NextGen nuclear DNA sequencing in cyclic vomiting syndrome reveals a significant association with the stress-induced calcium channel (RYR2)

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Abstract

Background Cyclic vomiting syndrome (CVS) is a common, frequently disabling, ‘functional’ condition characterized by recurring, stereotypical attacks of intense nausea, vomiting, and lethargy, with the essential absence of these symptoms between episodes. Although the pathogenesis of CVS is yet unexplained, evidence has accumulated which suggest pathogenic roles for stress-related, autonomic, neuroendocrine, and mitochondrial factors. The objective of this pilot study was to elucidate mechanism(s) by identifying genes involved in the presumed multifactorial pathogenesis of CVS. Methods In this pilot study, DNA from 75 unrelated CVS cases and 60 healthy controls were assayed by Courtagen Life Science’s next-generation sequencing platform (nucSEEK™), including over 1100 nuclear-encoded genes involved with mitochondria, metabolism, or ion channels. Significant sequence variants were defined as evolutionary conservation at least to Xenopus (frog) per the UCSC Genome Browser. Key Results The RYR2 gene, encoding a stress-induced calcium channel present in many neurons, was the only gene demonstrating a statistically significant difference in the proportion of conserved sequence variants among the groups (18/75 CVS, 24%, vs 3/60 controls, 5%; p = 0.0018, OR = 6.0, 95% CI = 1.7–22). Conclusions & Inferences We propose a mechanism in which RYR2 sequence variants result in aberrant stress-induced calcium release into the mitochondria of autonomic neurons, resulting in an increased risk to develop autonomic/functional disease such as CVS, and related conditions such as migraine and gut dysmotility. This model incorporates the existing hypotheses regarding CVS pathogenesis into a cohesive mechanism, and might have treatment implications.

Keywords CVS, LETM1, Mitochondria, PNKD, POLG, Ryanodine, TRAP1.
**INTRODUCTION**

Cyclic vomiting syndrome (CVS, OMIM: 500007)\(^1\) is a common, frequently disabling, ‘functional’ condition characterized by recurring, stereotypical attacks of intense nausea, vomiting, and lethargy, with the essential absence of these symptoms between episodes.\(^2\) Cyclic vomiting syndrome is an incapacitating condition as episodes usually last for days and often require intravenous fluid therapy for dehydration.\(^2\) Cyclic vomiting syndrome is likely common, being present in up to 2% of children, and may lead to academic disability due to frequent and prolonged school absences.\(^3\)

Scientific investigations have yet to explain fully the pathogenesis of CVS. Evidence has accumulated which suggest pathogenic roles for autonomic, neuroendocrine, and mitochondrial dysfunction in which triggering stressors \(e.g.,\) psychological, infectious, or infradian rhythms \(n\) initiate the vomiting cascade in patients with specific susceptibility factors.\(^4\) Stressors are known to initiate the cascade of hypothalamic-pituitary-adrenal axis activation, leading to autonomic alterations via corticotropin-releasing factor \(\text{CRF}\). Corticotropin-releasing factor acts to mediate autonomic alteration including sympathetic activation, vagal inhibition and sacral parasympathetic activation responses to stress.\(^5\) Tache has proposed that altered CRF receptor-mediated signaling plays a key role in triggering emesis in patients with CVS; this notion is supported by the resemblance in clinical features between patients with CVS and those resulting from brain CRF hyperactivity.\(^6\)

Comorbid symptoms commonly experienced by patients with CVS include migraine headaches, anxiety, gut dysmotility \(e.g.,\) constipation, and postural orthostatic tachycardia syndrome,\(^7\)\(^8\) all of which are conditions associated with aberrant autonomic control. The autonomic nervous system plays an essential role in the responses to emetic activators. The input from vagal and sympathetic afferent nerves during emetic stimulation are integrated in a series of brainstem nuclei and efferent signals are then generated, thereby initiating coordinated muscular actions involved in vomiting. Three groups of investigators have observed increases in sympathetic tone, and to a lesser degree decreased parasympathetic activity, in children with CVS and have hypothesized that autonomic imbalance may render patients more predisposed to emetic signals.\(^9\)\(^10\)

