Supplementary Online Content

Tester DJ, Bombei HM, Fitzgerald KK, et al. Identification of a novel homozygous multi-exon duplication in *RYR2* among children with exertion-related unexplained sudden deaths in the Amish community. *JAMA Cardiol*. Published online January 8, 2020. doi:10.1001/jamacardio.2019.5400

This supplementary material has been provided by the authors to give readers additional information about their work.

SUPPLEMENTAL METHODS

Exome molecular autopsy

Exome sequencing and subsequent variant annotation was performed on genomic DNA derived from all four SUDY victims, unaffected father, and the unaffected mother by the Mayo Clinic Advanced Genomics Technology Center and Bioinformatics Core facilities. Exome sequencing was performed as previously described.[1] Following exome sequencing, single nucleotide variants (SNVs) and insertion/deletions (INDELs) were filtered to identify variants which followed a recessive inheritance pattern using Ingenuity Variant Software (Qiagen, Redwood City, CA). All variants were first filtered for a call quality score ≥ 20 and a read-depth of 10.

To be considered a candidate mutation following a recessive inheritance model, the variant identified in the affected individuals had to be non-synonymous (i.e. amino acid altering: missense, nonsense, splice-error, frame-shift INDEL, or in-frame INDEL). Given a recessive inheritance model, only rare (allele frequency ≤ 1% in the genome aggregation database [gnomAD])[2] variants present as either homozygotes (same mutation inherited from each parent) or compound heterozygotes (two unique mutations in the same gene each inherited from a different parent) were considered.

Copy number variant (CNV) analysis by PatternCNV

Exon-level copy number variants (CNVs) were called from the exome sequencing data using PatternCNV, a freely available downloadable software (http://bioinformaticstools.mayo.edu/research/patterncnv/) which calls CNVs based on the observation that read-depth coverage may vary between exons but exist in consistent patterns between samples.[3]

Chromosomal microarray

Genome chromosome microarray was performed using reagents, equipment, and software designed for the CytoScanHD microarray platform (ThermoFisher Scientific, Waltham, MA). Array preparation consists of enzymatic digestion, adapter ligation, PCR, magnetic bead purification of PCR products, fragmentation of DNA into 25-100bp segments, and biotinylation of DNA fragments. Biotinylated DNA was hybridized onto a CytoScanHD array, washed and stained in a GeneChip® Fluidics Station 450, and scanned in a GeneChip® scanner using the CytoScanHD_Array_450 protocol. The resulting CEL file was converted into an analyzable format, and data were analyzed using Chromosome Analysis Suite (ChAS) version 3.3.

Mate-pair sequencing

One microgram of DNA was processed using Illumina Nextera Mate Pair Library Kit (Illumina, San Diego, CA). Library preparation consists of tagmentation - simultaneous fragmentation of the genome into 2-5kb fragments and biotinylation of cleaved ends, strand displacement, circularization, and fragmentation of circularized DNA. Biotinylated DNA fragments were then processed through end repair, a-tailing, adapter ligation, and PCR processes. Samples were multiplexed at two samples per lane, and sequenced on the Illumina HiSeq 2500 in Rapid Run mode. Data were aligned to the reference genome (GRCh38) using BIMAv3,[4] and abnormalities identified by the mate pair assay[5,6] were identified and visualized using Ingenium, an in-house developed bioinformatics tool designed to analyze mate pair data.[7] Sanger sequencing using forward (CTAGCCCTTTTCTAACCTATACCATTGG) and reverse (CATTGAAAGCTACATACCATTCTGGAG) PCR primers specific to the unique junction formed at the site of the tandem duplication were used to further validate the precise nucleotide breakpoints of the CNV.

RYR2 duplication genotyping in the extended pedigree

RYR2 exon 1-4 duplication genotyping of additional family member samples was performed using the commercially available TaqMan® Copy Number Assay, Hs00134396_cn (ThermoFisher Scientific, Catalog number 4400291) which maps to *RYR2* exon 2 and the TaqMan® Copy Number Reference Assay, human, RNAse P (ThermoFisher Scientific, Catalog number 4403326) according to the manufacturer's instructions using an Applied Biosystems ViiA 7 Real-Time PCR System with ViiA7 Software v1.2. Briefly, each patient sample underwent real-time PCR amplification using four replicates per sample using TaqMan® Genotyping Master Mix. Three reference samples with known duplication status (i.e. homozygous wild-type, heterozygous *RYR2* duplication, and homozygous *RYR2* duplication) were included in each assay run. Following amplification, the data was first analyzed using QuantStudio™ Real-Time PCR system Software v1.3 (Applied Biosystems) set to a manual CT threshold of 0.2 and auto baseline set to on and then transferred to CopyCaller™ software for automated CNV calling.

