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Importance of Variant Interpretation in Whole-Exome Molecular Autopsy

Population-Based Case Series

Editorial, see p 2727

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BACKGROUND: Potentially lethal cardiac channelopathies/cardiomyopathies may underlie a substantial portion of sudden unexplained death in the young (SUDY). The whole-exome molecular autopsy represents the latest approach to postmortem genetic testing for SUDY. However, proper variant adjudication in the setting of SUDY can be challenging.

METHODS: From January 2012 through December 2013, 25 consecutive cases of SUDY from 1 to 40 years of age (average age at death 27±5.7 years; 13 white, 12 black) from Cook County, Illinois, were referred after a negative (n=16) or equivocal (n=9) conventional autopsy. A whole-exome molecular autopsy with analysis of 99 sudden death-susceptibility genes was performed. The predicted pathogenicity of ultrarare, nonsynonymous variants was determined using the American College of Medical Genetics guidelines.

RESULTS: Overall, 27 ultrarare nonsynonymous variants were seen in 16/25 (64%) victims of SUDY. Among black individuals, 9/12 (75%) had an ultrarare nonsynonymous variant compared with 7/13 (54%) white individuals. Of the 27 variants, 10 were considered pathogenic or likely pathogenic in 7/25 (28%) individuals in accordance with the American College of Medical Genetics guidelines. Pathogenic/likely pathogenic variants were identified in 5/16 (31%) of autopsy-negative cases and in 2/6 (33%) victims of SUDY with equivocal findings of cardiomyopathy. Overall, 6 pathogenic/likely pathogenic variants in 4/25 (16%) cases were congruent with the phenotypic findings at autopsy and therefore considered clinically actionable.

CONCLUSIONS: Whole-exome molecular autopsy with gene-specific surveillance is an effective approach for the detection of potential pathogenic variants in SUDY cases. However, systematic variant adjudication is crucial to ensure accurate and proper care for surviving family members.

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Clinical Perspective

What Is New?

- The first population-based, case-series study involving the whole-exome sequencing-based cardiac channelopathy/cardiomyopathy gene-specific molecular autopsy of sudden unexplained death in the young cases within the United States.

What Are the Clinical Implications?

- Although the American College of Medical Genetics guidelines are useful, careful evaluation of the decedent's autopsy findings and premortem clinical phenotype remains critical before adjudicating a variant as the definitive cause of death.
- The goal of these investigative studies is to provide closure to families surrounding the loss of their loved one, but perhaps the only thing worse than no answer is to give a false answer prematurely.

Sudden cardiac death is a major worldwide public health burden, with an estimated annual incidence ranging from 180 000 to 450 000[1] in the United States and as many as 3.7 million deaths globally.[2] The majority of these deaths are because of coronary artery disease among the elderly.[3] However, ≈2000 to 5000 young people between 1 and 35 years of age die suddenly each year in the United States.[4] For many of these sudden deaths in the young, a comprehensive medicolegal investigation, including a conventional autopsy examination, elucidates a clear cause of death. However, in ≤40% of these cases, gross and microscopic inspection of the heart and other organs fails to reveal a definite cardiac/noncardiac etiology,[5] and therefore these cases are categorized as autopsy-negative sudden unexplained death in the young (SUDY).

Potentially lethal and heritable cardiac channelopathies such as long QT syndrome, catecholaminergic polymorphic ventricular tachycardia, and Brugada syndrome are typically associated with grossly and histologically normal hearts and may account for a significant portion of SUDY. Additionally, heritable cardiomyopathies, including hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and arrhythmogenic cardiomyopathy (ACM), may present with a mild structural phenotype that could escape detection at autopsy. Together these genetic heart diseases may be the underlying etiology for a significant percentage of SUDY cases.

Recently, guidelines for autopsy investigations of SUDY cases stipulate procurement and retention of tissue suitable for DNA extraction as a class I recommendation and advise that postmortem genetic testing (ie, the molecular autopsy) be considered as the new standard of care in the decedent's evaluation.[6–8] With ≥99 sudden death-susceptibility genes to date, postmortem genetic testing with whole-exome sequencing (WES) and targeted gene analysis represents a cost- and time-effective approach for performing the molecular autopsy. Although there is increasing evidence to support the use of a whole-exome molecular autopsy (WEMA),[5,9–15] standardization of the procedure for characterizing putative pathogenic mutations is crucial to enable proper counseling of surviving family members and accurate publishing for scientific progress. Recently, the American College of Medical Genetics (ACMG) has provided guidelines for the interpretation of sequence variants,[16] which may be helpful in delineating the predicted pathogenicity of variants identified by WEMA in SUDY cases.

Here, we describe a cohort of 25 unrelated SUDY victims referred consecutively by the Office of the Medical Examiner, Cook County, Illinois. We performed a WEMA to determine the spectrum and prevalence of ultrarare, nonsynonymous variants (NSVs) within sudden death-susceptibility genes and demonstrate use of both the ACMG guidelines and the necropsy-derived phenotype data for proper variant adjudication.

METHODS

The data, analytical methods, and study materials will not be made available to other researchers for the purposes of reproducing the results or replicating the procedure.

Study Subjects

From January 2012 to December 2013, 25 consecutive, unrelated SUDY cases were referred to the Windland Smith Rice Sudden Death Genomics Laboratory at the Mayo Clinic in Rochester, Minnesota, to undergo WEMA after Mayo Clinic Institutional Review Board approval. All 25 cases underwent a comprehensive autopsy by the Office of the Medical Examiner from Cook County, Illinois. Enrollment criteria required sudden death of an individual between 1 and 40 years of age, which remained unexplained or equivocal after a comprehensive autopsy.

Control Population

A total of 973 European white control exomes (509 females, 464 males) from the ICR1000 UK exome series and the 1958 Birth Cohort study were included for a case:control subset analysis of genetic variation amid these 99 genes between white decedents and these controls.[17] As previously reported, exome sequencing was performed using the Illumina TruSeq and Illumina instruments.[17]

DNA Isolation

Genomic DNA was isolated from autopsy whole blood or frozen tissue using the Gentra Puregene Blood Kit (Qiagen) following the manufacturer's protocol.

