Novel rare variants in congenital cardiac arrhythmia genes are frequent in drug-induced torsades de pointes

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Marked prolongation of the QT interval and polymorphic ventricular tachycardia following medication (drug-induced long QT syndrome, diLQTS) is a severe adverse drug reaction (ADR) that phenocopies congenital long QT syndrome (cLQTS) and is one of the leading causes for drug withdrawal and relabeling. We evaluated the frequency of rare non-synonymous variants in genes contributing to the maintenance of heart rhythm in cases of diLQTS using targeted capture coupled to next-generation sequencing. Eleven of 31 diLQTS subjects (36%) carried a novel missense mutation in genes with known congenital arrhythmia associations or with a known cLQTS mutation. In the 26 Caucasian subjects, 23% carried a highly conserved rare variant predicted to be deleterious to protein function in these genes compared with only 2–4% in public databases (P < 0.003). We conclude that the rare variation in genes responsible for congenital arrhythmia syndromes is frequent in diLQTS. Our findings demonstrate that diLQTS is a pharmacogenomic syndrome predisposed by rare genetic variants.

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INTRODUCTION

Adverse drug reactions (ADRs) are common and have been implicated as frequent causes of morbidity and mortality. Marked prolongation of the QT interval and polymorphic ventricular tachycardia following medication (drug-induced long QT syndrome, diLQTS) is a severe ADR that phenocopies congenital long QT syndrome (cLQTS). Prolongation of the QT interval and the resultant polymorphic ventricular tachycardia, termed torsades de pointes, can precipitate ventricular fibrillation and sudden cardiac death and is one of the leading causes for drug withdrawal and relabeling. The incidence of diLQTS is estimated at 1–5% of patients receiving antiarrhythmic therapy with QT interval-prolonging antiarrhythmic drugs. This important ADR is also recognized, albeit at a much lower frequency, with a wide range of ‘non-cardiovascular’ therapies, including antibiotics, antipsychotics and methadone. Inhibition of a key repolarizing potassium current (termed IKr) is the common mechanism across drug classes.

Rare and common genetic variation is well-recognized as one source of variable response to drug therapy, including variable susceptibility to ADRs. Genome-wide association studies in large populations have described common loci influencing QT interval duration in the absence of medications. Genome-wide association studies for diLQTS have involved far fewer subjects and preliminary data have not consistently implicated loci with high degrees of statistical confidence. One missense variant in the KCNQ1 gene, D85N, has been observed more frequently in cases with diLQTS. To date, mutations in 13 genes encoding ion channel pore-forming proteins or function-modifying subunits have been identified in cLQTS, revealing striking incomplete penetrance in some families. Mutations in ion channel and associated genes have also been implicated in other congenital arrhythmia syndromes, such as catecholaminergic polymorphic ventricular tachycardia, which is also characterized by incomplete penetrance and by susceptibility to serious arrhythmias. Previous studies have used Sanger sequencing to screen diLQTS subjects for mutations in cLQTS genes. These analyses have been confined to less than five most commonly identified disease genes, and have identified possible causative variants in 10–20% of subjects, with one recent study from Japan having identified mutations in 8/20 (40%) of diLQTS cases.

Here we test the hypothesis that rare variants in arrhythmia genes contribute to risk for diLQTS, by a comprehensive analysis of rare non-synonymous variants across a large set of genes involved in the maintenance of heart rhythm in cases of diLQTS using targeted capture coupled to next-generation sequencing, and compare frequencies with publicly available databases. Elucidating the predictors of ADRs could lead to safer use of currently available drugs and enable development of newer drugs with decreased potential for toxicity.

MATERIALS AND METHODS

Study subject ascertainment

Drug-associated torsades de pointes was diagnosed in patients receiving a recognized culprit drug who developed the typical electrocardiographic features, including QT prolongation or deformity, pause-dependent onset and polymorphic ventricular tachycardia lasting >10 beats in the 150–240 beats per min range. More rapid polymorphic ventricular tachycardia was classified as ventricular fibrillation, and such patients were not included. Most cases included are from Vanderbilt University Medical Center; in all cases electrocardiographic documentation of the event and

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of the inciting drug was required. A blood sample was obtained from each patient for extraction of DNA from lymphocytes. For Vanderbilt patients, informed consent using a method approved by the Institutional Review Board was obtained. For non-Vanderbilt patients, local Human Subjects approval was obtained.

