Circulation

ORIGINAL RESEARCH ARTICLE

Primary Myocardial Fibrosis as an Alternative Phenotype Pathway of Inherited Cardiac Structural Disorders

Editorial, see p 2727

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BACKGROUND: Myocardial fibrosis is a common postmortem finding among young individuals with sudden cardiac death. Because there is no known single cause, we tested the hypothesis that some cases of myocardial fibrosis in the absence of identifiable causes (primary myocardial fibrosis [PMF]) are associated with genetic variants.

METHODS: Tissue was obtained at autopsy from 4031 consecutive individuals with sudden cardiac death in Northern Finland, among whom PMF was the only structural finding in 145 subjects with sudden cardiac death. We performed targeted next-generation sequencing using a panel of 174 genes associated with myocardial structure and ion channel function when autopsies did not identify a secondary basis for myocardial fibrosis. All variants with an effect on protein and with a minor allele frequency <0.01 were classified as pathogenic or variants of uncertain significance on the basis of American College of Medical Genetics consensus guidelines.

RESULTS: Among the 96 specimens with DNA passing quality control (66%), postmortem genetic tests identified 24 variants of known or uncertain significance in 26 subjects (27%). Ten were pathogenic/likely pathogenic variants in 10 subjects (10%), and 14 were variants of uncertain significance in 11 genes among 16 subjects (17%). Five variants were in genes associated with arrhythmogenic right ventricular cardiomyopathy, 6 in hypertrophic cardiomyopathy–associated genes, and 11 in dilated cardiomyopathy–associated genes; 2 were not associated with these disorders. Four unique variants of uncertain significance cosegregated among multiple unrelated subjects with PMF. No pathogenic/likely pathogenic variants were detected in ion channel–encoding genes.

CONCLUSIONS: A large proportion of subjects with PMF at autopsy had variants in genes associated with arrhythmogenic right ventricular cardiomyopathy, dilated cardiomyopathy, and hypertrophic cardiomyopathy without autopsy findings of those diseases, suggesting that PMF can be an alternative phenotypic expression of structural disease–associated genetic variants or that risk-associated fibrosis was expressing before the primary disease. These findings have clinical implications for postmortem genetic testing and family risk profiling.

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Key Words: cardiomyopathies ■ death, cardiac, sudden ■ fibrosis ■ genetics

Sources of Funding, see page 2724

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Clinical Perspective

What Is New?

- Postmortem genetic studies identify potentially relevant genetic variants in cardiac structure encoding genes in almost one third of individuals with sudden cardiac death with primary myocardial fibrosis as the only significant finding at autopsy.
- Variants that have been previously detected in families with dilated, hypertrophic, or arrhythmogenic right ventricular cardiomyopathy are seen in these individuals with primary myocardial fibrosis sudden cardiac death, suggesting that primary myocardial fibrosis can be an alternative phenotypic expression of structural disease–associated genetic variants or that risk-associated fibrosis was expressing before the primary disease.

What Are the Clinical Implications?

- Postmortem genetic studies in individuals with sudden cardiac death associated with primary myocardial fibrosis, for the purpose of assessing a possible heritable component and subsequent family screening, should include a panel inclusive of multiple known inherited structural diseases, regardless of the presence or absence of classic anatomic findings of arrhythmogenic right ventricular cardiomyopathy, hypertrophic cardiomyopathy, or dilated cardiomyopathy.

Sudden cardiac death (SCD) remains a major cause of death in Western societies despite the many strategies that have been explored in attempts to predict and prevent cardiac arrest.[1] Up to 50% of SCDs are first cardiac events occurring in the absence of previously identified cardiac disease. Although the cumulative incidence of SCD has not decreased, the proportion of SCDs resulting from coronary artery disease has decreased, and the proportion of nonischemic causes has increased.[2]

Secondary myocardial fibrosis is common among a diverse group of diseases that associate with SCD risk such as coronary heart disease, hypertensive left ventricular hypertrophy, healed myocarditis, dilated cardiomyopathies (DCMs) of various causes, myotonic dystrophy and related disorders, and inherited structural disorders. However, we have observed that the most common nonischemic cause of SCD among young subjects in Northern Finland was myocardial fibrosis in the absence of another associated cause (ie, primary myocardial fibrosis [PMF]).[3] A recent study from the United Kingdom also identified left ventricular fibrosis as a major cause of SCD among young athletes.[4] The cause of PMF in subjects with SCD, in the absence of disorders in which fibrosis is defined as secondary or is a component of a specific structural disease, is unknown. Causes may include unapparent or healed acquired diseases and genetically based disorders.

Major advances have been made in the genetic basis for rare cardiovascular syndromes, including both structural and ion channel causes. The strategy of postmortem genetic testing after SCD and a subsequent negative autopsy improves the identification of the cause of death,[5–7] benefiting diagnosis in both probands and identification of family members at potential risk. High-throughput next-generation sequencing (NGS) can generate data on many genes, and even on the whole exome, in a matter of a few days. With current methods, such studies can be done even with DNA from origins such as formalin-fixed paraffin-embedded samples, recognizing that the yield of high-quality DNA will be lower from these sources than from DNA-preserving blood samples or tissue storage or blood spot cards. Nevertheless, challenges remain as to whether observed variants are properly classified as disease-causing versus variants having undetermined or uncertain significance (VUS). The disease-causing label is more reliable when multiple unrelated subjects have disease associated with the same variant or there is cosegregation of the variant associated with disease in single families.