Whole-Exome Next-Generation DNA Sequencing

Genomic DNA samples were submitted to Mayo Clinic's Advanced Genomics Technology Center for WES. The Bravo liquid handler and Aligent's protocol was used to prepare paired-end libraries, and DNA was fragmented using a Covaris E210 sonicator. Agencourt AMPure SPRI beads were used to purify the constructs. SureSelect forward and Agilent SureSelect ILM Pre-Capture Indexing reverse primers were used to enrich the DNA fragment libraries, which were analyzed with Agilent Bioanalyzer DNA 1000 chip.

Exome capture was performed with the SureSelect XT Human All Exon V5 plus UTR Target Enrichment System (Agilent). Dynal Dynabeads MyOne Streptavidin T1 captured the DNA:RNA hybrids, and Agencourt Ampure XZP beads eluted DNA from the beads, which were amplified with Agilent Sure Select Post-Capture Indexing forward and Index polymerase chain reaction reverse primers. Sequencing of the exome libraries was completed with Illumina HiSeq 2000 platform and TruSeq SBS sequencing kit V3 reagents.

Variant Filtering and Pathogenicity Assessment

After WES, variants were filtered using Qiagen's Ingenuity Variant Analysis software (Qiagen Bioinformatics). Variants were included only if they (1) had a high-quality score (read depth >10 reads, call quality >20, genotype quality >20, and present in genes outside the top 1% of exonically variable genes and top 5% of exonically variable 100 base windows), (2) were NSVs (ie, missense, nonsense, frameshift insertion/deletion [INDEL], in-frame INDEL, or splice error), and (3) met our rarity threshold (minor allele frequency [MAF] ≤0.00005 in any ethnic group within Exome Aggregation Consortium, n=60 706],[18] 1000 Genome Project [1KG, n=1094],[19] and the National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project[n=6503] databases). Variants meeting these criteria underwent a further gene-specific surveillance for all known cardiac channelopathy-, cardiomyopathy-, and sudden unexplained death in epilepsy-susceptibility genes (N=99) (Table I in the online-only Data Supplement).

The ACMG guidelines for the interpretation of sequence variants were used to classify identified variants as pathogenic (P), likely pathogenic (LP), or variant of uncertain significance (VUS).[16] To be considered clinically actionable, the variant had to meet ACMG guideline criteria for a P or LP variant designation and be congruent with the presence/absence of disease- or gene-suggested autopsy findings. Herein we consider a clinically actionable variant as an LP or a P variant that should immediately prompt the physician to perform mutation-specific cascade genetic testing besides the standard cardiology clinical evaluation in all first-degree relatives of the deceased. Further, among those relatives who test positive for the implicated variant, periodic reassessment of potential disease manifestation related to the identified disease-susceptibility variant should be performed.

Candidate disease-causing variants identified through WEMA were confirmed in the decedents' genomic DNA using standard polymerase chain reaction and Sanger sequencing methods. Polymerase chain reaction primers, conditions, and sequencing methods are available on request.

Statistical Analysis

Fisher's exact tests were performed to determine statistical significance between 2 groups. A $P<0.05$ was considered to be significant.

RESULTS

SUDY Cohort

The demographics for the 25 SUDY cases are summarized in Table 1. The average age at death was 27±5.7 years. There were 17 males (68%) and 8 (32%) females. Thirteen (52%) cases were white and 12 (48%) were black. Sixteen (64%) cases were autopsy negative and 9 (36%) cases had equivocal findings or inconclusive cardiac abnormalities, with 6 (24%) having equivocal findings for a possible cardiomyopathy. The 25 SUDY victims died during these circumstances: unwitnessed (10; 40%), sleep (9; 36%), nonspecific (3; 12%), and exertion or auditory trigger (3; 12%). Eleven (44%) SUDY cases had a personal history of either illicit drug use (24%) or mental illness (20%). A prior history of arrhythmia or syncope was noted in 2 (8%) cases. No cases had a known family history of arrhythmia, syncope, seizure, or cardiac arrest.

Yield of NSVs in Sudden Death-Susceptibility Genes

After WEMA, we identified 27 ultrarare (MAF <0.00005) NSVs within the 99 sudden death-susceptibility genes in 16/25 (64%) individuals overall, including 9/12 (75%)

Table 1. Sudden Unexplained Death in the Young Cohort Demographics (N=25)

Cases	Value
Mean age, y	27.0±5.7
Male	17 (68)
Female	8 (32)
White	13 (52)
Black	12 (48)
Autopsy negative	16 (64)
Equivocal	9 (36)
Equivocal cardiomyopathy	6 (24)
Event	
Unwitnessed	10 (40)
Sleep	9 (36)
Nonspecific	3 (12)
Exertion/auditory	3 (12)
Illicit drug use	6 (24)
Mental illness	5 (20)
Arrhythmia or syncope	2 (8)

Values indicates n (%) or mean.

black and 7/13 (54%) white decedents (Figure 1 and Table 2). Compared with the 54% yield observed in white cases, ultrarare NSVs were identified in 281/973 (28.9%, *P*=0.064) European white controls (Figure 2). It is interesting to note that 4/13 (30.8%) white SUD cases hosted multiple ultrarare NSVs amid these 99 genes versus 43/973 (4.4%, *P*=0.002) of the European controls (Figure 2). Of the 27 NSVs identified, 4 (p.R783H-MYH7, p.L567Q-SCN5A, p.Y462S-RYR2, and p.N4763S-RYR2) were present in major genes (ie, strong evidence for disease association) for cardiac channelopathies and cardiomyopathies (*KCNQ1*, *KCNH2*, *SCN5A*, *RYR2*, *MYH7*, and *MYBPC3*) and 23 variants were in "minor" genes (ie, limited evidence genes) (Table 2 and Figure 3). None of the variants was identified within any of the 3 sudden unexplained death in epilepsy-susceptibility genes (*KCNA1*, *SCN1A*, and *SCN8A*).

