Multiplex ligation-dependent probe amplification copy number variant analysis in patients with acquired long QT syndrome

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Aims

Thirteen genetic loci map to families with congenital long QT syndrome (cLQT) and multiple single nucleotide mutations have been functionally implicated in cLQT. Studies have investigated copy number variations (CNVs) in the cLQT genes to ascertain their involvement in cLQT. In these studies 3–12% of cLQT patients who were mutation negative by all other methods carried CNVs in cLQT genes. Prolongation of the QT interval can also be acquired after exposure to certain drugs [acquired LQT (aLQT)]. Single nucleotide mutations in cLQT genes have also been associated with and functionally implicated in aLQT, but to date no studies have explored CNVs as an additional susceptibility factor in aLQT. The aim of this study was to explore the contribution of CNVs in determining susceptibility to aLQT.

Methods and results

In this study we screened the commonest cLQT genes (KCNQ1; KCNH2; SCN5A; KCNE1, and KCNE2) in a general population of healthy volunteers and in a cohort of subjects presenting with aLQT for CNVs using the multiplex ligation-dependent probe amplification method. Copy number variants were detected and confirmed in 1 of 197 of the healthy volunteers and in 1 of 90 subjects with aLQT. The CNV in the aLQT subject was functionally characterized and demonstrated impaired channel function.

Conclusion

Copy number variation is a possible additional risk factor for aLQT and should be considered for incorporation into pharmacogenetic screening of LQTS genes in addition to mutation detection to improve the safety of medication administration.

Keywords

Long QT syndrome • Genes • Copy number variation • MLPA • Multiplex ligation-dependent probe amplification

Introduction

Long QT syndrome (LQTS) is a condition characterized by abnormal cardiac repolarization leading to a prolonged QT interval on the surface electrocardiogram (ECG) and the morphologically distinct ventricular tachycardia termed tordes de pointes (TdP).¹ If not self-limiting or treated, TdP can degenerate into ventricular fibrillation and cause sudden cardiac death.

For congenital forms of LQTS (cLQT), 13 causative loci have been mapped in families presenting with the condition.²,3 Of these loci the majority of families carry mutations in the genes encoding KVLT1 (gene name KCNQ1); hERG (KCNH2); Nav1.5 (SCN5A); MinK (KCNE1); and MIRP1 (KCNE2). Presently, while over 900 mutations (most often as single nucleotides) have been associated with cLQT, up to 30% of familial cases remain mutation negative.⁴ If these familial cases are due to genetics and not environmental factors, this unexplained fraction implies that additional genetic loci remain to be identified and/or complex variations in these genes which are not detected by current technologies are responsible for altered gene function.

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What’s new?

- This study represents the first exploration of copy number variations (CNVs) as screening targets in acquired LQT (aLQT) in addition to single nucleotide mutations.
- A KCNQ1 CNV was identified in an aLQT subject, and this was functionally characterized and demonstrated impaired channel function.

To address the latter issue, several studies have used multiplex ligation-dependent probe amplification (MLPA; a multiplexed probe-based method of copy number analysis) and array-based comparative genomic hybridization (array CGH) approaches to explore larger genetic deletions/insertions and copy number variation (CNV) in the LQT genes as additional genetic determinants of cLQTS. These studies have indicated that 3–12% of otherwise mutation negative cLQT patients carry CNVs in their LQT genes.5–9 QT prolongation is the regulatory accepted surrogate biomarker for the arrhythmic potential of a drug. Acquired (or drug-induced) QT prolongation (aLQT) with or without resultant TdP has been reported for many drugs across multiple drug classes. During the last decade, QT prolongation (with or without arrhythmia) has been the most common cause of the withdrawal or restriction of use of drugs on the market.10 Although some risk factors for the development of aLQT are described, such as female gender, hypokalaemia, and underlying heart conditions, the individual development of TdP remains unpredictable and idiosyncratic. The idiosyncratic nature of aLQT, and the parallels between the phenotypic presentations of cLQT and aLQT have led to the hypothesis that there is a genetic predisposition to aLQT, and that susceptible individuals carry ‘forme fruste’ (or clinically in-apparent) mutations in cLQT genes.11 These individuals remain otherwise asymptomatic until challenged with a drug that affects cardiac repolarization. Multiple studies have explored mutations in cLQT genes as susceptibility factors to aLQT and these studies suggest that 5–15% of aLQT cases carry mutations in cLQT genes.12,13 Again these data suggest that other factors (genetic and otherwise) are an important component of susceptibility to aLQT and/or other types of other, as yet unexplored genetic variation in the known cLQT genes are contributing. These aLQT studies have concentrated on exploring single nucleotide changes in the gene sequences. With the emerging data that CNVs play a role in cLQT,5–9 our hypothesis follows that CNVs may also contribute to aLQT. In order to address this question we used MLPA with probes covering the KCNQ1; KCNH2; SCN5A; KCNE1, and KCNE2 genes to establish the background frequency of CNVs in a panel of volunteer subjects and a cohort of aLQT cases.

