Original Article

Technical Advances for the Clinical Genomic Evaluation of Sudden Cardiac Death

Verification of Next-Generation Sequencing Panels for Hereditary Cardiovascular Conditions Using Formalin-Fixed Paraffin-Embedded Tissues and Dried Blood Spots

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Background—Postmortem genetic testing for heritable cardiovascular (CV) disorders is often lacking because ideal specimens (ie, whole blood) are not retained routinely at autopsy. Formalin-fixed paraffin-embedded tissue (FFPET) is ubiquitously collected at autopsy, but DNA quality hampers its use with traditional sequencing methods. Targeted next-generation sequencing may offer the ability to circumvent such limitations, but a method has not been previously described. The primary aim of this study was to develop and evaluate the use of FFPET for heritable CV disorders via next-generation sequencing.

Methods and Results—Nineteen FFPET (heart) and blood (whole blood or dried blood spot) specimens underwent targeted next-generation sequencing using a custom panel of 101 CV-associated genes. Nucleic acid yield and quality metrics were evaluated in relation to FFPET specimen age (6 months to 15 years; n=14) and specimen type (FFPET versus whole blood and dried blood spot; n=12). Four FFPET cases with a clinical phenotype of heritable CV disorder were analyzed. Accuracy and precision were 100% concordant between all sample types, with read depths >100× for most regions tested. Lower read depth, as low as 40×, was occasionally observed with FFPET and dried blood spot. High-quality DNA was obtained from FFPET samples as old as 15 years. Genomic analysis of FFPET from the 4 phenotype-positive/genotype unknown cases all revealed putative disease-causing variants.

Conclusions—Similar performance characteristics were observed for next-generation sequencing of FFPET, whole blood, and dried blood spot in the evaluation of inherited CV disorders. Although blood is preferable for genetic analyses, this study offers an alternative when only FFPET is available. **(*Circ Cardiovasc Genet*. 2017;10:e001844. DOI: 10.1161/CIRCGENETICS.117.001844.)**

Key Words: autopsy ■ cardiomyopathies ■ genetic testing ■ genotype ■ paraffin

Heritable cardiovascular disorders (HCVDs) are a broad group of conditions that include cardiomyopathies, cardiac channelopathies, and connective tissue disorders. These conditions come to attention in myriad ways, the most tragic of which is sudden unexpected death. Sudden cardiac deaths (SCDs) are broadly defined as nonviolent, nonaccidental, unexpected deaths that are attributed to CV causes occurring within 24 hours from the onset of symptoms. They may or may not be witnessed. There are ≈180 000 to 450 000 cases of SCD each year in the United States, with striking male (3:1) and black (nearly 2:1, compared with whites) predilections.[1]

In the United States, sudden and unexpected deaths are investigated by medical examiners and coroners (ME/C), who are statutorily responsible for determining the cause and manner of death. Components of a competent death investigation of a sudden and unexpected natural death typically include scene investigation, autopsy, toxicology testing, histological examination of autopsy tissue, and consultation with subspecialists such as cardiac pathologists and neuropathologists, as appropriate.[2] When no cause of death is identified after all appropriate studies, postmortem genetic testing for HCVDs may reveal a pathogenic variant in ≈30% of cases, though the yield rate varies widely from <5% to 45% depending on how the subjects are selected and which testing methodology is used.[3–5]

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Unfortunately, however, postmortem genetic testing is not performed routinely in cases of sudden unexpected death

