Nonsense Mutations in hERG Cause a Decrease in Mutant mRNA Transcripts by Nonsense-Mediated mRNA Decay in Human Long-QT Syndrome

Qiuming Gong, MD, PhD; Li Zhang, MD; G. Michael Vincent, MD; Benjamin D. Horne, PhD, MPH; Zhengfeng Zhou, MD, PhD

Background—Long-QT syndrome type 2 (LQT2) is caused by mutations in the human ether-a-go-go-related gene (hERG). More than 30% of the LQT2 mutations result in premature termination codons. Degradation of premature termination codon–containing mRNA transcripts by nonsense-mediated mRNA decay is increasingly recognized as a mechanism for reducing mRNA levels in a variety of human diseases. However, the role of nonsense-mediated mRNA decay in LQT2 mutations has not been explored.

Methods and Results—we examined the expression of hERG mRNA in lymphocytes from patients carrying the R1014X mutation using a technique of allele-specific transcript quantification. The R1014X mutation led to a reduced level of mutant mRNA compared with that of the wild-type allele. The decrease in mutant mRNA also was observed in the LQT2 nonsense mutations W1001X and R1014X using hERG minigenes expressed in HEK293 cells or neonatal rat ventricular myocytes. Treatment with the protein synthesis inhibitor cycloheximide or RNA interference–mediated knockdown of the Upf1 protein resulted in the restoration of mutant mRNA to levels comparable to that of the wild-type minigene, suggesting that hERG nonsense mutations are subject to nonsense-mediated mRNA decay.

Conclusions—These results indicate that LQT2 nonsense mutations cause a decrease in mutant mRNA levels by nonsense-mediated mRNA decay rather than production of truncated proteins. Our findings suggest that the degradation of hERG mutant mRNA by nonsense-mediated mRNA decay is an important mechanism in LQT2 patients with nonsense or frameshift mutations. (Circulation. 2007;116:17-24.)

Key Words: arrhythmia ■ ion channels ■ long-QT syndrome ■ myocytes

Long-QT syndrome is a disease associated with delayed cardiac repolarization and prolonged QT intervals on the ECG, which can lead to ventricular arrhythmias and sudden death. The inherited long-QT syndrome type 2 (LQT2) is caused by mutations in the human ether-a-go-go-related gene (hERG), which encodes the pore-forming subunit of the rapidly activating delayed rectifier K⁺ channel (I_Kr) in the heart. More than 250 hERG mutations have been identified in patients with LQT2. The mechanisms of hERG channel dysfunction in LQT2 mutations have been studied extensively in the last 10 years. Most previous studies, however, have focused on the analysis of mutant proteins and channel function. More than 30% of LQT2 mutations are nonsense or frameshift mutations that introduce premature termination codons (PTCs). These PTC mutations generally are assumed to result in truncated dysfunctional channel proteins, and several nonsense and frameshift mutations have been studied at the protein level. However, it is now becoming clear that nonsense and frameshift mutations bearing PTCs can destabilize mRNA transcripts via a mechanism known as nonsense-mediated mRNA decay (NMD) in many human diseases, resulting in decreased abundance of mutant mRNA transcripts rather than in production of truncated proteins.

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NMD is an RNA surveillance mechanism that selectively degrades mRNA transcripts containing PTCs resulting from nonsense or frameshift mutations. The role of NMD as a disease-causing mechanism of PTC mutations is becoming increasingly evident. According to the proposed rule, NMD occurs when translation terminates >50 to 55 nt upstream of the 3′-most exon-exon junction. The molecular mechanisms of NMD have been studied extensively. These studies have shown that pre-mRNA splicing deposits the exon junction complex ∼20 to 40 nt upstream of the exon-exon junction in spliced mRNA. The exon junction

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complex can recruit Upf proteins, which are required for NMD. Several Upf proteins (Upf1, Upf2, Upf3a, Upf3b) have been identified. The Upf1 protein appears to play a key role in the distinction between proper and improper translation termination. Upf1 is a group 1 helicase that has RNA-dependent ATPase and ATP-dependent 5' to 3' helicase activities. Knockdown of Upf1 by RNA interference (RNAi) has been shown to inhibit NMD.

