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Stop-codon and C-terminus nonsense mutations are associated with lower risk of cardiac events in Long QT Syndrome Type 1 patients

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Abstract

Background—In Long QT Syndrome Type 1 (LQT1), the location and type of mutations have been shown to affect the clinical outcome. Although haploinsufficiency, including stop-codons and frameshift mutations, has been associated with lower risk of cardiac events in LQT1, nonsense mutations have been presumed functionally equivalent.

Objective—To evaluate clinical differences among patients with nonsense mutations.

Methods—The study sample comprised 1090 patients with genetically confirmed mutations. Patients were categorized into 5 groups depending on mutation type and location: missense not located in the high risk cytoplasmic-loop (c-loop) (n=698) used as reference, missense c-loop (n=192), stop-codons (ST) (n=67), frameshift (FS) (n=39), and others (n=94). Primary outcome was a composite end point of syncope, aborted cardiac arrest (ACA) and LQTS related death (cardiac events (CE)). Outcomes were evaluated with multivariate Cox regression analysis. Standard patch clamp techniques were used.

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Results—When compared to missense non c-loop mutations, the risk of CE was reduced significantly in patients with ST mutations (HR 0.57 CI 0.34–0.96 $p=0.035$), but not in patients with FS mutations (HR 1.01 CI 0.58–1.77 $p=0.97$). Our data suggest that for the most common stop-codon mutant channel (Q530X), currents were larger than haploinsufficient channels (pA/pF: WT, 42 ± 6 , $n=20$; Q530X+WT, 79 ± 14 , $n=20$, $P<0.05$) and voltage dependence of activation was altered.

Conclusions—Stop-codon mutations are associated with lower risk of cardiac events in LQT1 patients, while frameshift mutations show the same risk as the majority of the missense mutations. Our data indicate functional differences between these previously considered equivalent mutation subtypes.

Keywords

LQT1; mutations; nonsense; stop codon; frame-shift; haploinsufficiency; cardiac events; syncope; aborted cardiac arrest; sudden death

Introduction

Long-QT syndrome type 1 (LQT1) is caused by mutations in the KCNQ1 gene resulting in a decrease in the repolarizing potassium current IKs. LQT1 is the most prevalent genetic form of Long QT syndrome accounting for approximately 50% of the genotyped patients^{1, 2}. Long QT syndrome is a dominant disease, with most patients carrying both normal (wild-type, WT) and mutant subunits^{3, 4}. The IKs channel is formed by four KCNQ1 alpha-subunits together with KCNE1 beta-subunits⁵. Thus, patients carrying one mutant KCNQ1 allele can have IKs channels formed by either a reduced number of wild-type subunits (haploinsufficiency) or channel where the mutant subunit co-assembles with the wild type and reduces channel function (dominant-negative).

Mutation type can broadly be categorized by type as missense and non-missense. Missense mutations (mutations resulting in a codon coding for an altered amino acid) produce erroneous proteins that, in addition to decreasing channel function, may have deleterious effects on the function of the wild-type protein in a dominant negative fashion^{6–8}. Missense mutations, in particular located in the cytoplasmic loops between the transmembrane domains of the channel (c-loops), have previously been shown to be associated with high rate of clinical events^{4, 8, 9}. Non-missense mutations defined as stop-codons, frameshift mutations, in-frame deletions, splice site alterations and intron mutations are expected to have consequences on the amino-acid sequence that extend beyond the site of the mutation itself. In some instances, however, studies have indicated that nonsense, frameshift and splice site mutations may actually have less of a phenotypic impact on protein function than missense mutations^{6, 7, 10–12}. First, despite nonsense mutations causing major changes in the protein sequence, and likely resulting in severe changes in protein assembly and function, they are thought to result in a complete loss of function of the mutated protein subunit, causing haploinsufficiency, with the unaffected WT allele contributing to the formation of normal functioning channels¹⁰. Second, nonsense mutations may be targeted for nonsense mediated decay, where transcription of proteins with premature terminations is prevented, decreasing the impact of these mutation on channel function and cell electrophysiology^{11, 12}.