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without an identifiable cause after a complete medicolegal death investigation. Perhaps the most significant obstacle is the prohibitive cost of genetic testing for most ME/C office budgets. An additional obstacle is that appropriate sample for postmortem genetic testing may not have been retained at the time of autopsy, or if retained at autopsy, the samples may have been discarded before recognition of the need for testing. The National Association of Medical Examiners Position Paper on Retaining Postmortem Samples for Genetic Testing recommends that, at a minimum, 5 to 10 mL of whole blood (WB) be retained in EDTA, in deaths involving sudden unexplained deaths in those younger than 40 years, as well as other deaths where HCVDs may be present.[6] To maintain DNA integrity, such samples would need to be refrigerated (<4 weeks) or frozen at −20°C to −70°C (<4 weeks). Though most ME/C offices should be able to accommodate these storage requirements, underfunded ME/C offices, overwhelmed by criminal and toxicological deaths, may find the requirement for long-term frozen storage overly burdensome. In addition, it is not infrequent that the request for postmortem genetic testing arises several months or years after the completion of an autopsy, at which time the retained WB from autopsy is likely to have been discarded because of limited storage capabilities.[7]

In contrast, alternative autopsy samples, such as dried blood spot (DBS) cards, Formalin-fixed paraffin-embedded tissue (FFPET), or formalin-fixed stock tissue, can be stored indefinitely at room temperature, although genetic testing on these alternative specimens is more technically challenging. Specific recommendations for the retention of such specimens for potential genetic testing are not addressed in the National Association of Medical Examiners position paper. Of note, however, FFPET and formalin-fixed stock tissue are retained routinely from autopsy for legal purposes, as recommended by the College of American Pathologists which stipulates retention of tissue blocks from nonforensic autopsies for a minimum of 10 years, and indefinitely for tissue from forensic autopsies.[8] Although many ME/C offices are not accredited by College of American Pathologists, most ME/C offices will retain FFPET and formalin-fixed stock tissue at room temperature for a significantly longer period of time than autopsy samples that require refrigeration or freezing—making the former ideally suited, from a practical standpoint, for archival molecular interrogation.

Formalin fixation tends to result in shearing of nucleic acids to produce fragments of an average length of ≈150 base pairs,[9] precluding development of efficient Sanger assays that rely on reads of substantially longer fragments (>250 base pairs) and high-quality DNA.[10] Next-generation sequencing (NGS) technology, on the other hand, is a complex massively parallel methodology that allows each base to be read hundreds of times in each direction. The read length is much shorter (on the order of 50–250 base pairs), making FFPET a viable source for nucleic acid interrogation. Herein, we describe a robust clinical-grade methodology, leveraging NGS technology, to interrogate FFPET for pathogenic variants underlying HCVDs. In addition, multiple clinical vignettes are provided from a validation cohort that demonstrates the practical use of this method of testing.

Methods

The data, analytic methods, and study materials beyond that described herein will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. This study was approved by an institutional review committee. Materials from living patients were appropriately anonymized.

DNA Extraction

DNA was extracted from FFPET samples using the QIAamp DSP DNA FFPE Tissue Kit with some modifications to the manufacturer's protocol. The initial step was to dissolve the paraffin from 10-µm section curls of FFPET in xylene, followed by removal of the xylene. The sample was then lysed under denaturing conditions with proteinase K and incubated at 90°C to reverse the formalin crosslinking. The sample was then placed into a QIAamp MinElute column and centrifuged, with the DNA binding to the membrane and the contaminants flowing through. A wash step was then performed to wash away residual contaminants, and an elution step took place to elute the DNA from the membrane.

For DBS DNA extraction, a 3-mm hole puncher was used to punch up to five 3 mm punches from the DBS card. The Qiagen AutoPure LS method was used to extract the DNA from 3 mL of EDTA-anticoagulated WB as per manufacturer's recommendations. After extraction, DNA was checked for purity and concentration by the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc). DNA purity was determined to be acceptable at an A260:A280 ratio of 1.6:2.0 and at a minimum concentration of 2 ng/uL.

