resistance gene was used as a loading control.
The polyclonal antibody against hygromycin B phosphotransferase was custom-generated by Genscript (Piscataway, NJ) and used at a 1:1000 dilution. The intensity of the protein bands was quantified using ImageJ.

**Immunofluorescence**

The surface expression of Kv11.1 channels containing an external HA epitope tag was determined by immunofluorescence as described by Ficker et al. Briefly, live cells were blocked with PBS containing 5% goat serum, probed with rat monoclonal anti-HA antibody for 1 hour at 4°C (3F10, Roche, 1:100). Cells were washed with PBS and fixed with 4% paraformaldehyde at 4°C. Fixed cells were re-blocked with PBS containing 5% goat serum and permeabilized with 0.1% Triton X-100. Permeabilized cells were re-probed with rabbit anti-Kv11.1 N-terminus antibody at 22-23°C (H175, Santa Cruz Biotechnology Inc., 1:300). Cells were washed and incubated with Alexa 488-conjugated goat anti-rat and Alexa 594-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Images were acquired with a Zeiss Axioskop 2 microscope.

**Patch-clamp recordings**

Membrane currents were recorded in whole-cell configuration using suction pipettes as previously described. The bath solution contained (in mmol/L) 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mmol/L) 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, and 10 HEPES (pH 7.2 with KOH). An Axopatch-200B patch-clamp amplifier was used to record membrane currents. Computer software (pCLAMP10) was used to acquire and analyze current signals. All patch-clamp experiments were performed at 22-23 °C.
RNA preparations from blood samples and allele-specific quantification of RNA transcripts

Total RNA was isolated from peripheral blood lymphocytes using the RiboPure-Blood kit (Ambion, Austin, TX). The isolated RNA was treated with RNase-free DNase to remove genomic DNA. Allele-specific quantification was performed by sequence analysis using the method described by Ge et al.\(^\text{19}\) Briefly, a common synonymous single-nucleotide polymorphism (SNP), c.1956T/C (p.Tyr652Tyr, rs1137617), was used as a marker for wild-type (WT) and mutant alleles (1956T and IVS9-2delA are on the same allele). RT-PCR was performed to amplify Kv11.1a, Kv11.1a-USO and total Kv11.1 transcripts using the same forward primer in exon 7 (5’-ATGTGACGGCGCTCTACTTC-3’), isoform specific reverse primers in exon 10 (for Kv11.1a, 5’-GGCCTTGCATACAGGTTCAG-3’), intron 9 (for Kv11.1a-USO, 5’-GAGGGCATTTCCAGTCCAGT-3’) and in exon 9 (for total, 5’-GGTGCATGTGTGCTTGGAA-3’). The PCR products were sequenced using BigDye chemistry and a 3730xl DNA sequencer. Each PCR product was sequenced three times. The sequence traces were analyzed using the PeakPicker program, a software developed for quantitative analysis of allele ratio using a marker SNP.\(^\text{19}\) Allele expression ratio for RNA samples were calculated by normalization to the averaged allele ratio of genomic DNA samples from three mutation carriers and three non-carriers in the family.

Statistical analysis

QTc, RPA, immunoblot, and allelic ratio data were presented as median and compared by Mann-Whitney U test using NCSS 2007 (Kaysville, UT) and patch-clamp data were presented as mean±standard error of mean (SEM) and analyzed by ANOVA with Bonferroni correction using SigmaPlot 12.0 (San Jose, CA). \(P<0.05\) is considered statistically significant.
Results

Identification of a novel splice site mutation from a large LQT2 family.

The proband of this family is a 70-year-old female (Figure 1A). At age 39 she suffered cardiac arrest in response to ingestion of diet pills and underlying hypokalemia. She remained event-free until age 70, when syncopal episodes occurred while taking citalopram and having hypokalemia (3.1 mmol/L). Her initial ECG on admission showed markedly prolonged QT intervals with frequent premature ventricular contractions (PVCs). Potassium supplement increased the serum [K+] to a normal range (3.9 to 4.2 mmol/L) and together with citalopram withdrawal abolished the PVCs. Nevertheless, her QT interval remained markedly prolonged (QTc 565 ms) with T wave morphology typical of LQT2 (Figure S1). Having a history of recurrent syncope, cardiac arrest and markedly prolonged QT prolongation she was treated with implanted cardioverter defibrillator (ICD). We sequenced four long QT syndrome-associated K+ channel genes and identified a novel splice site mutation at the 3’ acceptor site of KCNH2 intron 9. This mutation, IVS9-2delA (c.2399-2delA), is a deletion of an A in the conserved AG dinucleotide of the 3’ acceptor site of intron 9 (Figure 1B). We also observed a common SNP in the KCNE1 gene, G38S (rs1805127). This common variant has been reported to have minor allele frequency of 44.8% in white persons. Furthermore, a large population-based study (Kora S4 population) did not find a significant association between G38S and the QT interval. Therefore, KCNE1 G38S was not further characterized. We have genotyped 14 family members and identified eight carriers of the IVS9-2delA mutation and six non-carriers (Figure 1A). This mutation was not present in 400 alleles from normal subjects and was not found in published sequence databases (1000 Genomes, dbSNP138, NHLBI ESP Exome Variant Server).