To date, LQT1 non-missense mutations have been presumed equivalent, and therefore were previously investigated clinically as a group and been found associated with lower risk².

Non-missense mutations account for about 20% of the LQT1 population². Recent studies have focused in repair as possible treatment for inherited channelopathies by read-through of nonsense mutations^{13–17}. In particular, with read-through of nonsense mutations also being a target of interest in LQT1¹⁸, it became important to further investigate the clinical significance of different subtypes of non-missense mutations in LQT1.

We hypothesized that non-missense mutations (stop-codon, frameshift and others) are not clinically equivalent. Our results suggest that stop-codon mutations are associated with the lowest risk, while other non-missense mutations are associated with the same clinical risk as non c-loop missense mutations. Our data indicates that low risk for stop-codon mutations may be due to increased channel function when compared to the haploinsufficient phenotype.

Methods

Population

The study included 1,090 patients from the International Long QT Registry¹⁹ with genetically confirmed mutations in KCNQ1 derived from 185 proband-identified families. All patients or guardians provided informed consent for both clinical and genetic studies. The study was approved by the Institutional Review Board. Clinical data of probands and first- and second-degree relatives of probands was prospectively collected from time of enrollment and retrospectively collected from birth to enrollment age through medical history and available ECGs. An enrollment packet was mailed, including enrollment questionnaire, study consent form and medical release form to the proband (or proband's parent, in the case of a minor, or health care proxy/next of kin, in case of a deceased proband). Personal medical history and family history were obtained by the patient's medical records, mailed questionnaire and/or telephone interview. Family members were contacted and, with informed consent, provided their own personal medical history. Patients were coded into the study regarding any family history of LQTS, syncope or cardiac arrest, sudden cardiac death, congenital deafness, and any known genetic disease. The current study involved an analysis of clinical and electrocardiographic data obtained historically at enrollment in the registry and updated annually. Subjects with Jervell and Lange-Nielsen syndrome with deafness and two known KCNQ1 mutations, as well as those with one known KCNQ1 mutation and congenital deafness were not included in the present study. Follow-up was continued and analyzed only until the age of 40 years of age to minimize the influence of cardiovascular disease on clinical outcomes. From the 91 total deaths in the study, only 1 non-Long QT related cardiac death occurred, so non-cardiac death was not a significant competing risk.

Clinical outcomes were defined as syncope categorized by abrupt onset and offset, aborted cardiac arrest (ACA), sudden cardiac death (SCD) or shock from an implantable cardioverter defibrillator (ICD). Cardiac events comprised a combined outcome of syncope, ACA, SCD or ICD shock. Fatal or near-fatal events were comprised of ACA, SCD or ICD

shock. The outcomes were verified through medical records with confirmation of arrhythmias if possible and documented.

Mutational characterization

Mutations were characterized and identified using available genetic tests in the molecular genetics laboratories. The amino acid sequence was characterized by type of mutation and classified as missense or non-missense. Because our previous study identified missense cytoplasmic loop (c-loop) as high risk⁸ we subdivided missense mutations into c-loop and non c-loop in order to compare the risk associated with non-missense mutations to the largest and lowest risk missense mutations. The non-missense group consisted of stop-codon mutations, splice site mutations, frameshift mutations and in-frame insertions or deletions as well as intron mutations. Non-missense mutations were further divided into sub groups based on location into C-terminus (C-term) and non-C-terminus (non-C-term).

Expression studies

The electrophysiological parameters were obtained from expression of wild-type and mutant human KCNQ1 channel subunit with the auxiliary KCNE1 subunit in HEK293T cells. Cells were transfected with two different ratios of KCNQ1 and KCNE1 subunits. To mimic normal IKs channels, WT and KCNE1 subunits were transfected at a ratio 1:1. To mimic haploinsufficient type channels, WT and KCNE1 subunits were transfected at a 0.5:1 ratio, using half of the amount of WT DNA. We also co-expressed mutant, WT and KCNE1 subunits at a 0.5:0.5:1 ratio to mimic the patient expression levels of each of these proteins. To assure a more homogeneous population of cells, all measurements were made 48–56 hours after transient transfection. Low amounts of cDNA were used for transfection so that the protein machinery of the cell was not saturated, enabling evaluation of differences between the wild-type (1 WT) or haploinsufficient (0.5 WT) subunits expression. The procedure is explained in detail in the supplementary data.