NGS Library Preparation and Sequencing

Three different capture panels were designed that included genes involved in multiple different HCVDs (**Table 1**). The SureSelect Custom Target Enrichment System (Agilent Technologies, Inc) was used for library preparation and involved mechanical shearing (ultrasonication) of 200 ng of genomic DNA with the LE220 (Covaris, Inc, Woburn, MA). Library preparation was automated on the Biomek FXp Laboratory Workstation (Beckman Coulter, Inc, Brea, CA) to include enzymatic mediated end repair, adenine addition (a-tailing), adapter oligonucleotide ligation, and enrichment of adapter-ligated fragments via a 6 cycle polymerase chain reaction. After each of these process steps, the libraries were purified with the AMPure XP Purification system (Agencourt, Beckman Coulter, Inc). Custom biotinylated RNA oligonucleotides complimentary to the target sequences were used for in-solution hybridization. After hybridization, Dynabead MyOne Streptavidin T1 beads (Life Technologies, Inc, Carlsbad, CA) were used to isolate (capture) the target regions and a 14 cycle polymerase chain reaction amplification added sample-specific index sequences. A final quantification of libraries was performed to ensure sufficient product (≥2.0 nmol/L) for sample normalization and pooling. Library quantity and quality were evaluated on the Qubit 2.0 Fluorometer (Life Technologies, Inc) and the 2100 Bioanalyzer (Agilent Technologies, Inc). After library preparation, samples were normalized to a concentration of 2.0 nmol/L, pooled, and loaded on the Illumina HiSeq 2000 instrument (Illumina, Inc, San Diego, CA) for cluster generation and multiplexed, paired-end massively parallel sequencing.

Bioinformatics

The base calling files (.bcl) generated from HiSeq were demultiplexed using bclfastq2 (Illumina, Inc) to generate FASTQ sequence files for each sample.[11] The raw sequencing quality was assessed using the FastQC algorithm (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; Babraham Institute, Cambridge, United Kingdom). Secondary analysis was performed using the CLC Genomics Server v4.1 (CLC Bio, Boston, MA), with read alignment to generate a BAM file completed using the standard aligner and variant calling using the quality score–based caller.[12] Variants require a minimum base quality of 20 and a neighborhood (5 bases to the left and right of variant position) mean quality value of 15.

Table 1. Next-Generation Sequencing Panels for Hereditary Cardiovascular Conditions

Capture Panel	Condition	Genes Sequenced
1	Hypertrophic cardiomyopathy	*ACTC1, ACTN2, ANKRD1, CAV3, CSRP3, DES, GLA, LAMP2, MYBPC3, MYH7, MYL2, MYL3, MYLK2, MYOZ2, NEXN, PLN, PRKAG2, RAF1, TCAP, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, VCL*
1	Dilated cardiomyopathy	*ABCC9, ACTC1, ACTN2, ANKRD1, CRYAB, CSRP3, CTF1, DES, LAMA4, LAMP2, LDB3, LMNA, MYBPC3, MYH6, MYH7, MYPN, NEXN, PLN, RBM20, SCN5A, SGCD, TAZ, TCAP, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, VCL*
1	Arrhythmogenic cardiomyopathy	*CASQ2, DES, DSC2, DSG2, DSP, JUP, PKP2, RYR2, TMEM43, TTN*
1	Noonan syndrome and related disorders	*BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, SHOC2, SOS1*
2	Marfan syndrome and related disorders	*ABCC6, ACTA2, CBS, COL3A1, FBN1, FBN2, LTBP2, MYH11, MYLK, SKI, SLC2A10, SMAD3, TGFB2, TGFBR1, TGFBR2*
2	Familial hypercholesterolemia	*ABCG5, ABCG8, APOB, LDLR, LDLRAP1, PCSK9*
3	Long-QT and Brugada syndromes	*AKAP9, ANK2, CACNA1C, CACNA2D1, CACNB2, CAV3, GPDIL, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNQ1, SCN1B, SCN3B, SCN4B, SCN5A, SNTA1*

Alignment was done against the complete hg19 version of the human reference genome, and subsequent variant calls reported back for the assay target region only. Postsecondary analysis was performed using custom scripts to annotate the variants and load them into a custom-built web application for quality review and results interpretation. Validation of the variant calling was performed using both synthetic and Sanger-confirmed samples. Detection of insertions or deletions of base pairs (indels) was profiled using synthetic data and shown to be accurate up to insertion and deletion lengths of 8 base pairs. The complete bioinformatics workflow was further validated through input/output testing by the Mayo Software Quality Assurance team. Target sequences with 100% redundant sequence found in off-target genomic regions were identified by a systematic analysis of all read-length fractions within the assay target, using the CLC aligner. Postsequencing metrics and their definitions are in **Table 2**.