Clinical phenotype was characterized in 13 genotyped subjects based on medical history...
and ECG assessment. The median value of QTc was significantly longer in mutation carriers (500 ms, n=8) versus non-carriers (430 ms, n=5, P<0.01, Mann-Whitney U test), and all mutation carriers presented typical LQT2 patterns on ECG. Other than the proband, two mutation carriers were also symptomatic. One with recurrent syncope and markedly prolonged QT interval was treated with ICD therapy and the other was treated with beta-blockers. The remaining mutation carriers were asymptomatic, however, one carrier with a QTc of 550 ms was treated with beta-blockers.

**IVS9-2delA caused isoform switch from the functional Kv11.1a to the non-functional Kv11.1a-USO isoform.**

In order to study how the IVS9-2delA mutation causes Kv11.1 channel dysfunction we first cloned the full-length KCNH2 gene as described in the Methods. Due to the large size, it is difficult to introduce mutations into the full-length gene construct. Therefore, we generated a shorter version of KCNH2 gene construct in which the two longest introns were shortened to 600 bp (Figure S2A). Immunoblot and patch clamp analysis revealed that the full-length and short KCNH2 gene constructs expressed Kv11.1a and Kv11.1a-USO proteins and generated comparable currents (Figures S2B and S3). We then introduced the IVS9-2delA mutation into the short KCNH2 gene construct. To determine the mRNA levels expressed from the WT and mutant short genes, we performed RPA analysis using a probe containing sequence complementary to the Kv11.1a-USO mRNA variant (Figure 2). This probe generated a protected fragment of 319 nt for Kv11.1a-USO and a 210 nt fragment for Kv11.1a. As shown in Figures 2B and 2C, IVS9-2delA completely abolished Kv11.1a-USO and concomitantly increased the level of Kv11.1a-USO mRNA.

We performed immunoblot blot analysis to determine whether the IVS9-2delA mutation
leads to isoform switch at the protein level. The WT short KCNH2 gene construct expressed three protein bands at about 155 kDa, 135 kDa and 100 kDa, consistent with the sizes of Kv11.1a and Kv11.1a-USO expressed from cDNA constructs (Figures 2D and S2B). The 155 kDa band represents the fully glycosylated mature form of Kv11.1a, the 135 kDa band represents the core-glycosylated immature form of Kv11.1a, and the 100 kDa band represents the core-glycosylated form of Kv11.1a-USO. The ISV9-2delA mutant short KCNH2 gene construct expressed the 100 kDa Kv11.1a-USO protein exclusively. The disappearance of the 155 kDa and 135 kDa Kv11.1a proteins was accompanied by an increase in the level of the 100 kDa Kv11.1a-USO protein (Figures 2D and 2E).

Previous studies have shown that the Kv11.1a-USO protein fails to traffic to the cell surface.\textsuperscript{10,12} We characterized the cell-surface expression of the ISV9-2delA mutant channels by immunostaining using short KCNH2 gene constructs containing a HA epitope inserted into the extracellular loop between the S1 and S2 transmembrane domains. The same HA-tag inserted in KCNH2 cDNA has been shown to have no effect on electrophysiological properties or trafficking phenotype of Kv11.1 channels.\textsuperscript{17} HEK293 cells transfected with HA-tagged WT and mutant short genes were probed with monoclonal anti-HA antibody prior to fixation and permeabilization. The cells were then permeabilized with Triton X-100 and re-probed with polyclonal anti-Kv11.1 antibody. Alexa 488-conjugated goat anti-rat antibody was used to detect the surface expression of Kv11.1 channels and Alexa 594-conjugated goat anti-rabbit antibody was used to detect both surface and intracellularly expressed channel proteins. As shown in Figure S4, only the WT Kv11.1 channels were detected at the cell surface. Intracellular staining revealed the expression of Kv11.1 proteins in both WT and IVS9-2delA mutant cells. These results suggest that the mutant IVS9-2delA proteins are expressed but fail to reach the cell
surface.