The objective of this work was to determine whether NMD occurs in hERG mutations that contain PTCs. We investigated 2 nonsense mutations, W1001X and R1014X, in the C-terminal region of the hERG channel. The W1001X and R1014X mutations have previously been studied at the protein level using hERG cDNAs. It was found that both mutations produced truncated hERG channel proteins and reduced hERG current amplitude. The R1014X mutation also caused a dominant-negative effect on the wild-type (WT) hERG current, which is expected to result in a severe clinical phenotype. However, the R1014X carriers have presented with a mild phenotype. In the present study, we demonstrate that rather than the production of truncated proteins, the primary defect of the W1001X and R1014X mutations is the degradation of mutant mRNA by NMD.

Methods

Subjects

The study was approved by the institutional review board and carried out on receipt of informed consent. The participants were blood-related members of a large family previously identified as having the R1014X mutation. Phenotyping was performed on the basis of the history of LQTS-related cardiac events, the assessment of QT intervals and T-wave morphology, and pedigree analysis. Genotyping was conducted by sequencing of DNA samples collected from buccal swabs. Normal control subjects were unrelated individuals.

RNA and DNA Preparations From Blood Samples

Total RNA was isolated from peripheral blood lymphocytes using the RiboPure-Blood kit (Ambion, Austin, Tex). The isolated RNA was treated with RNase-free DNase to remove genomic DNA. Genomic DNA was isolated from lymphocytes or Epstein Barr virus–transformed lymphoblastoid cells with the DNeasy tissue kit (Qiagen, Valencia, Calif).

Allele-Specific Quantification of RNA Transcripts and Genomic DNA

The relative abundance of RNA transcripts from WT and R1014X alleles was determined by a modified “hot-stop” polymerase chain reaction (PCR) method. In this assay, the regular reverse-transcription PCR was carried out using the primers in exon 13 (E13-F, forward 5'-GCCTTCTCAGGAGTGTCCAA-3') and exon 14 (E14-R, reverse 5'-GAAAGCGAGTCCAAGGTTGAG-3'). After 35 cycles, [32P]-dCTP was added and subjected to a single cycle of PCR. With hot-stop PCR, only homoduplexes incorporated [32P]-labels and any heteroduplexes formed during previous cycles were unlabeled. Thus, hot-stop PCR will prevent the detection of WT/mutant heteroduplexes, which are resistant to restriction enzyme digestion. Because hot-stop PCR analysis yields a relative measure of transcripts from 2 alleles, normalization to a reference housekeeping gene is unnecessary. The hERG genomic DNA was analyzed by hot-stop PCR with the same forward primer as used in reverse-transcription PCR and a reverse primer in intron 13 (I13-R, 5'-CTCCGCGCTAGAGGTGTG-3'). For analysis of allelic variation in hERG mRNA expression in normal subjects, the ratio of a common polymorphism 1692A/G was determined by hot-stop PCR using the primers in exon 6 (E6-F, forward 5'-ATCAACTTCCGCACCCCTA-3') and exon 7 (E7-R, reverse 5'-TGTGTGGCTGCTCCATGT-3'). The labeled PCR products were treated with TaqI or NheI restriction enzyme and analyzed by 5% PAGE and autoradiography. For quantitative analysis, the intensity of each band was quantified with Scion Image software (Scion Corp, Frederick, Md). The ratio of 2 alleles was calculated, and a correction factor according to the respective GC content of each digested product was applied to the ratio.