Variant Adjudication With ACMG Guidelines

Of the 27 ultrarare NSVs, 10 NSVs (37%) were classified as P variants or LP variants based on the ACMG guideline criteria (Figure 1 and Table 2). These 10 NSVs were found in 7/25 (28%) individuals [5/13 (38%) white and 2/12 (17%) black]. Compared with the white SUDY cases, 36/973 (3.7%) white controls hosted an ultrarare NSV in ≥1 of the 99 genes that would be graded as either an LP or a P variant (*P*=0.000098). Multiple LP or P variants were identified in 2/13 (15.4%) of the white sudden death victims versus 2/973 (0.21%, *P*=0.00094) white controls (Figure 2). P or LP variants were identified in 5/16 (31%) autopsy-negative SUDY victims and in 2/6 (33%) SUDY victims who had an equivocal finding of cardiomyopathy at autopsy. Nine (36%) decedents hosted an ultrarare VUS. These VUSs are potentially informative given their ultrarare status. However, all 17 VUSs identified occurred in so-called minor genes where the strength of evidence for disease association is limited (Figure 3). Furthermore, only 5 of the 17 VUSs matched the phenotype at autopsy. Although there appears to be genotype-phenotype concordance potentially, these variants could not be considered the underlying cause of death until further evidence for pathogenicity is satisfied with either functional validation studies or other criteria in the ACMG guidelines. Twelve VUSs were discordant with the phenotype observed at autopsy and therefore were dismissed as likely benign (Figure 3).

Clinically Actionable Variants

The ACMG has proposed that the use of LP (and therefore also pathogenic) should mean that the variant of interest has a ≥90% certainty of being disease-causing.[16] However, some NSVs identified through

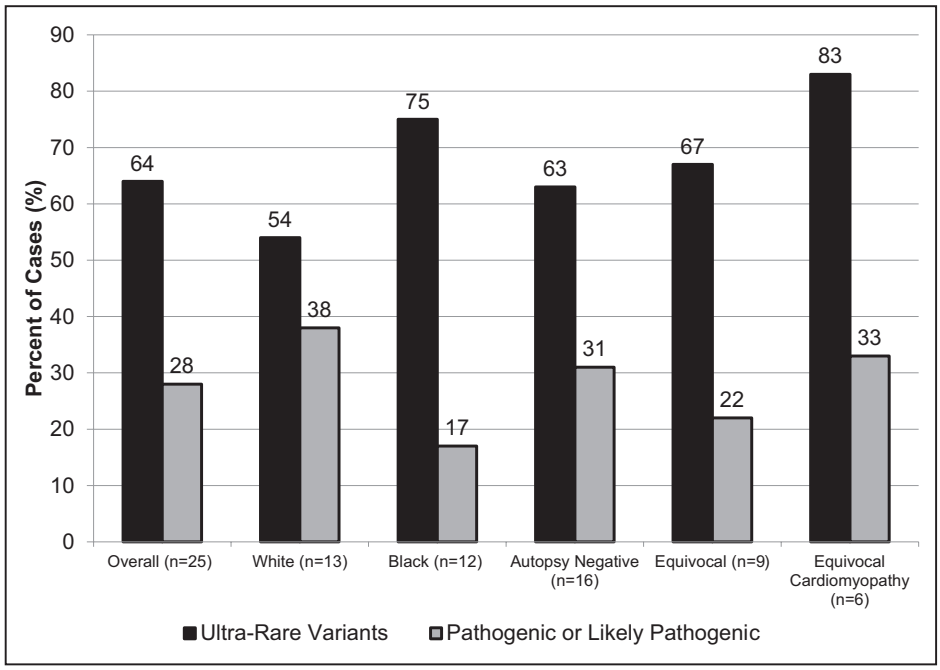

Figure 1. Yield of ultrarare nonsynonymous variants and ACMG guideline-designated pathogenic/likely pathogenic variants identified in sudden unexplained death in the young.
Shown is a bar graph indicating the percentage yield of ultrarare (minor allele frequency <0.005%) nonsynonymous variants and ACMG guideline-predicated pathogenic/likely pathogenic variants detected among 99 cardiac channelopathy/cardiomyopathy/SUDEP-associated genes for our overall cohort and the ethnic-specific (white or black), autopsy-negative, and autopsy-equivocal subsets. ACMG indicates American College of Medical Genetics; and SUDEP, sudden unexplained death in epilepsy.

Table 2. Sudden Unexplained Death in the Young Case Summary and Variant Adjudication

Case	Sex	Age, y	Race	Setting	Autopsy Findings	Autopsy Classification	Equivocal Cause	Variant(s)	Associated Disease(s)	ACMG Criteria Met	ACMG Classification	Clinically Actionable?
1	M	14	White	Exertion	Contraction band necrosis	Autopsy negative	—	p.L567Q-SCN5A	LQTS, BrS, DCM	PS1, PS3, PS4, PM1, PM2, PP3	Pathogenic	Yes
								p.N4763S-RYR2	CPVT, ACM	PM1, PM2, PP2	Likely pathogenic	Yes
								p.Y332C-PDLIM3	DCM	PM2	VUS	No
								p.G28A-MYPN	HCM, DCM	PM2	VUS	No
2	M	19	Black	Auditory	Interventricular septum hypertrophy, right atrial enlargement with endocardial fibroelastosis, right ventricular elongated chordae	Autopsy negative	—	p.S688P-PKP2	ACM	PM2	VUS	No
3	F	19	White	Unwitnessed	None	Autopsy negative	—	p.I591V-ACTN2	HCM, DCM	PM2	VUS	No
4	F	19	White	Sleep	None	Autopsy negative	—	—	—	—	—	—
5	F	21	White	Unwitnessed	None	Autopsy negative	—	c.903+1G>A-NEBL	DCM	PVS1, PM2	Likely pathogenic	No
6	F	23	White	Unwitnessed	None	Equivocal	Possible drug toxicity	—	—	—	—	—
7	M	25	Black	Sleep	Thin LV wall (0.6 cm), scattered hypertrophic myocytes	Equivocal	Cardiomyopathy	p.K120T-KCNJ2	LQTS	PM2	VUS	No
8	M	26	White	Sleep	None	Autopsy negative	—	—	—	—	—	—
9	F	27	Black	Nonspecific	LV hypertrophy (1.8 cm), LV posterior MI scarring	Equivocal	Ischemic cardiomyopathy	p.R783H-MYH7	HCM, DCM	PS1, PM1, PM2, PM5, PM6, PP2, PP4	Pathogenic	Yes
								p.V27236fs-TTN	HCM, DCM	PVS1, PM2	Likely pathogenic	Yes
								p.V190M-JUP	ACM	PM2	VUS	—
10	F	27	Black	Unwitnessed	Interstitial and perivascular fibrosis	Autopsy negative	—	p.R25W-LAMP2	HCM	PM2	VUS	—
11	M	27	Black	Unwitnessed	None	Equivocal	Possible sarcoidosis	—	—	—	—	—
12	M	27	White	Sleep	Focal perivascular fibrosis, probe-patent foramen ovale	Autopsy negative	—	p.K942X-MYH6	HCM, DCM	PVS1, PM2	Likely pathogenic	No
13	M	28	Black	Unwitnessed	Focal myocyte hypertrophy	Autopsy negative	—	p.E44Q-ILK	DCM	PM2	VUS	No
14	M	28	Black	Unwitnessed	Intraventricular septum hypertrophy (2.0 cm)	Equivocal	Cardiomyopathy	p.A52P-MYOM1	HCM	PM2	VUS	No