The aim of this study was to test the hypothesis that some SCDs with only PMF at autopsy may have unique genetic backgrounds that associate with myocardial fibrosis. Furthermore, our aim was to determine whether variants were identified in ion channel coding genes to assess whether some of the SCDs might actually be caused by inherited ion channelopathies independently of myocardial fibrosis.

METHODS

The data, analytical methods, and study materials will be made available to other researchers for the purposes of reproducing the results or replicating the procedure. Inquiries can be directed to the corresponding author.

Study Population (The Fingesture Study)

The study population was derived from the Fingesture study, which has stored both clinical and autopsy data from 4031 consecutive individuals with SCD between 1998 and 2012 in Northern Finland (Figure 1). In all cases, medicolegal autopsy was performed at the Department of Forensic Medicine, University of Oulu, Oulu, Finland, by experienced forensic pathologists, each performing >100 autopsies a year, using contemporary guidelines for the diagnosis of cause of death.[8] In this autopsy-based study, sudden death was defined as a witnessed death within 6 hours of the onset of symptoms or an unwitnessed death within 24 hours of the individual last being seen in a stable state of health. Only those whose sudden deaths were attributable to cardiac disease, on the basis of clinical and autopsy data, were included in Fingesture. The study complies with the Declaration of

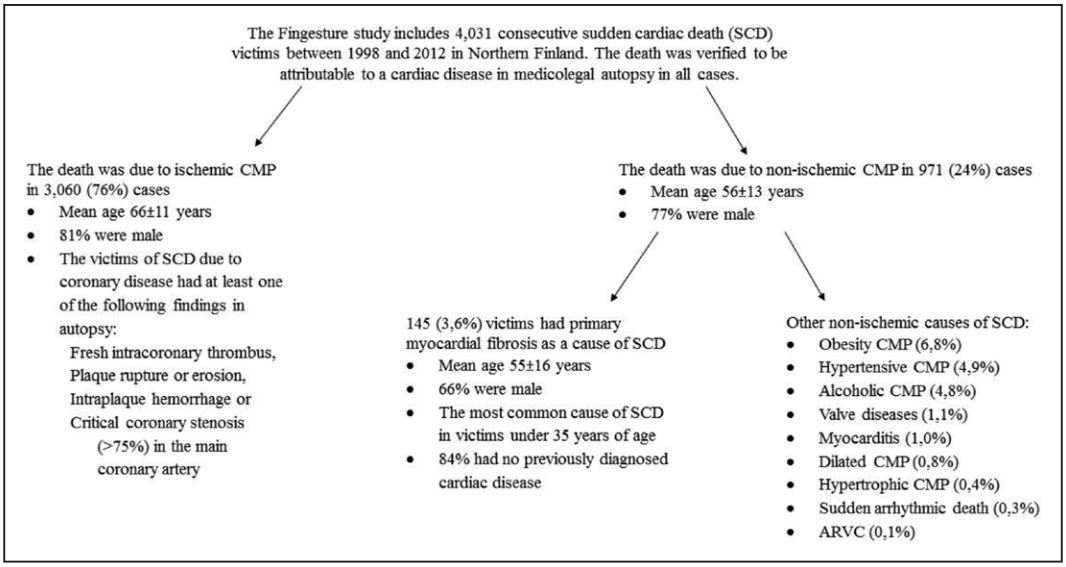

Figure 1. Description of autopsy findings in the Fingesture study.
ARVC indicates arrhythmogenic right ventricular cardiomyopathy; and CMP, cardiomyopathy.

Helsinki and was approved by the Ethics Committee of the University of Oulu. The National Authority for Medicolegal Affairs (Valvira) approved the review of postmortem data by the investigators.

In Finland, all sudden deaths are investigated by law if the death is not the result of known disease, the individual has not been treated by physician during his/her last illness, or the death is otherwise unexpected. Hence, Finland has the highest sudden death autopsy rate in Western societies,[9] with Fingesture including the vast majority of individuals with unexpected SCD. The hearts of those with SCD were meticulously examined, including heart weight and wall thickness measurements, extent of coronary artery disease, histological examinations, and identification and characterization of myocardial fibrosis. A toxicology investigation was performed if there was suspicion of exposure or autopsy findings were insufficient to define the cause of death. The determination of the cause of death was also contributed to by medical records and questionnaires sent to the next of kin. The criteria for each postmortem diagnosis were described by Hookana et al.[3]

Subjects With PMF as a Cause of Death

Among the 4031 individuals with SCD, death was attributed to coronary heart disease in 3060 (ischemic SCD, 76%). The remaining 971 (24%) were classified as nonischemic SCD and subcategorized into 11 specific diagnoses. The present study includes only those with nonischemic SCD with PMF (n=145) adjudicated as the cause of death. PMF was defined by the presence of interstitial, diffuse, or patchy fibrotic replacement of myocytes in the absence of healed myocardial infarction, chronic coronary artery disease, anatomy associated with inherited structural cardiac diseases (arrhythmogenic right ventricular cardiomyopathy [ARVC], hypertrophic cardiomyopathy [HCM], DCM), myocarditis, valve disease, or hypertensive hypertrophy of the left ventricular myocardium with or without secondary scarring. Classification as PMF was limited to those with heart weight <420 g and the absence of hypertrophied myocytes to limit overlap with hypertensive left ventricular hypertrophy. There were no other noncardiac organ changes or diseases that may also have myocardial fibrosis such as systemic sclerosis, Fabry disease, or myotonic dystrophy. Demographic and clinical characteristics of subjects with PMF are presented in Table 1. Typical histological findings are presented in Figure 2.