High-throughput genotyping
A set of 79 genes important for regulating heart rhythm (the ‘Rhythmonome’\textsuperscript{25}) was targeted (Supplementary Table 1) and included known cLQTS genes, other genes associated with congenital arrhythmia syndromes (for example, \textit{RYR2}\textsuperscript{26}, \textit{GPD1L}\textsuperscript{27}), genes encoding known or suspected partners of disease gene proteins (for example, \textit{KCNE5}, \textit{SCN8A}) and genes identified in genome-wide studies as modulators of normal QT intervals (for example, \textit{NOS1AP}). A custom Nimblegen array was designed to capture exon and flanking sequences of the 79 genes, totaling 260 kb of targeted DNA. A barcoding approach using unique 7-bp sequences to multiplex four samples in a single lane was developed and implemented. Single-end 36-bp plus 7-bp barcode reads with four samples per lane were generated on an Illumina Genome Analyzer II (San Diego, CA, USA).

Barcodes of 7 bp were stripped from the short reads using a custom Perl script. Short-read sequences were aligned to the hg18 reference genome with BWA.\textsuperscript{28} The Genome Analysis Toolkit\textsuperscript{29} base quality score recalibration, indel realignment, duplicate removal, SNP calling and genotyping were performed simultaneously across all 31 samples using standard hard filtering parameters.\textsuperscript{30}

The data from 1000 Genomes low coverage genome sequencing pilot project was downloaded for 60 CEU subjects.\textsuperscript{31} A program written in C++ was used to extract variants in the targeted region of interest.

The NHBLI GO Exome Sequencing Project (ESP) (http://snp.gs.washington.edu/EVS/) provided allele frequencies for variants detected in the regions of interest in 1351 individuals of Caucasian ancestry. Individual genotypes were not available due to confidentiality constraints.

Variants from all three sources were annotated using the Seattle Seq Annotation tool,\textsuperscript{32} novelty ascertained using KAVIAR,\textsuperscript{33} conservation scores determined using PhastCons\textsuperscript{34} and GERP,\textsuperscript{35} and \textit{in silico} prediction of function determined using PolyPhen\textsuperscript{26} and SIFT.\textsuperscript{37} A database for storage was created using MySQL and named ‘Variation’.

Sanger sequencing
Confirmatory genotyping was performed using Sanger sequencing for all novel rare missense variants and those conserved and deleterious in high-priority arrhythmia genes passing all filters or failing one filter. PCR primers were designed using the NCBI Primer Blast program to eliminate non-specific targets. Amplicons were sequenced in one direction using an Applied Biosystems 3730 sequencer. If a variant was identified, the sequencing reaction was repeated with the opposite primer for confirmation.

Statistical analysis
The two-tailed heteroscedastic \( t \) test was used to compare variants per subject in the 1000 Genomes data and diLQTS case data. The two-tailed Fisher’s exact test was used for all \( 2 \times 2 \) table comparisons. Statistical calculations were performed using Stata.

**RESULTS**

Custom capture and high-throughput sequencing
We designed and utilized a custom exon capture strategy targeting 79 genes related to heart rhythm, previously dubbed the ‘Rhythmonome’\textsuperscript{25} (Supplementary Table 1), including the 13 genes previously implicated as disease genes in cLQTS, as well as 9 other genes related to familial arrhythmia syndromes congenital short QT syndrome, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia. Capture of exons and flanking sequences totaling 260 kb was successful in 31 of 33 subjects with diLQTS (Supplementary Table 2). A high-quality next-generation sequence was generated with an average read depth of \( 27 \times \) across the targeted region. Additional alignment metrics are reported in Supplementary Table 3.

Variant calling and annotation
The targeted region contained 6267 variants across all subjects, with variant counts by genotype, subject and ethnicity presented in Supplementary Table 4. Of the identified variants, 633 were in unique locations and annotations for function and novelty are given in Supplementary Table 5. Novelty was determined using KAVIAR, including annotation of dbSNP132 and 1000 Genomes Pilot and Phase 1 data.\textsuperscript{33} We used Sanger sequencing to confirm all missense variants in the 22 congenital arrhythmia genes and novel, highly conserved or deleterious variants in the other 57 genes (Supplementary Table 6). There were 32 novel missense variants, of which 26 were highly conserved or predicted to be deleterious to protein function and 11 that occurred in the 22 congenital arrhythmia syndrome genes. Three variants had been previously reported in cLQTS, and each was a heterozygous substitution. The novel mutations in the 22 congenital arrhythmia genes and the novel, highly conserved or deleterious variants in the remaining 57 Rhythmonome genes are reported in Table 1. In addition to three previously described mutations associated with cLQTS, we confirmed missense variants across the whole set of 79 genes in 20 of 31 subjects (64.5%, Table 2). Those in congenital arrhythmia syndrome genes occurred in 11 of 31 subjects (36%).

