Clinical and genetic spectrum of hereditary cardiac arrhythmia syndromes

Bhuiyan, Z.A.

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Recurrent Intrauterine Fetal Loss due to Near Absence of HERG: Clinical and Functional Characterization of a Homozygous Non-sense HERG mutation

Zahurul A. Bhuiyan,1 Tarek S. Momenah,2 Qiuming Gong,3 Ahmad S. Amin,4,5 Saleh Al Ghamdi,2 Julene S. Carvalho,6,7 Tessa Homfray, 6 Marcel M.A.M. Mannens,1 Zhengfeng Zhou,3 Arthur A. M. Wilde4

Department of Clinical Genetics1 and Heart Failure Research Centre,4 Academic Medical Center, University of Amsterdam, Netherlands
Department of Pediatric Cardiology,2 Prince Sultan Cardiac Centre, Riyadh, Saudi Arabia
Division of Cardiovascular Medicine,3 Department of Medicine, Oregon Health and Science University4, Portland, Oregon, USA
Cellular and Molecular Arrhythmia Research Program,5 Department of Medicine, University of Wisconsin, Madison, USA
Department of Fetal Medicine,6 St. Georges Hospital Medical School, London, UK
Brompton Fetal Cardiology,7 Royal Brompton Hospital, London, UK

Abstract

Background:
Inherited arrhythmias may underlie intrauterine and neonatal arrhythmias. Resolving the molecular genetic nature of these rare cases provides significant insight into the role of the affected proteins in arrhythmogenesis and (extra-) cardiac development.

Objectives:
We have performed clinical, molecular and functional investigation in a consanguineous Arabian family with repeated early miscarriages and two intrauterine fetal losses in the early part of the 3rd trimester of pregnancy due to persistent arrhythmias.

Methods:
In-depth clinical investigation was performed in two siblings, both developed severe arrhythmia during the 2nd trimester of pregnancy. Homozygosity mapping with microsatellite repeat polymorphic markers encompassing various cardiac ion channel genes linked to electrical instability of the heart was performed. Screening of the candidate gene in the homozygous locus was done. Biochemical and Electrophysiology analysis was performed to elucidate the function of the mutated gene.

Results:
Screening of the HERG gene in the homozygous locus detected a homozygous non-sense mutation Q1070X in the HERG C-terminus in the affected children. Biochemical and functional analysis of the Q1070X mutant showed that the mutant HERG though have the properties to traffic to the plasma membrane and could form functional channels, are destroyed by the Non-sense Mediated Decay (NMD) pathway before its translation. NMD leads to near absence of HERG in the homozygous Q1070X mutation carriers causing debilitating arrhythmias (already prior to birth) in the homozygous carriers and apparently without any phenotype in the heterozygous carriers.

Conclusions:
Homozygous HERG Q1070X is equivalent to a near functional knockout of HERG and clinical consequences appear early, originating at the early stages of embryonic life. HERG Q1070X is rendered functionless by the NMD pathway before it could form a functional ion channel.
Clinical and Genetic Spectrum of Hereditary Cardiac Arrhythmia Syndromes

Key Words
Long QT Syndrome, Sudden Cardiac Death, Electrophysiology, HERG, NMD

Abbreviation list
LQT, long QT syndrome; ECG, electrocardiogram; AV, atrioventricular; QTc, corrected QT interval; HERG, human ether-a-go-go–related; NMD, Non-sense mediated decay.

Introduction
Congenital LQT S is an inherited disorder defined by prolongation of the QT interval. Patients with LQTS are predisposed to the ventricular tachyarrhythmia torsade de pointes (TdP) leading to recurrent syncope or sudden cardiac death. LQTS affects an estimated 1 in 2,000 people worldwide. The molecular basis of LQTS is heterogeneous and caused by mutations in one of several genes including KCNQ1, HERG, KCNJ2, KCNE1, and KCNE2 encoding potassium channel subunits, the cardiac sodium channel gene SCN5A, L-type calcium channel gene CACNA1C, and ANK2, a membrane anchoring gene. Mutations involving the gene HERG are responsible for the LQT2 form of LQTS. In cellular expression studies, HERG mutations are associated with reduction in the rapid component of the delayed rectifier repolarizing current (I\textsubscript{Kr}). Diminution in the repolarizing I\textsubscript{Kr} current contributes to lengthening of the QT interval, the electrocardiographic hallmark of LQT2 patients.