Several reports have linked CVS to mitochondrial dysfunction. The apparent maternal inheritance of comorbid symptomatology \(e.g.,\) pain syndromes \(\text{migraine, complex regional pain syndrome, gut dysmotility [GERD, constipation], and depression}\) has been reported in up to two-thirds of CVS families. As the mitochondrial DNA \(\text{mtDNA}\) is exclusively maternally inherited, this observation suggests that mtDNA mutations may be contributing factors in the pathogenesis of CVS.\(^12\)\(^13\) Additionally, defects in mitochondrial energy production in CVS are postulated based on abnormal body fluid metabolite screening during vomiting episodes, electron transport chain activities in some cases,\(^14\) and clinical efficacy of therapeutic L-carnitine and coenzyme Q10,\(^15\) including in the subset of patients not exhibiting apparent maternal inheritance.

The various hypotheses in terms of CVS pathogenesis are not distinct, as stress results in heightened demands for energy,\(^16\) and dysautonomia is common in patients with genetic mitochondrial disorders.\(^10\) In order to elucidate the mechanisms of CVS beyond the mtDNA, 75 CVS cases and 60 controls were assayed by Courtagen Life Science’s next-generation sequencing platform \(\text{nucSEEK}\)\(^\text{TM}\), in order to sequence over 1100 nuclear-encoded genes involved with energy production, metabolism, mitochondria, or ion channels.

**METHODS**

Given the published literature regarding the connection between CVS and mitochondrial dysfunction, many patients with CVS are referred for Courtagen’s sequencing assays on a clinical basis. nucSEEK\(^\text{TM}\) is a nuclear mitochondrial NextGen sequencing panel which includes the 1034 gene MitoCarta \(\text{TM}\) \(\text{all genes encoding proteins with known mitochondrial location,}\) and additional genes including ion channel genes, peroxisomal genes, cytosolic ‘metabolic’ genes, and potential mitochondrial disease phenocopies. The genes included in the panel are available online at Courtagen’s website \(\text{www.courtagen.com}\).

Subjects for this analysis were retrospectively aggregated for the present study based on the presence of ‘cyclic vomiting’ or ‘CVS’ in the clinical information provided with the sample. Limited clinical information was provided with the samples in order to facilitate clinical sequence interpretations, and only this pre-existing information was used for this study. We defined ‘GI dysmotility’ to be the presence of any symptomatic diagnosis suggestive of abnormal gut transit speed including gastrointestinal reflux disease, gastroparesis, constipation, or diarrhea. No formal motility testing was performed. Control samples were obtained from self-declared ‘healthy’ Courtagen employees and their personal contacts; no clinical information was available beyond child vs adult, gender, and race/ethnicity. The above-described limited data set, containing no personal identifiers, was used for the present study. Families were not contacted in regard...
to the current study. All patient and control subjects were unrelated.

Saliva samples were obtained in commercially available Oragene collection kits by DNA Genotek and sent by regular mail directly from the families to the laboratory. DNA was extracted from the samples using the SPRI-TE nucleic acid extractor and the SPRI-TE gDNA extraction kit according to manufacturer’s protocols. Sequence-ready libraries for the MiSEQ DNA sequencer [Illumina, San Diego, CA, USA] were prepared using the HaloPlex V2 library preparation kit [Agilent, Santa Clara, CA, USA]. Sensitivity for detection of known variants exceeded 98% for 95% of the exonic sequence. Variants suspected to be related to disease were confirmed by Sanger sequencing.

For this study, a significant sequence variant was defined as one that is highly predicted to alter protein number or function (e.g., start, stop, frameshift, and clear splicing variants corresponding to ACMG categories 4 or 5) or highly conserved missense variants, which we defined as evolutionary conservation at least to Xenopus (frog) per the UCSC Genome Browser. Poorly aligned sequences were discounted, and a single species with a different amino acid sequence was allowed to account for potential sequence error in the database. With the understanding that lesser conserved missense variants are likely to have little to no biological relevance, yet highly conserved missense variants may or may not be relevant, we attempted to be inclusive in the identification of potential disease-related variants. Statistical testing (http://www.vassarstats.net) was performed by Fisher exact test. Identifying potential protective effects [lower prevalence of conserved variants in CVS than in controls] was infeasible given this study design as such genes would likely be numbered among the >1000 genes in which no conserved variants were identified among the CVS subjects, and thus analyses were conducted with one-tail. Odds ratios are given with 95% confidence interval without correction. Correction for multiple testing (http://www.vassarstats.net) was performed by Fisher exact test. Identifying potential protective effects [lower prevalence of conserved variants in CVS than in controls] was infeasible given this study design as such genes would likely be numbered among the >1000 genes in which no conserved variants were identified among the CVS subjects, and thus analyses were conducted with one-tail. Odds ratios are given with 95% confidence interval without correction. Correction for multiple testing was not employed as appropriate for a pilot study.