(*Continued*)

Table 2. Continued

Case	Sex	Age, y	Race	Setting	Autopsy Findings	Autopsy Classification	Equivocal Cause	Variant(s)	Associated Disease(s)	ACMG Criteria Met	ACMG Classification	Clinically Actionable?
15	M	28	White	Sleep	Enlarged heart (610g), LV hypertrophy, enlarged tricuspid valve with abnormal chordae, fusion of aortic valve cusps, myocyte hypertrophy	Equivocal	Hypertrophic cardiomyopathy	p.Y462S-RYR2	CPVT, ACM	PM1, PM2, PM5, PP2	Likely pathogenic	No
								p.R350Q-DES	DCM	PM1, PM2, PM5, PP2	Likely pathogenic	Yes
16	M	29	Black	Exertion	Focal interstitial and perivascular fibrosis, myocyte nuclear hypertrophy	Equivocal	Excited delirium (schizophrenia)	p.R1899H-MYH6	HCM, DCM	PM2	VUS	No
17	M	29	Black	Unwitnessed	Subendocardial fibrosis	Autopsy negative	—	p.Q1289X-DSP	ACM	PVS1, PM2, PP3	Pathogenic	No
								p.F1198L-LAMA4	DCM	PM2	VUS	No
								p.R3Q-CTF1	DCM	PM2	VUS	No
18	M	29	White	Unwitnessed	Focal interstitial and perivascular fibrosis	Equivocal	Cardiomyopathy	—	—	—	—	—
19	M	29	White	Sleep	None	Autopsy negative	—	p.G8D-SCN4B	LQTS	PM2	VUS	No
20	M	30	Black	Sleep	None	Autopsy negative	—	—	—	—	—	—
21	M	30	White	Nonspecific	None	Autopsy negative	—	—	—	—	—	—
22	M	30	White	Unwitnessed	None	Autopsy negative	—	—	—	—	—	—
23	F	36	White	Sleep	Interstitial fibrosis, myocyte hypertrophy	Autopsy negative	—	p.D22167fs-TTN	HCM, DCM	PVS1, PM2	Likely pathogenic	Yes
								p.V585M-CACNA1C	BrS, LQTS	PM2	VUS	No
24	M	37	Black	Sleep	Enlarged heart (625 g), LV hypertrophy (2.2 cm), intraventricular septum hypertrophy (2.0 cm), myocyte fibrosis and disarray	Equivocal	Hypertrophic cardiomyopathy	p.W427R-RBM20	DCM	PM2	VUS	No
								p.A531T-MYLK2	HCM	PM2	VUS	No
								p.P486L-TBX1	HCM	PM2, PP2	VUS	No
25	F	39	Black	Nonspecific	Tunneling of LAD (1 mm deep)	Autopsy negative	—	—	—	—	—	—

ACM indicates arrhythmogenic cardiomyopathy; ACMG, American College of Medical Genetics; BrS, Brugada syndrome; CPVT, catecholaminergic polymorphic ventricular tachycardia; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LAD, left anterior descending; LQTS, long QT syndrome; LV, left ventricle; and VUS, variant of uncertain significance.

WES that meet the ACMG criteria for a P or an LP designation may not be consistent with the autopsy findings for the SUDY. Of the 10 P/LP variants identified, 6 variants (p.R350Q-DES, p.R783H-MYH7, p.N4763S-RYR2, p.L567Q-SCN5A, p.V2736fs-TTN, and p.D22167fs-TTN) identified in 4/28 (14.3%) were congruent with the SUDY's phenotype at autopsy and therefore deemed clinically actionable (Table 2 and Figure 3).

The remaining 4 P/LP variants (p.Q1289X-DSP, p.K942X-MYH6, c.903+1 G>A-NEBL, and p.Y462S-RYR2) were not congruent (Table 2 and Figure 3) with autopsy findings. Although p.Q1289X-DSP, p.K942X-MYH6, and c.903+1 G>A-NEBL all satisfy the ACMG