Cardiac I\textsubscript{Kr} channels are composed of HERGa and HERGb subunits, which differ in their amino terminal sequence. Functional HERG channels result from the co-assembly of 4 HERG subunits (HERGa and HERGb) into a tetrameric protein. Proposed molecular mechanisms that may account for reduced I\textsubscript{Kr} current in patients with HERG mutations are disruption of protein synthesis, protein trafficking, gating, or permeation, and degradation of mutant mRNA by the nonsense-mediated mRNA decay (NMD) pathway. Homozygous missense mutations in HERG are associated with a severe form of LQT, with symptoms appearing in the early years of life. Natural human knockout of the larger isoform, HERGa had more severe effects, with intrauterine fetal death and also severe fetal arrhythmias in a new born, where tachyarrhythmias were diagnosed in the utero. Bi-allelic non-sense mutations affecting both HERG isoforms (HERGa and HERGb) have not yet been described.

In this report, we have identified a family with recurrent stillbirths and neonatal/intrauterine long QT syndrome, the disease locus was mapped to HERG, a HERG mutation segregating with disease was identified, but the cell surface expression and electrophysiology were amazingly normal. Because the mutation truncated the distal C-terminus of the protein, we then examined whether loss of interaction with a HERG C-terminus interacting protein,
14-3-3ε, might contribute to the phenotype. In addition, we investigated the hypothesis that Q1070X results in accelerated mRNA degradation and thus failure of protein translation, the phenomenon of “nonsense mediated decay” recently implicated as a new loss-of-function of mechanism in ion channel and other diseases.

Methods

Clinical data
The study was performed according to a protocol approved by the local ethics committee. The patients studied constitute the offspring of asymptomatic, consanguineous, Arabian parents (Fig. 2A). There were two miscarriages at 8 (II:1) weeks and 10 (II:2) weeks of pregnancy, followed by the delivery of a stillborn (II:3) hydropic baby who had reported irregular heart beat, at 29th weeks of pregnancy. Autopsy was performed, complete work-up for still birth, and chromosome culture was also performed. Fourth pregnancy was uneventful with the delivery of a healthy male infant (II:4). The fifth pregnancy (II:5) was complicated by brady and tachyarrhythmia in the second trimester followed by fetal demise at 29 weeks. Autoantibody screening was undertaken, which was negative for antinuclear antibodies, Anti SS-A, Anti SS-B, Anti Sm and Anti RNP.

The mother was 29 years old when she conceived the proband (II-6). She was closely followed throughout the pregnancy. The first fetal echocardiogram was performed at 16 weeks and from 20 weeks the fetus was monitored weekly. Brady and tachyarrhythmias were seen from 22 weeks. The proband was followed closely after birth, with continuous monitoring, ECG and echocardiogram. Resting ECG, exercise induced ECG and 24 hours Holter monitoring were performed in both parents.

Haplotype and Mutation analyses
Considering the consanguinity of the parents, and the absence of cases of sudden death in the family, we have performed homozygosity mapping around several candidate genes SCN5A, KCNH2, KCNQ1, CLCN2, ANK-B, CALNA2, KIR6.2, CACNA1C, KCNJ12, KCNE1, MIRP1, and KCNJ4, in which mutations were reported to cause electrical instability of the heart. Homozygosity mapping utilises the concept that affected individuals will be homozygous by descent for a mutation and polymorphic markers nearby. Microsatellite repeat markers from ABI-Prism Linkage Mapping Set (PE Biosystems) encompassing these genes were used. After PCR, the amplified fragments were separated using the ABI Prism 377 automatic DNA sequencer (PE Biosystems), and analyzed with the Genotyper program (PE Biosystems). DNA from peripheral blood lymphocytes and amniotic fluid cells was
isolated by PUREGENE kit (Gentra Systems, Minneapolis, MN 55447). All 15 exons of the \textit{KCNH2} gene were amplified from genomic DNA by the PCR primers described by Itoh et al (1998).\textsuperscript{12} PCR products were purified using QIAquick PCR purification kit (Qiagen), and were sequenced bi-directionally on an ABI 377 sequencer.