Construction of Minigenes

Human genomic DNA was used as a template for PCR amplification of fragments spanning from hERG exons 12 to 15. The PCR products were cloned into pCRII vector with the TA cloning kit (Invitrogen, Carlsbad, Calif) and verified by DNA sequencing. The minigenes were then subcloned into a mammalian expression vector pcDNA5/FRT (Invitrogen). The N-terminus of the minigene was tagged by Myc epitope, which is in frame with the hERG translation sequence. The W1001X and R1014X mutations in the minigenes was determined by a modified “hot-stop” polymerase chain reaction (PCR) method. In this assay, the regular reverse-transcription PCR was carried out using the primers in exon 13 (E13-F, forward 5'-GCCTTCTCAGGAGTGTCCAA-3') and exon 14 (E14-R, reverse 5'-GAAAGCGAGTCCAAGGTTGAG-3'). After 35 cycles, [32P]-dCTP was added and subjected to a single cycle of PCR. With hot-stop PCR, only homoduplexes incorporated [32P]-labels and any heteroduplexes formed during previous cycles were unlabeled. Thus, hot-stop PCR will prevent the detection of WT/mutant heteroduplexes, which are resistant to restriction enzyme digestion. Because hot-stop PCR analysis yields a relative measure of transcripts from 2 alleles, normalization to a reference housekeeping gene is unnecessary. The hERG genomic DNA was analyzed by hot-stop PCR with the same forward primer as used in reverse-transcription PCR and a reverse primer in intron 13 (I13-R, 5'-CTCCGCGCTAGAGGTGTG-3'). For analysis of allelic variation in hERG mRNA expression in normal subjects, the ratio of a common polymorphism 1692A/G was determined by hot-stop PCR using the primers in exon 6 (E6-F, forward 5'-ATCAACTTCCGCACCCCTA-3') and exon 7 (E7-R, reverse 5'-TGTGTGGCTGCTCCATGT-3'). The labeled PCR products were treated with TaqI or NheI restriction enzyme and analyzed by 5% PAGE and autoradiography. For quantitative analysis, the intensity of each band was quantified with Scion Image software (Scion Corp, Frederick, Md). The ratio of 2 alleles was calculated, and a correction factor according to the respective GC content of each digested product was applied to the ratio.

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were generated with the pAlter in vitro site-directed mutagenesis system (Promega, Madison, Wis) and verified by DNA sequencing.

Stable Expression of Minigene Constructs in HEK293 Cells

The minigenes in pcDNA5/FRT vector were stably transfected into HEK293 cells by using the Flip-In method (Invitrogen). In this approach, an FRT site sequence is integrated into the genome of HEK293 cells and recombined by Flp recombinate with the FRT site of the pcDNA5/FRT vector. The pcDNA5/FRT vector carries the hygromycin resistance gene, which is used for the selection of stable cell lines.

RNase Protection Assay of mRNA Transcripts From Minigene Transfected Cells

RNA isolation and RNase protection assay (RPA) were performed as previously described. Briefly, cytoplasmic RNA was isolated from HEK293 cells or neonatal rat ventricular myocytes expressing hERG minigenes with the RNeasy kit (QiaGen). The antisense RNA riboprobes were transcribed in vitro in the presence of biotin-16-UTP (Roche, Indianapolis, Ind). RNA (30 μg) was analyzed with the riboprobes using the RPAIII and BrightStart BioDetect kits (Ambion). Yeast RNA was used as control for the complete digestion of riboprobes by RNase. The expression level of the hygromycin resistance gene from the pcDNA5/FRT vector or the E2 gene from adenovirus was used as a loading control for normalization. The intensity of each band was quantified with Scion Image software.

RNA Interference

Two plasmids, pSUPERpuro-hUpf1/I and pSUPERpuro-hUpf1/II (kindly provided by Dr Oliver Mühlemann), were used to inhibit expression of Upf1 as described by Paullussen et al. These plasmids contain short hairpin RNAs targeting 2 sequences of hUpf1 (5′-GAGAATCGCCTACTTCACT-3′ for pSUPERpuro-hUpf1/I and 5′-GATGCAGTTCCGCTCCATT-3′ for pSUPERpuro-hUpf1/II). The HEK293 cells stably expressing WT or R1014X minigenes were transfected with a mixture of 1 μg pSUPERpuro-hUpf1/I and 1 μg pSUPERpuro-hUpf1/II or 2 μg pSUPERpuro with scrambled sequence of hUpf1/I using Lipofectamine 2000 (Invitrogen). At 24 hours after transfection, puromycin was added to the final concentration of 1.5 μg/ml for 48 hours to eliminate the untransfected cells. Before analysis, the cells were cultured without puromycin for at least 24 hours to avoid potential effects of this translation inhibitor on NMD. The knockdown of the Upf1 protein was analyzed by Western blot as described.