Comparison to publicly available data
To estimate the prevalence of similar, novel, rare variants in the general population detected with next generation sequencing, data from the subset of 26 Caucasian diLQTS subjects were compared to those from the pilot phase of the 1000 Genomes project obtained from 60 Caucasian subjects, as well as exome sequence data from 1351 Caucasian individuals provided by the ESP. For this comparison only the most conserved and deleterious variants were considered.

In the 1000 Genomes pilot data with an average read depth of \( 2–4 \times \), the same 260-kb targeted region contained 21 novel, rare, missense variants, of which only 1 (a homozygous \textit{RYR2} variant) was predicted to be deleterious and in a highly conserved region. No mutations previously associated with cLQTS were observed in these 60 subjects. While a similar percentage of variants were novel and missense in both the 1000 Genomes data and the diLQTS data (3.1 vs 5.0%, respectively; \( P = 0.76 \)), fewer total variants per subject were identified in the 1000 Genomes data than in the diLQTS cases (166 vs 269.2, respectively; \( P < 0.0001 \)).

Data from 1351 Caucasian individuals provided by the ESP were obtained in genomic intervals overlapping the targeted region with an average read depth of 83 \( \times \). In this data set, only minor allele frequency, rather than individual genotype, is reported, and therefore allele and carrier frequencies cannot be directly compared. Of the 2216 exomic variants discovered, 710 were novel missense alleles, of which 33 were at sites conserved across species and were predicted to be deleterious in the 22 congenital arrhythmia genes. In this set, 14 previously detected cLQTS mutations were also found. A comparison of both the 1000 Genomes data and ESP data to the 26 Caucasian diLQTS variant carriers is shown in Table 3. More than 23% of Caucasian diLQTS subjects carry a previously identified cLQTS mutation or a novel, missense, conserved, deleterious variant, while less than 2% of subjects in 1000 Genomes carry a similar variant (\( P = 0.0027 \)).
Table 1. Novel, rare, missense variants confirmed by Sanger sequencing

| Gene   | Association | chr | Position | Amino-acid change | Protein position | Conserved<sup>a</sup> | Predicted deleterious<sup>b</sup> |
|--------|-------------|-----|----------|-------------------|------------------|-----------------------|-------------------------------|
| KCNH2  | cLQT2<sup>c</sup> | 7   | 150275404 | ARG, TRP | 1033/1160 | No | Yes |
| CACNA1C| cLQT8<sup>c</sup> | 12  | 2658977  | ALA, VAL | 1733/2139 | Yes | NA |
| AKAP9  | cLQT11<sup>d</sup> | 7   | 91565028  | GLN, GLU | 353/3908 | Yes | No |
| SNTA1  | cLQT12<sup>e</sup> | 20  | 31490264 | THR, ASN | 147/506 | Yes | Yes |
| KCNBD3 | Brugada     | 1   | 112121241 | ARG, CYS | 566/656  | Yes | No |
| GPD1L  | Brugada     | 3   | 32175498 | ALA, VAL | 249/352 | Yes | No |
| RYR2   | CPVT<sup>f</sup>  | 1   | 235699065 | LEU, VAL | 555/4968 | Yes | Yes |
| RYR2   | CPVT<sup>f</sup>  | 1   | 235881420 | LEU, PRO | 2607/4968 | Yes | Yes |
| CACNB2 | cLQT11<sup>c</sup> | 10  | 18469672 | MET, VAL | 1/623 | Yes | No |
| KCND3  | Brugada     | 1   | 112121241 | ARG, CY | 147/506 | Yes | Yes |
| GPD1L  | Brugada     | 3   | 32175498 | ALA, VAL | 249/352 | Yes | No |
| RYR2   | CPVT<sup>f</sup>  | 1   | 235699065 | LEU, VAL | 555/4968 | Yes | Yes |
| RYR2   | CPVT<sup>f</sup>  | 1   | 235881420 | LEU, PRO | 2607/4968 | Yes | Yes |
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| RYR2   | CPVT<sup>f</sup>  | 1   | 235881420 | LEU, PRO | 2607/4968 | Yes | Yes |
| CACNB2 | cLQT11<sup>c</sup> | 10  | 18469672 | MET, VAL | 1/623 | Yes | No |

Abbreviation: NA, not applicable.

<sup>a</sup>NERP<sup>X</sup> or PhastCons<sup>X</sup> 0.9.
<sup>b</sup>Polyphen2 'probably damaging' or SIFT 'DAMAGING'.
<sup>c</sup>Congenital long QT syndrome.
<sup>d</sup>Catecholaminergic polymorphic ventricular tachycardia.
<sup>e</sup>Congenital short QT syndrome.