\textbf{cDNA and Minigene constructs}

Mutant \textit{KCNH2} cDNA (Q1070X) was generated using PCR-based site-directed mutagenesis and subcloned into the eukaryotic expression vector pCGI.\textsuperscript{13} The minigene was constructed by PCR amplification of genomic DNA fragment spanning from HERG exons.
12 to 15 as previously described. The N-terminus of the minigene was tagged by Myc epitope, which is in-frame with the HERG (encoded by KCNH2) translation sequence. The Q1070X mutation in the minigene was generated using the pAlter in vitro site-directed mutagenesis system (Promega, Madison, WI). Human 14-3-3ε cDNA containing expression vector was kindly provided by Dr. Dirk Isbrandt, Institute for Neural Signal Transduction, ZMNH, Germany.

**Transfection**

HEK-293 cells were incubated at 37 °C in 5% CO2 and cultured in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml, Invitrogen), streptomycin (100 μg/ml, Invitrogen), and non-essential aminoacids (0.1 mM, Invitrogen). Transient transfections were performed with Superfect (Qiagen) using 3 μg HERG cDNA (homomeric channels) or 1.5 μg WT and Q1070X cDNA (heteromeric channels). 14-3-3ε is member of a protein family that participates in wide variety of biological processes including potentiating HERG activity by stabilizing the HERG in its phosphorylated state. To study the effect of 14-3-3ε on biophysical properties of Q1070X HERG channels (as it lacks the C-terminus S1137), co-transfection of 1.5 μg HERG cDNA and 14-3-3ε cDNA was performed. Transfected cells were identified under epifluorescent microscopy using 1 μg of green fluorescence protein (GFP) as a reporter gene. All measurements were done 48 hours after transfection. For Western blot and RNAse protection assay (RPA) analyses, the HERG cDNAs and minigenes in pcDNA5/FRT vector were stably transfected into Flp-In HEK293 cells as previously described.

**Electrophysiology & Statistical Analysis**

HERG currents were measured using the whole-cell configuration of the patch-clamp technique as described previously. Detailed method has been described in the supplementary section.

**Western blot analysis**

Western blot procedures were described previously. Briefly, Flp-In HEK293 cells stably transfected with wild-type HERG or the Q1070X mutant cDNAs were lysed in a buffer containing 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors. The cell lysates were subjected to SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. HERG proteins were detected with an anti-HERG antibody against the N-terminus (Santa Cruz) and visualized with ECL detection kit. RNAse protection assay of mRNA transcripts from minigene transfected cells
RNA isolation and RPA were performed as previously described. Briefly, cytosolic RNA was isolated from Flp-In HEK293 cells stably expressing HERG minigenes using the RNeasy kit (Qiagen) and analyzed with the riboprobes using the RPAIII and BrightStart BioDetect kits (Ambion). Yeast RNA was used as a control for the complete digestion of the probes by RNAs. The expression level of the hygromycin B resistance gene from the pcDNA5/FRT vector was used as a loading control for normalization. The intensity of each band was quantified using Scion Image software.