Construction and Use of Recombinant Adenovirus

The AdEasy vector kit was used to generate WT and R1014X minigene recombinant adenoviruses (Strategene, La Jolla, Calif). First, the WT and R1014X minigenes were subcloned into pShuttle-CMV vector and recombined with the pAdEasy plasmid in Escherichia coli strain BJ5183. The pAdEasy/miniplasmids were amplified in HEK293 cells, and purified over a discontinuous CsCl gradient.

Primary Culture of Neonatal Rat Ventricular Myocytes

Neonatal rat ventricular myocytes were prepared as described. Briefly, 1- to 3-day-old Sprague-Dawley rat pups were killed under ether anesthesia by decapitation, and hearts were removed through a sternotomy. The ventricles were trimmed free of atria, fat, and connective tissues. Myocytes were dissociated by several 20-minute cycles of collagenase/pancreatin treatment and serum neutralization. Myocytes were cultured in Dulbecco’s modified Eagle’s medium with 17% Media 199, 10% horse serum, 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). After 1 day in culture, myocytes were infected with the recombinant adenoviruses.

Statistical Analysis

Data are presented as mean±SEM for QTc intervals or mean±SD for QTc intervals and typical LQT2 ECG pattern with the subtle bifid T waves. The mean interval QTc interval in the mutation carriers was 461±7 ms (n=7) versus 420±13 ms (n=8) in noncarriers (P<0.001). Four mutation carriers had exercise tests, with maximum QTc value of 510±10 ms. In this family, 89% (8 of 9) of the R1014X mutation carriers were asymptomatic. The only person with a history of cardiac events is the 71-year-old proband. From 32 to 42 years of age, she had multiple syncopal episodes and 1 cardiac arrest that were associated with the presence of hypokalemia (serum K+ 2.7 mEq/L) caused by taking a dietary supplement containing potassium-wasting diuretics or taking QT-prolonging antihistamines. Since then, she has remained asymptomatic by stopping the potassium-wasting diet, avoiding QT-prolonging drugs, and taking β-blockers.

Analysis of mRNA Isolated From Blood Samples

The R1014X mutation causes premature termination of the hERG channel protein. This mutation has previously been studied at the protein level. However, it has been known that nonsense and frameshift mutations that contain PTCs can lead to the degradation of mRNA transcripts by NMD in many diseases. To determine the underlying pathogenic mechanism of the R1014X mutation, it is important to study this mutation at the mRNA level. Because the affected heart tissue from the mutation carriers was not available for this study, we analyzed hERG mRNA transcripts isolated from the lymphocytes of patients carrying the R1014X mutation.
To rule out possible allelic variation in hERG expression in the general population, we examined the allele-specific expression of hERG mRNA in normal subjects. We analyzed 3 normal subjects who are heterozygous for a common polymorphism, 1692A/G. To distinguish between 1692A and 1692G alleles, the relative levels of mRNA transcripts from 1692A and 1692G alleles were measured by the hot-stop PCR assay. The 1692A allele contains an NheI restriction site, which is absent in the 1692G allele. Thus, digestion with NheI should allow us to determine the relative ratio of the 2 WT alleles. After digestion of the PCR products with NheI, the 1692A allele should be cut into 2 fragments of 286 and 46 bp, and the 1692G alleles should remain uncut (332 bp). As shown in Figure 2B, in subjects 3, 4, and 5 (lanes 3 to 5), there are 2 bands of 268 and 332 bp, suggesting that they are heterozygous for the 1692A/G polymorphism. In these 3 normal subjects, the average ratio of 1692G to 1692A was 0.97 ± 0.07. This result suggests that there is no significant allelic variation in hERG mRNA expression in normal subjects. Subjects 1 and 2 (lanes 1 and 2) are homozygous for 1692A and 1692G, respectively.