Statistics

Baseline characteristics of the study population were reported as frequencies or mean values as appropriate by mutation type and location by groups as defined above. The cumulative probability of cardiac events was analyzed by Kaplan-Meier analysis by the mutation type and location using the log-rank test to statistically compare the groups. Multivariate Cox proportional hazard regression models were used to determine the risk of the outcome as defined above. We used missense non c-loop as reference presenting results of significant factors associated with risk of cardiac events. The models used sex as a time-dependent covariate for 0–15 years and 16–40 years because of the known age-sex interaction in risk of cardiac events in LQT1 patients²⁰. In addition, Since almost all subjects were first- and second-degree relatives of probands, the effect of potential lack of independence between subjects was evaluated by refitting the Cox model using the robust sandwich estimator for family²¹.

Results

The study comprised 1,090 patients with genetically confirmed LQT Syndrome Type 1 from the International Long QT Registry. Among these patients 890 (82%) had a missense mutation, of which 192 (22%) were located in the cytoplasmic loop, and 200 patients had a non-missense mutation. The overall comparison of baseline characteristics between missense and non-missense mutations is shown in Table 1. List of mutations, number of patients and number of families with each mutation is given on supplementary table 1.

A total of 67 patients had a stop codon mutation dominated by Q530X (n=28, 42%) and R518X (n=20, 30%). Both these mutations are located in the C-term region of the KCNQ1 channel, while Y171X is located in the non C-term region. A total of 39 patients had a frameshift mutation dominated by P448fsX14 (n=11, 28%), L191fsX93 (n=10, 26%) and P400fsX62 (18%). Figure 1 shows the proposed location of non-missense mutations. Splice site mutations comprised 61 patients (30% of non-missense) dominated by the A344A/sp (n=40, 65%) mutation, while in-frame deletions and insertions and intron intervening sequences (IVS) mutations comprised the remaining non-missense (n=33) with IVS7 +5G>5 being most prevalent (n=17, 24%).

Clinical outcome

A total of 440 (40%) patients reached the composite endpoint of cardiac events, while 121 (11%) patients had a near-fatal or fatal event. Figure 2A shows the cumulative probability of cardiac events for the various groups. We show that non-missense stop codons are associated with the lowest risk (40-year event rate of 27%), and all missense non c-loop, frameshift, splice and all other non-missense mutations as intermediate risk (40-year event rate of 44%, 46%, 43%, and 39% respectively). Our results from this analysis also show that missense c-loop mutations are associated with high-risk (40-year event rate of 70%), as previously reported. The results were confirmed in the multivariable analysis (Table 2) where stop codon mutations were associated with significantly lower risk (HR 0.57, CI 0.34–0.96, p=0.035) when compared to non c-loop missense mutations independent of known clinical risk factors. As expected, c-loop missense mutations were associated with significantly higher risk (HR 1.95, CI 1.56–2.43, p<0.001). Because occurrence of cardiac events for the lower risk mutations may be due in part to their shorter QTc, we repeated the analysis without QTc adjustment. Stop-codon mutations association with lower risk was slightly stronger without QTc adjustment (HR 0.52, CI 0.31–0.88, p=0.015), suggesting a shorter QTc may partially explain the decrease in risk observed. Analysis was repeated using the robust sandwich estimator for family membership²¹. All significant predictors of risk maintained significance using this robust measure of variance. In addition, analyses was performed further adjusting for proband status (i.e. whether patients were probands or family members) and found consistent results which were similar to the primary analysis (HR for stop-codons vs. non-c loop missense = 0.54, p= 0.02). However, this analysis may be biased by the inclusion of only symptomatic patients who are those with a prolonged QTc, and may therefore not represent the true penetrance of events in the LQTS population.