Results

Clinical Analysis

II-5: Fetal bradycardia with a heart rate of ~90bpm without fetal hydrops was noted at 22 weeks, and the patient (I:2) was referred to a tertiary fetal medicine unit. Fetal echocardiography carried out at 23+2 weeks of gestation showed persistent ventricular tachycardia, and hydrops with mild ascites, pericardial and pleural effusions, and skin oedema. Ventricular and atrial rates were 210-220bpm and 120-130bpm respectively. The fetal heart was structurally normal. There was however, important circulatory compromise as shown by almost cessation of flow through the right heart. Maternal treatment with oral flecainide was commenced which led to a decrease in the rate of tachycardia (~170–190bpm). At 24 weeks episodes of sinus rhythm (rate ~120bpm) were documented. These were short-lived and alternated with 2:1 AV block (atrial rate = 120 bpm, ventricular rate = 60 bpm). A fetal ECG performed at 24+5 weeks demonstrated a VT and showed the presence of persistent and periodic waveforms at 3Hz (rate ~180bpm). Despite therapeutic flecainide levels, the fetus continued to show frequent and increasing episodes of tachycardia alternating with episodes of 2:1 block. At 26+3 weeks, sotalol was added, without effect. Fetal hydrops had progressed considerably with massive ascites, pericardial effusion and skin edema. Amiodarone was commenced at 27 weeks and sotalol and flecainide stopped. 18 hrs after commencing amiodarone mother complained of shortness of breath and chest pains. An ECG showed 1st degree heart block with a QTc of 461ms. Maximal QTc = 519 ms at 36h, normal QTc = 416 ms a week later. By 28 weeks gestation there was evidence of a maternal pericardial effusion. In view of the maternal health and poor prognosis for the fetus, labour was induced. A stillborn female baby was delivered. At autopsy fetal hydrops was confirmed. Detailed examination of the heart revealed patchy endocardial fibroelastosis, compatible with chronic cardiac failure. No structural abnormalities were noted. There was no evidence of a metabolic disease. Histological examination of the cardiac conduction system showed a well formed sinus node, AV node, AV bundle and bundle branches.
II-6: During this pregnancy, the first fetal echocardiogram was performed at 16 weeks which showed normal rhythm and normal myocardial function. Mother (I:2) was followed weekly from 20 weeks on, and at 22 weeks, persistent bradycardia at 60 bpm was noted. The mother was placed on dexamethasone 4mg daily, and one week later, with ongoing bradycardia, on ritodrine 10 mg Q4H. One week later, the fetal heart rate showed a mixed pattern, with approximately 50% sinus rhythm, and the remainder episodes of brady and tachycardia. There was no hydrops present. Ritodrine was discontinued, and the mixed pattern continued for the next few weeks.

At 29 weeks ventricular ectopy was noted, with rhythm changes from 120 to 220 bpm, most likely due to ventricular tachycardia, with dissociation noted on fetal echo M-mode. At 30 weeks, with the possible diagnosis of LQT syndrome, propanolol was started at a low dose, with gradual increase to 10mg Q6H. Dexamethasone was continued until 32 weeks.