Table 1. Demographic and Clinical Features of Individuals With Sudden Cardiac Death With Primary Myocardial Fibrosis (N=145)

Characteristic	Value
Sex, n (%)	
Women	50 (34.5)
Men	95 (65.5)
Age, y	55±16
Women	60±17
Men	52±15
Body mass index, kg/m^2	23.4±4.0
Location at the time of sudden cardiac death,* n (%)	
Home	122 (91.7)
Public location	11 (8.3)
Before cardiac arrest,† n (%)	
Cardiac disease	22 (15.9)
Diabetes mellitus	8 (5.5)
Heart failure	0 (0)
Hypertonia	18 (13.0)
Morbus cordis coronarius	0 (0)
Dyslipidemia	5 (3.6)
Dyspnea	0 (0)

Values are expressed as mean±SD or number of subjects (percent).
*Data were missing for 12 subjects.
†Data were missing for 7 subjects.

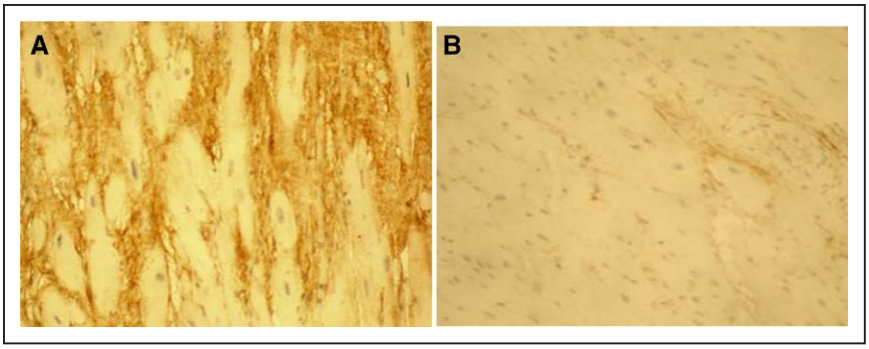

Figure 2. Typical histological findings of primary myocardial fibrosis in sudden cardiac death. Immunohistochemical staining with procollagen I antibodies in myocardial tissue of a subject with primary myocardial fibrosis sudden cardiac death (**A**) and an age-matched control (**B**). Areas stained brown represent fibrosis.

Tissue Samples and Gene Sequencing

We carried out genetic studies in the 96 of 145 individuals (66%) with SCD with PMF whose DNA passed the quality control for further analysis. DNA was isolated from formalin-fixed paraffin-embedded tissue samples obtained during autopsy. The TruSight Cardio gene panel kit, composed of 174 genes with associations with inherited cardiac conditions most affected by a genetic predisposition (http://support.illumina.com/downloads/trusight-cardio-product-files.html), was used for library preparation (Illumina, San Diego, CA; Table 2). Samples were bead purified with Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN). The quality of the samples selected for NGS was confirmed with quantitative polymerase chain reaction–based formalin-fixed paraffin-embedded quality control kit (Illumina), and the samples passing quality control, that is, with a quantitative polymerase chain reaction ΔCq value ≤2.3, were selected for gene panel sequencing with NextSeq550 platform (Illumina). Within the BaseSpace Genomics computing environment (Illumina), BWA Enrichment (BWA Genome Aligner Software and the GATK Variant Caller) was used for sequence alignment and variant calling; VariantStudio for annotation, filtering, and classification of the variants; and Integrative Genomics Viewer[10] for data visualization to exclude falsely annotated variants and sequencing artifacts. All variants classified as pathogenic, likely pathogenic, or VUS and with read depth <100 were confirmed by Sanger Sequencing (ABI3130xl, Applied Biosystems, Foster City, CA). The in silico prediction tools PolyPhen[11] and SIFT[12] were used to predict the effect of amino acid alterations on protein function within BaseSpace.