Methods

Subjects

Volunteer panel

DNA was collected from 206 volunteers at the Clinical Pharmacology Unit, AstraZeneca Alderley Park. The collection was established for use in genetic technology development and as controls for genetic research only and as such no subject, clinical, or demographic information was collected. All samples were anonymized. The collection and use of the samples were approved by the local ethics committee.

Acquired LQT cohort

Drug-associated TdP was diagnosed in patients receiving a recognized culprit drug and developing typical electrocardiographic features, including QT prolongation or deformity, pause-dependent onset, and polymorphic ventricular tachycardia lasting ≥10 beats in the 150–240 beats/min range. Faster polymorphic ventricular tachycardia was classified as ventricular fibrillation, and such patients were not included. Cases included here are from both the Vanderbilt University Medical Center (n = 60) and elsewhere (n = 32); in all cases, electrocardiographic documentation of the event and of the inciting drug was required as well as availability of 5 μg DNA. These 97 patients include 27 patients displaying marked QT prolongation from normal values to 600 ms shortly after initiation of a culprit drug that normalized with drug withdrawal. A blood sample was obtained from each patient for extraction of DNA from lymphocytes. DNA was harvested by, and archived by, the Vanderbilt Program in human genetics. For Vanderbilt patients, informed consent approved by the Institutional Review Board was obtained. For non-Vanderbilt patients, local human subjects’ approval was obtained. Table 1 displays a summary of clinical features.

The scientists performing analysis of the subjects by MLPA and further CNV characterization were blinded to all clinical data for the subjects.

Multiplex ligation dependent probe amplification

Multiplex ligation dependent probe amplification analysis was performed with the SALSA MLPA P114-A2 probe mix as described by MRC Holland (see http://www.mlpa.com). The MLPA probes cover LQTS genes KCNQ1, KCNH2, SCN5A, KCNE1, and KCNE2. Reactions were performed as recommended by MRC Holland with the following modifications: 7 μL of 5 μL of genomic DNA (concentration 20 ng/μL) was used in the initial hybridization which was performed for 16–18 h. Polymerase chain reaction cycle number was increased from 35 to 40 to optimize probe signal intensity.

Table 1  Clinical characteristics of the 90 subjects successfully screened by MLPA

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Female</td>
<td>69.7%</td>
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<tr>
<td>HTN</td>
<td>72.3%</td>
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<tr>
<td>LVH</td>
<td>27.0%</td>
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<tr>
<td>CHF</td>
<td>40.0%</td>
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<tr>
<td>CAD</td>
<td>32.2%</td>
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<tr>
<td>Antiarrhythmics</td>
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<tr>
<td>Class 1a</td>
<td>35.6%</td>
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<tr>
<td>Class 1c</td>
<td>1.1%</td>
</tr>
<tr>
<td>Class III</td>
<td>46.7%</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>17.8%</td>
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<tr>
<td>Psychotropics</td>
<td>5.6%</td>
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<tr>
<td>Antihistamines</td>
<td>3.3%</td>
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<tr>
<td>Immunosuppressants</td>
<td>1.1%</td>
</tr>
<tr>
<td>Opiods</td>
<td>4.4%</td>
</tr>
<tr>
<td>Antiemetics</td>
<td>2.2%</td>
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</table>

Fifteen subjects had more than one drug exposure.
Multiplex ligation-dependent probe amplification analysis was performed on a 3730 x 1 DNA Analyser (Applied Biosystems). A detection threshold of blue fluorescence of at least 150 units was applied. Multiple ligation-dependent probe amplification data analysis was performed using protocols developed specifically for MRC Holland MLPA by the National Genetics Reference Laboratory (Manchester, UK; http://www.ngf.org.uk/Manchester/projects/informatics/mlpa). Samples passed QC for MLPA analysis if the standard deviation for the control ligation products was $< 0.1$, if this threshold was exceeded the data were excluded from further analysis.