Analyzing near-fatal or fatal events (Figure 2B and Table 3) the results were consistent with very low risk in stop codon mutations (HR 0.15, CI 0.02–1.11, p=0.063) with only one

aborted cardiac arrest during follow-up. High risk was seen in c-loop (HR 1.77, CI 1.17–2.67, $p=0.007$), as expected. For the composite endpoint of all cardiac events and for near-fatal or fatal events we were unable to demonstrate a statistically significant difference among the other non-missense mutations when compared to non c-loop missense mutations. When specifically comparing stop codons to other non-missense mutations we did not have sufficient power to show a statistically significant lower risk (all cardiac events: HR 0.60, CI 0.33–1.07, $p=0.086$; ACA/LQT death: HR 0.16, CI 0.02–1.18, $p=0.07$); these findings are not shown in Tables.

To evaluate whether location mattered among patients with non-missense mutations we carried out a secondary analysis as shown in Figure 3 and Table 4. In the multivariable analysis we demonstrate the importance of location in the subgroups of stop codon mutations and found that C-terminal location was associated with significantly lower risk of cardiac events when compared to non C-terminal location (HR 0.27, CI 0.10–0.75, $P=0.012$). Due to the very low event rate for stop codons in general this analysis could not be carried out for near-fatal or fatal events. The results for stop codon mutations were also consistent for frameshift mutations where we found a borderline significant lower risk associated with C-term location (HR 0.35, CI 0.11–1.06, $p=0.064$) when compared to non c-term location and when comparing all non-missense C-term locations to non C-term location (HR 0.47, CI 0.28–0.79, $p=0.005$). We had insufficient power to show significantly reduced risk for the C-term locations among the mutations categorized as splice and others (Table 4). Thus the reduced risk associated with C-term location was driven by the low risk in stop codon and frameshift mutations.

Cellular expression studies results

To explore the mechanism underlying the reduced risk associated with stop-codon mutations compared to other mutations, we measured channel function of the two most prevalent stop codon mutations, R518X and Q530X, which account for the majority of patients with stop codon mutations in LQT1 (see table 1). To analyze whether mutant channel subunit could modulate WT I_{Ks} current, we co-expressed I_{Ks} subunits (KCNQ1 and KCNE1) in HEK293T cells for the two mutations (R518X and Q530X) (Figure 4A, B, C and D). The currents of channels expressing the Q530X subunit (Q530X+WT) were significantly larger than that of the haploinsufficient channels (WT) (pA/pF: WT, 42 ± 6 ; Q530X+WT, 79 ± 14 , $P<0.05$), and not significantly different from that of the wild-type channels (WT+WT) (pA/pF, 57 ± 8 , $p=0.07$) (Figure 4E). For cells expressing the R518X mutant subunit, difference did not reach statistical significance (pA/pF, R518X+WT, 48 ± 10 . $P=0.23$ compared to WT+WT, $p=0.29$ compared to WT, Figure 4E).

Conductance was significantly altered for the channels expressing Q530X subunits (Q530X+WT) when compared to haploinsufficient channels (WT) (nS/pF, WT, 26 ± 5 ; Q530X+WT, 47 ± 10 , $p<0.05$), while not significantly larger than that of wild-type (WT+WT) channels (nS/pF, WT+WT, 32 ± 5 , $p=0.07$) (Figure 4F). Voltage dependence of activation was left shifted compared to those of both wild-type (WT+WT) and haploinsufficient (WT) channels (mV, WT+WT, 38 ± 4 ; WT, 41 ± 4 ; Q530X+WT, 26 ± 4 , $p<0.05$) (Figure 4G). On the other hand, both conductance and voltage dependence of activation of the channels expressing

R518X (R518X+WT) were not statistically different from that of either haploinsufficient or wild-type channels (Figure 4E, F and G). Our results suggest that expression of stop-codon mutant proteins can be rescued by the wild-type subunit, explaining the lower arrhythmic risk and mild QT prolongation for patients with stop codon mutations.