RESULTS

A total of 75 CVS subjects were identified, including 18 adults (18+ years), 29 adolescents [12–17 years] and 28 children [<12 years]. This cohort of CVS patients had a mean age of 15 years at the time of the study [range 2–57]; 31/75 were male. The control group consisted of 60 ‘healthy’ subjects, over 90% of which are adults, 35/60 were male. Both groups were >90% of European heritage and self-identified as ‘White’. The remaining 10% of both populations were Americans of very diverse origins.

RYR2 was the only one among the 1100+ genes that demonstrated a statistical difference between the groups [Table 1]. Six of the 18 subjects with RYR2 variants were the common [1.6% on ESP6500] polymorphism p.Glu1400Gly, which individually was statistically more common in CVS than in controls. Based on the available, yet limited, clinical information, the common phenotype among the cases with heterozygous RYR2 variants was stress-related symptomatology and the triad of chronic pain [usually migraine headache], fatigue, and GI dysmotility [at different, often multiple, levels], all of which are common in CVS in general [Table 2].

Due to moderately low subject numbers, only quite high effects [odds ratios >5] are likely to be statistically significant, so the data were analyzed additionally for genes with possible trends to evaluate in future studies. Potential candidate genes identified were POLG and LETM1. Five of the 9 subjects with POLG variants were the common [2.8% on ESP6500] polymorphism p.Glu1143Gly, which displayed borderline statistical significance. Additionally, the TRAP1 gene was evaluated based on the corresponding author’s experience of conserved variants in this gene being associated with CVS and chronic pain, chronic fatigue, and gastrointestinal dysmotility. Lastly, the PNKD gene was evaluated based on the corresponding author’s clinical experience.

DISCUSSION

In this pilot study, the type 2 ryanodine receptor [RYR2] was the only gene in which conserved sequence variants were statistically more common in the CVS population than in our healthy control population. As only 75 CVS patients were ascertained, this study is limited to identifying genes and/or common variants with a major effect on CVS pathogenesis. Thus, this study does not yet exclude a potential contribution of the many other ion channels and metabolic enzymes in the sequencing panel.

RYR2 functions as a calcium channel that is required for excitation-contraction coupling in the heart, but is also abundant in the central and peripheral/autonomic nervous systems. Intracellular calcium [Ca2+] homeostasis plays a crucial role in neuron function and survival, and neuronal Ca2+ influx is mediated via RYRs and inositol [1,4,5]-trisphosphate receptors [IP3Rs] on the endoplasmic reticulum [ER] by the action of neurotransmitters and membrane voltage.
Acute stress escalates mental and physical function via activation of the sympathetic nervous system, and deactivation of the parasympathetic nervous system, as part of the ‘fight-or-flight’ response, characterized by a rapid increase in concentrations of the catecholamines norepinephrine and epinephrine. Mutations in RYR2 are reported to be a cause of stress-triggered cardiac arrhythmias, including ventricular arrhythmias and resultant sudden death. Downstream signaling of catecholamines leads to cAMP-mediated stress-induced oxidation and nitrosylation of RYR2 and protein kinase A-hyperphosphorylation depletion of calstabin2 from brain RYR2, resulting in leaky channels that contribute to stress-induced cognitive dysfunction in mice. RYR2 dysfunction and pathologic intracellular Ca\(^{2+}\) leakage occur due to a combination of sympathetic hyperactivity via beta-adrenergic stimulation and gain-of-function RYR2 mutations. Sympathetic activation down-regulates gut motility at the same time as it up-regulates muscle and nerve, providing a link with the dramatic shutdown of foregut activity often seen in CVS episodes.

