Genotype-phenotype correlations

ORIGINAL ARTICLE

KCNQ1 p.L353L affects splicing and modifies the phenotype in a founder population with long QT syndrome type 1

Jamie D Kapplinger,1,2 Anders Erickson,3 Sirisha Asuri,4 David J Tester,5 Sarah McIntosh,4 Charles R Kerr,6 Julie Morrison,7 Anthony Tang,8 Shubhayan Sanatani,9 Laura Arbour,3,4 Michael J Ackerman1,2,5,10

ABSTRACT

Background Variable expressivity and incomplete penetrance between individuals with identical long QT syndrome (LQTS) causative mutations largely remain unexplained. Founder populations provide a unique opportunity to explore modifying genetic effects. We examined the role of a novel synonymous KCNQ1 p. L353L variant on the splicing of exon 8 and on heart rate corrected QT interval (QTc) in a population known to have a pathogenic LQTS type 1 (LQT1) causative mutation, p.V205M, in KCNQ1-encoded LQT1.

Methods 419 adults were genotyped for p.V205M, p. L353L and a previously described QTc modifier (KCNH2-p.K897T). Adjusted linear regression determined the effect of each variant on QTc, alone and in combination. In addition, peripheral blood RNA was extracted from three controls and three p.L353L-positive individuals. The mutant transcript levels were assessed via qPCR and normalised to overall KCNQ1 transcript levels to assess the effect on splicing.

Results For women and men, respectively, p.L353L alone conferred a 10.0 (p=0.064) ms and 14.0 (p=0.014) ms increase in QTc and in men only a significant interaction effect in combination with the p. V205M (34.6 ms, p=0.003) resulting in a QTc of ~500 ms. The mechanism of p.L353L’s effect was attributed to approximately threefold increase in exon 8 exclusion resulting in ~25% mutant transcripts of the total KCNQ1 transcript levels.

Conclusions Our results provide the first evidence that synonymous variants outside the canonical splice sites in KCNQ1 can alter splicing and clinically impact phenotype. Through this mechanism, we identified that p.L353L can precipitate QT prolongation by itself and produce a clinically relevant interactive effect in conjunction with other LQTS variants.

BACKGROUND

Long QT syndrome (LQTS) is characterised by a prolongation of the heart rate corrected QT interval (QTc) on 12-lead ECG and can predispose to syncope, seizures and sudden death if its trademark arrhythmia of torsades de pointes occurs.1 Tragically, sudden death may be the first manifestation. Approximately 80% of congenital LQTS is caused by mutations in at least 17 LQTS susceptibility genes which predominantly encode for either pore-forming ion channel subunits or channel-interacting proteins.2 KCNQ1 encodes for the α-subunit of the slow delayed rectifier potassium channel, IKs (Kv7.1), and is the gene responsible for LQTS type 1 (LQT1).3 To date, hundreds of KCNQ1 mutations have been identified, including missense, nonsense, canonical splice site, in-frame insertions/deletions and frameshift mutations. Incomplete penetrance and variable expressivity are well recognised within LQTS1 families.4–7 The genetic underpinnings for the heterogeneity in expressivity and penetrance largely remain unexplained.4–8

Although LQTS is relatively rare (1:2000),9 it is highly prevalent (~1:125)10 in a remote Canadian First Nations community of 5500 people in northern British Columbia (the Gitxsan). Through community initiation, those diagnosed with LQTS and their relatives (~800) have participated in our research which identified a novel c.613 G>A missense variant in KCNQ1. As previously described, this mutation results in a valine to methionine substitution at position 205 (p.V205M) in Kv7.1’s S3 transmembrane region and negatively impacts IKs by slowing activation and accelerating deactivation.11–12 Besides the functional evidence for loss-of-function, a prolonged QTc segregates with p.V205M-positive status compared with variant-negative relatives and associated sudden cardiac death (SCD) further supports its designation as a LQT1 pathogenic variant.7–10 Typical for LQTS, the phenotype is variable in this population, with some presenting earlier and more severely than others and sex differences in presentation have been noted. Although inherited LQTS traditionally has been considered to be a single gene disorder, evidence supports that the presentation is determined by additional genes and interactions with exogenous triggers, such as QT-prolonging drugs, physical activity and emotion. Indeed, LQTS is considered a model for complex inheritance.15 Therefore, this founder population provides a unique opportunity to identify genetic or environmental modifiers of disease in LQTS.