The fetus showed episodes of ventricular arrhythmia, however with decreased frequency and better controlled heart rate. On repeated fetal echo there was no hydrops, and no evidence of haemodynamic dysfunction. At 32 weeks, an elective Cesarean delivery was performed, with the delivery of an 1800 g boy with Apgar scores of 7 at one and 9 at five minutes. At delivery, the irregular heart rate was confirmed. On admission at the neonatal ICU, the baby had normal vital signs. Cardiac monitoring showed short runs of polymorphic ventricular tachycardia and (functional) AV block with variable escape rhythms with different QRS morphology and a QTc of 605ms (Fig. 1D). Lidocaine loading dose was given twice and maintenance therapy of 20mg/kg/min was established without any effect on the aberrant rhythm. Lidocaine i.v, and magnesium sulphate all proved ineffective in controlling the arrhythmia. Therefore, central access was obtained, a temporary pacemaker was inserted, and a β-blocker started. At 12 hours of age, the child underwent placement of a permanent epicardial pacemaker (VVI), resulting in a stable rate of 150bpm. The baby was discharged home after a month in stable condition, on β-blocker treatment. On followup examinations, the child is healthy and growing well. He is now 14-months of age. The pacemaker is functioning well, and the ECG shows ventricular pacing, with a rate of 100-120bpm. The brother (II:4) of the proband (II:6) is now six years old, growing well, and his ECG is normal (Fig. 1C). Mother is now 24 weeks pregnant with a son with no signs of intrauterine arrhythmia. No other organ is found to be affected in the proband (II:6), as well as in the heterozygous carrier brother and parents. ECG of both parents showed borderline QTc intervals (Fig. 1A and 1B), without having any symptoms, but the mother showed several premature extra systoles during 24-hour Holter monitoring.
Figure 2A: Pedigree of the studied family and homozygosity mapping with the microsatellite repeats encompassing the HERG gene. Affected individuals are shown as filled circles (female) and squares (male). Obligate carriers by genotyping are denoted by with black dots in symbols. Deceased individuals are indicated by slashes, proband is indicated by an arrow. There were two miscarriages at 8th and 10th weeks, respectively, which are shown as triangle shaped symbols. Results of genotypic analysis are shown for 4 different markers D7S661, D7S636, D7S798 and D7S2465, two on each side of the HERG gene are shown below each individual. The disease haplotype is denoted with a filled vertical bar, and normal haplotypes are indicated by open vertical bar (I:1) and hatched vertical bar (I:2). Consanguineous marriage is indicated by =. Note that the affected children (II:5, II:6) inherited two disease carrying HERG alleles, one from each parent.

Figure 2B: Screening of the HERG gene shows substitution of nucleotide “C” for a “T” at cDNA position 3208 (NCBI ref: NM_000238) which creates a stop codon at this position. Left panel shows homozygous changes in the proband (II:6) and the still born sister (II:5) of the proband. Right panel shows heterozygous changes of the same nucleotide in the father (I:1), mother (I:2) and in another son (II:4) and the recently conceived child (II:7).
**Genetic Analysis**

Homozygosity mapping around the Candidate genes (mentioned in materials and methods) showed homozygosity of the microsatellite-repeat markers encompassing the HERG gene in the proband (II:6) and his still-born sister (II:5) (Fig. 2A), which led us to screen the HERG gene in the proband. We have identified a homozygous non-sense mutation in exon 14 of HERG at nucleotide position 3208 (c.3208 C>T; NCBI Ref. NM_000238) in II:6 (Fig. 2B), which causes a premature stop codon at peptide position p.1070 that eliminates the distal C-terminal domain of the protein. The same mutation was found heterozygously in both parents (I:1 and I:2) (Fig. 2A and 2B), and also in the other living brother (II:4) (Fig. 2A and 2B). It was not present in 200 control individuals. Analysis of DNA isolated from amniotic fluid cells derived from the stillborn sister (II:5) (Fig. 2A) revealed that she also carried the homozygous mutation in HERG (Fig. 2B). Mother is now 24th weeks pregnant with a son (II:7), who is also a heterozygous carrier for the Q1070X mutation.

**Western blot analysis of the Q1070X mutant protein**

To study biochemical properties of the Q1070X mutant, we performed Western blot analysis of HERG channel proteins. As shown in Fig. 3, wild-type HERG expressed two protein bands: a lower band of 135 kDa and an upper band of 155 kDa. The 135 kDa band represents the core-glycosylated immature form of the channel protein located in the ER, and the 155 kDa band represents the complex-glycosylated mature form of the channel protein located in the plasma membrane.18 Similar to wild-type HERG, the Q1070X mutant also generated two protein bands of 125 kDa and 145 kDa, reflecting a truncation of 90 amino acids by the mutant termination codon. The relative level of the two protein bands in the Q1070X mutant was comparable to that of wild-type HERG, suggesting that the Q1070X mutant protein also having complex-glycosylated mature form comparable to what found in the WT HERG in the plasma membrane.

