Multiple splicing defects caused by hERG splice site mutation 2592+1G>A associated with long QT syndrome

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Stump MR, Gong Q, Zhou Z. Multiple splicing defects caused by hERG splice site mutation 2592+1G>A associated with long QT syndrome. Am J Physiol Heart Circ Physiol 300: H312–H318, 2011. First published November 5, 2010; doi:10.1152/ajpheart.00818.2010.—Long QT syndrome type 2 (LQT2) is caused by mutations in the human ether-a-go-go-related gene (hERG). Cryptic splice site activation in hERG has recently been identified as a novel pathogenic mechanism of LQT2. In this report, we characterize a hERG splice site mutation, 2592+1G>A, which occurs at the 5′ splice site of intron 10. Reverse transcription-PCR analyses using hERG minigenes transfected into human embryonic kidney-293 cells and HL-1 cardiomyocytes revealed that the 2592+1G>A mutation disrupted normal splicing and caused multiple splicing defects: the activation of cryptic splice sites within exon 10 and intron 10 and complete intron 10 retention. We performed functional and biochemical analyses of the major splice product, hERGΔ24, in which 24 amino acids within the cyclic nucleotide binding domain of the hERG channel COOH-terminus is deleted. Patch-clamp experiments revealed that the splice mutant did not generate hERG current. Western blot and immunostaining studies showed that mutant channels did not traffic to the cell surface. Coexpression of wild-type hERG and hERGΔ24 resulted in significant dominant-negative suppression of hERG current via the intracellular retention of the wild-type channels. Our results demonstrate that 2592+1G>A causes multiple splicing defects, consistent with the pathogenic mechanisms of long QT syndrome.

MATERIALS AND METHODS

hERG minigenes and cDNA constructs. Human genomic DNA was used as a template for PCR amplification of exons 8–12 from the hERG gene. PCR products were cloned into the pcRII vector using TA cloning (Invitrogen, Carlsbad, CA) and verified by DNA sequencing. The NH2-terminus of the hERG minigene was preceded by a Kozak sequence and translation start codon. The 2592+1G>A mutation was generated using the pAlter site-directed mutagenesis system (Promega, Madison, WI). A hERG cDNA construct with an in-frame deletion of 72 nt from exon 10 was made using overlap extension PCR. This construct, hERGΔ24, was designed to generate channels in which 24 amino acids from the cyclic nucleotide binding domain were deleted. For hemagglutinin (HA)-tagged hERG cDNA constructs, the HA epitope (YPYDVPDYA) was inserted in-frame at the COOH-terminus of hERG. The design of the Flag-tagged hERG cDNA construct has been previously described (12). The hERG minigenes and cDNA constructs were subcloned into the pcDNA3 vector (Invitrogen). Minigene and cDNA constructs were transiently and stably transfected into human embryonic kidney (HEK)-293 cells, as previously described (14, 30, 31). Green fluorescent protein cDNA (1 μg) was cotransfected with hERG cDNA (5 μg) to serve as an indicator in patch-clamp experiments. For the coexpression studies, a HEK-293 cell line stably expressing WT hERG was transiently characterized of LQT2 (7, 14). Over 20 LQT2 mutations are predicted to disrupt the splicing of hERG pre-mRNA, and, to date, only a few hERG splice site mutations have been characterized (7, 13, 29).

In normal eukaryotic pre-mRNA processing, the consensus sequence for the 5′ splice site is defined by a 9-bp region at the exon-intron boundary, in which the +1 and +2 positions are 100% conserved as a guanine and thymine, respectively. The LQT2 mutation 2592+1G>A disrupts the invariant +1 position of the 5′ splice site sequence of intron 10. To investigate the pathogenic mechanisms associated with 2592+1G>A, we performed mRNA analysis using wild-type (WT) and mutant minigenes to determine the specific splicing defects. Our results indicated that the 2592+1G>A mutation induces multiple splicing defects, including the activation of three cryptic 5′ splice sites and complete intron 10 retention. Three of the mutant splice products contained a premature termination codon (PTC), while the fourth transcript leads to an in-frame deletion of 24 amino acids from the highly structured, cyclic nucleotide binding domain in the COOH-terminus of the hERG channel. Biochemical and patch-clamp studies revealed trafficking and functional defects in the 2592+1G>A splice mutant channels. Importantly, mutant channels containing the large COOH-terminal deletion coassembled with WT channels, trapping them in the endoplasmic reticulum, which led to the dominant-negative suppression of hERG current. This study demonstrates that the 2592+1G>A mutation induces multiple splicing defects that can contribute to several pathogenic mechanisms associated with long QT syndrome.