Genetic Analysis

The panel of 174 genes were associated with known structural and molecular cardiac conditions, with and without prior associations with secondary fibrosis. Genes associated with noncardiac diseases that may be accompanied by myocardial fibrosis such as Fabry disease and myotonic dystrophy were not tested for, but there were no histories or documentation of such disorders, and there are no candidate genes known to be associated with true PMF. The study group was derived

Table 2. Cardiac Structure- and Function-Related Genes Sequenced in the Panel

ABCC9	*CACNB2*	*DOLK*	*GJA5*	*KCNJ5*	*MYH11*	*PRDM16*	*SGCG*	*TNNC1*
ABCG5	*CALM1*	*DPP6*	*GLA*	*KCNJ8*	*MYH6*	*PRKAG2*	*SHOC2*	*TNNI3*
ABCG8	*CALR3*	*DSC2*	*GPD1L*	*KCNQ1*	*MYH7*	*PRKAR1A*	*SLC25A4*	*TNNT2*
ACTA1	*CASQ2*	*DSG2*	*GPIHBP1*	*KLF10*	*MYL2*	*PTPN11*	*SLC2A10*	*TPM1*
ACTA2	*CAV3*	*DSP*	*HADHA*	*KRAS*	*MYL3*	*RAF1*	*SMAD3*	*TRDN*
ACTC1	*CBL*	*DTNA*	*HCN4*	*LAMA2*	*MYLK*	*RANGRF*	*SMAD4*	*TRIM63*
ACTN2	*CBS*	*EFEMP2*	*HFE*	*LAMA4*	*MYLK2*	*RBM20*	*SNTA1*	*TRPM4*
AKAP9	*CETP*	*ELN*	*HRAS*	*LAMP2*	*MYO6*	*RYR1*	*SOS1*	*TTN*
ALMS1	*COL3A1*	*EMD*	*HSPB8*	*LDB3*	*MYOZ2*	*RYR2*	*SREBF2*	*TTR*
ANK2	*COL5A1*	*EYA4*	*ILK*	*LDLR*	*MYPN*	*SALL4*	*TAZ*	*TXNRD2*
ANKRD1	*COL5A2*	*FBN1*	*JAG1*	*LDLRAP1*	*NEXN*	*SCN1B*	*TBX20*	*VCL*
APOA4	*COX15*	*FBN2*	*JPH2*	*LMF1*	*NKX2-5*	*SCN2B*	*TBX3*	*ZBTB17*
APOA5	*CREB3L3*	*FHL1*	*JUP*	*LMNA*	*NODAL*	*SCN3B*	*TBX5*	*ZHX3*
APOB	*CRELD1*	*FHL2*	*KCNA5*	*LPL*	*NOTCH1*	*SCN4B*	*TCAP*	*ZIC3*
APOC2	*CRYAB*	*FKRP*	*KCND3*	*LTBP2*	*NPPA*	*SCN5A*	*TGFB2*	
APOE	*CSRP3*	*FKTN*	*KCNE1*	*MAP2K1*	*NRAS*	*SCO2*	*TGFB3*	
BAG3	*CTF1*	*FXN*	*KCNE2*	*MAP2K2*	*PCSK9*	*SDHA*	*TGFBR1*	
BRAF	*DES*	*GAA*	*KCNE3*	*MIB1*	*PDLIM3*	*SEPN1*	*TGFBR2*	
CACNA1C	*DMD*	*GATAD1*	*KCNH2*	*MURC*	*PKP2*	*SGCB*	*TMEM43*	
CACNA2D1	*DNAJC19*	*GCKR*	*KCNJ2*	*MYBPC3*	*PLN*	*SGCD*	*TMPO*	

from the total of 96 qualifying cases and resulted in mean read depth of ×960 per sample. On average, 99.4% of the captured region (0.572 Mb) was covered at least by 20 reads and 98.8% at least by 50 reads for the analyzed samples. All variants with a potential effect on protein were selected for analysis (missense, frameshift, stop gained/lost, initiator codon, in-frame insertion, in-frame deletion, and splice-site alterations) and filtered further according to their prevalence in dbSNP or Exome Aggregation Consortium database by excluding variants with minor allele frequency >0.01 among Finnish subjects. Further assessments for pathogenicity were based on American College of Medical Genetics consensus guidelines.[13]

CTF1 p.Ala92Thr Genotyping

Because of poor coverage of the genomic region around *CTF1*{p.Ala92Thr} (Chr16: 30913528, Human GRCh37/hg19) in the Exome Aggregation Consortium database and the lack of population frequency information in other public databases (Ensembl, dbSNP), *CTF1*{p.Ala92Thr} was genotyped in 448 geographically matched control subjects with Sanger Sequencing (ABI3130xl, Applied Biosystems). Genomic region was amplified with TaKaRa LA Taq with the GC Buffer kit (Takara Bio USA, Inc, Mountain View, CA) using the following primers: forward, 5′-GGGCTGCCAGTGCACGAG-3′; and reverse, 5′-GGCCAGCAAGGCCTCCACG-3′.[14]

Statistical Analysis

On the basis of the minor allele frequency of the identified variants in the GnomAD database (http://gnomad.broadinstitute.org/) in the Finnish population, the number of expected carriers in the analyzed patient cohort (n=96) was calculated. The number of expected carriers is based on the Hardy-Weinberg equation, where 2pq represents the frequency of the heterozygote genotype (p=major allele frequency, q=minor allele frequency, and p+q=1).

RESULTS

Potentially relevant variants were detected in 26 of the subjects (27%) with analyzable DNA with PMF, including 24 unique variants in 16 genes. Ten variants in 10 subjects (10%) were classified as either pathogenic or likely pathogenic according to American College of Medical Genetics guidelines. The remaining 14 variants were classified as VUS. Four unique VUS coexpressed in multiple unrelated subjects with SCD were associated with PMF (3 unique variants present in 2 subjects each and 1 unique variant in 3 subjects; see Table 3, which includes all nonsynonymous mutations detected with a minor allele frequency <0.01 in the dbSNP and ExAc exome databases).