Discussion

The present study is the first to specifically evaluate differences in clinical outcome among patients with genetically confirmed non-missense mutations in Long QT Syndrome Type 1. We have shown that patients with stop-codon mutations in *KCNQ1* have relatively low rate of fatal and near-fatal events and a significantly lower risk of combined cardiac events when compared to both missense and other non-missense mutations in *KCNQ1*. We also found that other non-missense mutations (i.e frameshift and splice mutations) carried the same risk as missense mutations in the non c-loop location. To further investigate the findings we evaluated the importance of mutation location within the non-missense mutation group and found that C-term location is associated with significantly lower risk than non C-term.

Stop-codon mutations cause an early termination of the protein, but the shorter protein may still participate in the formation of functional channels^{22, 23}. The limited experimental data that currently exists for functional effects of stop-codon mutations. First, previously published data show that when expressed without the WT subunit, Q530X and R518X do not produce any functional channels^{18, 24} and consistently are associated with deafness in patients²⁵. Second, in contrast to our data, when expressed together with wild-type subunits, these mutant channels do not show significant difference from haploinsufficient expression of the channel. These studies did not compare currents of the WT and the haploinsufficient channel¹⁸. In our hands, low levels of DNA transfection are necessary to probe differences between WT and haploinsufficient channels in heterologous expression systems. We believe that our data clearly shows, in cells expressing wild-type and the two most common channel mutations, currents for these two mutants are not significantly different than WT channels. In addition, we show that for cells expressing the wild-type together with the longer R530X subunits, currents are larger than haploinsufficient channels, explaining the mild phenotype in patients with these stop-codon mutations. Our data is consistent with other findings that report that the R518X causes relatively benign phenotypic changes due to the impaired ability of the truncated protein to participate in and disrupt channel formation and function^{1, 26–30}.

Although the data we show relates to a ratio 1:1 of wild-type to stop-codon mutant subunits, a ratio of 3:1 mutant to wild type was shown to decrease wild-type channel current²⁴. Patients with stop-codon mutant channels show on average a mild QTc prolongation, but variable expression of the mutant subunit may explain the QTc prolongation observed for some patients with these mutations. Indeed, *KCNQ1* expression has been suggested to be influenced by the presence of certain 3'UTR variants³¹, the presence of these polymorphisms are not known for these patients, but may account for the variability of patient phenotype.

Our data is also consistent with the notion that C-term non-missense mutations have a milder phenotype than non-C-term mutations. Non-C-term mutants are not expected to produce functional channel proteins, because they do not have the potassium permeation domain of the protein. Our data indicates that stop-codon mutant channels are not all equivalent to haploinsufficient channels and that non-missense mutant proteins can contribute to functional channels despite large alterations in the channel protein. Although our data suggests that the two most common stop-codon mutations have a decrease in risk due to relatively increased function, other mechanisms of increased function were not ruled out. Nonsense-mediated decay is an RNA quality control mechanism that selectively degrades mRNA harboring premature stop codons³². It eliminates abnormal mRNA transcripts and prevents the production of truncated proteins that may have dominant-negative effects¹¹. Stop-codon mutations may be particularly prone to cell facilitated nonsense-mediated decay^{18, 33}.

Our data suggest that, contrary to previous belief^{2, 8}, non-missense proteins are not all equivalent, producing a mild-haploinsufficient phenotype in patients. Indeed, we show that although stop-codon mutations have a milder phenotype, other non-missense mutations are associated with the same risk as non-c loop missense mutations. Other non-missense mutations may result in either a non-functional or even deleterious proteins. We hypothesize that the increased clinical risk could be explained by the inherent ability of these mutation types to alter the amino-acid sequence, possibly acting as an antagonist, inhibiting channel function. This is in contrast to stop-codon mutations that simply truncate the protein and may have a milder influence on the functional currents⁸. We also show that non-missense mutations in the C-terminus have a lower associated risk than other non-missense locations. This lower risk associated with the downstream C-terminal location is consistent for both stop-codon and frameshift mutations and can be explained by the production of a truncated, but functional, protein subunit that has an overall function higher than haploinsufficiency.