![Figure 3: Western blot analysis of the Q1070X mutation. Flp-In HEK293 cells were stably transfected with wild-type HERG or Q1070X. The cell lysates were subjected to SDS-PAGE and immunoblotted with anti-HERG antibody against the N-terminus. Results shown are representative of three independent experiments.](image-url)
Figure 4: Biophysical properties of WT and Q1070X-HERG currents. (A) Current density: representative HERG I_{\text{tail}} traces recorded at -120 mV. The graph shows the mean peak I_{\text{tail}} for WT and Q1070X, normalized to the cell capacitance. (B). Activation: representative families of HERG current traces of WT and Q1070X transfected cells. Corresponding I-V curves show the mean I_{\text{tail}} (indicated by arrow), normalized to maximum value, and plotted as a function of the prepulse voltage. The solid lines represent Boltzmann equation fits. (B) Inactivation: representative families of HERG current traces and corresponding mean inactivation time constants (\tau) plotted as a function of the test pulse voltage. Arrows indicate channel inactivation. The time constants were calculated using a monoexponential fit. (C). Recovery from inactivation: representative families of HERG current traces and corresponding mean time constants of recovery from inactivation plotted as a function of the test pulse voltage. Arrows indicate channel recovery. Time constants were calculated using a monoexponential fit. (D) Deactivation: representative families of HERG current traces and corresponding mean fast (squares) and slow (rectangles) deactivation time constants plotted as a function of the test pulse voltage. Arrows indicate channel deactivation. Deactivation time constants were calculated using a double-exponential fit. "n" indicates number of cells. Time scales and protocols shown as insets.
**Electrophysiology**

Electrophysiologic properties of WT and Q1070X channel are presented in Fig 4. In Fig 4A, representative *I*_{\text{tail}} from WT and Q1070X transfected cells are shown. Mean peak *I*_{\text{tail}}, normalized for cell capacitance, for WT and Q1070X channels were not significantly different (-171±10 and -164±16 pA/pF, respectively). Activation I-V relations of WT and Q1070X, as shown in figure 4B, matched closely. Accordingly, V1/2 and k values for WT and Q1070X activation were not significantly different (-15.9±1.8 and -15.2±1.8 for V1/2, and 6.9±0.2 and 7.1±0.3 for k value, respectively). Next, we studied voltage-dependence of inactivation of WT and Q1070X channels. Both channels showed a voltage-dependent decrease in inactivation time constant. However, inactivation of Q1070X was faster than WT at all test voltages, and statistical significance was reached at voltages between 0 to 60 mV (P<0.05; Fig. 4C). Recovery from inactivation showed a voltage-dependent increase in WT and Q1070X channels. Although no significant differences in recovery time constants were found at voltages between -120 to -60 mV, at higher voltages (-50 to -30 mV) Q1070X channels recovered significantly faster from inactivation than WT (P<0.05). Finally, fast and slow time constants of deactivation were measured, and showed no significant difference between the two channels (P>0.05 for comparisons at all voltages).

To determine whether Q1070X has a dominant negative effect, we have measured HERG current density after co-expression of WT and Q1070X in HEK-293 cells. Compared to WT, co-expression of WT and Q1070X did not alter mean peak *I*_{\text{tail}} at -120 mV: 150±13 and 153±18 pA/pF for WT and WT+Q1070X respectively (n=5 for each group).

14-3-3ε protein has been shown to play an important role in the β-adrenergic stimulation of the HERG channel activity.\textsuperscript{14,15} In the case of HERG channels, 14-3-3ε binding stabilizes a phosphorylated state and enhances HERG activity by shifting channel activation toward hyperpolarizing membrane potentials.\textsuperscript{15} Previous findings suggest that β-adrenergic stimulation induces phosphorylation of particular serine (S) residues in the HERG channel.\textsuperscript{19} In vitro experiments have shown that phosphorylation of the N-terminal S283 and C-terminal S1137 residues in the HERG channel stimulated binding of 14-3-3ε.\textsuperscript{15} This binding produced a hyperpolarizing shift in the V1/2 activation, and consequently producing a possible increase in the HERG channel activity. Furthermore, particular C-terminal HERG mutations, lacking the 1137 residue and clinically associated with stress-induced arrhythmias in LQT2, have been shown to disrupt 14-3-3ε induced enhancement of the HERG channel activity.\textsuperscript{14} Therefore, we studied the effect of 14-3-3ε on V1/2 activation of the Q1070X channel, which also lacks the S1137 residue (Fig. 5). After co-expression of WT HERG with 14-3-3ε, V1/2 showed, as expected, a hyperpolarizing shift to -23.2±1.0 mV (n=8; P<0.05). In contrast, co-expression of 14-3-3ε with Q1070X induced a depolarizing shift of V1/2 to 10.0±1.7 (n=12; P<0.05).
Minigene analysis of the Q1070X mutation