The PMF-associated variants were detected predominantly in myocardial structure–coding genes. No variants were identified in ion channel–coding genes. Two VUS were detected in *RYR2*, which has previously been associated with both primary arrhythmia syndromes and cardiomyopathies. The disease-associated variants were identified in genes associated with ARVC (*DSP, PKP2, DSG2*), HCM (*TPM1, MYH7, MYBPC3*), and DCM (*TTN, CRYAB, RBM20, LMNA, ABCC9, RYR2, LAMA4, CASQ2*[15,16]). All pathogenic/likely pathogenic variants detected either were directly associated with the inherited structural abnormality or were null variants in regions of genes that are commonly mutated in patients with these inherited disorders.

ARVC-Associated Variants

Two of 5 variants in desmosomal genes (*DSP, PKP2, DSG2*) have been described in patients with ARVC, and the other 3 were novel. Of the 2 variants previously identified in patients with ARVC, the Arg808Cys variant causes local conformational alterations to desmoplakin.[17] A missense substitution Ala372Pro in the *PKP2* gene results in a change in conserved residue.[18] These variants were considered as probably/likely disease causing.

HCM-Associated Variants

We observed 4 pathogenic/likely pathogenic variants in HCM-related genes. Of these variants, Met982Thr in a highly conserved *MYH7* region was previously described in subjects with HCM,[19,20] and an Ala833Thr variant in *MYBPC3* was observed in familial HCM.[21] We also observed a pathogenic frameshift variant in *MYBPC3* (Tyr1100Val) that was previously reported in patients with HCM (ClinVar database) and another pathogenic variant in the *TPM1* gene (Asp175Asn)[22] that is relatively common in Finnish patients with HCM.[23]

DCM-Associated Variants

Four pathogenic/likely pathogenic variants identified in our study (3 in TTN, 1 in *CRYAB*) have been associated with DCM, which is an expected result considering the frequency of fibrosis in DCM.[15,16] In addition, we detected 7 VUS in genes previously associated with DCM (*RBM20, LMNA, ABCC9, RYR2, LAMA4, CASQ2*). One particular variant has previously been described in familial DCM.[24] Two novel truncating variants in *TTN* in our subjects are considered likely disease causing because those truncating variants appeared in the A band in which mutations leading to truncating Titin variants exist predominantly among patients with DCM.[25–27] The third alteration in the *TTN* gene also was a novel frameshift mutation in the A band that is predicted to lead to a truncated or absent protein; we classified it as likely pathogenic in the absence of sufficient data on its pathogenic capabilities.

CTF1{p.Ala92Thr} was observed in 3 cases (3.1%). It had also previously been described in 1 patient (1 of

208) with DCM and was absent in healthy controls (0 of 204).[14] *CTF1* {p.Ala92Thr} had no population frequency in public databases (Ensembl, dbSNP, and Exome Aggregation Consortium database). However, according to the Exome Aggregation Consortium database, the genomic region surrounding it is poorly covered; thus, its absence in databases might not reflect the true population incidence. To resolve this, we genotyped *CTF1*{p.Ala92Thr} in 448 geographically matched control subjects with direct sequencing and found a relatively high carrier frequency in these controls (in 5 of 448). All of the control carriers had normal echocardiograms, suggesting that it is unlikely that this mutation is disease causing. Thus, we did not consider this variant disease related and excluded it from Table 3.

DISCUSSION

Our postmortem registry of subjects with SCD in Northern Finland has demonstrated that PMF is a common finding in individuals <40 years old.[3] This observation aligns with another study that explored the causes of SCD in young athletes in United Kingdom[4] but does not parallel other surveys in which HCM and ARVC have been the most common autopsy findings among young individuals with SCD.[28–30] Although the pathologies reported in autopsy-based studies have geographical variability between each study population, the inherited structural diseases have overlapping features in that they all commonly express myocardial fibrosis as a component of the anatomic pathology.

Table 3. Summary of Myocardial Structure Gene Variants in Sudden Cardiac Death Subjects With Primary Myocardial Fibrosis