Quantitative linkage analysis and eLOD scores

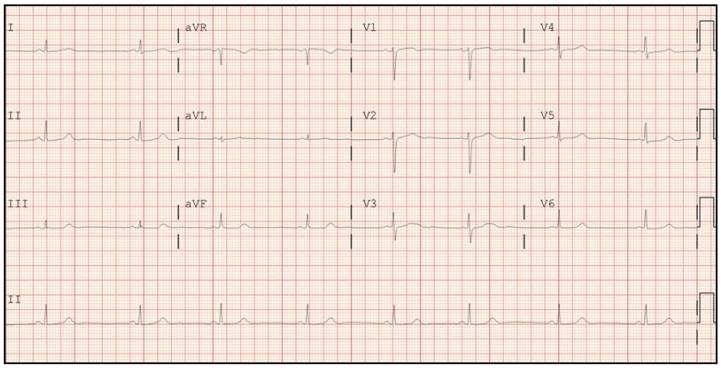
An estimated logarithm of the odd score (eLOD) was generated for each pedigree using a formula/approach endorsed by the Clinical Genome Resource Consortium.[8] The standard operating procedure developed by the Clinical Genome Resource Consortium can be found at https://www.clinicalgenome.org/site/assets/files/2165/gene_curation_sop_version_6_aug_2018_final.pdf.

The eLOD score for pedigree 1 was based on the genotype information available on 6 affected and 6 unaffected offspring of heterozygous parents. The eLOD score for pedigree 2 was based

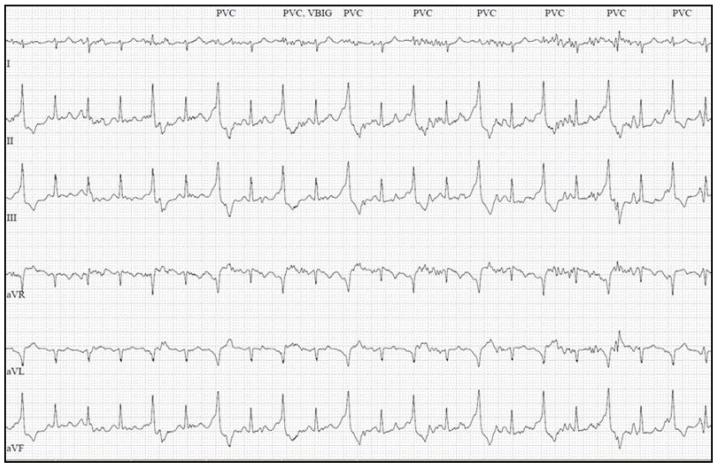
on the genotype information available on 4 affected and 3 unaffected offspring of heterozygous parents.