Although our results suggest a higher risk associated with frameshift mutation compared to stop-codon mutations, one LQT1 frameshift mutant channel was recently shown to be the target of strong nonsense mediated decay in iPS derived cardiomyocytes in the recessive form of LQT1³⁴. This may not be the case for frameshift mutations associated with the dominant form of the disease studied here. Huge variation in clinical severity of LQT1 is well-known and some of these genotype-phenotype differences may be explained by variable expression levels of the mutant subunit caused by nonsense-mediated decay. This repair system can affect clinical outcome by several mechanisms that are not fully understood such as causing distinct traits to manifest from mutations in the same gene, changing the pattern of inheritance, and by altering the specific clinical phenotype. Nonetheless, our results suggest that read-through of stop-codon mutations, as suggested for repair of the genetic disease as a possible treatment of Long QT syndrome, including LQT1, and other channelopathies^{13, 14, 17, 18}, may not be advisable for the dominant form of LQT1. Read-through of the amino acid would generate a missense mutation instead of the premature stop-codon, a result our work suggests, is associated with an increased clinical risk.

Limitations

The International Long QT Registry contains clinical patient history largely based on retrospectively collected data from birth to enrollment. Prospective information was collected at yearly intervals after enrollment. The number of patients with non-missense mutations is limited and relatively small compared to the large number of patients with missense mutations. Further, since almost all subjects were first- and second-degree relatives of probands, the effect of potential lack of independence between subjects was evaluated by refitting the Cox model using the robust sandwich estimator for family membership. We also carried out a secondary analysis in which additional adjustment was made for proband status. All significant predictors of life threatening event risk remained significant using this robust measure of variance with and without proband adjustment. Nonetheless, the high prevalence of specific C-terminus stop-codon mutations R518X and Q530X may overestimate the strength of the risk reduction effect in this class. Even with statistical efforts to minimize a possible family membership effect it is unknown to what degree other genetic modifying confounders exist and the relative effects of nonsense mediated decay is unknown. In addition, because of their high prevalence, the lower risk observed is likely driven by the two most common benign mutations. Our results do not exclude the possibility that some stop-codon mutations are associated with higher cardiac risk. Individual mutation characterization at the cell level may help clarify individual mutation risk. Validation of the results is warranted in another LQT1 population. Expression of mutant subunits in mammalian cells may not fully recapitulate the cardiac phenotype of the mutants. We rely in this study on the clinical phenotype as the main outcome and had no information on the status of the genetic variants that may affect clinical phenotype.

Conclusion

Stop-codon mutations are associated with significantly lower risk of cardiac events in a large registry of LQT1 patients and a C-terminal mutation location was associated with lower cardiac risk among non-missense mutations. Cellular electrophysiological studies of the biophysical function of stop-codon mutant channels suggest that these are not equivalent to haploinsufficient channels and that non-missense mutant proteins can contribute to functional channels despite large deletion in the channel protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LQT1	Long QT Syndrome type 1
WT	Wild type
C-loops	Cytoplasmic loops
ACA	Aborted cardiac arrest
SCD	Sudden cardiac death
ICD	Implantable cardioverter-defibrillator

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Clinical Perspectives

Long-QT syndrome type 1 (LQT1) is caused by mutations in the KCNQ1 gene resulting in a decrease in the repolarizing potassium current I_{Kr} . Missense mutations, in particular those located in the cytoplasmic loops between the transmembrane domains of the channel have previously been shown to be associated with high rate of clinical events, while non-missense mutations have been presumed equivalent in function and clinical relevance. In this study of 1,090 patients from the International Long QT Registry our results suggest that non-missense stop-codon mutations are associated with the lowest risk, while other non-missense mutations are associated with the same clinical risk as non c-loop missense mutations. Our results suggest that the relatively lower risk for stop-codon mutations may be due to increased channel function when compared to the haploinsufficient phenotype.

Figure 1. Schematic drawing of the KCNQ1 potassium channel subunit

Frequency and location of the mutations including the A344sp in the membrane spanning unit and R518X and Q530X in the C-terminus region of the channel. The size of the circles represent the number of subjects with mutations at the respective locations.