Copy number variation confirmation and characterization

In all cases, putative CNVs identified by MLPA were confirmed by a second independent method, quantitative PCR (qPCR), using commercially available gene specific assays, or custom-made assays.

Sequencing was performed to delineate the breakpoints of confirmed CNVs. Sequencing was performed using BigDye™ Dye terminator sequencing (Applied Biosystems) and the ABI 3730™ capillary sequencer. Sequence data analysis was carried out using Chromas 2.33 (Technelysium Pty Ltd).

Polymerase chain reaction and sequencing primers and methods are all provided in the Supplementary material online.

Copy number variation functional characterization

Copy number variations detected in subjects with aLQT were synthesized in vitro using the GC-RICH PCR System (Roche) according to the manufacturer’s instructions. The amplicon was then cleaved with NheI and Xmal and subcloned into the pCI vector (Promega) for expression.

The construct sequence was verified by direct sequencing. Electrophysiological modelling to determine the impact of the variation on channel function was performed. The methods used for cell culture, ion channel transfection, and voltage-clamp experiments have been described previously.14 Construct PCR primers are provided in the Supplementary material online, Table S4.

Results

Volunteer panel

Successful MLPA results were obtained for 90 out of 97 aLQT samples. Multiplex ligation-dependent probe amplification pass rate was closely correlated with DNA quality (see Supplementary material online, Figure S3).

Two deletions were detected in the aLQT panel; a single copy loss of KCNQ2 was identified in sample aLQT1 (Figure 1B) and a single copy loss of KCNQ1 exon 13 was found in aLQT2 (Figure 1C).

The KCNQ2 deletion was confirmed by qPCR. Whole genome analysis SNP data were available for this patient and these data showed loss of heterozygosity for all SNPs along the entire arm of chromosome 7q, implying a deletion of one copy of chromosome 7q. Examination of the case record revealed that the subject had leukaemia in addition to aLQT. It is therefore likely that this deletion reflects a somatic event linked to leukaemia. This mutation was not considered to be related to aLQT and was not considered further in interpretation of the results in relation to aLQT.

The single copy loss of exon 13 of KCNQ1 in aLQT2 was confirmed by qPCR. The MLPA probe sets around this region covered exons 12, 13, and 15, and it was therefore uncertain whether exon 14 was also deleted. Sequence analysis was performed in order to delineate the break points associated with the KCNQ1 exon 13 loss and to confirm whether exon 14 was also deleted. In order to further investigate this, long range PCR was performed across the region with primers designed in exons 12 and 15 of the gene, and the region sequenced by ‘primer walking’. Gel electrophoresis of the PCR product showed that the sample was heterozygous for a deletion in this region. Sequencing confirmed a deletion of 2.7 kb encompassing both exons 13 and 14 of KCNQ1 with the breakpoints identified 822 bp upstream of exon 13 and 813 bp downstream of exon 14 (see Supplementary material online, Figure S4).

Mapping of the deleted exons on to the KCNQ1 amino acid and protein structure shows that this CNV results in deletion of amino acids Gin531 to Ser676 in the C-terminal domain of the protein, and the addition of a novel, truncated 14-aa sequence (Figure 2).

In order to explore and characterize any electrophysiological perturbation resulting from the KCNQ1(del531-676) mutant the deletion was constructed using PCR primers designed such that the reverse primer would not only cleave the tail end of KCNQ1 but would also include the 14 new amino acids appended onto the end of the channel (primers reported in Supplementary Information). The KCNQ1(del531-676) exon 13 deletion was introduced into the wild-type KCNQ1 gene construct and. CHO cells were transfected with wild-type or mutant KCNQ1, along with KCN1 as previously described.15 Figure 3 shows the results of electrophysiological study comparing wild-type KCNQ1 to the KCNQ1(del531-676) with the alternative truncated C-terminal. The wild-type experiment yielded typical slowly-activating delayed rectifier currents ($I_{Ks}$), while the current generated by the mutant showed much smaller activating current (panel C) and virtually absent tail currents (panel D). This result confirms that the large deletion mutant identified here is a loss of function allele.