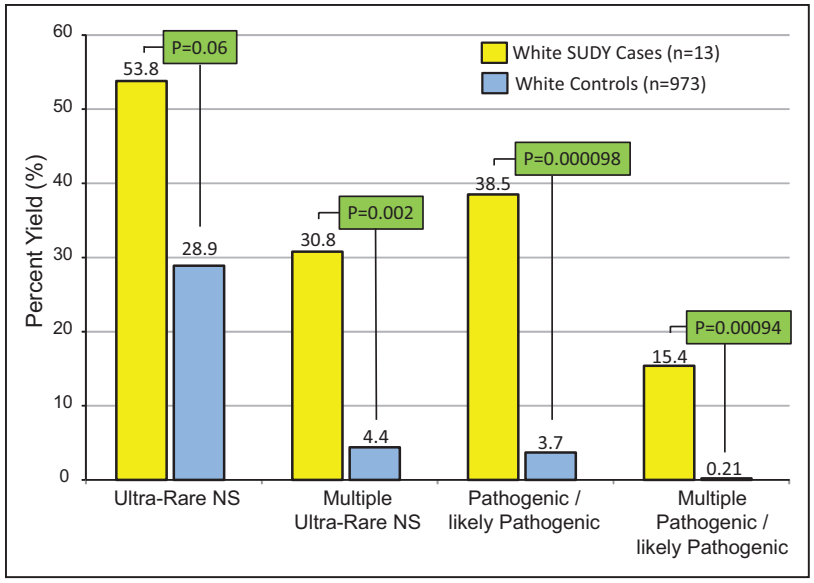

Figure 2. White case: control comparative analysis of ultrarare nonsynonymous variants and ACMG guideline-designated pathogenic/likely pathogenic variants.
Shown is a bar graph indicating the percentage yield of ultrarare (minor allele frequency <0.005%) nonsynonymous (NS) variants and ACMG guideline-predicated pathogenic/likely pathogenic variants detected among 99 cardiac channelopathy/cardiomyopathy/SUDEP-associated genes for white sudden unexplained death in the young cases (n=13) and European white controls (n=973). ACMG indicates American College of Medical Genetics; and SUDEP, sudden unexplained death in epilepsy.

guideline criteria for P or LP designation based on being an ultrarare and null variant (ie, nonsense or splice-error), these variants in cardiomyopathy-associated genes were identified in SUDY victims, with no autopsy findings suggestive of a structurally abnormal heart. Therefore, these variants may require additional lines of evidence before assigning them as being disease-causing and relevant to surviving family members. The p.Y462S-RYR2 variant was identified in a patient with autopsy findings consistent with HCM. RYR2 mutations cause catecholaminergic polymorphic ventricular tachycardia, a structurally normal heart-associated arrhythmia syndrome. This SUDY case also hosted a desmin (p.R350Q-DES) LP variant that would be consistent with his autopsy findings and most likely represents the pathogenic basis for the decedent's sudden death.

Case Summaries for SUDY Victims With a Clinically Actionable Variant

Overall, 4/25 (16%) of the SUDY cases hosted ≥1 clinically actionable variant. A 14-year-old white male (Case 1, Table 2) who experienced an exertion-related autopsy-negative sudden death hosted a p.L567Q-SCN5A P variant and a p.N4763S-RYR2 LP variant. *SCN5A* encodes a voltage-gated cardiac sodium channel, and mutations in the gene have been associated with LQT3[20] and Brugada syndrome 1.[21] Electrophysiological studies of the p.L567Q-SCN5A mutation have demonstrated a significant effect on sodium channel inactivation,[22] and the patients with this specific mutation are particularly prone to sudden death.[23] *RYR2* gene mutations cause catecholaminergic polymorphic ventricular tachycardia,

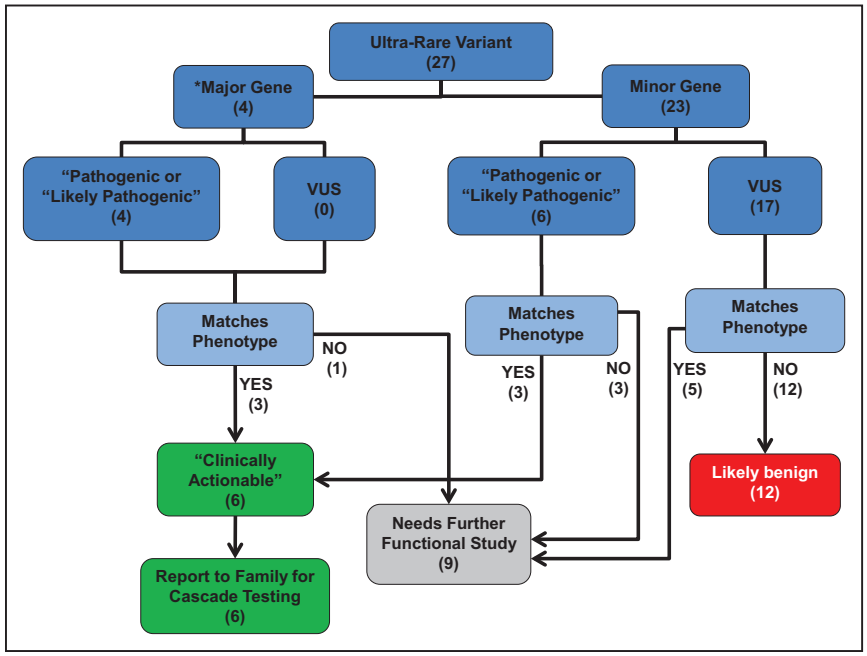

Figure 3. Variant interpretation flow diagram.
Shown is a flow-diagram algorithm used to determine whether an identified ultrarare nonsynonymous variant was considered clinically actionable, needs further functional study, or presumed likely benign. Numbers in parentheses indicate the number of variants. Major genes include *KCNQ1*, *KCNH2*, *SCN5A*, *RYR2*, *MYH7*, and *MYBPC3*. Minor genes include all other genes in 99 gene panel. VUS indicates variant of uncertain significance.

a structurally normal heart-associated arrhythmia syndrome that often manifests during exertion.