Mutated Gene	Nucleotide Change	Effect on Protein	Predicted Effect	n	NGS Coverage, n	ExAC >3000 Finnish Controls MAF	GnomAD >10 000 Finnish Control Subjects MAF	Expected Carriers in 96 Subjects	ACMG Score
Pathogenic variants									
TPM1	523G>A	Asp175Asn	Missense	1	412	Not detected	0.0001554	0.03	PS1+PS4
MYBPC3	3297dupG	Tyr1100Valfs	Frameshift	1	593	Not detected	0.0001534	0.03	PVS1+PM2+PP5
TTN	88421G>A	Trp29474Stop	Truncating	1	234	Not detected	0.0003142	0.06	PVS1+PM1+PM2
TTN	87394C>T	Arg29132Stop	Truncating	1	50*	Not detected	Not detected	Not detected	PVS1+PM1+PM2
Likely pathogenic variants									
TTN	77971delTG	Thr25991Serfs	Frameshift	1	75*	Not detected	Not detected	Not detected	PM1+PM2+PM4
DSP	2422C>T	Arg808Cys	Missense	1	115	0.0001512	0.00003877	0.007	PS3+PM2+PP5
PKP2	1114G>C	Ala372Pro	Missense	1	73*	0.001663	0.002335	0.45	PM6+PP2+PP3+PP4
MYBPC3	2497G>A	Ala833Thr	Missense	1	262	0.002268	0.002289	0.44	PS1+PP1+PP2
MYH7	2945T>C	Met982Thr	Missense	1	440	0.0009072	0.0005816	0.11	PS1+PM1+PP4 (HCM)
CRYAB	460G>A	Gly154Ser	Missense	1	42*	0.000756	0.001202	0.23	PS1+PM1+PP3+PP5
VUS									
DSP	6295-6296CC>AT	Pro2099Ile	Missense	2	252/114	0.00348	0.003684	0.7	
DSP	6307A>G	Lys2103Glu	Missense	1	76*	Not detected	0.0001551	0.03	
DSG2	2906C>T	Ala969Val	Missense	1	47*	Not detected	Not detected	Not detected	
MYH7	3116A>G	Glu1039Gly	Missense	2	98*/46*	0.001059	0.0007366	0.14	
MYH7	4510A>T	Asn1504Tyr	Missense	1	70*	Not detected	Not detected	Not detected	
RBM20	1958C>T	Thr653Ile	Missense	2	343/318	Not detected	0.0007982	0.15	
LMNA	77T>A	Ile26Asn	Missense	1	179	Not detected	Not detected	Not detected	
ABCC9	1320+1G>A		Affects canonical splicing	1	385	0.0004555	0.0006660	0.13	
RYR2	7495G>A	Ala2499Thr	Missense	1	104	0.001176	0.0007064	0.14	
RYR2	7552C>T	Arg2518Trp	Missense	1	128	Not detected	0.0004037	0.08	

(*Continued*)

Table 3. Continued

Mutated Gene	Nucleotide Change	Effect on Protein	Predicted Effect	n	NGS Coverage, n	ExAC >3000 Finnish Controls MAF	GnomAD >10 000 Finnish Control Subjects MAF	Expected Carriers in 96 Subjects	ACMG Score
LAMA4	3110G>A	Arg1037Gln	Missense	1	43*	0.001512	0.002249	0.43	
CASQ2	874G>T	Ala292Ser	Missense	1	174	0.001969	0.002520	0.48	
MYLK2	1444T>G	Phe482Val	Missense	1	163	0.0004535	0.0003588	0.07	
DTNA	92G>A	Arg31Gln	Missense	3	231/153/175	0.005464	0.005855	1.1	

Expected carriers in 96 subjects according to Hardy-Weinberg equation calculated with GnomAD MAF.

ACMG criteria: very strong evidence of pathogenicity: PVS1=null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single exon or multiexon deletion) in a gene in which loss of function is a known mechanism of disease; strong evidence of pathogenicity: PS1=same amino acid change as a previously established pathogenic variant regardless of nucleotide change; PS2=de novo mutation in a patient with the disease and no family history; PS3=well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product; PS4=the prevalence of the variant in affected individuals is significantly increased compared with the prevalence in control subjects; moderate evidence of pathogenicity: PM1=located in a mutational hot spot and/or critical and well established functional domain (eg, active site of an enzyme) without benign variation; PM2=absent from control subjects (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes, or ExAC; PM3=for recessive disorders, detected in trans with a pathogenic variant; PM4=protein length changes resulting from in-frame deletions/insertions in a nonrepeat region or stop-loss variants; PM5=novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before; PM6=assumed de novo but without confirmation of paternity and maternity; supporting evidence of pathogenicity: PP1=cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease; PP2=missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease; PP3=multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc); PP4=patient's phenotype or family history is highly specific for a disease with a single genetic pathogenesis; PP5=reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation.

The following are the rules for combining criteria to classify sequence variants:

Pathogenic:

1. 1 Very strong (PVS1) AND
   a. ≥1 Strong (PS1–PS4) OR
   b. ≥2 Moderate (PM1–PM6) OR
   c. 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) OR
   d. ≥2 Supporting (PP1–PP5)
2. ≥2 Strong (PS1–PS4) OR
3. 1 Strong (PS1–PS4) AND
   a. ≥3 Moderate (PM1–PM6) OR
   b. 2 Moderate (PM1–PM6) AND ≥2 supporting (PP1–PP5) OR
   c. 1 Moderate (PM1–PM6) AND ≥4 supporting (PP1–PP5)

Likely pathogenic:

1. 1 Very strong (PVS1) AND 1 moderate (PM1–PM6) OR
2. 1 Strong (PS1–PS4) AND 1–2 moderate (PM1–PM6) OR
3. 1 Strong (PS1–PS4) AND ≥2 supporting (PP1–PP5) OR
4. ≥3 Moderate (PM1–PM6) OR
5. 2 Moderate (PM1–PM6) AND ≥2 supporting (PP1–PP5) OR
6. 1 Moderate (PM1–PM6) AND ≥4 supporting (PP1–PP5)

ACMG indicates American College of Medical Genetics; MAF, minor allele frequency; NGS, next-generation sequencing; PMF, primary myocardial fibrosis; SCD, sudden cardiac death; and VUS, variants of uncertain significance.