The subject with the KCNQ1 exon 13 deletion was a 26-year old male who had a witnessed cardiac arrest and regained consciousness after 1 to 2 min of cardiopulmonary resuscitation, with QTc interval prolonged to 600 ms. His past medical history was significant for

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gastrectomy surgery 4 years prior with a current body mass index of 26.6 kg/m² down from over 45 kg/m². He was taking trazodone, spironolactone, furosemide, clonazepam, and venlafexine with blood levels of potassium 2.4 mEq/L (normal 3.5–5 mEq/L) and magnesium 1.7 mg/dL (normal range 1.5–2.5 mg/dL) and normal kidney function after arrest. The patient’s arrest took place in the setting of a recent increase in his furosemide dose without taking prescribed potassium supplementation which accounts for his profound hypokalaemia and hypomagnesaemia. Despite electrolyte repletions to normal levels, the patient continued to have prolonged QTc interval.

Discussion

Acquired LQT (with or without resultant TdP) has been the greatest cause of drug withdrawal and labelling restrictions during the last decade. This has prompted greater regulatory pressure on pharmaceutical companies to determine the cardiovascular safety of their products prior to marketing and has led the FDA to issue their ICH...
E14 guidelines ‘Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs’. Nevertheless cases of aLQT continue to occur. This potential genetic basis of aLQT is recognized within the ICH E14 guidelines which recommend that ‘Genotyping patients who experience marked prolongation of the QT/QTc or TdP while on drug therapy should be considered’, although any further recommendations on which genes and what variants should be pursued are not given.

With emerging evidence that CNVs are also a determinant of cLQT, then there is clearly a rationale to test for CNVs as additional genetic susceptibility factors in aLQT. The present study is, therefore, the first to assess the likely contribution of CNVs of susceptibility to aLQT in an attempt to broaden our understanding of this area and to help better define our pharmacogenetic screening strategies for this event.

In order to achieve this, the background frequency of CNVs in LQT genes was first established for a volunteer population. The finding of a CNV in a common cLQT gene in only 1 of 197 volunteers (0.5%) establishes a low frequency of such variants in the population. The CNV was a single copy gain of probes within the KCNE1 and KCNE2 genes, which are adjacent to each other in the genome (21q22.1 and 21q22.12, respectively). Since there were no clinical data available for the volunteer panel, it is not possible to assess any effect on any ECG measurements or phenotypic presentation. Due to the lack of clinical data for this subject and the challenges of locating the genomic position of a copy number gain CNV, functional analysis was not performed for this CNV, and it is unclear whether this CNV could have a functional effect.

Copy number gain of KCNE1 and KCNE2 has also been identified in one individual from a panel of 1200 North-American control subjects, and copy number gain of KCNE1 alone was found by another study in one subject from a panel of 2026 Caucasian and African American subjects as reported in the Database of Genomic Variants. The aCGH data from these large studies show that copy number gain CNV in these genes is a rare event.

**Figure 2** Diagram of the protein structure of KCNQ1 with the wild-type c-terminal sequence (A) and the resultant amino acid sequence change; deletion of Gln531 to Ser676l and the addition of a new 14aa sequence at the C-terminal (B).
In the Database of Genomic Variants, CNV deletions are also reported in KCNH2, and there are several CNV gains and losses reported in SCN5A. These are all rare events observed at 0.5% frequency in large populations. Fifty structural variations are reported in KCNQ1, and these are mostly small rare CNV deletions. These data from a wealth of array studies confirm that copy number changes are rare but observed in these genes within a ‘normal’ population.

One of the 90 subjects (1%) with verified drug-induced LQT and successfully screened by MLPA carried a confirmed LQT CNV. In this study of CNVs, this subject was found to carry a single copy loss of a probe situated in KCNQ1 exon 13. Probes in exons 12 and 15 of KCNQ1 were normal for this subject, although the probe set did not contain any probes for exon 14. Additional sequencing work using primer walking on a long PCR product spanning exons 12–15 of the gene was used to characterize the extent of and to delineate the break points of this deletion. This work characterized the CNV as a 2.7 kb deletion within the KCNQ1 gene, resulting in the loss of both exons 13 and 14.