NGS Assay Verification and Performance

The NGS assays were verified previously using WB genomic DNA to satisfy NGS assay–specific validation requirements.[13–17] For the purposes of verification of FFPET and DBS sample types, a total of 19 DNA samples were assessed, including 5 FFPET and 5 DBS matched samples, 6 aged FFPET samples ranging from 6 to 10 years old, 1 Coriell DNA control sample (NA12878), and 2 WB samples that were matched to 2 of the matched FFPET and DBS samples. For intra- and interassay precision/reproducibility, 3 FFPET DNA samples and 3 matched DBS DNA samples were run in duplicate or triplicate, depending on sample volume. Analytic sensitivity was tested by varying the amount of initial DNA input. An additional 14 archived FFPET samples (aged 6 months to 15 years) were assessed

Table 2. Quality Parameter Terms and Definitions

Term	Definition
Average depth of coverage	Average coverage depth represented at each position in a specific region of interest
Depth of coverage	No. of sequence reads aligning to overlapping positions in a specific region of interest
Mapping quality	A measure of the confidence that a sequencing read comes from the position it is aligned to by the mapping algorithm
Maximum depth of coverage	The highest number of reads that align to specific regions of interest
No. of reads	Total number of reads produced by the sequencer
No. of reads mapped to human genome	No. of sequencing reads that align to the human genome reference sequence (HS37D5)
Percent reads mapped to human genome	(No. of reads mapped to human genome)/(No. of reads)×100
Quality score	Per base quality score calculated in the bioinformatics pipeline that indicates the probability of the base call being correct/incorrect at a specific base location (common example: Phred score)
Unique read	Sequencing read that aligns specifically to 1 location in a genome

to determine whether age of samples would impact quality of DNA extraction. Formalin fixation time (time tissue as in formalin before processing and paraffin infiltration) was available for 12 of the 14 FFPET samples and ranged from 1 to 10 days (mean, 3.6 days; median, 2.5 days).

For verification of accuracy, different matched samples were run (depending on sample volume) for each of the different capture sets. For capture set 1, 5 matched FFPET and DBS samples were run with 2 cases also including matched WB DNA. For capture sets 2 and 3, 4 matched FFPET and DBS samples were run with one of these sets also including matched WB DNA.

Analytic sensitivity studies were performed to establish the minimum amount of genomic DNA input that will provide acceptable results for the test system. The established protocol requires 200 ng total FFPET DNA and DBS DNA. This concentration is tightly controlled during the library preparation process. To establish a lower limit of DNA, samples from 3 individuals (DBS and FFPET sources) were processed with 200 ng and again with 100 ng DNA. Bases mapped to target with >100× coverage for 200 and 100 ng inputs were highly comparable.

The Agilent SureSelect custom capture probes were assessed for specificity using the UCSC Genome Browser BLAT Search Tool. The results from BLAT indicated which regions other than the intended target region would be captured with the probes. In general, the specificity of variant calling relies on correct bioinformatics alignment of reads in those regions. Reads that aligned to >1 region because of homology had mapping quality scores <60. Reads with mapping quality <60 were not used by the variant caller to count toward the coverage of a potential variant position. Thus, variants were called using only the reads that were unique for each position. Assessment of bioinformatics specificity included review of the depth of coverage of unique reads (as opposed to total reads). Unique read depth of coverage was assessed and deemed acceptable (>100×) for most regions tested.