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transfected with hERGΔ24 or pcDNA3 empty vector (13, 21). HEK-293 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum at 37°C in 5% CO2. HL-1 murine cardiomyocytes (6) were also transiently transfected with the WT and mutant minigenes using Lipofectamine 2000 (Invitrogen). HL-1 cells were cultured in Claycomb medium (Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum and 0.1 mM norepinephrine (Sigma-Aldrich) at 37°C in 5% CO2.

Reverse transcription-PCR analysis of mRNA splicing. In minigene splicing assays, cytoplasmic mRNA was isolated from transfected HEK-293 and HL-1 cells using the Qiagen RNeasy kit (Qiagen, Valencia, CA). After reverse transcription (RT) using the SuperScript III First-Strand DNA Synthesis kit (Invitrogen), PCR was performed with primers in exon 9 (forward 5′-GTGCTGAAGGCCCTCCT-GAG-3′) and exon 11 (reverse 5′-CCGACTGAAGCCACCC-TCTAAC-3′). The PCR products were analyzed by agarose gel electrophoresis and cloned into pCRII vector for sequence analysis.

Patch-clamp recordings. Membrane currents were recorded in whole cell configuration using suction pipettes, as previously described (30). All patch-clamp experiments were performed at −22°C. The bath solution contained 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH 7.4). The pipette solution contained 130 mM KCl, 1 mM MgCl2, 5 mM EGTA, 5 mM MgATP, and 10 mM HEPES (pH 7.4). An Axopatch-200B patch clamp amplifier was used to record membrane currents, and the computer software pCLAMP8 was used to analyze current signals. Data are presented as means ± SE and are analyzed by Student’s t-test. P < 0.05 is considered statistically significant.

Immunofluorescence microscopy. The characterization of hERG channels by immunofluorescence has been described previously (11, 30). To detect the presence of hERG at the cell surface, intact cells were blocked using a blocking buffer without Triton X-100 and then probed with an anti-hERG antibody specific to the extracellular loop between the S1 and S2 membrane-spanning domains of the channel (Alomone Laboratories, Jerusalem, Israel). Cells were fixed with 4% paraformaldehyde and reprobed with an Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR). To detect hERG in permeabilized HEK-293 cells, the cells were first fixed with 4% paraformaldehyde and blocked with PBS containing 0.2% Triton X-100. Cells were then incubated with the anti-hERG S1-S2 antibody for 1 h at room temperature and then probed with the Alexa Fluor 594-conjugated secondary antibody. Images were acquired with a Zeiss Axioskop 2 microscope.

Western blot and immunoprecipitation. Western blot analysis and immunoprecipitation were performed, as previously described (30, 31). Briefly, proteins from whole cell lysates were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, detected with anti-hERG, anti-HA, and anti-Flag antibody, and visualized with the Plus-ECL (PerkinElmer, Waltham, MA) detection kit. For proteinase K treatment, transfected cells were incubated with 2 ml of buffer containing 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl2 (pH 7.4), with or without 200 μg/ml proteinase K (Sigma-Aldrich) for 30 min at 37°C. The proteinase K activity was stopped by adding ice-cold PBS containing 6 mM PMSF and 25 mM EDTA. In the immunoprecipitation experiments, cotransfected cells were lysed with immunoprecipitation buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mg/ml BSA, and protease inhibitors), and hERG channels were immunoprecipitated with anti-HA antibody. Proteins were detected by Western blot with anti-Flag and anti-HA antibody, as described above.

RESULTS

LQT2 2592+1G>A causes multiple splicing defects. To determine the effect of the 2592+1G>A mutation on pre-mRNA splicing, we performed a minigene splicing assay. HEK-293 cells were transfected with WT and 2592+1G>A minigenes containing exon 8 to exon 12 of hERG genomic DNA (Fig. 1). Transcripts were analyzed by RT-PCR using primers in exons 9 and 11. The RNA isolated from the WT minigene yielded a single PCR product of 504 base pairs. In contrast, four unique splice products (432, 569, 622, and 816 bp) were generated in cells expressing the mutant minigene.