Figure 2.
A: Cumulative probability of cardiac events in LQT1. Kaplan-Meier estimates of cumulative probability of cardiac events in LQT1 patients based upon location and type of mutation. Stop codon mutations are associated with reduced risk of cardiac events and missense c-loop mutations are associated with increased risk. **B: Title: Cumulative probability of aborted cardiac arrest or sudden cardiac death related to LQT1.** Kaplan-Meier estimates of cumulative probability of cardiac events in LQT1 patients based upon

location and type of mutation. Stop codon mutations are associated with reduced risk of cardiac events and missense c-loop mutations are associated with increased risk.

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Figure 3.

Left Panel: Cumulative probability of cardiac events in LQT1 with stop codon mutations. Kaplan-Meier estimates of cumulative probability of cardiac events in LQT1 among patients with a stop codon mutation. A C-term location was associated with reduced risk of cardiac events as compared to a non c-term location. **Right Panel: Cumulative probability of cardiac events in LQT1 with non-missense mutations.** Kaplan-Meier estimates of cumulative probability of cardiac events in LQT1 among patients with non-missense mutations. A C-term location was associated with reduced risk of cardiac events as compared to a non C-term location.

Figure 4. Functional effect of the two most common stop-codon mutations

A–D, Typical current traces of from holding potential -80 mV to a series of 3 second voltage steps between -40 mV and $+100$ mV in 20 mV intervals followed by a step to -20 mV, of (WT+WT)/E1 (**A**), WT/E1 (**B**), (R518X+WT)/E1 (**C**), and (Q530X+WT)/E1 (**D**), subunits expressed on HEK293T cells. Traces at $+40$ mV voltage step are shown in red. **E,** Summary data of peak current density at $+40$ mV. **F,** Summary data of Gmax of the tail current-voltage plot. Gmax was normalized to cell membrane capacitance. **G,** Summary data of $V_{1/2}$ of the tail current-voltage plot. Error bars are s.e.m. Number of cells measured for each mutant are indicated in the graphs.

Table 1

Location/type	Missense		Non-missense			
	C-loop	Non c-loop	Stop codons	Frameshift	Splice	Others
Patients, (n%)	192 (17.5)	698 (64.0)	67 (6.2)	39 (3.6)	61 (5.6)	33 (3.0)
Female, (n%)	105 (54.7)	379 (54.3)	39 (58.2)	26 (66.7)	35 (57.4)	25 (63.6)
Age at 1st cardiac event, mean $\pm$ SD, y	12 $\pm$ 8	13 $\pm$ 9	20 $\pm$ 12	16 $\pm$ 11	13 $\pm$ 7	13 $\pm$ 7
Most frequent mutations, (n%)	V254M (60.4)	G168R (13.0)	Q530X (41.8)	P448fsX14 (28.2)	A344A/sp (65.6)	IVS7 +5G>5 (24.2)
	Y184S (7.3)	G269S (6.2)	R518X (29.6)	L191fsX93 (25.6)	K598K (27.9)	DelF340 (21.2)
	R243C (6.8)	L266P (4.7)	Y171X (13.4)	P400fsX62 (18.0)	M159 /sp (6.6)	IVS10-1 G>T (21.2)
Proposed location						
• C-term	0 (0.0)	196 (28.1)	48 (71.6)	24 (61.5)	17 (27.9)	15 (45.5)
• Non C-term	192 (100.0)	502 (71.9)	19 (28.4)	15 (38.5)	44 (72.1)	18 (54.6)
QTc at enrollment, mean $\pm$ SD, ms	497 $\pm$ 55	477 $\pm$ 54	460 $\pm$ 37	464 $\pm$ 46	483 $\pm$ 39	468 $\pm$ 46
QTc 500, ms (n%)	74 (44.6)	177 (29.3)	5 (8.1)	7 (20.0)	22 (40.7)	8 (25.0)
QTc missing ECG, (n%)	25 (13.1)	94 (13.5)	5 (7.5)	4 (10.3)	7 (11.5)	1 (3.0)
QTc with imputations, mean $\pm$ SD, ms	497 $\pm$ 52	477 $\pm$ 50	460 $\pm$ 36	465 $\pm$ 45	483 $\pm$ 37	469 $\pm$ 46
Therapy during follow-up						
• Beta-blockers, (n%)	98 (51.6)	305 (45.2)	33 (50.0)	24 (61.5)	28 (45.9)	12 (36.4)
• Pacemaker, (n%)	2 (1.0)	12 (1.7)	0 (0)	1 (2.6)	3 (4.9)	1 (3.0)
• Defibrillator, (n%)	13 (6.8)	51 (7.3)	4 (6.0)	5 (12.8)	7 (11.5)	3 (9.1)
• Sympathectomy, (n%)	1 (0.5)	4 (0.6)	0 (0)	0 (0)	2 (3.3)	1 (3.0)
Cardiac events during follow-up						
• Syncope, (n%)	106 (55.5)	226 (32.7)	14 (20.9)	12 (30.8)	17 (27.9)	9 (28.1)
• Aborted cardiac arrest, (n%)	12 (6.3)	22 (3.2)	0 (0)	1 (2.56)	1 (1.64)	3 (9.4)
• Sudden cardiac death, (n%)	25 (13.0)	56 (8.0)	1 (1.5)	3 (7.7)	4 (6.6)	1 (3.0)
• Any cardiac event, (n%)	118 (61.5)	262 (37.5)	15 (22.4)	13 (33.3)	21 (34.4)	11 (33.3)