SUPPLEMENTAL RESULTS

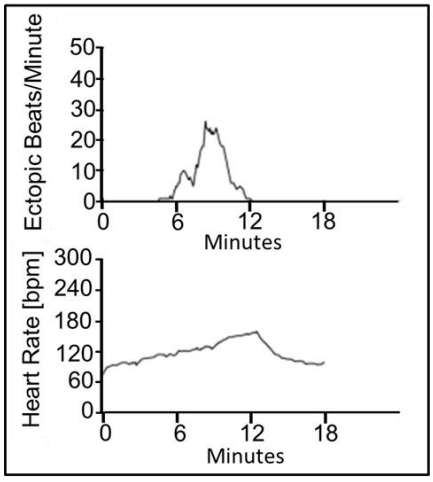
A) 12-Lead ECG at rest, Pedigree 1



B) Exercise stress test, Pedigree 1

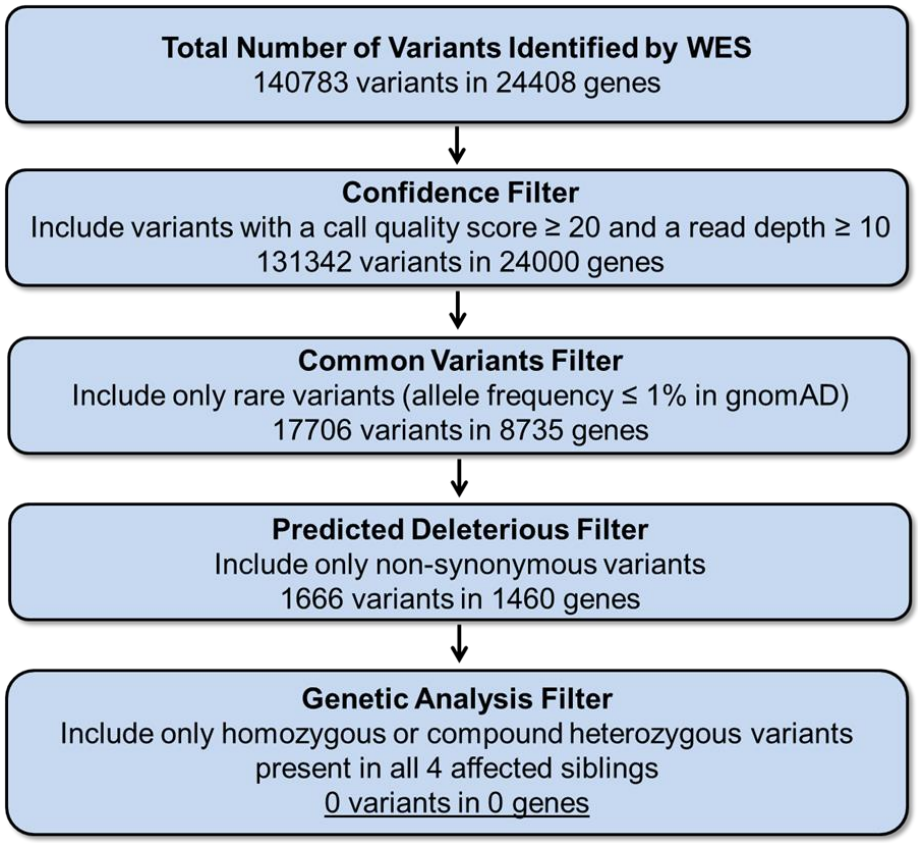


C) Exercise stress test (VE vs HR), Pedigree 1



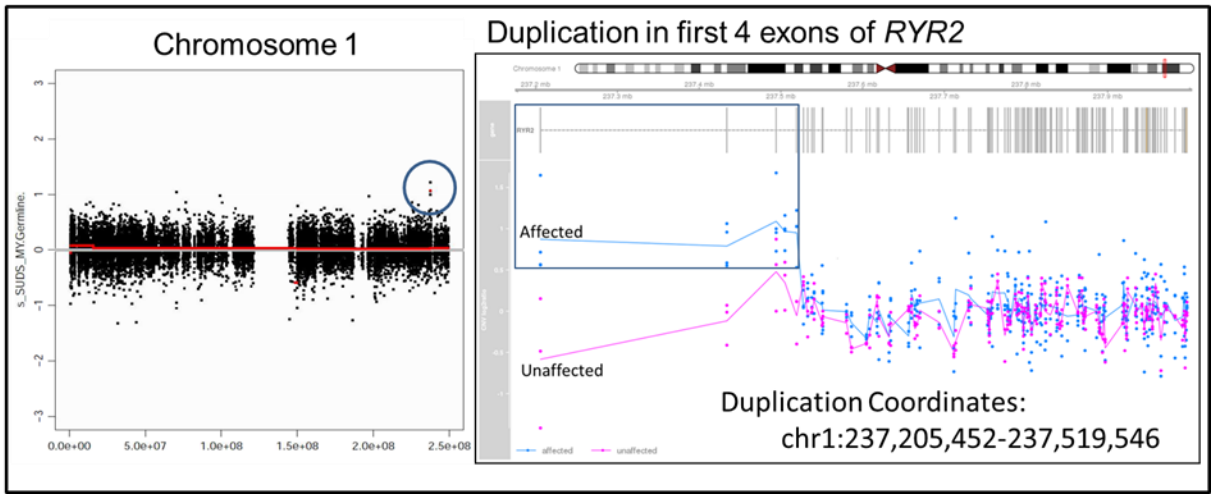
eFigure 1. Cardiologic testing of Patient 1 hosting the homozygous *RYR2* duplication.