A 27-year-old black female (Case 9, Table 2) who collapsed suddenly with seizure-like activity had a p.R783H-MYH7 P variant and a p.V27236fs-TTN LP variant. She had a history of a cardiac blood clot 4 years before death, and her autopsy was equivocal for ischemic cardiomyopathy. The *MYH7* gene is associated with familial HCM and DCM. Although the p.R783H variant results in a conservative amino acid substitution, this specific variant has been observed previously in 2 individuals with cardiomyopathy.[24] *TTN* frameshift mutations have been associated with DCM. The TTN frame-shift variant identified in this SUDY case localizes to the sequence segment encoding for the A-band region of the protein, where such frameshift mutations are overrepresented in cases of DCM compared with controls.[25]

A 28-year-old white male (Case 15, Table 2) found dead in bed hosted both a p.R350Q-DES and p.Y462S-RYR2 LP variant. He had a history of arrhythmias and was scheduled for cardioversion later that year. Additionally, his autopsy was equivocal for HCM, with microscopic findings of myocyte hypertrophy and gross findings of an enlarged tricuspid valve with abnormal chordae, enlarged chambers, left ventricle hypertrophy, and fusion of right and noncoronary cusps of the aortic valve. His heart was enlarged, weighing 610 g. His mother was diagnosed previously with HCM. *DES*-encoded desmin is a class III intermediate filament specific to muscle cells. Mutations in desmin have been associated with HCM, DCM, myofibrillar myopathy, and sudden death.[26–29] *RYR2* encodes the cardiac ryanodine receptor found in the sarcoplasmic reticulum of cardiac muscle. The receptor facilitates calcium release that is crucial for cardiac contraction.

A 36-year-old white female (Case 23, Table 2) found unresponsive in bed hosted a p.D22167fs-TTN frameshift variant designated as LP. She had a history of heart murmur of unknown type. Although originally considered autopsy-negative, microscopic findings at autopsy included mild to moderate interstitial fibrosis as well as myocyte hypertrophy. The mutation results in a frameshift mutation within the region encoding for the A-band portion of the TTN protein where frame-shift mutations are overrepresented in DCM cases when compared with controls.[25]

DISCUSSION

Since providing the first ever proof-of-principle case report of a WES-based comprehensive molecular autopsy of a previously healthy 16-year-old victim of SUDY , where we identified a pathogenic *MYH7* mutation,[30] the utility of the WEMA has been increasingly recognized as an efficient and cost-effective method for comprehensive postmortem genetic evaluation of SUDY cases.[5,9–15]

Here, we present the first population-based study involving WEMA of SUDY cases within the United States. Using WES and a gene-specific analysis involving 99 cardiac channelopathy-, cardiomyopathy-, and sudden unexplained death in epilepsy-associated genes, we identified an ultrarare (MAF <0.00005; 1 in 20 000 alleles) amino acid-altering variant in a sudden death-susceptibility gene in 54% of our white and 75% of our black victims of SUDY.

In 2016, Bagnall and colleagues[5] reported on the genetic analysis of 59 cardiac channelopathy/cardiomyopathy genes in a similar SUDY cohort of 113 cases from Australia or New Zealand. Using an MAF <0.1% (ie, 1 in 1000 alleles), 27% of their cases hosted what they termed a clinically relevant, pathogenic, or likely pathogenic cardiac gene mutation. Of the 36 clinically relevant variants identified, 30 had an MAF <0.00005 (the threshold used in our study). Thus, 6 of the variants identified would not have been considered to be potentially disease-causing using our more stringent MAF cutoff. In fact, 4 of these variants have a higher prevalence in Exome Aggregation Consortium (1 in 500 to 1 in 1000 subjects) than the estimated disease prevalence (eg, 1 in 2000 for long QT syndrome) with which they would be associated.

Although both studies support the utility of WEMA to identify potential sudden death-causing variants within sudden death-susceptibility genes, the challenge of the WES-based molecular autopsy does not lie in the identification of variants but, rather, in the adjudication of their predicted pathogenicity. Accurate variant classification is crucial to enable proper counseling of surviving family members and accurate publishing for scientific progress.

Erroneously or prematurely adjudicating ambiguous variants as pathogenic has the potential to harm patients and their families. Tragically, this became a reality for 1 family described by Ackerman et al[29] as they dealt with the disastrous consequences of unnecessary treatment based on an erroneously interpreted variant in *KCNQ1*. Our group and others have estimated previously that as much as 10% of the variants published as long QT syndrome-associated mutations may be classified incorrectly.[31,32] This number is likely to be higher when accounting for all sudden death genes because a recent study has indicated that as many as 30% of all disease-causing genetic variants in the literature may have been reported incorrectly.[33]

To assist in the interpretation of identified variants, the ACMG guidelines provide a framework for variant classification by incorporating a variety of weighted factors that lead to a final delineation of P, LP, benign, or

VUS.[16] Using strict criteria, variants are assessed for very strong (ie, a null variant), strong (ie, the same amino acid change as a previously established pathogenic variant, confirmed as de novo when there is no family history, or is associated with well-established functional studies demonstrating a deleterious effect), moderate (ie, absence from large control populations like Exome Aggregation Consortium), or supporting (ie, multiple lines of computational evidence predicting a deleterious effect) evidence for pathogenicity. Points earned in each category are combined in a variety of ways to reach a final variant classification.

Recently, after interrogation of 77 cardiac channelopathy/cardiomyopathy genes, Lahrouchi and colleagues[15] reported a yield of ACMG guideline-predicated P/LP variants in 13% of their mostly European decent (88%) cohort of 302 SUDY cases without structural heart disease or nonspecific cardiovascular changes. Congruent with a phenotype of negative-autopsy and suspected sudden cardiac arrhythmia death, 85% of the L/LP variants identified by Lahrouchi and colleagues[15] were in major cardiac channelopathy-susceptibility genes (ie, *KCNQ1*, *KCNH2*, *SCN5A*, and *RYR2*).

While using their own interpretation for labeling variants as P or LP, Bagnall and colleagues[5] reported finding such variants in 27% of their SUDY cases. However, when applying the ACMG guideline criteria to their identified variants, this yield decreases to only 7.1% (8/113). In fact, based on the ACMG guidelines, 28 of their 36 P or LP variants would be demoted to a VUS. The difference in variant interpretation stems largely from the overcalling of missense variants as being LP. In their study, missense variants with an MAF <0.1% that were predicted to be damaging by ≥2 out of 3 in silico tools (SIFT, Polyphen, Mutation Taster) and involving conserved nucleotides (genomic evolutionary rate profiling score ≥2) were considered as LP.[5] However, according to ACMG, this level of evidence alone would be deemed insufficient to promote a variant to an LP designation.