Case Studies

To assess the clinical use of NGS FFPET analysis, archived cases were selected for pilot sequencing and analysis. Tissue Registry archives of Mayo Clinic (Rochester, MN) were queried for cases of

SCD occurring between January 1, 2013, and June 1, 2015. Cases (including clinical history and autopsy findings) were screened to evaluate for sufficiently compelling phenotype to have warranted a confident diagnosis of cardiomyopathy or a connective tissue disease. NGS was performed on myocardial FFPET on 4 selected phenotype-positive cases involving sudden death. All variants were classified, by consensus, according to American College of Medical Genetics and Genomics guidelines.[18]

Results

Accuracy and Precision

Accuracy was 100% concordant between WB-, FFPET-, and DBS-derived DNA for all variant and wild-type position calls in each capture set. For capture set 1, 737 single-nucleotide variants and 167 indels were concordant; for capture set 2, 139 single-nucleotide variants and 14 indels were concordant; and for capture set 3, 171 single-nucleotide variants and 49 indels were concordant. There were no false-positive or false-negative variants in all regions that met the established quality parameters.

Inter- and intra-assay precision was assessed through the use of 3×3×3 experiments, which included 3 FFPET- and 3 DBS-derived DNA samples run across 3 separate instrument runs (interassay precision) or run in triplicate within the same instrument run (intra-assay precision). In all cases, the acceptance criteria (100% concordance of variant calls within the regions of interest and a repeat rate of <10%) were met (**Table 3**).

Analytic Sensitivity

Concordance rates for both input amounts were 100%, except for 1 sample that failed sequencing. This sample was a DBS sample, with 100 ng input, that failed for the cardiac channelopathy panel because of a high duplication rate.

Analytic Specificity

Average depth of coverage was correlated inversely to capture panel size, as was expected, and was mostly comparable between all 3 sample types (Figure). It is interesting to note that FFPET depth of coverage for capture panel 3 is higher than the other sample types, especially the WB samples. This may be because there were fewer WB sample runs on that capture panel, and one of the samples had decreased depth of coverage, likely skewing the average depth of coverage down. Regionally decreased depth of coverage (as low as 40×) was occasionally observed with the FFPET and DBS sample sources when compared with WB DNA. Average depth of coverage overall (all capture panels combined) between the 3 sample types was comparable, as were maximum depth of coverage and percent reads mapped to the human genome.

In clinical practice, regions with lower depth of coverage can be analyzed additionally with an alternative methodology, typically Sanger sequencing. For the assay described here, it was determined that, in clinical practice, supplemental Sanger sequencing because of low coverage issues may need to be performed for *CBS* exon 7, *APOB* exon 1, *LDB3* exon 12, *SKI* exon 7, and *TGFBR1* exon 1. These regions consistently demonstrated lower coverage with all 3 sample types (FFPET, DBS, and WB).

Table 3. Inter- and Intra-Assay Precision of Sequencing

Capture Panel	Sample	Source	No of SNVs	SNV Concordance (%)	No. of Indels	Indel Concordance (%)
Interassay precision*						
1	1	FFPET	125	100	10	100
1	1	DBS	127	100	11	100
1	2	FFPET	101	100	7	100
1	2	DBS	108	100	7	100
1	3	FFPET	143	100	11	100
1	3	DBS	149	100	12	100
2	1	FFPET	39	100	5	100
2	1	DBS	39	100	5	100
2	2	FFPET	33	100	2	100
2	2	DBS	33	100	3	100
2	3	FFPET	31	100	3	100
3	1	FFPET	37	100	3	100
3	2	FFPET	45	100	4	100
3	2	DBS	45	100	5	100
3	3	FFPET	45	100	3	100
3	3	DBS	46	100	5	100
Intra-assay precision						
1	1	FFPET	125	100	10	100
1	1	DBS	127	100	10	100
1	2	FFPET	101	100	7	100
1	2	DBS	108	100	7	100
1	3	FFPET	143	100	11	100
1	3	DBS	149	100	12	100
2	1	FFPET	38	100	4	100
2	1	DBS	39	100	4	100
2	2	FFPET	33	100	1	100
2	2	DBS	33	100	1	100
2	3	FFPET	31	100	1	100
3	1	FFPET	37	100	2	100
3	2	FFPET	44	100	5	100
3	2	DBS	45	100	5	100
3	3	FFPET	46	100	4	100
3	3	DBS	46	100	5	100

DBS indicates dried blood spot; FFPET, formalin-fixed paraffin-embedded tissue; and SNVs, single-nucleotide variants.