![Fig. 1](http://ajpheart.physiology.org/). Analysis of the 2592+1G>A mutation (Mut) using minigenes transiently transfected into human embryonic kidney (HEK)-293 and HL-1 cells. Wild-type (WT) and 2592+1G>A minigenes were transfected into HEK-293 cells (A) and HL-1 cardiomyocytes (B). Isolated RNAs were amplified by RT-PCR. C: schematic of the WT and 2592+1G>A minigenes used in the transfection experiments. Arrows indicate primers used in RT-PCR experiments. The 2592+1G>A mutation activates cryptic splice sites in exon 10 and intron 10 and also can trigger complete intron 10 retention. Results shown are representative of three independent experiments. Unt, untransfected.
and normal splicing was completely abolished (Fig. 1A). The PCR products were sequenced to determine the identity and origin of the aberrant splicing events observed with the mutant minigene (Supplemental Fig. 1; the online version of this article contains supplemental data). Sequence analyses confirmed that the WT PCR product was generated by normal splicing of intron 10. The major splice product from the mutant minigene was found to be generated by the activation of a 5′ cryptic splice site 72 nt upstream of the normal intron 10 donor site within exon 10. The three minor splice products arose from the activation of 5′ cryptic splice sites within intron 10, 65 nt and 118 nt downstream from the WT splice site, and another from the complete retention of intron 10. The inclusion of intron 10 sequence in these three transcripts results in the introduction of a PTC that is located >50–55 nucleotides upstream of the 3′-most exon-exon junction. According to the proposed rule, these transcripts are predicted to be degraded by NMD, precluding the generation of truncated hERG channels (15, 19). NMD has previously been shown to degrade several hERG nonsense mutations associated with long QT syndrome (5, 14). To determine whether the 2592+1G>A mutation also induced multiple splicing defects in the heart, the WT and mutant minigenes were transiently transfected into the HL-1 cardiomyocytes (Fig. 1B). RT-PCR analyses confirmed the same splicing pattern that was observed in the HEK-293 cell, suggesting that the mutation results in multiple splicing defects in the heart.

hERGΔ24 channels are nonfunctional. The major mutant splice transcript resulted from the activation of a cryptic splice site (GAG/gtgctg) within exon 10 and is predicted to delete 24 amino acids that normally encode the B and C α-helices of the cyclic nucleotide binding domain of hERG channels. To study the functional expression of this splice mutant, hERGΔ24, we performed patch-clamp experiments using HEK-293 cells transiently transfected with a cDNA construct lacking the corresponding 72 nt from the hERG cDNA (Fig. 2). The hERG channels were activated by 4-s depolarizing steps to voltages between −70 and 60 mV from a holding potential of −80 mV, and tail current was recorded on repolarization to −50 mV. The current-voltage plot of hERG current density, recorded at the end of each depolarizing step, indicated that WT channels exhibit typical hERG current with maximal outward current of 31.4 ± 4.9 pA/pF (n = 10) at 0 mV and a steep negative slope conductance at more positive voltages consistent with inward rectification due to voltage-dependent hERG inactivation (Fig. 2B) (23, 28, 31). In contrast, the 24-amino acid deletion within the cyclic nucleotide binding domain completely abolished hERG current.

hERGΔ24 channels have trafficking defect. To investigate the mechanism underlying the absence of current from hERGΔ24 channels, we performed Western blot analysis of WT and mutant channels stably expressed in HEK-293 cells (Fig. 3). WT hERG was expressed as two bands at 135 and 155 kDa, which represent the core-glycosylated, immature channel and the mature, fully glycosylated channel expressed at the plasma membrane, respectively (31). In contrast, hERGΔ24 only expressed the immature form of the protein, indicating that the 24-amino acid deletion disrupted hERG trafficking. To further test trafficking, we treated HEK-293 cells stably expressing WT hERG or hERGΔ24 with a serine protease, proteinase K, which degrades the extracellular domains of proteins expressed at the cell surface. As shown in Fig. 3, the 155-kDa band of WT hERG was sensitive to proteinase K treatment, as digested fragments were observed as a 40- to
80-kDa smear. In contrast, the immature bands of both WT and hERGΔ24 channels were resistant to proteinase K treatment. We then further examined the cell surface expression of WT and mutant channels by immunofluorescence staining (Fig. 4). Permeabilized and nonpermeabilized transfected HEK-293 cells were treated with an antibody specific to the extracellular S1 and S2 domains of hERG. In nonpermeabilized cells, the immunofluorescent signal of WT hERG was detected predominantly along the plasma membrane, while no immunofluorescence was observed in cells transfected with hERGΔ24 (Fig. 4, A and C). In permeabilized cells, WT hERG exhibited a diffuse staining pattern visible throughout the cell, whereas hERGΔ24 distribution was restricted to perinuclear regions and was absent in the cell processes (Fig. 4, E and G). The combined results from our Western blot analysis, proteinase K digestion, and immunofluorescence strongly suggest that hERGΔ24 channels do not traffic to the cell surface.