As the Q1070X mutant has a normal membrane localisation and functional properties attributable to functional IKr and which could not explain the mechanism of fatal arrhythmias in the homozygous Q1070X carriers, we ought to search for an alternative mechanism as described in various disease pathology. NMD is an RNA surveillance mechanism that selectively degrades mRNA transcripts containing Premature Termination Codons (PTC) resulting from nonsense or frameshift mutations. The role of NMD as a disease-causing mechanism of PTC mutations is becoming increasingly evident. (new) According to the proposed rule, NMD occurs when translation terminates > 50 to 55 nucleotide upstream of the 3’-most exon-exon junction. The molecular mechanisms of NMD have been studied

Figure 5: Co-expression of WT and Q1070X with 14-3-3ε. Representative families of HERG current traces and corresponding I-V curves from WT or Q1070X transfected cells in the absence or presence of 14-3-3ε.
extensively into the pathogenicity of various diseases (for detailed please see review 20-22). Recently, KCNH2 nonsense mutations have been shown to cause a decrease in the level of mutant mRNA by the NMD pathway. To study whether the Q1070X mutation is subject to NMD, we used a minigene construct containing the KCNH2 genomic sequence spanning from exon 12 to exon 15. In these experiments, Flp-In HEK293 cells were stably transfected with either the wild-type or Q1070X mutant minigenes, and the expressed mRNA was analyzed by RPA. Fig. 6A shows the structure of the minigene and the mRNAs after splicing. The Q1070X mutation results in a PTC in exon 14, which is expected to trigger NMD. As shown in B, the mRNA level of the Q1070X minigene was significantly (88%) lower than that of the wild-type minigene. To test whether NMD is responsible for the decrease in Q1070X mutant mRNA, we examined the effect of the protein synthesis inhibitor cycloheximide (CHX), which has been shown to abrogate NMD. The cells expressing wild-type or Q1070X minigenes were treated with CHX for 3 hours before RNA isolation.

**Figure 6:** Analysis of the Q1070X mutation using minigene constructs. A: The structure of the Myc-tagged minigene and spliced mRNAs. The positions of wild-type termination codon (TER) and mutation-induced PTC are indicated. B: Analysis of mRNA by RNAse protection assay (RPA). Flp-In HEK293 cells were stably transfected with wild-type HERG or Q1070X minigenes, and the expressed mRNA was analyzed by RPA. Cells expressing wild-type and mutant minigenes were treated (+) or not treated (-) with 100 μg/ml of CHX for three hours before RNA isolation. The level of hygromycin B resistance gene (Hygro) served as a loading control. The quantitative data after normalization using protected hygromycin B resistance gene mRNA are plotted as percentage of wild-type control from three independent experiments.
Treatment with CHX had no effect on the level of wild-type mRNA, but significantly increased the level of Q1070X mutant mRNA, suggesting that the mutant mRNA is degraded by NMD.