*Each sample analyzed in 3 interassay replicates.

DNA Quality From Archived FFPET Samples

To assess whether the age of FFPET archived samples would affect the quality of extracted DNA for NGS analysis, we assessed the quality of extracted DNA from 14 archived FFPET blocks aged 6 months to 15 years. All 14 of the blocks were from transmural sections of left ventricle. Eleven of the blocks were taken at a postmortem interval of <2 days,

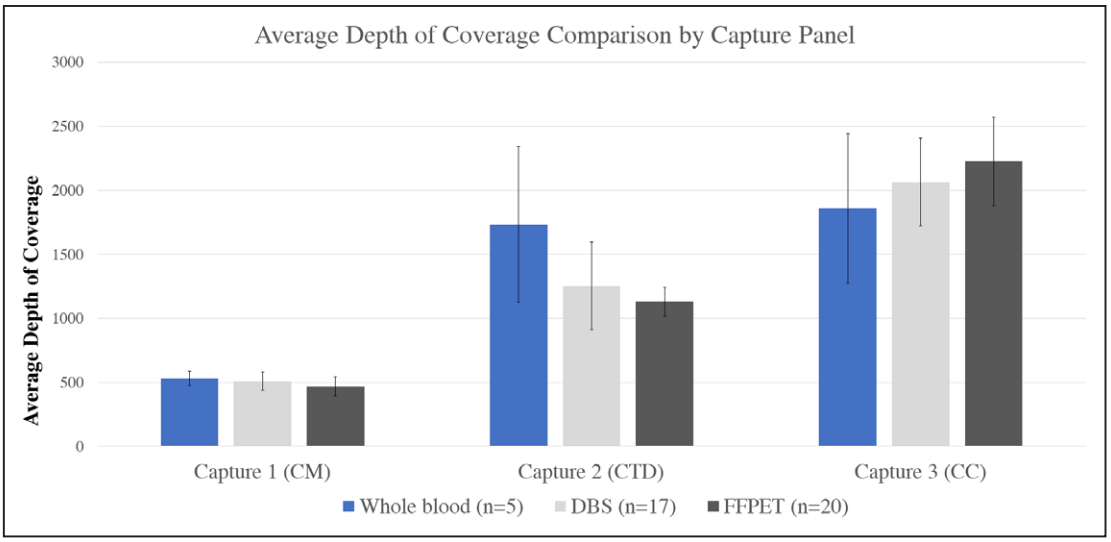

Figure. Average depth of coverage comparison by capture panel. Each assay demonstrated similar depth of coverage across specimen types (whole blood; dried blood spot [DBS]; and formalin-fixed paraffin-embedded tissue [FFPET]). Differences in depth of coverage between the assays (cardiomyopathies [CM]; connective tissue disorders [CTD]; and cardiac channelopathies [CC]) are because of assay size (genes covered).

whereas 3 of the blocks were taken at a postmortem interval of >2 days. Precise postmortem intervals beyond 2 days are difficult to establish because precise time of deaths is not known. Although the 3 cases with postmortem intervals >2 days were estimated to be ≈3 to 5 days, none are known definitively. No cases exhibited tissue decomposition by light microscopy. In comparing average DNA concentration, volume, and yield between the sample types, as expected, DBS, FFPET, and WB DNA had, respectively, increasing values (**Table 4**). Acceptable DNA quantity (>200 ng) and quality (meeting the quality metrics described in the Methods section) were obtained from all blocks (**Table 5**).

Case Studies

Four compelling phenotype-positive cases were selected from a genotype query, including 2 cases of hypertrophic cardiomyopathy (HCM), 1 case of Noonan syndrome, and 1 case of Marfan syndrome. The details of the clinical histories and sequencing results in these cases are described below.