hERGΔ24 induced dominant-negative effects. Patients with LQT2 mutations carry both WT and mutant alleles, which can result in dominant-negative effects. To determine whether the hERGΔ24 mutant can also suppress WT hERG current in a dominant-negative manner, we performed patch-clamp studies in HEK-293 cells coexpressing both WT hERG and hERGΔ24 (Fig. 5). In these experiments, a HEK-293 cell line stably

Fig. 4. Immunofluorescence staining of WT hERG and hERGΔ24 channels. Nonpermeabilized (A–D) and permeabilized (E–H) HEK-293 cells transfected with WT hERG (A, B, E, and F) and hERGΔ24 (C, D, G, and H) were stained using an antibody specific to the S1-S2 extracellular domain of hERG. The phase-contrast image corresponding to the immunofluorescence image is shown for each transfection.

Fig. 5. Dominant-negative effects. A: representative currents from a WT hERG stable cell line transiently transfected with pcDNA3 vector or hERGΔ24 cDNA. hERG current was recorded using the protocol described in the Fig. 2 legend. B: current-voltage plot of hERG tail current amplitude measured at −50 mV following a depolarizing voltages from −70 to 60 mV for WT hERG + pcDNA3 vector (circles, n = 6) and WT hERG + hERGΔ24 (triangles, n = 8).
expressing WT hERG was transiently transfected with hERGΔ24 cDNA or control plasmid (pcDNA3 vector) (13, 21). We found a significant decrease in the hERG current when the WT hERG stable cell line was transfected with hERGΔ24 compared with the empty vector control (Fig. 5A). Current-voltage plots of the peak tail current densities measured at −50 mV following depolarizing voltages for WT hERG + vector and WT hERG + hERGΔ24 are shown in Fig. 5B. The average peak tail current density following test voltages of 30 mV of WT hERG + vector and WT hERG + hERGΔ24 were 18.8 ± 2.3 pA/pF (n = 6) and 7.4 ± 1.4 pA/pF (n = 8, P < 0.05), respectively, a decrease of ~61% (Fig. 5B). These results suggest that hERGΔ24 is able to coassemble with WT channels to suppress hERG channel function.

**Heterotetrameric WT/hERGΔ24 channels are trapped within the cell.** To determine the mechanisms associated with the dominant-negative suppression of hERG current, we performed immunoprecipitation studies on HEK-293 cells coexpressing differentially tagged WT hERG and hERGΔ24 channels. We transiently transfected HEK-293 cells stably expressing Flag-tagged WT hERG with HA-tagged WT hERG or hERGΔ24. The coassembly of hERG channels was determined by immunoprecipitation with anti-HA antibody, followed by Western blot analysis with anti-Flag antibody (Fig. 6). WT-Flag hERG coimmunoprecipitated with WT-Flag and hERGΔ24-Flag channels. The association between WT-FL and WT-HA channels was observed in the immature and mature bands of the channel. The association between WT-FL and hERGΔ24-HA channels, however, was only observed in the immature band of the channel. This suggests that the WT hERG and hERGΔ24 channels were able to coassemble as core-glycosylated channels, but did not undergo complex glycosylation and trafficking to the cell surface. The membrane was also probed with anti-HA antibody, demonstrating the efficiency of immunoprecipitation of the HA-tagged channels. Western blot analysis with anti-Flag antibody without immunoprecipitation revealed that the coexpression of WT and hERGΔ24 led to the decrease in the expression of the mature band of WT-Flag and the concomitant increase in the expression of the immature band. These results suggest that the coassembly with hERGΔ24 causes a significant trafficking defect of WT hERG channels and thus contributes to the observed dominant-negative suppression of hERG current (Fig. 5).

