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Original article

A splice site mutation in hERG leads to cryptic splicing in human long QT syndrome

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Abstract

Mutations in the human ether-a-go-go-related gene (hERG) cause type 2 long QT syndrome. In this study, we investigated the pathogenic mechanism of the hERG splice site mutation 2398+1G>C and the genotype–phenotype relationship of mutation carriers in three unrelated kindreds with long QT syndrome. The effect of 2398+1G>C on mRNA splicing was studied by analysis of RNA isolated from lymphocytes of index patients and using minigenes expressed in HEK293 cells and neonatal rat ventricular myocytes. RT-PCR analysis revealed that the 2398+1G>C mutation disrupted the normal splicing and activated a cryptic splice donor site in intron 9, leading to the inclusion of 54 nt of the intron 9 sequence in hERG mRNA. The cryptic splicing resulted in an in-frame insertion of 18 amino acids in the middle of the cyclic nucleotide binding domain. In patch clamp experiments the splice mutant did not generate hERG current. Western blot and immunostaining studies showed that the mutant expressed an immature form of hERG protein that failed to reach the plasma membrane. Coexpression of the mutant and wild-type channels led to a dominant negative suppression of wild-type channel function by intracellular retention of heteromeric channels. Our results demonstrate that 2398+1G>C activates a cryptic site and generates a full-length hERG protein with an insertion of 18 amino acids, which leads to a trafficking defect of the mutant channel.

Keywords: Long QT syndrome; Splicing mutation; Arrhythmia; Sudden death; Myocytes

1. Introduction

Long QT syndrome type 2 (LQT2) is caused by mutations in the human ether-a-go-go-related gene (hERG) [1]. hERG encodes the pore-forming subunit of the rapidly activating delayed rectifier K^+ channel (I_{Kr}), which is one of the major ion channel currents contributing to the repolarization of the cardiac action potential [2–4]. A wide spectrum of hERG mutations has been identified including missense, nonsense, in frame insertion or deletion, frameshift and splice site mutations [5–9]. The effects of missense, nonsense, and frameshift mutations on hERG channel function have been studied experimentally [10–14]. However, less is known about the pathogenic mechanisms of splice site mutations in LQT2. To date, more than 10 splice site mutations have been identified in hERG from LQT2 patients [5–9]. For most splice site mutations in hERG, the alterations in the mRNA transcripts were not reported. Although splice site mutations are expected to cause abnormal splicing, it is not possible to predict with certainty how the splice site mutations affect pre-mRNA splicing by genomic sequence analysis alone. Studies of splicing defects in a variety of diseases have shown that splice site mutations can result in different abnormal splicing patterns including exon skipping, use of nearby cryptic splice sites, and whole intron retention [15,16]. Cryptic splice sites are silent splice sites that are activated when the authentic

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splice site is destroyed by mutations. In order to understand the causative role of splice site mutations in LQT2, it is crucial to perform functional analysis of these mutations at the mRNA level. To date, only the 1945+6T>C splice site mutation in hERG has been experimentally characterized at the mRNA level [8]. This splice site mutation has been shown to cause intron 7 retention and exon 7 skipping. Intron 7 retention resulted in the truncation of the hERG channel protein and nonfunctional channels [8].

In this study we investigated the pathogenic mechanism of the hERG splice site mutation 2398+1G>C and genotype–phenotype relationship of the mutation carriers. The 2398+1G>C mutation has been identified in three unrelated LQT2 kindreds [1,5,7]. This mutation affects the consensus sequence of the donor splice site of intron 9. The donor splice site is defined by a consensus sequence of 9 bp at the exon–intron boundary. The 2398+1G>C mutation involves the G at +1 position, which is 100% conserved. Although this mutation is predicted to disrupt normal splicing of intron 9 [1], how it alters splicing is unknown. Our results demonstrate that the 2398+1G>C mutation disrupts the normal splicing and leads to the activation of a downstream cryptic splice site. The cryptic splicing results in a full-length hERG protein with an insertion of 18 amino acids, leading to a trafficking defect of the mutant channel.