Table 2. Characteristics of 20 subjects with confirmed novel<sup>a</sup> rare variants and previously associated cLQTS variants

| Age  | Gender  | Ethnicity   | Offending drug       | Baseline QTc | Previously observed cLQTS variants | Novel congenital arrhythmia gene variants | Novel remaining rhythmonome gene variants |
|------|---------|-------------|----------------------|--------------|-----------------------------------|----------------------------------------|------------------------------------------|
| 75   | Male    | Caucasian   | Amiodarone           | 465          | KCNH2 Arg784Trp                    | GPD1L Val249Met                         | JPH3 Arg656Trp                           |
| 60   | Male    | Caucasian   | Quinidine            | 320          | CAV3 Thr78Met                      | SCN5A Gly615Glu                         |                                          |
| 75   | Male    | Caucasian   | Quinidine and sotalol| 428          | AKAP9 Gly533Glu                    |                                      |                                          |
| 18   | Female  | Caucasian   | Metoclopramide       | 431          | CACNB2 Met1Val                     |                                      |                                          |
| 80   | Female  | Caucasian   | Trimethoprim-sulfamethoxazole | 384  | AKAP6 Phe1000Leu                   |                                      |                                          |
| 68   | Male    | Caucasian   | Sotalol              | NA           | KCND3 Arg566Cys                    |                                      |                                          |
| 39   | Female  | Caucasian   | Encainide and bretyllium | 394  | RYR2 Gly4361Gln                    |                                      |                                          |
| 60   | Male    | Caucasian   | Dofetilide           | 436          | CACNA1C Ala1733Val                 | CALR Asp418Gly                         |                                          |
| 54   | Male    | African     | Ganciclovir and sirolimus | 490  | CACNB2 Ile170Val                  | ZFH3 Gly117Ser                         |                                          |
| 72   | Female  | Caucasian   | Sotalol              | 399          | KCNH2 Arg1033Trp                   | RYR2 Leu555Val                         | PPR2R3A Phe1000Leu ZFH3 His3611Tr        |
| 59   | Male    | Caucasian   | Dofetilide           | 436          | KCND3 Arg566Cys                    |                                      |                                          |
| 60   | Female  | Asian       | Disopyramide         | 440          | AKAP6 Val839Ala                    |                                      |                                          |
| 78   | Male    | Caucasian   | Azithromycin         | 398          | AKAP7 Gly533Glu                    |                                      |                                          |
| 44   | Female  | Caucasian   | Sotalol and quinidine| NA           | APPL2 Arg504Leu                   |                                      |                                          |
| 63   | Female  | Asian       | Disopyramide         | 438          | ATP2A2 Arg504Leu                   |                                      |                                          |
| 73   | Female  | Caucasian   | Quinidine and procainamide | 420  | JPH2 Thr286Ala                     |                                      |                                          |
| 67   | Male    | Caucasian   | Quinidine           | 399          | JPH2 Val345Leu                     |                                      |                                          |
| 73   | Female  | Caucasian   | Quinidine           | 431          | KCND3 Phe315Leu                    |                                      |                                          |
| 66   | Female  | Asian       | Disopyramide         | 383          | ZFH3 Leu741Phe                     |                                      |                                          |
| 75   | Male    | Caucasian   | Quinidine           | 440          | ZFH3 Lys3689Glu                    |                                      |                                          |

Abbreviation: NA, not applicable.

<sup>a</sup>Novel means not previously reported in KAVIAR<sup>26</sup> including dbSNP132 and 1000 Genomes Pilots and Phase 1.
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| Table 3. Comparison of gene variants in diLQTS Caucasians and controls |
|-------------------------|-----------------|-----------------|----------------------|
|                         | diLQTS Genomes  | 1000 Genomes    | ESP Caucasians       |
|                         | Caucasians n = 26 | CEU n = 60 | NA                |
| Total variants          | 5168            | 9974            | NA                  |
| Average variants per subject | 198.8          | 166.2           | NA                  |
| Variants in unique locations | 528            | 424             | 2216               |
| Variants in unique locations per subject | 20.3           | 7.1             | NA                  |
| Novel variants          | 44 (8.3%)       | 73              | 1326                |
| Novel, missense or nonsense variants | 146 (27.7%)   | 84              | 1043                |
| Novel, missense or nonsense, conserved, predicted deleterious all 79 genes | 25 (4.7%)       | 21 (5.0%)        | 710                 |
| Novel, missense or nonsense, conserved, predicted deleterious in 22 congenital genes | 4 (0.8%)      | 1 (0.2%)         | 33 (1.5%)           |
| Previously detected lQTS mutations | 9 (1.7%)        | 1 (0.2%)         | 118 (5.3%)          |
| Subjects with lQTS or novel, missense or nonsense, conserved, predicted deleterious in 22 congenital arrhythmia genes | 6 (23.1%)       | 1 (1.7%)         | 47 (3.5%)           |

Abbreviations: clQTS, congenital long QT syndrome; diLQTS, drug-induced long QT syndrome; ESP, Exome Sequencing Project; NA, not applicable.