In patch-clamp recordings, the WT short KCNH2 gene construct generated typical Kv11.1 current (Figure 3). The IVS9-2delA mutation completely abolished Kv11.1 current. This result is consistent with previous studies showing that Kv11.1a-USO isoform failed to generate Kv11.1 current when expressed in mammalian cells.9-12 Together, these experiments strongly suggest that the ISV9-2delA mutation leads to a switch from the functional Kv11.1a to the non-functional Kv11.1a-USO isoform.

**IVS9-2delA caused cryptic splicing and intron retention in the absence of intron 9 poly(A) signal.**

We have previously shown that the relative expression of Kv11.1a and Kv11.1a-USO mRNA variants is regulated by the competition between splicing and polyadenylation of intron 9.11 To determine the role of the intron 9 poly(A) signal in the isoform switch caused by the IVS9-2delA mutation, we eliminated the intron 9 poly(A) signal in the short KCNH2 gene. RT-PCR analysis using primers in exon 9 and exon 11 revealed a single 504 bp band in both WT short KCNH2 genes with or without intron 9 poly(A) signal (Figure 4, lanes 1 and 3). Sequence analysis confirmed that this band represents normal splicing of exon 9 and exon 10 in the Kv11.1a mRNA transcript (Figure S5A). In the IVS9-2delA mutant short KCNH2 gene containing an intact intron 9 poly(A) signal, RT-PCR failed to detect any spliced product (Figure 4, lane 2). This is consistent with the RPA results that showed a complete switch from Kv11.1a to Kv11.1a-USO. However, when the intron 9 poly(A) signal is deleted from the IVS9-2delA mutant construct, RT-PCR revealed the presence of two faint bands at 499 bp and 1596 bp (Figure 4, lane 4). Sequence analysis shows that the 499 bp band represents the splicing at a cryptic site 5 nt downstream of the normal intron 9/exon 10 splice site (Figure S5A) and the 1596 bp band
represents complete intron 9 retention (Figure S5B). These results suggest that IVS9-2delA-induced isoform switch is due to the presence of the poly(A) signal in intron 9. In the absence of the competing poly(A) signal, the IVS9-2delA mutation would cause cryptic splicing and intron retention.

**Isoform switch in HL-1 cells.**

To determine whether the ISV9-2delA mutation could cause isoform switch in heart cells we transiently transfected the HL-1 murine cardiomyocyte cell line with the WT and mutant short KCNH2 gene constructs and perform RPA, immunoblot and patch-clamp experiments. In RPA analysis the IVS9-2delA mutation completely abolished Kv11.1a and increased the level of Kv11.1a-USO mRNA in HL-1 cells (Figures 5A and 5B). The immunoblot experiments shows that the IVS9-2delA mutation completely abolished the 155 kDa and 135 kDa Kv11.1a protein and resulted in increased expression of the 100 kDa Kv11.1a-USO protein (Figures 5C and 5D). Patch-clamp experiments show that HL-1 cells expressed an endogenous E-4031-sensitive $I_{Kr}$ current (Figure 6). In cells transfected with the WT short KCNH2 gene the E-4031-sensitive current was significantly increased. However, in cells transfected with the IVS9-2delA mutant short gene the level of E-4031-sensitive current was similar to the untransfected cells suggesting that the IVS9-2delA mutation has no dominant-negative effect on endogenous $I_{Kr}$ current in HL-1 cells. The maximum tail E-4031-sensitive current densities for untransfected HL-1 cells, transfected with WT and IVS-2delA were 2.6±0.3 pA/pF, 10.3±1.9 pA/pF and 2.7±0.4 pA/pF, respectively, ($P<0.001$, untransfected vs WT, $P>0.05$, untransfected vs IVS-2delA, $P<0.001$ WT vs IVS-2delA, ANOVA with Bonferroni correction). These results indicate that the ISV9-2delA mutation leads to an isoform switch in HL-1 cardiomyocytes.
Isoform switch in patient lymphocytes.