2. Materials and methods

2.1. Subjects

The study was approved by the institutional review board and carried out on receipt of informed consent from all study participants. The participants were from three kindreds previously identified as having the 2398+1G>C mutation. Phenotyping was performed based on the history of LQTS-related cardiac events, clinical assessment according to the enhanced ECG criteria in regard to QT intervals and T-wave morphology, and pedigree analysis [8]. Normal unrelated individuals served as controls.

2.2. hERG minigenes and cDNA constructs

Human genomic DNA was used as a template for PCR amplification of a fragment spanning from hERG exons 8 to 11. The PCR products were cloned into pCRII vector using TA cloning kit (Invitrogen, Carlsbad, CA), and verified by DNA sequencing. The minigene was then subcloned into a mammalian expression vector pcDNA5/FRT (Invitrogen), containing a cytomegalovirus (CMV) promoter. The N-terminus of the minigene was tagged by the Myc epitope preceded by a Kozak sequence with a translation start codon, which is in-frame with the hERG translation sequence. The 2398+1G>C mutation in the minigene was generated using pAlter in vitro site-directed mutagenesis system (Promega, Madison, WI). hERG cDNA with the in-frame insertion of 18 amino acids was made by subcloning the RT-PCR fragment containing the 54 bp insertion into the hERG cDNA backbone by SphI and StuI sites. The Flag- and Myc-tagged hERG cDNA constructs were previously described [12,17].

2.3. Transfection of HEK293 cells

The minigene constructs in pcDNA5/FRT vector were stably transfected into HEK293-Flp-In cells as previously described [18]. The hERG cDNA constructs in pcDNA3 vector were stably or transiently transfected into HEK293 cells as previously described [4,11].

2.4. Construction and use of recombinant adenovirus

The AdEasy vector kit was used to generate WT and 2398+1G>C minigene recombinant adenoviruses (Stratagene, La Jolla, CA). First, the WT and 2398+1G>C minigenes were subcloned into pShuttle-CMV vector and recombined with the pAdEasy plasmid in *Escherichia coli* strain BJ5183. The pAdEasy/minigene plasmids were transfected into HEK293 cells. After 2 days, the transfected cells were cultured in growth medium containing 1.25% Seaplaque-agarose to promote the formation of recombinant viral plaques. Approximately two to three weeks later, individual plaques were picked, amplified in HEK293 cells, and purified over a discontinuous CsCl gradient.

2.5. Primary culture of neonatal rat ventricular myocytes

Neonatal rat ventricular myocytes were prepared as described [18]. Briefly, hearts were removed from 1 to 3-day-old Sprague–Dawley rat pups. The ventricles were trimmed free of atria, fat and connective tissues. Myocytes were dissociated by several 20-minute cycles of collagenase/pancreatin treatment. Myocytes were cultured in DMEM with 17% Media 199, 1% penicillin/streptomycin, 10% horse serum and 5% fetal bovine serum. After one day in culture, myocytes were infected with the recombinant adenoviruses.

2.6. RT-PCR analysis of RNA splicing

For endogenous hERG RNA splicing assays, total RNA was isolated from lymphocytes of normal subjects and patients carrying the 2398+1G>C mutation using the RiboPure Blood kit (Ambion, Austin, TX). Contaminating genomic DNA was removed by DNase I treatment according to the protocol provided by the supplier. For minigene splicing assays, cytoplasmic RNA was isolated from transfected HEK293 cells or neonatal rat ventricular myocytes using the Qiagen RNeasy kit. After reverse transcription (RT) using the SuperScript III First-Strand DNA Synthesis kit (Invitrogen), PCR was performed with primers in exon 8 (forward 5′-CTCGAGGAGTACTTCCAGCACG-3′) and exon 10 (reverse 5′-TTGCCAGGCCTTGCATACAG-3′) for RNA isolated from lymphocytes and HEK293 cells. For RT-PCR analysis of RNA isolated from neonatal rat ventricular myocytes, a reverse primer complementary to the sequence in the recombinant adenovirus (reverse 5′-GATCCGGTGGATCGGATATCT-3′) was used to distinguish between the mRNA transcripts of the infected minigene and endogenous rat ERG. The PCR products were analyzed by electrophoresis on agarose gels, and cloned into pCRII vector for sequence analysis.