Recent studies have shown that Ca\(^{2+}\) conducting channels of both the sarcoplasmic/ER such as RYR/IP3R and those of the outer mitochondrial membrane [VDACs] form multimolecular complexes that can facilitate local ion exchange between the two organelles. RYR/IP3R-mediated increases in mitochondrial [Ca\(^{2+}\)] are followed by an increase in mitochondrial [ATP] and the reactive oxygen species [ROS] superoxide (\(\text{O}_2^-\)). As RYR2 carries 21 reactive thiolos out of 89 cysteine moieties per subunit, the net effect of thiol oxidation by ROS is increased channel conductance of Ca\(^{2+}\). Although mitochondria can buffer elevated [Ca\(^{2+}\)], after a point, the [Ca\(^{2+}\)] increase activates the opening of the permeability transition pore, allowing nonselective exchange of ions between the mitochondrial matrix and the cytosol, thereby initiating the mitochondria-dependent apoptotic cascade. Thus, mechanisms of RYR2-related disease vulnerability are likely complex, with both acute (e.g., increased sympathetic and/or decreased parasympathetic activity) and chronic (e.g., ROS-mediated mitochondrial damage, and even apoptosis) effects leading to aberrant autonomic function.

Central nervous system-based mechanisms cannot be excluded as well, such as RYR2 effects on emotion processing pathways. Indeed, patients with CVS commonly (47%) meet the criteria for a generalized anxiety disorder but the direction of causality is unclear: central anxiety resulting in autonomic/visceral nervous system changes, or autonomic dysfunction perceived as anxiety by the person based on autonomic afferent signaling.

The association between RYR2 and CVS was ascertained in this pilot study for which significance was not corrected for multiple comparisons. As over 1100 genes were sequenced, any resultant associations should be viewed as hypothesis building, to be validated, or not, following additional studies. However, RYR2 had been identified by our group because of high prevalence in patients with pediatric acute-onset neuropsychiatric syndrome, a condition that overlaps with CVS, and we had therefore analyzed our data on RYR2.

### Table 2

Clinical presentation of 18 subjects and 3 controls with conserved RYR2 variants

<table>
<thead>
<tr>
<th>Patient</th>
<th>Variant*</th>
<th>Provided functional co-morbidities†</th>
<th>Control</th>
<th>Variant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p.Ser1400Gly</td>
<td>Fatigue</td>
<td>1</td>
<td>p.Arg1119His</td>
</tr>
<tr>
<td>3</td>
<td>p.Ser1400Gly</td>
<td>GI dysmotility</td>
<td>3</td>
<td>p.Gly1885Glu</td>
</tr>
<tr>
<td>4</td>
<td>p.Ser1400Gly</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>p.Ser1400Gly</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>p.Ser1400Gly</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>p.Gly1885Glu</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>p.Gly1885Glu</td>
<td>Chronic pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>p.Gly1885Glu</td>
<td>Chronic pain, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>p.Arg3506Ter</td>
<td>Chronic pain, fatig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>p.Asn4736Asp</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>p.Ile1925Thr, p.Ile2721Thr</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>p.Met1564Ile</td>
<td>Chronic pain, fatig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>p.Arg1051Cys</td>
<td>Fatig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>p.Ile217Val</td>
<td>Fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>p.Phe4022Tyr</td>
<td>Fatig</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>p.Ala1136Val</td>
<td>Chronic pain, fatig, GI dysmotility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>p.Ala1136Val</td>
<td>Fatig</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All variants listed are heterozygous. †Per medical records provided with the samples.