In 2004, sequencing of the most common genes causing LQTS in a First Nations index case (see figure 1) detected the p.V205M variant,11 along with a novel synonymous variant, a c.1059 G>A substitution in the KCNQ1 gene that maintains the original amino acid (Leucine) at position 353.
The significance of p.L353L was not clear, but it emerged again within the population when further molecular studies of the variable phenotype were initiated. Through pedigree analysis, p.L353L was confirmed to be inherited in trans to the p.V205M mutation. Population studies have confirmed the variant to be prevalent in the community, and to date, we have identified more than 70 adults with the p.L353L variant. This variant is not documented in Ensembl, dbSNP and ExAC.14 We sought to determine the underlying role of the p.L353L variant on the splicing of KCNQ1’s exon 8, its potential QT-prolonging effect alone and as a genetic modifier in patients with p.V205M-mediated LQTS1.

METHODS

Enrolment

As part of a larger study and in keeping with participatory methods,11 participants were invited to enrol if they had clinical features of LQTS or were related to an individual with a diagnosis of LQTS. Referrals for study entry were through affected family members and physicians. Community level approval was obtained as well as individual informed consent from all participants. Health information was documented upon enrolment through questionnaire and medical records to confirm clinical diagnosis or suspicion of LQTS. Chronic diseases such as cardiovascular disease, diabetes, autoimmune disease, medications, alcohol use and street drug use were also recorded when possible.

Genetic analysis

Blood or saliva samples were collected and DNA was extracted and stored by standard methods and in keeping with DNA on Loan agreement with the community.15 Genotyping was carried out for the known KCNQ1 c.613 G>A (p.V205M) LQTS1-causing variant as previously described11 and for a second KCNQ1 known disease-causing variant (p.R591H) documented in an adjacent First Nations community10 in the BC Provincial Health Services Authority (PHSA) lab of the BC Children’s Hospital, Vancouver. Expanded LQTS gene sequencing was carried out initially on five participants with a diagnosis of p.V205M-mediated LQTS1 and as well for another 37 participants with a borderline or increased QTc without the p.V205M mutation. The KCNQ1 p.L353L variant was observed in several of those who had expanded sequencing in this initial study, and the previously published and controversial KCNH2 p.K897T variant16–18 was also noted frequently. Genotyping for the KCNQ1 p.V205M, p.R591H, p.L353L and KCNH2 p.K897T variants was then carried out on all participants over the age of 16.

QTc determination

At enrolment, an ECG was performed and all other available 12-lead ECGs were collected from medical files and reviewed. Heart rate QTc measurements were assessed blinded to variant and clinical status on all available 12-lead ECGs. QT intervals were determined in all leads by the tangent method19 and the longest QT interval in any lead for an individual in any available ECG recording, corrected for rate, was considered the peak QTc.20,21 The peak QTc for each participant was recorded and used for analysis. Twelve hundred and sixty-nine manually read ECGs (mQTc) accounted for 61% of the eligible study population (257 persons). For those without mQTcs (162 persons), the Stata 13-IC program was used for the linear interpolation of mQTc on cQTcs (ECG computer calculated QTc) for the missing values of the mQTcs.

In silico splicing predictions

Four in silico predictive algorithms (Sroogle22 (http://sroogle.tau.ac.il/), ESEfinder23 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), RESCUE-ESE24 (http://genes.mit.edu/burgelab/rescue-ese/) and Human Splicing Finder25 (http://www.umd.be/HSF3/)) were used to predict the impact of the p.L353L on splicing regulatory motifs. All tools were used with default settings.
RNA extraction and RT-PCR
Total blood RNA was obtained with the PAXgene System (Qiagen, Hilden, Germany) from three p.L353L-positive and two p.L353L-negative individuals from the First Nations population and one control from outside the First Nations population. cDNA was generated using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany). Previously published primers26 were used to PCR amplify the KCNQ1 cDNA from exons 5 (5′: 5′GCGCATCCGCCTTCC TGGAGGTGCTATGCT) and the exon 10 reverse primer (9F: 5′ACGG) was used to assess the KCNQ1 transcript levels and were reported as mean±SD.