*Confirmed with Sanger sequencing.

Our motivation for this study was based on the hypothesis that at least some of the SCDs characterized as PMF at autopsy might be associated with genetic variants associated with a unique syndrome characterized by myocardial fibrosis, and our data demonstrated PMF-associated variants in the same genes that associate with specific inherited structural disorders in a substantial subgroup of the subjects. Because there are no known genes that associate with isolated PMF, the data suggest that PMF may represent, in large part, a unique expression among a spectrum of phenotypes of ARVC, HCM, and DCM rather than uniform expression of genotype-phenotype associations,[31] in effect an alternative phenotypic expression pathway in specific inherited structural diseases rather than genetic variants unique to PMF. It is reasonable to consider the possibility that the structural disease variants identified might be nonexpressed genotypes of the structural disorders and that PMF is independent of these genotypes. However, the fact that 10 of the genotypes identified, spanning multiple examples of all 3 disorders, were disease causing or likely disease causing and that 4 of the unique VUS cosegregated among >1 individual with PMF mitigates strongly against that possibility. The presence of PMF in the absence of even subtle phenotypic features associated with ARVC, HCM, or DCM strongly supports variable expression of these genotypes as the most likely conclusion. Because late expression of the classic phenotypes of these disorders may occur,[32,33] it is also possible that PMF may represent an earlier anatomic expression in some of these cases.

In the case of ARVC, although the right ventricle is predominantly involved in the majority of cases, the left ventricle may be substantially involved with fibrofatty replacement even with mild changes in the right

ventricle.[34] This suggests that some ARVC genotypes may be expressed as PMF. Fibrosis is also a hallmark of HCM, and a recent observation suggests that myocardial fibrosis may precede the characteristic hypertrophy of the left ventricular septum or apex,[33] thus making the separation between early-stage HCM and PMF challenging. In addition, excessive myocardial fibrosis is a prominent feature in DCM and an important predictor of arrhythmic events in such patients.[35] These shared structural features of genetically distinct entities prompted us to clarify whether the overlapping property in these diseases extends beyond phenotype to the genetic background.

The nature of our data source does not permit an estimate of the proportion of ARVC, HCM, and DCM genotypes that will express as isolated PMF phenotypes. Typical phenotypes of these disorders are more likely to be identified and treated in advance of SCD, as well as being classified as preexisting disease. Finnish law limits mandated medicolegal autopsy to cases of unexpected death, and hence, classic ARVC, HCM, and DCM phenotypes are likely underrepresented. In contrast, selection bias in Fingesture is negligible for conditions unlikely to be recognized before SCD such as PMF.

Overall, we observed 24 variants in 16 genes among 27% of the individuals with SCD associated with PMF with analyzable DNA. This is in the general range in which postmortem genetic studies have identified variants in young subjects.[5–7] The actual number might be higher because of the limited number of variants identified as associated with the inherited structural disorders to date. For the specific structural disorders, relevant genetic variants have been identified in ≈50% of those with ARVC,[36–39] <50% of those with HCM,[40–42] and 30% to 50% of individuals with DCM,[16] supporting the hypothesis that the fibrosis in PMF is, at least in some cases, the primary expression associated with a defect in myocyte structural proteins, not a secondary response to a specific acquired or inherited condition characterized by myocyte loss and secondary replacement. In ARVC, HCM, and DCM, the genetic background in Finland has been shown to be rather homogeneous. In previous studies, variants in a few ARVC- and HCM-related genes account for most of the genetic background for clinical disease, and the apparent founder mutation of ARVC (*PKP2* {Q59L}) has a relatively high prevalence in the general population (0.3%), introducing questions about relevance or variable penetrance.[23,43,44] In DCM, the genetic background is more heterogeneous in Finland, but the *LMNA* variant p.Ser143Pro seems to be overrepresented among subjects with DCM with worse outcomes.[45] Only 1 founder mutation in the HCM gene (*TPM1{p.*Asp175Asn}) was detected in this study, and notably, none of the ARVC-related mutations observed previously in Finland were seen in this study.

In addition, speculation has been raised previously about whether PMF could be an innocent bystander and some of these young subjects with SCD could have an ion channelopathy, as observed in several other sudden unexplained death populations, or even whether ion channel mutations might cause myocardial fibrosis.[32] We did not detect any pathological mutations in ion channel–encoding genes in our PMF population. Although 2 VUS were found in *RYR2*, the gene has also been associated with cardiomyopathies, in addition to catecholaminergic polymorphic ventricular tachycardia. Mutations in the N-terminal part have been shown to associate with experimental cardiomyopathies.[46]

PMF, HCM, DCM, and ARVC are traditionally classified as distinct phenotypes. However, increased knowledge about the disease pathologies, in addition to traditional classifications, has provided information on their overlapping features, thus making it challenging to recognize precisely the end of 1 entity and the beginning of another. Therefore, these entities likely represent different expressions of a disease spectrum in which genetic predisposition to excessive myocardial fibrosis is an important component of disease development and adverse prognosis. This concept is supported by the present study, which demonstrates that myocardial fibrosis, in the absence of other structural abnormalities, may be a variable phenotypic expression of a wide spectrum of specific structural diseases that are considered inherited. Whether the PMF variation in expression of the classic disease phenotypes (ie, ARVC, HCM, DCM) results from interactions with modifier genes or epigenetic influences cannot be determined from this study. However, the concept of genetic modification of expression is a hypothesis applicable to these observations, justifying further study.[31]