Based on ACMG guideline criteria, 28% of our overall cohort hosted ≥1 P/LP variant. The yield of P/LP variants was ≈30% for both the autopsy-negative and equivocal cases with cardiomyopathic findings at autopsy. This reflects a 20% higher yield of ACMG guideline-predicated variants in our cohort than what was observed in the phenotypically and demographically similar cohort investigated by Bagnall and colleagues.[5] The large difference in yield may stem from differences in the next-generation sequencing platforms and bioinformatics-based variant annotation and analytic pipelines that were used, as well as our inclusion of 40 additional genes that were not included in the Bagnall study.[5] In fact, 3 of our 25 (12%) cases had either a TTN frameshift (2 cases) or NEBL splice-error (1 case) LP variant. However, these 2 genes were not included in the Bagnall study.[5]

Although 28% of our SUDY cases hosted a P or an LP variant based on the ACMG guidelines, the decedent's phenotype at autopsy was congruent with the genetic finding in only 14% of cases and therefore considered clinically actionable. These clinically actionable P/LP variants have sufficient genotype-phenotype evidence to warrant cascade genetic testing of surviving family members. However, other ultrarare variants that do not rise to our present consideration of clinically actionable might be deemed as nevertheless worthy of careful research/clinical-based investigations to see whether the variant cosegregates with the disease phenotype. If it did, then those variants might be elevated to a clinically actionable status that would warrant cascade genetic testing for future family members.

It is interesting to note that 3 cardiomyopathy-associated genes variants that satisfied the ACMG guideline criteria for P or LP designation based on being an ultrarare and null variant (ie, nonsense or splice-error) were identified in victims of SUDY with no autopsy findings suggestive of a structurally abnormal heart. Although some cardiomyopathy-associated gene variants can provide a potentially lethal arrhythmic substrate before the development of overt cardiomyopathic changes, because these identified variants were not congruent with the observed phenotype, the designation of these variants as ACMG guideline-predicated P or LP should be rendered with some residual skepticism.

It is noteworthy that one-third of the ultrarare variants identified were categorized as needs further functional study. These variants are potentially informative clinically given their presence in disease-associated genes and their ultrarare status. However, they currently lack sufficient clinical evidence (ie, cosegregation with disease phenotype) and research-based evidence (ie, in vitro functional validation assay) to be put forward as a disease-causing, clinically relevant variant.

CONCLUSIONS

Despite 64% of this population-based cohort of consecutive, unrelated SUDY cases having ultrarare, nonsynonymous variants within sudden death-susceptibility genes, 28% had a variant classified as either P or LP based on the ACMG guidelines. Furthermore, 14% of cases had a congruent genotype-phenotype correlation enabling the variant to be clinically actionable for cascade genetic testing of surviving family members. The substantial number of VUSs demonstrates the necessity for further standardizing the adjudication of putative pathogenic variants. Although the current ACMG guidelines are useful, careful evaluation of the decedent's autopsy findings and premortem clinical phenotype remains critical before adjudicating a variant as the definitive cause of death in SUDY cases. The goal of

these investigative studies is always to provide closure to families surrounding the loss of their loved one, but perhaps the only thing worse than no answer is to give a false answer prematurely.

ARTICLE INFORMATION

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M.J.A. and D.J.T. designed the study. D.J.T. performed DNA extraction. G.W.S., J.P.A., and D.J.T. performed variant filtering and variant analysis. S.M.W. performed conventional autopsies. G.W.S., J.P.A., and D.J.T. wrote the initial drafts of the article. M.A.S. and E.R.B. provided the control data.

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Dr M. Ackerman is a consultant for Audentes Therapeutics, Boston Scientific, Gilead Sciences, Invitae, Medtronic, MyoKardia, and St Jude Medical. Dr M. Ackerman and Mayo Clinic have an equity/royalty relationship with AliveCor, Blue Ox Health Corporation, and StemoniX. However, none of these entities was involved in this study in any way. The other authors report no conflicts of interest.

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Importance of Variant Interpretation in Whole-Exome Molecular Autopsy: Population-Based Case Series

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SUPPLEMENTAL MATERIAL

Table 1. List of 99 strong and limited evidence cardiac channelopathy-, cardiomyopathy, and sudden unexplained death in epilepsy (SUDEP)-associated genes

Number	Gene	Protein Name	Disease Association
1	*ABCC9*	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	DCM
2	*ACTC1*	actin, alpha, cardiac muscle 1	HCM, DCM
3	*ACTN2*	actinin, alpha 2	HCM, DCM
4	*AKAP9*	A kinase (PRKA) anchor protein (yotiao) 9	LQTS
5	*ANK2*	ankyrin 2	LQTS
6	*ANKRD1*	ankyrin repeat domain 1 (cardiac muscle)	HCM, DCM
7	*BAG3*	Bcl2-associated athanogene 3	DCM
8	*CACNA1C*	calcium channel, voltage-dependent, L type, alpha 1C subunit	BrS, LQTS
9	*CACNA2D1*	calcium channel, voltage-dependent, alpha 2/delta subunit 1	BrS
10	*CACNB2*	calcium channel, voltage-dependent, beta 2 subunit	BrS
11	*CALM1*	calmodulin	LQTS, CPVT
12	*CALM2*	calmodulin	LQTS
13	*CALM3*	calmodulin	LQTS
14	*CALR3*	calreticulin 3	HCM
15	*CASQ2*	calsequestrin 2 (cardiac muscle)	CPVT
16	*CAV3*	caveolin 3	LQTS
17	*CRYAB*	crystallin, alpha B	DCM
18	*CSRP3*	cysteine and glycine-rich protein 3 (cardiac LIM protein)	HCM, DCM
19	*CTF1*	cardiotrophin 1	DCM
20	*DES*	desmin	DCM
21	*DMD*	dystrophin, muscular dystrophy	DCM
22	*DSC2*	desmocollin 2	ACM
23	*DSG2*	desmoglein 2	ACM
24	*DSP*	desmoplakin	ACM
25	*EMD*	emerin (Emery-Dreifuss muscular dystrophy)	DCM
26	*EYA4*	eyes absent homolog 4 (Drosophila)	DCM
27	*FCMD*	fukuyama type congenital muscular dystrophy (fukutin)1	DCM
28	*FHL2*	four and a half LIM domains 2	DCM
29	*FXN*	frataxin	HCM
30	*GATA4*	GATA-binding protein 4	HCM
31	*GATAD1*	GATA zinc finger domain containing 1	DCM
32	*GLA*	galactosidase, alpha	HCM
33	*GPD1L*	glycerol-3-phosphate dehydrogenase 1-like	BrS
34	*HCN4*	hyperpolarization activated cyclic nucleotide-gated potassium channel 4	BrS
35	*ILK*	integrin-linked kinase	DCM
36	*JAG1*	jagged 1	HCM
37	*JPH2*	junctophilin 2	HCM
38	*JUP*	junction plakoglobin	ACM
39	*KCNA1*	potassium voltage-gated channel, shaker-related subfamily, member 1	SUDEP
40	*KCND3*	potassium voltage gated channel, Shal-related family, member 3	BrS
41	*KCNE1*	potassium voltage-gated channel, Isk-related family, member 1	LQTS
42	*KCNE2*	potassium voltage-gated channel, Isk-related family, member 2	LQTS