Bivariate and multivariate analyses were carried out considering drug use and cardiovascular disease. The p.L353L variant alone increased the QTc by 29.3 ms (p<0.001) above baseline. However, when the p.V205M*p.L353L variant combination was inherited, the QTc was predicted to increase by another 34.6 ms (p=0.003) above that dictated by each variant alone. When holding all variants at their mean QTc and controlling for the above covariates (also held at their mean), the predicted adjusted means demonstrate that those men with the combination of p.V205M*p.L335L variants will have a QTc approaching 500 ms (see figure 2A). This result was significantly different than for p.L335L or p.V205M variants alone (p<0.014, p<0.001, respectively) and for those with no documented variants. These results remained significant after Bonferroni correction for multiple testing (see table 3A).

For women, after adjustment for age and other covariates, the increase in QTc based on the p.L335L variant alone was

### Table 1

| Variant status of participants included in the regression analyses and their unadjusted average QTc |
|---|---|---|
| Variant status of participants (n=419) | Men (n=137) | Women (n=282) |
| | N | QTc (ms) Mean (SD) | N | QTc (ms) Mean (SD) |
| Negative for all variants | 91 | 425.9 (29.3) | 169 | 447.8 (26.2) |
| V205M only | 21 | 457.0 (17.6) | 40 | 477.8 (30.3) |
| L335L only | 15 | 435.3 (19.6) | 40 | 461.7 (33.7) |
| K897T only | 10 | 430.9 (38.6) | 18 | 441.7 (24.7) |
| V205M*L335L | 5 | 520.1 (64.7) | 7 | 476.3 (31.3) |
| V205M*K897T | 0 – | 3 | 451.0 (12.8) |
| L335L*K897T | 0 – | 3 | 486.3 (5.5) |
| V205M*L335L*K897T | 0 – | 2 | 470.5 (2.1) |

QTC, corrected QT.
10.0 ms (p=0.064) and 31.5 ms (p<0.001) for the p.V205M and p.L353L respectively. Although the QTc effect of the p.V205M*K897T combination was not statistically significant (p=0.064) and 0.007, respectively), there is no longer evidence of statistical significance for the p.V205M and p.L353L alone. The predicted QTc associated with p.L353L*p.K897T conferred a positive effect (p=0.02) resulting in an increase of 68.9±4.2%, p=0.03; Δ8: 9.2±0.6%) and significantly less skipping of exon 8 than p.L353L-positive individuals (WT: 68.9±4.2%, p=0.03; Δ8: 25.3±2.1%, p=0.003, figure 4C).

This Δ8 transcript has been identified previously and shown to generate non-functional channels which exert a dominant-negative effect on WT channels.26 Given the increase in the Δ8 transcripts, statistical modelling fitted to a binomial distribution was used to predict the likelihood of functional Kv7.1 tetramer formation. The increase in Δ8 transcripts from 9.2% to 25.3% results in a 2.3-fold reduction in the likelihood of functional protein tetramer formation from 65.6% to 28.6% (figure 4D).

DISCUSSION

Our study suggests that the KCNQ1 p.L353L synonymous variant impacts splicing efficiency resulting in the generation of alternatively spliced transcripts and ultimately decreasing functional Kv7.1 tetramer formation. While it has a moderate independent effect on the QTc, it is unlikely to increase the QTc sufficiently to result in a LQTS phenotype alone. However, our results suggest a potential modifying and sometimes synergistic effect on the QTc when inherited with other variants.

LQTS as a complex condition

A prolonged QTc interval on 12-lead ECG (≥450 for men and ≥460 for women) is a marker of increased risk for arrhythmia and SCD30 34 and may be a result of genetic and/or non-genetic factors. Since the time the first LQTS gene (LQTS1) was mapped to 11p in 1992 (subsequently confirmed as KCNQ1), the variability in the phenotype has been highlighted.32 At that time, Vincent et al compared QTc values of those linked to the chromosome 11p15 locus (carriers) and their unlinked relatives (non-carriers), demonstrating that carriers had a significantly higher QTc. However, they also showed that there was an overlap in QTc values between carriers (range 410–590 ms) and non-carriers (380–470 ms) and not all carriers were symptomatic. Ongoing evaluation of LQTS cohorts and families has yielded consistent trends of variable clinical presentation.4 It is well established that a combination of mutations confers a more severe phenotype, with greater risk of SCD33 34 and common variants combined with mutations can modulate the overall effect on phenotype and the channel or channels involved.18 35–37 For example, the LQTS1 phenotype in a South American LQTS family had an increased QTc interval compared to the QTc interval of the non-carrier parent,18 suggesting a modifying effect of the KCNQ1 p.M273V variant on the phenotype.