Table 2

Multivariable Analysis. Risk factors for cardiac events

	Hazard Ratio	95% Confidence Interval	P-value
Female : male			
15	0.62	0.49–0.78	<0.001
15–40	1.46	1.01–2.13	0.046
QTc per 20 ms increase*	1.26	1.17–1.36	<0.001
Mutation type and location			
Missense non c-loop	Reference	N/A	N/A
Missense c-loop	1.95	1.56–2.43	<0.001
Non-missense stop codon	0.57	0.34–0.96	0.035
Non-missense frameshift	1.01	0.58–1.77	0.97
Non-missense splice	0.95	0.61–1.48	0.82
Non-missense others	0.90	0.49–1.65	0.74
Separate analysis			
Non-missense stop codon vs other non-missense	0.60	0.33–1.07	0.085

Adjusted for time-dependent beta-blocker use,

* including imputed QTc

Table 3

Multivariable analysis. Risk for aborted cardiac arrest or sudden cardiac death

	Hazard Ratio	95% Confidence Interval	P-value
Female : male			
15	0.38	0.23–0.66	<0.001
15–40	1.11	0.66–1.86	0.71
QTc per 20 ms increase*	1.28	1.11–1.48	
Mutation type and location			
Missense non c-loop	Reference	N/A	N/A
Missense c-loop	1.77	1.17–2.67	0.007
Non-missense stop codon	0.15	0.02–1.11	0.063
Non-missense frameshifts	1.19	0.43–3.82	0.73
Non-missense splice	0.79	0.32–1.96	0.61
Non-missense others	1.17	0.43–3.21	0.76
Separate analysis			
Non-missense stop codon vs other non-missense	0.16	0.02–1.18	0.072

Adjusted for time-dependent beta-blocker use,

* including imputed QTc

Table 4

Multivariable analysis. Risk for cardiac events within non-missense mutations only based on location

	Hazard Ratio	95% Confidence Interval	P-value
Non-missense stop codon C-term vs non c-term	0.27	0.01–0.75	0.012
Non-missense frameshift C-term vs non c-term	0.35	0.11–1.06	0.064
Non-missense splice C-term vs non c-term	0.97	0.35–2.64	0.95
Non-missense others C-term vs non c-term	0.64	0.19–2.20	0.48
Frameshift or stop codons C-term vs non c-term	0.28	0.13–0.59	<0.001
Any non-missense mutation C-term vs non c-term	0.47	0.28–0.79	0.005

Adjusted for age, QTc incl imputed and time-dependent beta-blocker