**Minigene Analysis of the R1014X and W1001X Mutations**

To study whether the decrease in the abundance of mRNA levels in the R1014X mutation is due to NMD, we constructed minigenes containing the hERG genomic sequence spanning from exon 12 to 15 and expressed the minigenes in HEK293 cells. In the minigene experiments, the 2 LQT2 nonsense mutations R1014X and W1001X were analyzed by RPA. Figure 3A shows the structure of the minigene and the mRNAs after splicing. The R1014X and W1001X mutations lead to a PTC in exon 13, which is expected to trigger NMD. As shown in Figure 3B, the mRNA level of the R1014X minigene was significantly lower than that of the WT minigene. Because degradation of mRNA by NMD depends on protein synthesis, we examined whether inhibition of protein synthesis by cycloheximide (CHX) abrogates NMD of the mutant mRNA, as has been shown for other PTC-containing transcripts. The cells expressing WT and R1014X minigenes were treated with CHX for 3 hours before RNA isolation. Treatment with CHX had no effect on the level of WT mRNA but significantly increased the level of R1014X mutant mRNA, suggesting that the mutant mRNA is degraded by NMD. Similar results were observed in the W1001X minigene (Figure 3C), suggesting that the degradation of PTC-containing mRNAs by NMD may represent a common mechanism in LQT2 patients with nonsense mutations.

**Effect of Suppression of Upf1 on NMD of the R1014X Mutation**

Recently, the Upf1 protein has been identified as a key factor for NMD. Reducing Upf1 expression by RNAi has been used as a functional assay to assess the NMD sensitivity of PTC-containing mRNA transcripts. To study the role of Upf1 in the reduced mRNA level of the R1014X mutation, we used the RNAi method to knock down Upf1 protein expression. In these experiments, HEK293 cells stably ex-
pressing the WT and R1014X minigenes were transfected with pSUPERpuro-hUpf1/I and pSUPERpuro-hUpf1/II. The Upf1 knockdown in the transfected cells was confirmed by Western blot analysis using anti-Upf1 antibody (a gift from Dr Jens Lykke-Andersen) (Figure 4A). Detection of tubulin with anti-tubulin antibody served as a loading control. In the RPA analysis of hERG minigene mRNA transcripts, the level of R1014X mutant mRNA was significantly increased in Upf1-siRNA–transfected cells (Figure 4B). These results suggest that mRNA transcripts of the R1014X mutation undergo NMD.

Analysis of NMD in Neonatal Rat Myocytes Using R1014X Adenovirus Minigene

The above experiments indicate that mRNA transcripts of the R1014X mutation are subject to NMD in lymphocytes and HEK293 cells. The noncardiac cells may behave differently from cardiac cells in the degradation of mutant mRNA by NMD. Therefore, it is important to evaluate whether the defects observed in noncardiac systems are present in cardiac myocytes. To test whether NMD of the R1014X mutation occurs in cardiac myocytes, we infected neonatal rat ventricular myocytes with WT or R1014X minigene adenovirus and performed RPA analysis. As shown in Figure 5, the mRNA level of the R1014X mutant was significantly lower than that of WT. Treatment with CHX had no effect on the level of WT mRNA but significantly increased the level of R1014X mutant mRNA, suggesting that the R1014X mutant mRNA is degraded by NMD in cardiac myocytes. No protected bands in the control lane indicate that the riboprobe is specific for exogenous hERG transcripts.

Discussion

The present results demonstrate that the W1001X and R1014X mutations lead to a reduction of mutant mRNA transcripts by NMD. Our findings provide the first evidence that PTC-containing mRNA transcripts in LQT2 are subject to NMD. NMD is an evolutionarily conserved mRNA surveillance pathway that detects and eliminates PTC-containing mRNA transcripts, thereby preventing the synthesis of truncating proteins.
NMD occurs when translation terminates at nonsense codons, usually due to the presence of premature termination codons (PTCs). This termination event occurs at an exon-exon junction, which is a feature common to most LQT2 mutations. NMD as a modifier of haploinsufficiency may explain the severity of LQT2 phenotypes, especially in the case of R1014X mutations. This specific mutation has been associated with a severe phenotype, which is consistent with the findings of this study.