In silico analysis

To determine if p.L353L could impact splicing, four in silico tools were used to determine if the variant may impact any predicted splicing regulatory elements. In support of the role of this variant on splicing, all four tools predicted exon splicing enhancer (ESE) motifs directly at the p.L353L position, and these sites were predicted to be disrupted by the synonymous nucleotide substitution (see table 4). Additionally, many of the tools predicted a change in the type of ESE from an alternative splicing factor/splicing factor 2 (ASF/SF2) ESE to a SRp40-binding site.

### Table 2

| Variable                  | Beta coefficient | 95% CI | p Value | R²  |
|---------------------------|-----------------|-------|---------|-----|
| (A) Men, interaction model, n=137, intercept=418.4 ms, adjusted R²=0.54 |                |       |         |     |
| V205M                     | 29.3            | 19.2  | 0.001   | 0.54|
| L353L                     | 14.0            | 2.9   | 0.014   |     |
| K897T                     | 5.7             | -19.3 | 0.651   |     |
| V205M*L353L               | 34.6            | 12.0  | 0.003   |     |
| L353L*K897T               | -               |       |         |     |
| V205M*K897T               | -               |       |         |     |
| V205M*L353L*K897T         | -               |       |         |     |
| (B) Women, interaction model, n=282, intercept=444.3 ms, adjusted R²=0.28 |                |       |         |     |
| V205M                     | 31.5            | 20.1  | <0.001  | 0.28|
| L353L                     | 10.0            | -0.6  | 0.064   |     |
| K897T                     | -4.2            | -16.1 | 0.481   |     |
| V205M*L353L               | -14.5           | -42.6 | 0.312   |     |
| V205M*K897T               | -27.2           | -43.1 | 0.001   |     |
| L353L*K897T               | 29.9            | 4.7   | 0.020   |     |
| V205M*L353L*K897T         | -14.0           | -51.3 | 0.461   |     |
| (C) Men and women, interaction model, n=419, intercept=422.3 ms, adjusted R²=0.40 |                |       |         |     |
| V205M                     | 30.0            | 21.8  | <0.001  | 0.40|
| L353L                     | 11.2            | 3.1   | 0.007   |     |
| K897T                     | -0.6            | -11.7 | 0.914   |     |
| V205M*L353L               | 7.2             | -17.4 | 0.558   |     |
| V205M*K897T               | -26.7           | -40.5 | <0.001  |     |
| L353L*K897T               | 19.3            | -6.5  | 0.142   |     |
| V205M*L353L*K897T         | -25.9           | -60.0 | 0.136   |     |

- Beta coefficients from OLS linear regression representing the baseline (intercept) and change in QTc (ms). Model was adjusted for age, past and current alcohol abuse, QT-prolonging drug use, cardiovascular disease and indicator variable for interpolated QTc.
- 95% CI using robust SEs.
- QTc, corrected QT.
African Founder population with the KCNQ1 p.A341V mutation was influenced by common variants in NOS1AP, a regulator of neuronal nitric oxide synthase. Since then, the effects of these NOS1AP variants on the QTc interval and clinical risk have been confirmed in a large heterogeneous LQTS cohort.

Furthermore, numerous additional genomic regions (16q21 near NDRG4 and GINS3, 6q22 near PLN, 1p36 near RNF207, 16p13 near LITAF and 17q12 near LIG3 and RFFL), containing genes not previously recognised to be associated with LQTS, contribute to a prolonged QT interval in population-based...
and leads to the skipping of exons 7 and 8. Several other splice site mutations situated in exon–intron junctions have been associated with LQTS with varying degrees of severity, including KCNQ1 c.477+1 G>A, inherited homozygously in a German family with LQTS and profound hearing loss, KCNQ1 c.1032 +3 A>G resulting in skipping of exon 7 and a mild LQTS phenotype and KCNQ1 c.1251+1 G>A causing exon 9 skipping and a mild LQTS phenotype that triggered ventricular tachyarrhythmias during periods of hypokalaemia in a Japanese cohort. An intron-1 mutation c.387-5 T>A in KCNQ1 resulted in incomplete skipping of exon 2 with 10% of WT mRNA still expressed and homozygous individuals had a severe cardiac phenotype but no hearing loss. These mutations highlight the potential role of splicing in the pathogenesis of LQTS.