In ESP, while genotypes are not provided, assuming each subject only carried one minor allele, and all were heterozygous, less than 4% of subjects carried such variants ($P = 0.0003$).

**DISCUSSION**

Our data support the hypothesis that rare variants in congenital arrhythmia syndrome genes contribute to diLQTS susceptibility given significantly higher occurrence in cases of diLQTS compared to general populations.

Severe ADEs such as diLQTS are challenging to study, as phased drug development and safety monitoring ensure such reactions are rare. Further, precise definition of the phenotype and ascertainment of cases may be difficult.

The collection of subjects studied here is valuable given its careful characterization and documentation of the most severe, life-threatening consequence of prolongation of the QT interval, torsades de pointes, in time course with exposure to a culprit medication and without obvious other clinical cause. While the offending agents across these subjects are diverse, the common mechanism of inhibition of a key repolarizing potassium current makes collective analysis appropriate. As sample size grows, subset drug-specific analysis may be possible.

Additionally, studies of ADEs must also discriminate between risk factors (including genetic variants) predisposing to the disease that were the indication for the therapeutic agent and the ADR itself. For example, several subjects carry mutations in ZFHAX, a transcription factor that has been implicated in susceptibility to atrial fibrillation, a common indication for QT prolonging antiarrhythmics. These novel rare variants may predispose to atrial fibrillation itself, or increase the susceptibility of individuals to diLQTS with exposure to $I_{Kr}$-blocking drugs, though defining any causal relationships will require functional investigation.

Limiting the comparison to the 22 genes known to contribute to congenital repolarization disease alterable by drug challenge is the conservative approach we adopted here.

This analysis makes use of publicly available data as population controls. While the clinical characteristics of these subjects are unknown, the rare nature of these drug reaction outcomes makes occurrence in these samples unlikely. As next-generation sequencing data increase exponentially, reuse of such data across studies will become increasingly important. Given variability between sequencing technologies, comparisons such as the variants per subject reported in Table 3 and average read depth are important to understand the coverage and technical merit of platforms being evaluated. In this case, the average read depth of ESP data was far greater (83×), while average read depth of the 1000 Genomes data (2–4×) was far less than the case subjects (27×). Alignment and variant-calling pipelines also differ; while currently most data are made public in the final variant format such as .vcf, additional studies are needed to evaluate the possible utility and privacy implications of publicly releasing more basic data at the read level for uniform direct comparisons.

Collapsing variants across genes is necessary as these rare, novel mutations are expected to be private to a family, and direct comparison of individual variant frequencies such as done in genome-wide association studies is not possible. Focusing analysis on the variants in the most evolutionarily conserved locations as well as those predicted to be damaging in silico also distinguishes these variants as more likely deleterious beyond background variation. Despite finding a potential explanation for the diLQTS outcome in more cases than controls, many cases did not carry such a variant. Future direction includes expanding this hypothesis to drug-specific pharmacokinetic and pharmacodynamic genes, as variants in such pathways may have caused supra-therapeutic concentrations of medications.

Drug-induced LQTS is particularly important to drug discovery and development, as many therapeutics are stopped in development due to this severe adverse event. The variants discovered here and this approach to characterizing predisposing variants may be of use in both retrospective analyses of adverse events in trials as well as in eventual prospective screening of trial subjects prior to participation. The rare nature of this variation also implies that genotyping a specific variant may only be appropriate for screening within a family. These observations may extend to the investigation of other complex diseases and ADRs.

Our data finding rare variants in cases of diLQTS in excess of population controls support the idea that diLQTS is a pharmacogenomic syndrome predisposed by rare genetic variation. The development of genomic approaches to identify individuals at high risk for severe ADRs may allow prediction of safe, targeted therapy and improve drug safety.

**CONFLICT OF INTEREST**

Drs George and Roden have received royalties for a US Letters Patent No. 6 458 542, issued 1 October 2002 for ‘Method of Screening for Susceptibility to Drug-Induced Cardiac Arrhythmia’.

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