To test whether the IVS9-2delA mutation leads to an isoform switch of endogenously expressed mRNA, we isolated RNA from patient lymphocytes and analyzed allele-specific expression of mutant and WT alleles. To do this, we took advantage of the fact that several mutation carriers and non-carriers in the family are heterozygous for a common SNP, 1956T/C (rs1137617), in KCNH2 exon 8. Pedigree analysis indicates that 1956T and the IVS9-2delA mutation are on the same allele (Figure 1A). Thus, 1956C represents the WT allele and 1956T represents the mutant allele in mutation carriers. We performed RT-PCR to amplify Kv11.1a and Kv11.1a-USO mRNA transcripts using isoform specific primers (Figure 7A). Sequence analysis of samples from the proband showed that there was only the 1956C peak in the Kv11.1a PCR products (Figure 7B). In the Kv11.1a-USO PCR products, however, 1956T peak was much higher than that of 1965C. We also used another pair of PCR primers, common for both Kv11.1a and Kv11.1a-USO, to amplify total Kv11.1 transcripts (Kv11.1a and Kv11.1a-USO). Similar levels of 1956C and 1956T were observed. Sequence analysis of a normal subject from the family who carries the 1956T/C SNP revealed similar levels of 1956C and 1956T in Kv11.1a and Kv11.1a-USO and total Kv11.1 RT-PCR products (Figure 7B). We analyzed the allele ratio of 1956C and 1956T using the PeakPicker program, a software developed for quantitative analysis of allele ratio using a marker SNP.19 Figure 7C shows the allele ratios of 1956T to 1956C from four mutation carriers and three normal subjects from the family. These results strongly suggest that IVS9-2delA causes a switch from Kv11.1a to Kv11.1a-USO in endogenously expressed mRNA.

Discussion

In this study we report a novel KCNH2 splice site mutation, IVS9-2delA, identified in a large family with LQT2. The disease penetrance shown on 12-lead ECG is 100% as all mutation
carriers presented with QT prolongation and typical LQT2 T wave morphology. Among four mutation carriers with QTc >500 ms, three were symptomatic and presented with syncope and cardiac arrest. We show that the IVS9-2delA mutation results in a switch from the functional Kv11.1a isoform to the non-functional Kv11.1a-USO isoform, thereby reducing Kv11.1 current. These findings provide the first evidence associating a KCNH2 mutation that causes isoform switch to the pathogenesis of long QT syndrome.

It has been shown previously that different LQT2 mutations cause Kv11.1 channel dysfunction by different mechanisms. Missense mutations may cause defective protein trafficking, abnormal gating and/or kinetics, and altered or absent channel selectivity or permeability. Nonsense and frameshift mutations that carry premature termination codons may result in degradation of mutant mRNA by nonsense mediated mRNA decay. Mutant channels with premature termination codons occurring early in the coding sequence may escape NMD by the reinitiation of translation and generate N-terminally truncated channels with gating and trafficking abnormalities. Splice site mutations may lead to a variety of aberrant splicing patterns including exon skipping, use of nearby cryptic splice sites, and intron retention. Our present findings suggest that isoform switch caused by the IVS9-2delA mutation represents a novel pathogenic mechanism in LQT2.

Recent genome-wide polyadenylation mapping studies based on high-throughput sequencing have indicated that two-thirds of all human genes undergo alternative polyadenylation, leading to the generation of alternate transcripts with different coding sequences and/or variable 3’ untranslated regions. As such, alternative polyadenylation is increasingly recognized as an important layer of gene regulation. It has been proposed that the generation of non-functional isoforms by alternative polyadenylation can be viewed as a
mechanism to turn-off gene expression post-transcriptionally. For example, the cleavage and polyadenylation factor CstF-77 has been shown to negatively autoregulate its own expression by stimulating the usage of an alternative poly(A) signal in intron 3 that leads to the production of a truncated, non-functional CstF-77 isoform. Similarly, we propose that the formation of full-length, functional Kv11.1 channels by intron 9 splicing and the use of the distal poly(A) site in exon 15, represents a turn-on pathway for Kv11.1 expression, whereas generation of the truncated, non-functional Kv11.1a-USO isoform by the use of the proximal intron 9 poly(A) site represents a turn-off pathway for Kv11.1 expression. Identification of a novel LQT2 mutation that results in Kv11.1 isoform switch underscores the importance of alternative polyadenylation in regulation of Kv11.1 channel function and the pathogenesis of long QT syndrome. Thus, the IVS9-2delA mutation disrupts normal splicing, leading to the elimination of the turn-on pathway for Kv11.1 expression and forcing the pre-mRNA processing toward the turn-off pathway for Kv11.1 expression.