However, these LQTS1 causative mutations have all occurred at either the first or the last nucleotide of the exon or within the intron’s canonical 5’ or 3’ splice sites. In contrast, this study has identified the first synonymous variant localising outside the terminal positions of the exon that exerts a role in disease pathogenesis. As p.L353L (c.1059G/A) is 27 nucleotides downstream of the start of the exon and 70 nucleotides upstream of the end of the exon, this is far outside of the critical 5’ and 3’ splice site motifs. The evidence presented within this study suggests that the p.L353L (c.1059G/A) nevertheless results in decreased recognition of exon 8 leading to increased dropping of this exon from the mature mRNA. Based on the in silico evidence, this is likely due to the disruption of the ASF/SF2 ESE or conversion of ASF/SF2 to a SRp40 ESE motif. There is evidence that while SRp40 acts primarily as an ESE, this is location dependent and likely is due to the disruption of the ASF/SF2 ESE or conversion from the mature mRNA. Based on the in silico evidence, this is likely due to the disruption of the ASF/SF2 ESE or conversion of ASF/SF2 to a SRp40 ESE motif. There is evidence that while SRp40 acts primarily as an ESE, this is location dependent and thus SRp40 can also act as a strong to mild silencer of the splice site activity. In this previous study, the SRp40-binding site was moved along an exon of the ADAR2 gene, which has suboptimal splice efficiency, and at approximately the position SRp40 appears in the p.L353L mutant of KCNQ1, splice efficiency dropped. While we see that p.L353L disrupts splicing incompletely (by 24%), previous studies have also correlated a similar level of splicing disruption with a mild QT phenotype, suggesting that even small splice disruptions may contribute to disease.

Table 4 In silico analysis of the KCNQ1 c.1059G>A (p.L353L) variant

| Algorithm tool used | Prediction performed by the tool | Interpretation of the KCNQ1 gene >ENST00000155840 transcript >exon number: 8, c.1059G>A variation |
|---------------------|----------------------------------|--------------------------------------------------------------------------------------------------|
| ESEfinder 3.0       | ESE finder for SRp40, SC35, SF2/ASF and SRp55 proteins | Identifies CTGAAAGG as ESE. Predicts the G>A change in this motif to result in splicing regulatory protein binding from SF2/ASF to SRp40 |
| RESCUE-ESE 1.0      | ESE Hexamer finder               | Identifies TGAAGG as ESE and predicts the G>A change resulting in enhancer motif sequence disruption |
| Human Splicing Finder | Combines 12 different algorithms to identify and predict mutations’ effect on splicing motifs | Predicts the alteration of an exonic ESE site leading to potential alteration of splicing |
| Sroogle             | Splicing regulatory sequences identifier | Predicts ESE motifs at the c.1059 position and predicts a loss of ESE site with the G>A change |

ESE, exon splicing enhancer.

Splicing in disease

While synonymous variants are often dismissed as unlikely contributors to phenotype, increasingly their role in the pathogenesis of disease is being recognised. Even within LQTS, synonymous mutations have been recognised to cause disease. The hot spot mutation KCNQ1 A344A is the result of a c.1032G>A transition involving the terminal codon in exon 7. This synonymous mutation alters the 5’ splice site of intron 7 and leads to the skipping of exons 7 and 8.44 Several other splice site mutations situated in exon–intron junctions have been associated with LQTS with varying degrees of severity, including KCNQ1 c.477+1 G>A, inherited homozygously in a German family with LQTS and profound hearing loss, KCNQ1 c.1032+3 A>G resulting in skipping of exon 7 and a mild LQTS phenotype and KCNQ1 c.1251+1 G>A causing exon 9 skipping and a mild LQTS phenotype that triggered ventricular tachyarrhythmias during periods of hypokalaemia in a Japanese cohort. An intron-1 mutation c.387-5 T>A in KCNQ1 resulted in incomplete skipping of exon 2 with 10% of WT mRNA still expressed and homozygous individuals had a severe cardiac phenotype but no hearing loss. These mutations highlight the potential role of splicing in the pathogenesis of LQTS.