The mechanisms underlying LQT2 mutations can be classified into five main categories, as illustrated in Figure 6. Class 1 mutations involve abnormal protein synthesis by defective transcription or translation. Class 2 mutations result from defective protein trafficking, and class 3 mutations cause abnormal gating and/or kinetics. Class 4 mutations are associated with altered or absent channel selectivity or permeability. Class 5 mutations are characterized by the presence of PTC-containing transcripts, which are targeted for degradation by NMD, thus preventing the production of truncated proteins.

In the present study, the R1014X mutation was shown to result in a severe phenotype due to the accumulation of truncated mutant hERG channels. This result is consistent with previous studies that have demonstrated the importance of NMD in the regulation of hERG mRNA levels. The findings from this study highlight the role of NMD in the modulation of LQT2 phenotypes and suggest potential therapeutic strategies for patients with LQT2 mutations.

The study also provides insights into the complex interplay between mutation type and phenotype, emphasizing the importance of comprehensive approaches in the study of LQT2. The results underscore the necessity for careful consideration of the underlying mechanisms when interpreting the clinical significance of LQT2 mutations and highlight the potential for targeted therapeutic interventions.
with the observed clinical presentation of this family. It is interesting to note that the W1001X mutation carriers also present with a mild LQT2 phenotype. Moss et al reported that LQT2 patients with mutations in the pore region of hERG have a significantly higher risk of arrhythmia-related cardiac events than patients with nonpore mutations. Although the difference may be explained by in vitro electrophysiological effects of reported hERG mutations, with pore mutations having a greater negative effect on hERG current than nonpore mutations, it also is possible that NMD may play a role. It is noted that only 6% of LQT2 mutations in the pore region are nonsense or frameshift mutations, whereas >40% of the mutations in nonpore regions are nonsense or frameshift mutations. Clearly, further genotype-phenotype correlation studies are required to test whether NMD contributes to the observed differences in clinical presentations of pore and nonpore LQT2 mutations.

There are potential limitations to the present study. Our present experiments analyzed endogenously expressed mRNA from patients carrying the R1014X mutation, but the RNA was isolated from lymphocytes rather than the affected heart tissue. Although we have shown that the R1014X mutant minigene expressed in neonatal rat ventricular myocytes leads to reduced mRNA levels by NMD, further studies are required to determine whether the endogenous PTC-containing mRNA in human heart tissue is subject to NMD. Verification of our findings in human heart would strengthen the conclusion that hERG mutations that contain PTCs can lead to degradation of the mutant mRNA by NMD.

In summary, our findings that nonsense mutations in hERG lead to a reduced level of mutant mRNA by NMD add to our understanding of the disease-causing mechanisms of hERG mutations in LQT2. Thus, in studies of hERG nonsense and frameshift mutations, it is important to first analyze the abundance of mRNA to determine whether these PTC mutations are targeted by NMD. Obviously, this important point had been overlooked in previous studies that analyzed hERG PTC mutations only at the protein and functional levels. Because PTC mutations account for >30% of LQT2 mutations, the RNA surveillance imposed by NMD is of fundamental importance in the pathogenesis of LQT2.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Congenital long-QT syndrome type 2 (LQT2) is caused by mutations in human ether-a-go-go related gene (*hERG*), which encodes a voltage-gated potassium channel (*I_{Kr}*). The present work demonstrates that LQT2 nonsense mutations show a decrease in mutant mRNA transcripts via nonsense-mediated mRNA decay (NMD), an RNA surveillance mechanism that selectively eliminates the mRNA transcripts that contain premature termination codons. These results indicate that, contrary to intuition, the predominant consequence of *hERG* nonsense mutations is not the production of truncated proteins but rather the degradation of mutant mRNA by NMD. Given that nonsense and frameshift mutations account for >30% of LQT2 mutations, the RNA surveillance imposed by NMD is of fundamental importance in the pathogenesis of LQT2. Our findings have important implications for genotype-phenotype correlation investigations in LQT2. By eliminating abnormal mRNA transcripts carrying premature termination codons, NMD prevents the production of truncated proteins that could act in a dominant-negative manner. The clinical significance of NMD is the protection against severe disease phenotypes by converting the dominant-negative effect to haploinsufficiency. Thus, NMD appears to be an important factor in modifying phenotypic severity in LQT2.
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