**Discussion**

HERG is the gene encoding I_{Kr}, the rapid component of the delayed rectifier. Heterozygous missense mutations, and mutations giving rise to premature truncations are causally involved in LQT2. The HERG non-sense mutation Q1070X described in the present study is expected to lead to a premature termination codon at the C-terminal region of HERG channels. The parents are consanguineous, and both are heterozygous for the same mutation. There is virtually no clinical phenotype in the heterozygous carriers, with only borderline prolonged QT interval in the father and the mother (I:1) (Fig. 1A and 1B), and no symptoms or ECG changes in the child with heterozygous Q1070X (Fig. 1C). In addition, the siblings of both parents are asymptomatic, and no suspected premature sudden cardiac death has occurred in 3 generations. Despite the heterozygous HERG truncating mutation carriers showed no apparent LQT phenotype, homozygosity led to a profound QT prolongation, with 2:1 (functional) AV block and severe ventricular arrhythmias well before and immediately after birth. In both II-5 and II-6 (Fig. 2A), homozygosity for the HERG mutation (Fig. 2B) was detected. In both individuals, significant fetal arrhythmias were observed, with severe hemodynamic deterioration.

Our Western blot and patch clamp analyses showed that the Q1070X mutant trafficked normally to the plasma membrane and generated near normal HERG current with minor kinetic changes (faster inactivation) and perturbation of 14-3-3ε regulation. Although attenuated 14-3-3ε activity has been proposed as a disease causing mechanism in LQT2 by Choe et al.14 this alone could not explain the absence of phenotypes in the heterozygous carriers, and severe (fatal) phenotypes in the homozygous carriers. Using a minigene construct, we showed that the Q1070X mutation led to a marked decrease in mutant mRNA transcripts by the NMD pathway. The degradation of mutant mRNA transcripts by NMD would preclude the formation of functional mutant HERG channels. Thus, in homozygous Q1070X patients, most HERG mRNA transcripts are expected to be destroyed by the NMD mechanism before they could form functional channels. We conclude that elimination of mutant HERG mRNA is the disease-causing mechanism for the severe clinical phenotypes in the homozygous carriers. In heterozygous Q1070X carriers NMD eliminates most of the mutant HERG mRNA, leading to haploinsufficiency, thereby reducing the dominant-negative effect of the mutant protein. This explains the normal clinical phenotype and borderline QT interval in them. Clinical phenotypes in homozygous Q1070X carriers presumably also includes 2 miscar-
riages at 8-10 weeks and 2 still birth with documented arrhythmias at 29th weeks and 28 weeks, respectively. Hence, these observations may extend the LQT phenotype into the early stages of life leading to miscarriages and still births. Mouse homologue of HERG (Merg) expresses as early as 2-cell stages of development and its distribution become localised following compaction at the 8-cell stage. This relocalization of Merg could be affected by treatment with specific inhibitors of Merg-channel function, and mice with a complete knockout of Merg gene (Merga and Mergb) die during early development. In a separate study antisense “knockdown” of the zebrafish HERG ortholog (Zerg) in the single cell stage elicited bradycardia to asystole depending on the dose.

Both experiments in mice and zebra fish suggest that HERG is essential for rhythmic propagation from the very early stages of life.

We have previously described a non-sense mutation that presumably lead to lack of the HERGa isoform, retaining the HERGb isoform. The phenotype compared reasonably well with the current phenotype although the arrhythmias seem to appear later in the intrauterine life and no still birth were present. Clinical phenotypes although severe, are relatively attenuated among homozygous missense mutation carrier. They include frequent ventricular premature beats in the late stage of pregnancy and in the neonatal period associated with prolonged QTc and 2:1 functional AV block.

In conclusion, this study based on homozygosity for the nonsense Q1070X mutant demonstrates that degradation of the mutant HERG mRNA by NMD causes almost complete loss of HERG, leading to a severe exclusively cardiac phenotype that presumably includes miscarriages and still birth. Because our data show that the Q1070X mutant can form HERG channels with near normal function, interventions to prevent degradation of the mutant mRNA transcripts might be a potential therapeutic approach for homozygous HERG Q1070X patients, as has been found effective in non-sense mutation carriers in CFTR (cystic fibrosis), dystrophin (Duchenne muscular dystrophy), and AVPR2 gene associated with X-linked nephrogenic diabetes insipidus.

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