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Position of premature termination codons determines susceptibility of hERG mutations to nonsense-mediated mRNA decay in long QT syndrome

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ABSTRACT

The degradation of human ether-a-go-go-related gene (hERG, KCNH2) transcripts containing premature termination codon (PTC) mutations by nonsense-mediated mRNA decay (NMD) is an important mechanism of long QT syndrome type 2 (LQT2). The mechanisms governing the recognition of PTC-containing hERG transcripts as NMD substrates have not been established. We used a minigene system to study two frameshift mutations, R1032Gfs*25 and D1037Rfs*82. R1032Gfs*25 introduces a PTC in exon 14, whereas D1037Rfs*82 causes a PTC in the last exon (exon 15). We showed that R1032Gfs*25, but not D1037Rfs*82, reduced the level of mutant mRNA compared to the wild-type minigene in an NMD-dependent manner. The deletion of intron 14 prevented degradation of R1032Gfs*25 mRNA indicating that a downstream intron is required for NMD. The recognition and elimination of PTC-containing transcripts by NMD required that the mutation be positioned >54–60 nt upstream of the 3′-most exon–exon junction. Finally, we used a full-length hERG splicing-competent construct to show that inhibition of downstream intron splicing by antisense morpholino oligonucleotides inhibited NMD and rescued the functional expression of a third LQT2 mutation, Y1078*. The present study defines the positional requirements for the susceptibility of LQT2 mutations to NMD and posits that the majority of reported LQT2 nonsense and frameshift mutations are potential targets of NMD.

1. Introduction

The long QT syndrome type 2 (LQT2) is caused by mutations in the human ether-a-go-go-related gene (hERG, KCNH2) (Curran et al., 1995). hERG encodes the pore forming subunit of the rapidly activating delayed rectifier K^+ channel in the heart. Over five hundred mutations have been identified in patients with LQT2 (Kapplinger et al., 2009; Lieve et al., 2013; Nagaoka et al., 2008; Napolitano et al., 2005; Splawski et al., 2000; Tester et al., 2005). More than 30% of LQT2 mutations are nonsense or frameshift mutations that introduce premature termination codons (PTCs). We previously reported that PTC-containing hERG mRNAs are degraded by nonsense-mediated mRNA decay (NMD) (Bhuiyan et al., 2008; Gong et al., 2007; Zarraga et al., 2011). NMD is an RNA quality control mechanism that selectively degrades mRNA harboring PTCs (Kuzmiak and Maquat, 2006). NMD eliminates abnormal mRNA transcripts harboring PTCs, and thereby preventing the production of truncated proteins that often have dominant-negative effects. Thus, NMD protects against severe disease phenotypes by converting dominant-negative effects to haploinsufficiency (Khajavi et al., 2006).

The identification of potential NMD targets has important implications in genotype–phenotype correlations in LQT2. The LQT2 mutation R1014* generates truncated hERG channel protein that exhibits a dominant-negative effect on the wild-type (WT) channel in the context of the hERG cDNA construct (Gong et al., 2004). However, when an intron-containing minigene is used, the R1014* mutant mRNA is decreased by NMD (Gong et al., 2007). Therefore, haploinsufficiency rather than dominant-negative effect is the underlying mechanism for the R1014* mutation. Most mutation carriers in this family have a mild clinical phenotype which is consistent with this mechanism (Gong et al., 2007). In some cases, NMD could be detrimental if it prevents the production of truncated proteins that are fully or partially functional. This is typified in the LQT2 Q1070* mutation in which NMD results in a nearly complete elimination of mutant mRNA, precluding the formation of functional, truncated channels (Bhuiyan et al., 2008). Although several LQT2 nonsense and frameshift mutations have been shown to induce NMD, the mechanisms by which the NMD machinery recognizes PTC-containing hERG transcripts have not been established.

There are currently several models that describe the recognition of NMD substrates in mammalian cells. The classic model posits that

Abbreviations: CHX, cycloheximide; EJC, exon junction complex; hERG, human ether-a-go-go-related gene; HPH, hygromycin B phosphotransferase; LQT2, long QT syndrome type 2; MO, morpholino oligonucleotide; NMD, nonsense-mediated mRNA decay; nt, nucleotide; PTC, premature termination codon; RPA, RNase protection assay; shRNA, short hairpin RNA; UPF1, up-frameshift protein 1; WT, wild-type.

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NMD occurs when translation terminates >50–55 nt upstream of the 3′-most exon–exon junction (Kuzmiak and Maquat, 2006). According to this model NMD is linked to splicing and translation. Pre-mRNA splicing results in deposition of a multi-protein complex, known as exon-junction-complex (EJC), 20–24 nt upstream of each exon–exon junction (Kuzmiak and Maquat, 2006). The EJCs are displaced by the ribosome during the pioneer round of translation. If translation terminates at a PTC that is located 50–55 nt upstream of an exon–exon junction the downstream EJC serves as a binding platform for NMD factors that trigger NMD. Recent studies, however, have challenged the EJC-dependent model of NMD. For instance, PTCs located as close as 8–10 nt upstream of the 3′-most exon–exon junction still elicit NMD in T cell receptor-β and Ig-μ transcripts (Bühler et al., 2006; Carter et al., 1996). Insertion of an intron downstream of β-globin termination codon does not elicit NMD, whereas some PTCs that are located in the last exon or even in intron-less mRNA trigger NMD, suggesting that the presence of a downstream intron is neither sufficient nor required for triggering NMD (Bateman et al., 2005; Bühler et al., 2006; Rajavel and Neufeld, 2001; Singh et al., 2008). These experimental data support an EJC-independent model in which the physical distance between the termination codon and the poly(A)-binding protein C1 is a crucial determinant for recognition of NMD substrates (Eberle et al., 2008). Clearly, the mechanisms that define the susceptibility of PTC-containing mRNA to NMD may vary in different genes. To understand the mechanisms by which the NMD machinery discriminates between premature and normal termination codons in hERG it is necessary to determine the positional requirements associated with NMD-sensitivity and NMD-resistance. In this study we used hERG minigene and full-length hERG splicing-competent constructs to investigate the role of LQT2 PTC position in susceptibility of LQT2 mutations to NMD.