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variants in CVS before analysis was performed on the full gene set. Two genes noted upon full gene-set analysis to have a possible trend with CVS, POLG and LETM1, should be considered to be candidate genes of interest to be further evaluated in future studies. This includes the p.Glu1143Gly variant of POLG, which is only borderline-statistically associated with CVS before multiple testing corrections. However, both genes are good candidates for a role in CVS pathogenesis for other reasons. Mutations in POLG, which is both the mtDNA polymerase and proof reading protein, induce mitochondrial disease through secondary induced mutations within the mtDNA itself, and increased mtDNA sequence variation has been reported in CVS. The other gene, LETM1, is responsible for much of the pathology in Wolf-Hirschhorn syndrome (WHS), caused by haplodeletion of the end of chromosome 4p. LETM1 encodes a mitochondrial inner-membrane ion transporter that shares possible similarities with RYR2 such as altered intracellular [Ca$^{2+}$] levels and dysfunctional mitochondrial transition-pore opening. This gene is also of potential interest as the last author has treated WHS cases with CVS. Putative disease-associated variants in the PINK1 and TRAP1 genes have been identified in a few to several patients with CVS, mostly outside of the present study group, and will be published separately.

We employed an in-house control group in order to match the experimental group in terms of analysis. Existing public sequence databases generally are based on exome/genome capturing methodologies that have moderate and variable sensitivity, while the sensitivity of our panel-based methodologies is very high. Thus, using public databases would call into question any positives that would favor the null hypothesis. Obviously, the associated clinical symptomatology is incomplete, but serves to show that CVS patients with conserved RYR2 variants have some of the same key co-morbidities as do CVS patients in general.

The treatment of choice for RYR2-associated cardiac disease is beta blockade, which interferes with catecholamine-induced RYR2 release of calcium in myocardium, and is thus a good candidate therapy for RYR2-associated CVS. One-half of the CVS subjects in the present study are followed by the corresponding author, and anecdotal data suggest that $\beta$-adrenergic blockade, provided at adequate dosage, has short and medium-term efficacy in many of these patients, often dramatically. Propranolol is a commonly used treatment in CVS, although some sources suggest that the proportion of CVS patients favorably responding is not great, suggesting that RYR2 gene analysis might be used to determine which CVS patients are likely to respond to propranolol therapy. In one child with CVS and healthy parents, the p.Met1564Ile variant was found to be de novo while paternity was confirmed, which further supports that the observed association of RYR2 variants with CVS.

An apparent association of CVS with conserved variants in the RYR2-encoded calcium channel was identified. We propose a mechanism in which RYR2 sequence variants result in altered stress-induced calcium release into autonomic mitochondria, resulting in autonomic dysfunction. In addition, this mechanism alters mitochondrial function, possibly via ROS-mediated mechanisms. These processes predispose toward the development of autonomic/functional disease such as CVS, migraine, and gut dysmotility. The elegance of this proposed model is that it incorporates essentially all existing hypotheses of the pathogenesis of CVS, including the triggering of attacks by environmental stress, autonomic dysfunction, prevalent comorbidities of migraine and gut dysmotility, and mitochondrial dysfunction. Given the degree of co-morbidity seen in most CVS patients, a RYR2 variant may be a marker for predicting more pervasive, chronic symptoms across multiple body systems.
Like essentially all common conditions, the genetic component in CVS pathogenesis is likely polygenic, in which various combinations of variants confer disease risk. Most CVS patients in this study did not have any RYR2 sequence variants, and there are likely several additional genes in which certain variants confer risk for the development of CVS, possibly including some of the other genes noted in this article. Identifying these disease-predisposing genes is important for improving treatment options, exemplified by the identification of the relationship between mtDNA sequence variation and CVS led to the successful introduction of carnitine and coenzyme Q10 treatment for CVS. The presently proposed model provides additional potential treatment options, in particular β-adrenergic blockade (propranolol), stress reduction, and antioxidants for the subset of CVS patients with conserved RYR2 sequence variants.

**REFERENCES**


**FUNDING**

No funding declared.

**CONFLICTS OF INTEREST**

Richard G. Boles, the corresponding author, is a consultant for Courtagen Life Sciences, which offers NextGen sequencing on a clinical basis, and the genes discussed herein are included on some of Courtagen’s panels. Stacey A. Wong is an employee of Courtagen Life Sciences. No competing interests declared for James Lee and B U.K. Li.

**AUTHOR CONTRIBUTION**

RGB, SW and JL designed the study; JL analyzed the raw data, under the guidance of SW and RGB; JL and RGB performed the statistical analyses; JL drafted the manuscript; BL provided expertise in physiology. All authors edited the manuscript.