However, these LQTS1 causative mutations have all occurred at either the first or the last nucleotide of the exon or within the intron’s canonical 5’ or 3’ splice sites. In contrast, this study has identified the first synonymous variant localising outside the terminal positions of the exon that exerts a role in disease pathogenesis. As p.L353L (c.1059G/A) is 27 nucleotides downstream of the start of the exon and 70 nucleotides upstream of the end of the exon, this is far outside of the critical 5’ and 3’ splice site motifs. The evidence presented within this study suggests that the p.L353L (c.1059G/A) nevertheless results in decreased recognition of exon 8 leading to increased dropping of this exon from the mature mRNA. Based on the in silico evidence, this is likely due to the disruption of the ASF/SF2 ESE or conversion of ASF/SF2 to a SRp40 ESE motif. There is evidence that while SRp40 acts primarily as an ESE, this is location dependent and thus SRp40 can also act as a strong to mild silencer of the splice site activity. In this previous study, the SRp40-binding site was moved along an exon of the ADAR2 gene, which has suboptimal splice efficiency, and at approximately the position SRp40 appears in the p.L353L mutant of KCNQ1, splice efficiency dropped. While we see that p.L353L disrupts splicing incompletely (by 24%), previous studies have also correlated a similar level of splicing disruption with a mild QT phenotype, suggesting that even small splice disruptions may contribute to disease.
While splicing can be tissue specific, blood RNA has been used as a surrogate in the majority of the splicing mutations previously assessed in LQTS and was used in our experiments. The difference between p.L353L-positive and p.L353L-negative individuals suggests that the p.L353L variant has an impact on splicing, an effect likely recapitulated in the heart and shown clinically with an increase in QTc.

Sex differences

Women and men are well known to present differently in LQTS. Several mechanisms have been proposed to explain the sex difference, such as sex hormones which are believed to have both genomic and non-genomic effects on the QT interval. Oestrogen reduces repolarisation reserve in women and is therefore believed to be responsible for higher premenopausal susceptibility to drug-induced QT prolongation.49 Although our sample size of those with the combination p.V205M*p.L353L genotype is small (seven women and five men), the consistently higher increase in QTc in men, above that seen with p.V205M alone is not evident, on average, in the women with the same combination genotype. This finding is of interest in that mechanisms that affect the QTc in men disproportionately are rarely speculated on.50 However, male-specific effect has been observed in studies of modifying variants in LQTS1 populations, such as in the study by Lahtinen et al,51 where the presence of the variant p.D85N in KCNE1 was shown to modify the QT interval in men with LQTS1, but not in women. More recently, this phenomenon was observed in two additional Swedish founder populations with LQTS1 where QTc prolongation with NOS1AP variants was seen in men but not in women.22 Our results further support these observations that in certain circumstances men may be at higher risk, although the underlying mechanism is unclear.

Does the p.L353L destabilise the protective effect of KCNH2 p.K897T?

The effect of the KCNH2 p.K897T variant remains controversial in that it has been shown to confer both risk for18 36 and protection from17 a prolonged QTc. Our regression analysis in women suggests that the p.K897T variant likely lowers the QTc overall, consistent with other published reports.17 53 Also consistent with published reports suggesting the p.K897T may impair repolarisation if inherited with other variants,18 the combination of the p.L353L*p.K897T was associated with an increase in QTc compared with those negative for all variants. The resultant QTc effect clinically was indistinguishable from those with the pathogenic p.V205M variant alone (see figure 1 and table 3B). We were unable to assess this in men. More study is required to confirm this finding.

CONCLUSION

Our study provides the first evidence that synonymous variants outside the canonical splice sites in KCNQ1 can alter splicing and reduce WT transcript levels. The First Nations population which hosts a LQTS1 founder mutation (p.V205M) provided the unique opportunity to explore the effect of this distinctive splice variant on the QTc and the LQTS phenotype. We identified that p.L353L can affect the QT interval alone and may...
produce a synergistic effect in conjunction with other variants in certain circumstances. Sex-specific effects were observed, an emerging phenomenon reported in other LQT51 Founder population studies.

Author affiliations
1 Mayo Medical School, Mayo Clinic, Rochester, Minnesota, USA
2 Department of Molecular Pharmacology & Experimental Therapeutics, Windland Smith Rice Sudden Death Genomics Laboratory, Mayo Clinic, Rochester, Minnesota, USA
3 Division of Medical Sciences, University of Victoria, Victoria, British Columbia, Canada
4 Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada
5 Division of Heart Rhythm Services, Department of Cardiovascular Diseases, Mayo Clinic, Rochester, Minnesota, USA
6 Division of Cardiology, University of British Columbia, Vancouver, British Columbia, Canada
7 Gitxsan Health Society, Hazelton, British Columbia, Canada
8 Department of Medicine, University of Western Ontario, London, Ontario, Canada
9 Division of Cardiology, Department of Pediatrics, University of British Columbia, BC Children’s Hospital, Vancouver, British Columbia, Canada
10 Division of Pediatric Cardiology, Department of Pediatrics, Mayo Clinic, Rochester, Minnesota, USA

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