43	*KCNE3*	potassium voltage-gated channel, Isk-related family, member 3	BrS
44	*KCNH2*	potassium voltage-gated channel, subfamily H (eag-related), member 2	LQTS
45	*KCNJ2*	potassium inwardly-rectifying channel, subfamily J, member 2	LQTS
46	*KCNJ5*	potassium inwardly-rectifying channel, subfamily J, member 5	LQTS
47	*KCNJ8*	potassium inwardly-rectifying channel, subfamily J, member 8	BrS
48	*KCNQ1*	potassium voltage-gated channel, KQT-like subfamily, member 1	LQTS
49	*LAMA4*	laminin, alpha 4	DCM
50	*LAMP2*	lysosome-associated membrane glycoprotein 2	HCM
51	*LBD3*	LIM binding domain 3 (ZASP)	HCM, DCM
52	*LMNA*	lamin A/C	DCM
53	*MYBPC3*	myosin binding protein C, cardiac	HCM, DCM
54	*MYH6*	myosin, heavy chain 6, cardiac muscle, alpha	HCM, DCM
55	*MYH7*	myosin, heavy chain 7, cardiac muscle, beta	HCM, DCM
56	*MYL2*	myosin, light chain 2, regulatory, cardiac, slow	HCM
57	*MYL3*	myosin, light chain 3, alkali; ventricular, skeletal, slow	HCM
58	*MYLK2*	myosin light chain kinase 2	HCM
59	*MYOM1*	myomesin 1, 185kDa	HCM
60	*MYOZ2*	myozenin 2	HCM
61	*MYPN*	myopalladin	HCM, DCM
62	*NEBL*	nebulette	DCM
63	*NEXN*	nexilin (F actin binding protein)	HCM, DCM
64	*NKX2.5*	NK2 transcription factor related 5	HCM
65	*PDLIM3*	PDZ and LIM domain 3	DCM
66	*PKP2*	plakophilin 2	ACM
67	*PLN*	phospholamban	HCM, DCM
68	*PRKAG2*	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	HCM
69	*PSEN1*	presenilin 1	DCM
70	*PSEN2*	presenilin 2	DCM
71	*PTPN11*	protein tyrosine phosphatase, non-receptor type 11	HCM
72	*RAF1*	v-raf-1 murine leukemia viral oncogene homolog 1	HCM
73	*RANGRF*	RAN guanine nucleotide release factor	BrS
74	*RBM20*	RNA binding motif protein 20	DCM
75	*RYR2*	ryanodine receptor 2 (cardiac)	CPVT, ACM
76	*SCN1A*	sodium channel, voltage-gated, type I, alpha subunit	SUDEP
77	*SCN1B*	sodium channel, voltage-gated, type I, beta	BrS
78	*SCN3B*	sodium channel, voltage-gated, type III, beta	BrS
79	*SCN4B*	sodium channel, voltage-gated, type IV, beta	LQTS
80	*SCN5A*	sodium channel, voltage-gated, type V, alpha	LQTS, BrS, DCM
81	*SCN8A*	sodium channel, voltage gated, type VIII, alpha subunit	SUDEP
82	*SGCD*	sarcoglycan, delta (dystrophin-associated glycoprotein)	DCM
83	*SNTA1*	syntrophin, alpha 1	LQTS
84	*TAZ*	tafazzin	DCM, FAOD
85	*TBX1*	T-box 1	HCM
86	*TBX5*	T-box 5	HCM
87	*TCAP*	titin-cap (telethonin)	HCM, DCM
88	*TGFB3*	transforming growth factor, beta 3	ACM
89	*TMEM43*	transmembrane protein 43	ACM
90	*TMPO*	thymopoietin	DCM
91	*TNNC1*	troponin C type 1	HCM, DCM

92	*TNNI3*	troponin I type 3 (cardiac)	HCM, DCM
93	*TNNT2*	troponin T type 2 (cardiac)	HCM, DCM
94	*TPM1*	tropomyosin 1 (alpha)	HCM, DCM
95	*TRDN*	triadin	CPVT
96	*TTN*	titin	HCM, DCM
97	*TTR*	transthyretin	HCM, DCM
98	*TXNRD2*	thioredoxin reductase 2	DCM
99	*VCL*	vinculin	HCM, DCM

Cardiac channelopathies: Brugada syndrome (BrS), catecholaminergic polymorphic ventricular tachycardia (CPVT), Long QT syndrome (LQTS). Cardiomyopathies: arrhythmogenic cardiomyopathy (ACM), dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), sudden unexplained death in epilepsy (SUDEP).