2. Materials and methods

2.1. Plasmid constructs and transfection

The hERG minigene spanning exons 12–15 and the full-length hERG splicing-competent construct composed of hERG cDNA exons 1–10 and hERG genomic DNA from intron 10 to poly(A) site were generated as previously described (Gong et al., 2007, 2011). The full-length splicing competent construct encodes the full-length hERG potassium channel which corresponds to the hERGa isoform (NM_000238.3). The hERG mutations were generated by pAlter mutagenesis system (Promega, Madison, WI). Flp-In HEK293 cells (Invitrogen, Carlsbad, CA) were stably transfected with pcDNA5 hERG minigenes or full-length hERG splicing-competent constructs and selected with 100 μg/ml hygromycin B. The Flp-In HEK293 cells contain the FRT site at a single genomic locus, allowing stable integration of hERG constructs via Flp recombinase-mediated DNA recombination at a specific genomic location in all cell clones.

2.2. RNase protection assay and immunoblot analysis

RNase protection assay (RPA) was performed as previously described (Gong et al., 2011). Briefly, antisense RNA riboprobes were transcribed in vitro in the presence of biotin-14-CTP. The probe contained 277 nt spanning the region of exons 12 and 13. The total length of the probe was 409 nt and contained sequences from the pCRII vector at both ends. The expression level of hygromycin B resistance gene in the pcDNA5 vector was used as an internal control for normalization. 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 RPA bands was quantified using ImageJ.

Immunoblot was performed as previously described (Gong et al., 2010). The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and the blots were probed with an antibody against the N-terminus of the hERG channel protein (H175, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The expression level of hygromycin B phosphotransferase (HPH) encoded by hygromycin B resistance gene in the pcDNA5 vector was used as an internal control for normalization. The polyclonal antibody against HPH was used at 1:1000 dilution as previously described (Gong et al., 2010).

2.3. RNA interference

An UPF1 short hairpin RNA (shRNA), targeting the coding sequence of hUPF1 (5′-GAGAATCGCCTACTTCACT-3′), was used to inhibit expression of UPF1 (Gong et al., 2007). A double stable mammalian expression system was used to express hERG minigenes and UPF1 shRNAs. The design of the Flp–Cre cell line has been previously described (Stump et al., 2012a). Briefly, a loxP2272/loxP target site was introduced into Flp-In HEK293 cells (Invitrogen, Carlsbad, CA) at a single genomic locus. Flp recombinase-mediated recombination and Cre recombinase-mediated cassette exchange reactions were performed to stably introduce a single copy of each gene of interest. The WT hERG and R1032Gfs*25 mutant minigenes were introduced into the Flp–Cre HEK293 cells at the FRT site, whereas the UPF1 and scramble shRNAs were introduced into the Flp–Cre HEK293 cells at the loxP2272/loxP site. The knockdown of the UPF1 protein was analyzed by immunoblot as previously described (Gong et al., 2007).

2.4. Morpholino oligonucleotide treatment

Morpholino oligonucleotides (MO) were synthesized by Gene Tools (Philomath, OR). The antisense MO (5′-AAGCAGGGCTGGAGCTTACCTG AGA-3′) was designed to target the 5′-splice site of intron 14 (Gong et al., 2011). An invert MO with the same sequence but in reverse orientation was used as control. The Endo–Porter method was used to deliver MOs into the cells.

2.5. Patch-clamp recordings

Membrane currents were recorded in whole-cell configuration as previously described (Gong et al., 2004). The bath solution contained (in mM) 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mM) 130 KCl, 1 $MgCl_2$, 5 EGTA, 5 MgATP, and 10 HEPES (pH 7.2 with KOH). hERG channel current was activated by depolarizing steps between −70 and 50 mV from a holding potential of −80 mV. Tail current was recorded following repolarization to −50 mV. All patch-clamp experiments were performed at 22–23 °C.

2.6. Statistical analysis

Data are presented as mean ± standard error of mean. Student's t-test was used for comparison between two groups. ANOVA with Bonferroni correction was used for comparisons between more than two groups. $P < 0.05$ is considered statistically significant.

3. Results

3.1. LQT2 mutation R1032Gfs*25 but not D1037Rfs*82 leads to NMD

To determine the role of PTC position of LQT2 mutations in susceptibility to NMD, we studied two frameshift LQT2 mutations, R1032Gfs*25 and D1037Rfs*82. The R1032Gfs*25 and D1037Rfs*82 mutations have been previously reported as G1301fs/24 (del3094) and G1036fs/82 (insG3107-3108), respectively (Splawski et al., 2000). We used a minigene containing the hERG genomic sequence spanning exon 12 to exon 15 (Fig. 1A) (Gong et al., 2007). R1032Gfs*25 introduces a PTC in exon 14, and D1037Rfs*82 results in a PTC in the last exon (exon 15) of the hERG gene. We stably expressed the WT and LQT2 mutant