Shown in 1a) is a representative normal 12- lead electrocardiogram (ECG) and 1B) an exercise stress test (before the second SCA) showing augmentation, then suppression of ventricular ectopy with exertion (on Nadolol) for an affected family member. This is the most remarkable exercise stress test to date for this family member. As shown in 1C), peak ventricular ectopy occurred at 8 min and then decreased as effort and heart rate increases.

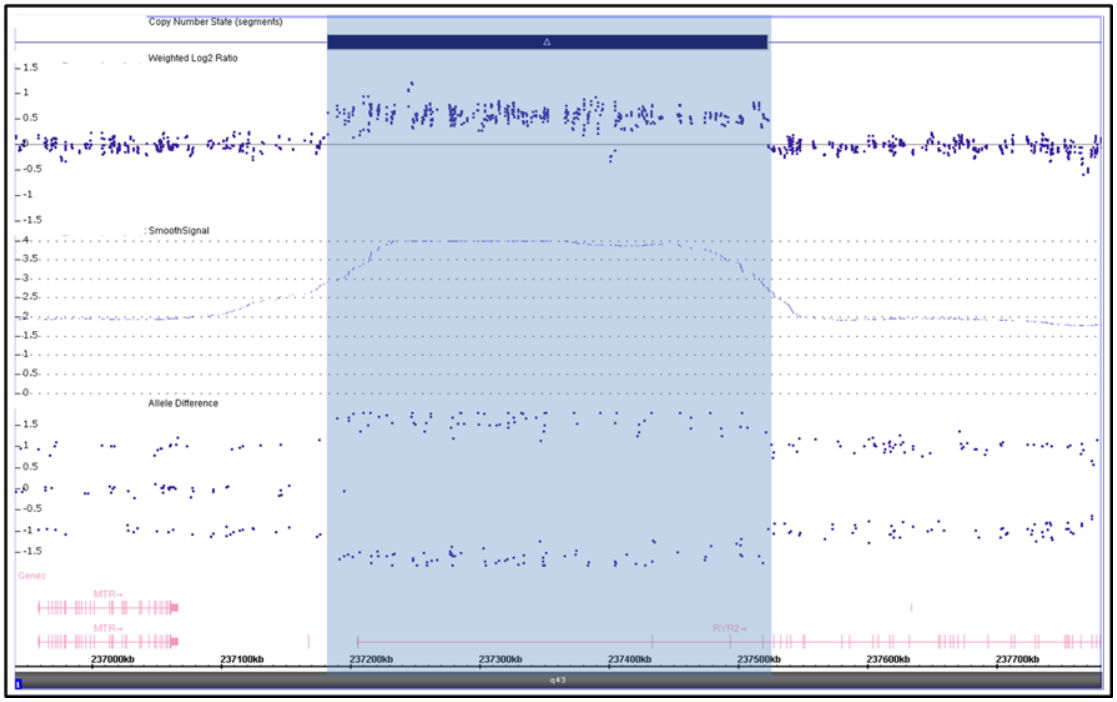


eFigure 2. Exome sequencing variant filtering strategy. Depicted is the recessive inheritance model rare variant filtering used in the exome sequencing familial triangulation strategy to identify potential pathogenic variants. Note that there were no non-synonymous variants identified by this strategy.

A)



B) CytoScanHD microarray – visualized in Chromosomal Analysis Suite (ChAS) version 3.3



eFigure 3. PatternCNV and CytoscanHD copy number variant (CNV) analysis. Depicted in panel A) are the results from the exome sequencing based-PatternCNV analysis for one of the 4 siblings from Pedigree 1 that died suddenly (Pedigree 1). In panel B) is the CytoscanHD microarray data that validates the identification of homozygous duplication occurring at chromosome 1 position chr1:237,205,452 to 237,519,546.

REFERENCES

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