The present experiments indicate that intron 9 poly(A) signal plays an important role in the isoform switch caused by the IVS9-2delA mutation. When intron 9 poly(A) signal was deleted, the mutation resulted in cryptic splicing and intron retention. However, these aberrant splicing patterns were not observed in the presence of intron 9 poly(A) signal. We have shown previously that relative expression of Kv11.1 isoforms is determined by the competition between splicing and alternative polyadenylation of intron 9. It is noted that the splicing of KCNH2 intron 9 is inefficient in the heart. About one-third of KCNH2 pre-mRNA is processed to Kv11.1a and two thirds undergo polyadenylation to form Kv11.1a-USO transcripts. The inefficient splicing of intron 9 is due to the presence of the weak 5’ splice site of intron 9. Thus, the polyadenylation of intron 9 is dominant over intron 9 splicing even in WT KCNH2. Because the
IVS9-2delA results in the deletion of an “A” in the invariant AG dinucleotide of the KCNH2 intron 9 acceptor splice site, it completely disrupts normal exon 9 to exon 10 splicing. In the absence of the competing intron 9 poly(A) signal, the mutation activates a cryptic 3’ acceptor splice site 5 nt downstream of the normal splice site. We calculated the consensus value (CV) scores of the normal and cryptic splice sites using the Human Splicing Finder program. The CV reflects the similarity of a splice site to the consensus sequence. The mean CV for 3’ splice sites for all human introns and exons is 86.8 with a standard deviation of 6.3. The CV of the cryptic splice site is 73.3 as compared to a CV of 88.6 in normal 3’ splice site of intron 9 indicating that the cryptic splice site is rated as very weak by Human Splicing Finder program. Thus, in the presence of the intron 9 poly(A) signal, intron 9 polyadenylation completely outcompetes the weak cryptic site, leading to exclusive formation of the Kv11.1a-USO mRNA.

The present study had some limitations. Although we have shown that the short KCNH2 gene containing the IVS9-2delA mutation results in Kv11.1 isoform switch when expressed in HL-1 murine cardiomyocytes, further studies are required to determine whether the mutation alters the relative expression of Kv11.1 C-terminal isoforms in human heart tissue. Furthermore, our present experiments analyzed mRNA endogenously expressed from patient lymphocytes rather than affected heart tissue. The verification of our findings in human heart tissue would strengthen our conclusion that the IVS9-2delA mutation causes long QT syndrome by an isoform switch mechanism.

In summary, we have identified a novel KCNH2 splice site mutation in a three generation family affected by LQT2. The mutation causes an isoform switch from the functional full-length Kv11.1a isoform to the non-functional Kv11.1a-USO isoform and results in long QT syndrome.
Our findings highlight the key role of Kv11.1 isoform regulation in Kv11.1 channel function and the pathogenesis of long QT syndrome.

**Acknowledgments:** We thank the LQT2 family members for their cooperation.

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**Conflict of Interest Disclosures:** None.

**References:**


2008;44:502-509.


**Figure Legends:**

**Figure 1.** A, The pedigree of a family with the IVS9-2delA mutation. The arrow indicates the proband. The QTc (ms), age at diagnosis and KCNH2 1956T/C SNP associated with each genotyped individual are shown. B, Sequence analysis revealing a deletion of an A (indicated by arrow) in the conserved AG dinucleotide of the 3’ acceptor site of intron 9. The sequences of WT and the IVS9-2delA mutation are shown below the sequence electropherogram.

**Figure 2.** Analysis of the IVS9-2delA mutation using the short KCNH2 gene construct. Flp-In HEK293 cells were stably transfected with WT and IVS9-2delA short KCNH2 gene constructs and analyzed by RPA and immunoblot. A, The structure of the short KCNH2 gene construct showing the position of the IVS9-2delA mutation in the 3’ acceptor site of intron 9. B, Analysis
of mRNA by RPA. C, RPA signals were quantified, normalized to hygromycin resistance gene (Hygro), and plotted as the relative expression of the total WT (1a+1a-USO) Kv11.1 mRNA. Data are presented as individual (WT, open symbols; mutant, filled symbols) and as median values (black bars). Circle, triangle and square symbols represent total, 1-a, and 1-a-USO mRNA respectively (n=4, *P<0.05, Mann-Whitney U test). D, Immunoblot analysis of WT the IVS9-2delA mutation. Cell lysates were subjected to SDS-PAGE and immunoblotted with an antibody against the N-terminus of Kv11.1. The expression level of hygromycin B phosphotransferase (HPH) encoded by hygromycin B resistant gene served as a loading control. E, The level of protein bands was quantified, normalized to HPH, and plotted as the relative expression of the total WT (1a+1a-OSU) Kv11.1 protein (n=4, *P<0.05, Mann-Whitney U test).