Accumulation of fibrotic tissue in myocardium at autopsy after SCD, without any other detectable cardiac abnormalities, in conjunction with emerging clinical imaging observations, suggests that myocardial fibrosis can be an important structural pathway for SCD risk. In addition to the observation that PMF shares genotype patterns with ARVC, DCM, and HCM, it seems to be a much more diverse pathophysiology than merely left-dominant ARVC or early-stage HCM/DCM. This conclusion derives from the observation that 73% of the subjects did not have variants in 174 myocardial genes in our panel, and we cannot exclude the possibility that some of these subjects have unknown variants associated with isolated myocardial fibrosis. An exome-wide association study would be required to identify candidate genes, either coexisting with the identified variants or unique to the remaining cases of PMF. However, this study population is not large enough for an association study, but our data invite a larger study of this type in the future. In addition, some of the cases of PMF are very likely the consequence of acquired con-

ditions such as previous myocarditis/toxic exposure or harmful drugs.

Only 16% of the individuals with SCD resulting from PMF had previously diagnosed cardiac disease, and SCD was the first manifestation in the remaining 84%. Although SCDs are common in Western societies,[47] medicolegal investigations have not been used uniformly in many communities. Recent recommendations emphasize the proper use of postmortem investigations in clinically unexplained SCDs.[48–50] Accurate postmortem studies in individuals with SCD are crucial for enhancing our knowledge about the causes of SCD and developing diagnostic tools to find subjects at risk.[51]

Recent studies have demonstrated that postmortem genetic analysis improves both the identification of the cause of death in young people, especially when autopsy findings are structurally negative or inconclusive and in the absence of a premortem diagnoses,[5,6] and the usefulness for screening families of individuals with SCD for carrier states.[7] In those with SCD, molecular autopsy is recommended in cases with suspected inherited disease or in those without any morphological abnormalities at autopsy.[48,49] The results of the present study suggest that molecular autopsy could also be important in PMF. Our ongoing study will explore inheritance patterns in the first-degree relatives and the potential for early diagnosis of PMF in mutation carriers with the use of cardiac ultrasound or magnetic resonance imaging.

Limitations

The major limitation of most NGS studies is the lack of evidence of the causative role between the observed gene variants and the disease. In addition, most of the variants we observed (17 of 24) were novel, of which 14 were considered VUS, although 4 unique VUS were observed in multiple subjects with a PMF phonotype. To verify the heritable component of PMF, the pathophysiological potential of affected proteins and mutations must be authenticated by meticulous functional studies and the cosegregation of mutation, and the disease must be confirmed in affected families. The diagnosis of PMF at autopsy may also depend on the expertise of the cardiovascular pathologist.[52] In the present study, only a few experienced forensic pathologists performed the autopsies from the geographical area covering almost one half of Finland, and uniform criteria were used for diagnosis. Nonetheless, the distinction between fatty-fibrotic replacement suggesting ARVC from PMF is sometimes difficult (Marja-Leena Kortelainen, MD, personal communication, 2017) because of a lack of specific differential diagnostic criteria in this field. In addition, there is always the possibility of ischemia caused by small vessel spasm or small vessel bridging, which, although very unlikely, could cause the fibrosis.

Conclusions

Disease-causing genetic variants associated with ARVC, HCM, and DCM were observed in the absence of the classic anatomic findings of these diseases with sufficient frequency among individuals with SCD who have no cardiac abnormalities other than PMF to generate a reasonable association argument. Because myocardial fibrosis is a common manifestation of each of these disorders, this observation supports the concept of variable phenotypic expression of specific genetic disorders.[33] PMF therefore may represent a phenotypically specific variant manifestation of ARVC, HCM, and DCM in which the expression of myocardial fibrosis alone may be responsible for prognosis. The absence of pathological mutations in ion channel genes in this population further supports the hypothesis that myocardial fibrosis is a variant phenotype expression specific for structural genetic disorders. The data also suggest that postmortem genetic studies in individuals with SCD associated with PMF, for the purpose of assessing a possible heritable component and subsequent family screening, should include a panel inclusive of multiple known inherited structural diseases, regardless of the presence or absence of classic anatomic findings of ARVC, HCM, or DCM.

ARTICLE INFORMATION

Received October 13, 2017; accepted February 15, 2018.

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Sources of Funding

This study was funded by Aatos and Jane Erkko Foundation, Helsinki, Finland; Academy of Finland; Finnish Foundation for Cardiovascular Research, Helsinki, Finland; Sigrid Juselius Foundation, Helsinki, Finland; Yrjö Jahnsson Foundation, Helsinki, Finland; American Heart Association Chair in Cardiovascular Research; Miami Heart Research Institute; and Leducq Foundation, Paris, France.

Disclosures

None.

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Circulation. 2018;137:2716-2726
doi: 10.1161/CIRCULATIONAHA.117.032175
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539