**DISCUSSION**

The hERG mutation 2592+1G>A was identified in a family in which two sudden deaths occurred at ages 9 and 20 yr, and one of the two living carriers underwent syncopes and a 2-day coma at age 4 yr (3). The present study demonstrates that this splice site mutation causes multiple defects that alter the function of hERG, including the dominant-negative suppression of WT hERG current, and may contribute to the observed severe phenotype. The hERG mutation 2592+1G>A disrupts the +1 position of the 5’ splice site of intron 10, GAT/gtgag, a position that is 100% conserved in mammalian 5’ splice sites. RNA analysis using mutant minigenes transfected into HEK-293 and HL-1 cardiomyocytes revealed that 2592+1G>A abolished normal splicing of hERG and triggered multiple splicing defects, including complete intron 10 retention and the activation of 5’ cryptic splice sites in exon 10 (GAG/gtgctg) and intron 10 (GCT/gtgtag and ATG/gtatac). The consensus value scores compare the intrinsic strengths of splice sites relative to the eukaryotic 5’ consensus splice site sequence; most 5’ sites have scores >70 (4, 24). The 2592+1G>A exon 10 and intron 10 cryptic splice site scores, 64.5, 57.0, and 63.1, are significantly lower than the WT splice site score, 82.3. The intrinsically weak cryptic splice sites are inactive in the presence of the WT intron 10 splice site. The 2592+1G>A mutation completely abolishes normal splicing, allowing activation of the cryptic splice sites, which, despite having low splice scores, all contain the 100% conserved +1 and +2 nucleotides. Inefficient splicing from the weak, cryptic splice sites also results in the generation of unspliced transcripts containing the entire intron 10.

Over 20 hERG splice site mutations have been identified in patients with LQT2 (17, 20, 26, 27). Only three of the mutations, however, have been functionally characterized. The first, T1945+6T>C mutation, occurs at the last position of the 5’ splice site of intron 7, disrupts the base pairing between the splice site and the U1 snRNA, and results in complete intron 7 retention (29). The second, 2398+1G>C, activates an intrinsic 5’ cryptic splice, resulting in the insertion of 18 amino acids into the cyclic nucleotide binding domain between the β5- and β6-sheets (13). This large, in-frame insertion within hERG results in trafficking defective channels that are competent to coassemble with WT channels. The third, the IVS9–28A>G mutation, was recently found to disrupt the branch point and 3’
splice site of intron 9, which activates an upstream cryptic 3′ splice site in a hybrid minigene construct (7). The present characterization of the splice site mutation 2592+1G>A represents the first hERG mutation found to cause multiple splicing defects. We found that the three minor splice products result in transcripts that contain PTGs and are predicted to be degraded by the NMD mechanism. The major cryptic splice product, hERGA24, which lacks the last two α-helices of the cyclic nucleotide binding domain, is trafficking defective, as observed by Western blot analysis, by their expression as core-glycosylated, proteinase K-insensitive proteins and by their perinuclear, intracellular distribution in immunostaining studies. Although this deletion disrupts channel trafficking, it does not preclude tetrameric assembly of mutant and WT channels. Furthermore, immunoprecipitation studies suggest that the dominant-negative suppression of hERG current by hERGA24 is due to the trapping of heterotetrameric channels within the cells.

It has been reported that LQT2 missense mutations located in highly structured regions, such as α-helices or β-sheets, correlate with a trafficking defective phenotype (2, 9, 10, 30). The cyclic nucleotide binding domain of hERG contains highly structured regions, and deletions of these regions have been shown to cause defective trafficking of the hERG channel (1). The present study on the LQT2 splice site mutation 2592+1G>A represents a relevant pathological example of large structural disruption of within the COOH-terminus of hERG. The mutant hERGA24 channel results in an in-frame deletion of the B and C α-helices of the COOH-terminal cyclic nucleotide binding domain. This mutant represents the largest deletion of highly structured domains caused by a disease-causing mutation in LQT2. A recent genotype-phenotype correlation study found that patients with mutations located in α-helical domains have significantly higher risk for arrhythmia-related cardiac events than do patients with mutations in either the β-sheet domains or other unaccredited locations (25). Our present finding that hERGA24 not only disrupts channel trafficking, but also leads to the dominant-negative suppression of hERG current, is consistent with the severe clinical phenotype observed in the patients carrying the 2592+1G>A mutation.

In summary, this report identifies multiple splicing defects induced by the 2592+1G>A mutation: the activation of a cryptic splice sites in exon 10 and in intron 10, and complete intron 10 retention. The activation of the cryptic splice site in exon 10 is the primary splicing defect and resulted in the in-frame deletion of 24 amino acids from the cyclic nucleotide binding domain. Functional and biochemical investigation of this splice mutant found that it gave rise to nonfunctional, trafficking defective channels, which coassembled with WT channels, trapped them in the endoplasmic reticulum, and resulted in the dominant-negative suppression of hERG current.

REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