Figure 3. Functional properties of WT and IVS9-2delA mutant Kv11.1 channels. A, Representative currents from Flp-In HEK293 cells stably transfected with WT and IVS9-2delA mutant short KCNH2 gene constructs. Kv11.1 current was activated from a holding potential of −80 mV with 4-s depolarizing test potentials between −70 and +50 mV. Tail current (indicated by arrows) was recorded at −50 mV following each depolarizing pulse. B, Current−voltage plot of tail current densities of WT (circles, n=10) and IVS9-2delA (squares, n=9). Data are plotted as mean±SEM.

Figure 4. RT-PCR analysis of the IVS9-2delA mutation. Flp-In HEK293 cells were stably transfected with WT and IVS9-2delA mutant in short KCNH2 gene (lanes 1 and 2) or short KCNH2 gene with intron 9 poly(A) signal deletion (del poly(A), lanes 3 and 4). RT-PCR was performed using primers in exon 9 and exon 11 as indicated by arrows. In the absence of intron 9
poly(A) signal, the IVS9-2delA mutation results in cryptic splicing (indicated by arrowhead) and complete intron 9 retention.

**Figure 5.** Analysis of the IVS9-2delA mutation in HL-1 cardiomyocytes. HL-1 cardiomyocytes were transiently transfected with HA-tagged WT and IVS9-2delA short KCNH2 gene constructs and analyzed by RPA and immunoblot. **A,** Analysis of mRNA by RPA. **B,** RPA signals were quantified, normalized to hygromycin resistance gene (Hygro), and plotted as the relative expression of the total WT (1a+1a-USO) Kv11.1 mRNA. Data are presented as individual (WT, open symbols; mutant, filled symbols) and as median values (black bars). Circle, triangle and square symbols represent total, 1-a, and 1-a-USO mRNA respectively (n=4, *P<0.05, Mann-Whitney U test). **C,** Immunoblot analysis of WT and IVS9-2delA mutation. Cell lysates were subjected to SDS-PAGE and immunoblotted with an antibody against the HA epitope. The expression level of hygromycin B phosphotransferase (HPH) encoded by hygromycin B resistant gene served as a loading control. **D,** The level of protein bands was quantified, normalized to HPH, and plotted as the relative expression of the total WT (1a+1a-USO) Kv11.1 protein (n=4, *P<0.05, Mann-Whitney U test).

**Figure 6.** Functional properties of WT and IVS9-2delA mutant Kv11.1 channels expressed in HL-1 cardiomyocytes. **A,** Representative currents from untransfected HL-1 cardiomyocytes, or HL-1 cardiomyocytes transiently transfected with HA-tagged WT and IVS9-2delA mutant short KCNH2 gene constructs. Kv11.1 current was activated from a holding potential of −50 mV with 2-s depolarizing test potentials between −40 and +40 mV. Tail current was recorded at −50 mV following each depolarizing pulse. Kv11.1 current was blocked by 5 μmol/L E-4031 and
expressed as E-4031 sensitive current. **B,** Current–voltage plot of E-4031 sensitive tail current of untransfected (triangles, n=7), WT (circles, n=8) and IVS9-2delA (squares, n=7) current. Data are plotted as mean±SEM.