Based on the delineation of the break points, we can show that there will be a deletion of amino acids 531–676 in the C-terminal domain of the KCNQ1 gene. This deletion will result in the truncation of glutamine 531 to serine 676 and the addition of a new 14aa sequence at the C-terminal (Figure 2). Synthesis of this mutation and electrophysiological characterization demonstrated a marked loss of function of the Iks repolarizing potassium current, an effect known to be associated with cLQTS.

A single copy loss of KCNQ1 exons 13 and 14 was also reported by Eddy et al. in a mutation negative cLQT case who had presented with two episodes of collapse during exercise and a QTc range from 410 to 580 ms. The authors postulated that this would result in the loss of a critical subunit interaction domain required for KCNQ1, resulting in a haploinsufficiency phenotype. Additionally, amino acids 610–620 of the KCNQ1 protein have been shown to be required for correct trafficking of the protein to the cell membrane. By studying serial C-terminus truncations it was identified that this region must be intact for normal channel processing and cell surface expression to occur. Other work has identified the region between aa589 and aa620 as the assembly domain for KCNQ1 subunits and no expression of fully functional K+ channels was seen with this domain missing. Our functional data add to the evidence that this portion of the gene is critical and likely underlies this subject’s adverse drug reaction. Recontact and return of results were not included in the original consent of this study, but should be considered in future case acquisition as these results may have clinical significance for treatment and family screening. As such screening becomes clinically indicated, a result like this one should lead to baseline screening of drug to determine if device treatment is warranted, as well as counselling of patients and possibly screening of family members.

Some of these case subjects were included in previous screening for variants, in which 10–15% of subjects carried DNA variants in the coding regions of congenital long-QT disease genes predisposing to aLQTS, though screening has not been comprehensive in all.
We have shown that MLPA can be used to detect CNV in the LQTS genes with secondary validation by qPCR and/or sequencing, multiplex ligation-dependent probe amplification is a relatively low cost method in comparison to other approaches such as aCGH and single plex qPCR. With the ability to multiplex up to 50 probes in one reaction, this allows high-resolution screening of regions of interest in a relatively high throughput manner. Due to the low frequency of CNV events in the LQT genes, we would not recommend MLPA screening of all aLQT patients, but this approach could be used to evaluate patients who are found to be mutation negative by other methods. As technological advances in the area of next generation sequencing (NGS) come on line, it is anticipated that the cost of the NGS approach will drop significantly, making it more amenable for use in routine pharmacogenetic screening, and could potentially be used to simultaneously investigate CNVs in aLQT patients where mutations are not identified.

This study has identified some important technical aspects relating to MLPA and CNV detection. The false-positive single copy loss of KCNQ1 exon 15 due to a heterozygous SNP in the probe region highlights the need for confirmation when using MLPA. The recognition that the KCNQ2 deletion was part of a larger chromosomal aberration related to a haematological malignancy not functional in the cardiac tissue of interest emphasizes the importance of context in MLPA result interpretation. These points should be noted in future CNV studies.

While the present study has identified CNVs as a potential additional risk factor for aLQT, there are particular limitations associated with the study that require additional work. First, only 5 out of 13 known cLQT genes were screened in this study and a broader screen to include additional genes would be warranted. Furthermore, this study has only focused on LQT genes, and genes related to individual handling of trigger drugs have not been studied (e.g. genes related to the ADME and PK of a compound). Further screening and functional characterization in a larger control population would be warranted and this should include the ability to follow-up on CNVs with a phenotypic assessment of carriers. Screening in larger aLQT cohorts is also required, along with electrophysiological and biophysical characterization of any variants identified in order to determine the functional consequences of the CNVs.

Conclusions
This study represents the first exploration of CNVs in aLQT as screening targets in addition to single nucleotide mutations, and should serve as the starting point for further analyses as we aim to fully understand susceptibility factors for aLQT, improve medication administration safety, and increase efficiency in bringing drugs to market.

Supplementary material
Supplementary material is available at Europace online.

Conflict of interest: None declared.

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References