**Figure 7.** Allele-specific expression of WT and mutant mRNA isolated from lymphocytes. **A,** Schematic diagrams are shown for RT-PCR primers for Kv11.1a (1a), Kv11.1a-USO (1a-USO) and total (1a+1a-USO). The position of the synonymous SNP, 1956T/C, is indicated. **B,** Sequence analysis of PCR products from the proband and a normal subject of the family. Arrow indicates the expression of only the 1956C peak in the 1a PCR product from the proband. **C,** Analysis of allele ratio of 1956C and 1956T using the PeakPicker program. The dot plot shows the allele ratios of 1956T to 1956C in 1a (triangle), 1a-USO (square) and total (circle) PCR products from three normal subjects (open symbols) and four mutation carriers (filled symbols) of the family (*P<0.05, Mann-Whitney U test).
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Identification of Kv11.1 Isoform Switch as a Novel Pathogenic Mechanism of Long QT Syndrome
Qiuming Gong, Matthew R. Stump, Vivianne Deng, Li Zhang and Zhengfeng Zhou

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**SUPPLEMENTAL MATERIAL**

**Figure S1.** ECG recordings of the proband. A standard 12-lead ECG of the proband shows marked QT prolongation with T wave morphology typical to LQT2. Her QTc (corrected QT interval by Bazett formula) in lead II or V5 is 565 ms. The T wave amplitude is low. Bifid T wave is present in leads III and V3 with second T wave component merged with U wave. The end of the T wave is marked by arrows.
**Figure S2.** Immunoblot analysis of full-length and short KCNH2 gene constructs. **A,** Structure of full-length KCNH2 gene and short KCNH2 gene. The total length of the full-length KCNH2 gene is 33460 bp spanning from 13 bp upstream of KCNH2 ATG start codon to 511 bp downstream of exon 15 AATAAA poly(A) signal. The length of short KCNH2 gene is 15249 bp with shortening of intron 2 (14974 bp) and intron 5 (4437 bp) to 600 bp. **B,** Flp-In HEK293 cells were stably transfected with full-length (FL) and short (ST) KCNH2 gene constructs. Flp-In HEK293 cells stably transfected with Kv11.1a (1a) and Kv11.1a-USO (1a-USO) cDNAs were used as controls. Cell lysates were subjected to SDS-PAGE and immunoblotted with an antibody against the N-terminus of Kv11.1 protein. The expression level of hygromycin B phosphotransferase (HPH) encoded by hygromycin B resistant gene served as a loading control. Results shown are representative of three independent experiments.
Figure S3. Voltage clamp recordings of Kv11.1 channel currents. A, Representative currents recorded from Flp-In HEK293 cells stably expressing full-length KCNH2 gene, short KCNH2 gene, Kv11.1a cDNA and Kv11.1a-USO cDNA as indicated. B, Histogram showing the averaged tail current density measured at −50 mV following a 4-s depolarizing pulse to 20 mV for full-length KCNH2 gene (FL, n=8), short KCNH2 gene (ST, n=10), Kv11.1a cDNA (1a, n=7) and Kv11.1a-USO cDNA (1a-USO, n=7). The tail current densities of full-length KCNH2 gene (6.9±0.5 pA/pF) and short KCNH2 gene (7.4±0.7 pA/pF) were reduced compared to that of Kv11.1a cDNA (17.8±1.4 pA/pF). $P<0.001$, FL vs 1a, $P<0.001$, ST vs 1a and $P>0.05$ FL vs ST (ANOVA with Bonferroni correction). The reduced current in the full-length and short KCNH2 gene constructs is consistent with the fact that only about one-third of mRNA transcripts are processed to the functional Kv11.1a isoform in Flp-In HEK293 cells. The data of short KCNH2 gene were also used as WT control in patch clamp experiments shown in Figure 3.
Figure S4. Immunofluorescence staining for cell-surface expression of WT and IVS9-2delA mutant channels. HEK293 cells were transiently transfected with WT (A-C) and IVS9-2delA mutant (D-F) short KCNH2 genes containing an extracellular hemagglutinin epitope inserted between the S1 and S2 transmembrane domains. The phase contrast image corresponding to the immunofluorescence image is shown for each transfection (A, D). The cell-surface staining of nonpermeabлизed cells using monoclonal anti-hemagglutinin antibody was visualized with Alexa 488-conjugated secondary antibody (green, B, E). Following surface labeling the cells were permeabilized and re-probed with polyclonal anti-Kv11.1 N-terminus antibody and Alexa 594-conjugated secondary antibody (red, C, F).
Figure S5. Sequence analysis of RT-PCR products shown in Figure 4 and schematic representation of splicing defects. A, Sequence analysis of the 504 bp product from lane 3 and the 499 bp product from lane 4, and schematic representation of normal and cryptic splicing. Arrow indicates the IVS9-2delA mutation and arrowhead indicates cryptic splice site 5 bp downstream of normal splice site of exon 10. B, Sequence analysis of the 1596 bp product from lane 4 and schematic representation of complete intron 9 retention.