Case 1

A 73-year-old man presented to the emergency room after several episodes of syncope. He had a longstanding clinical diagnosis of HCM (diagnosed at 32 years of age) and treatment with β-blockade and septal myectomy (47 years of age). He also had significant systolic dysfunction and biventricular dilatation in the setting of ischemic heart disease requiring coronary artery bypass grafting, intracardiac defibrillator placement, and mitral valve replacement and tricuspid valve annuloplasty for regurgitation. There was no definitive family history of cardiomyopathy or SCD.

Interrogation of his intracardiac defibrillator revealed discharge at the time of the described syncope. While in hospital, he had multiple intracardiac defibrillator shocks in association with episodes of ventricular tachycardia. Despite maximal medical therapy, he progressed to cardiogenic shock and multiorgan failure and died ≈11 days after presentation.

At autopsy, examination of the heart revealed findings in keeping with HCM including asymmetrical septal hypertrophy, marked gross and microscopic biventricular hypertrophy (1042 g heart; expected mean 330 g), mild interstitial fibrosis, and moderate myocyte disarray. There were also findings of ischemic heart disease including significant coronary artery atherosclerosis and multiple remote infarcts in the ventricular septum and left ventricular free wall.

FFPET NGS analysis revealed pathogenic frameshift variant because of duplication of a guanine in *MYBPC3*, in addition to 177 benign and likely benign variants. The pathogenic variant, *MYBPC3* c.2373dup (p.Trp792Valfs*41), was confirmed subsequently by Sanger sequencing on postmortem WB. This variant causes loss of function via haploinsufficiency and has been reported in numerous individuals and families with HCM.[19–22] The family was counseled and referred for appropriate screening including mutation-specific cascade testing.[23]

Case 2

A 71-year-old man was found prone and unresponsive on his garage floor after being seen alive and asymptomatic ≈1 hour earlier. He had a longstanding history of systemic hypertension and HCM with third-degree heart block requiring dual-chamber pacemaker. There was no definitive family history of cardiomyopathy or SCD.

Examination of the heart at autopsy confirmed findings consistent with HCM including increased ventricular septal:free wall ratio (1.6:1), moderate biventricular gross and microscopic myocyte hypertrophy (504 g heart; expected 335 g), moderate microscopic myocyte disarray, and mild interstitial (pericellular type) fibrosis. Bilateral renal arteriosclerosis and glomerulosclerosis were also noted, in keeping with a history of systemic hypertension.

FFPET NGS analysis of the genes in capture panel 1 revealed a duplicated thymine at position 2490 of *MYBPC3*, which was confirmed subsequently by Sanger sequencing

Table 4. Preanalytic and Postsequencing Quality Metrics

	FFPET*	DBS*	WB*
Preanalytic quality metrics			
DNA concentration, ng/uL	43.9 [20–106]	4.7 [2–9]	250 [250–250]
DNA volume, uL	74.6 [25–96]	74.3 [70–75]	233.3 [150–350]
Yield total DNA, ng	2652 [888–5420]	344.8 [179–433]	58 333 [37 500–75 000]
Postsequencing quality metrics			
No. of reads	4 348 631 [2 948 062–5 912 932]	4 489 067 [2 548 246–7 243 718]	5 599 742 [4 830 962–6 206 698]
No. of reads mapped to human genome	4 311 426 [2 919 015–5 855 372]	4 456 174 [2 531 381–7 155 629]	5 558 558 [4 788 983–6 154 718]
Percent reads mapped to human genome (%)	99.2 [98.3–99.5]	99.3 [98.8–99.5]	99.3 [99.2–99.5]
Maximum depth of coverage	2692 [1704–4262]	2571 [1647–3096]	2908 [2136–3536]
Average depth of coverage	1258 [832–2387]	1371 [924–1800]	1562 [847–2256]

DBS indicates dried blood spot; FFPET, formalin-fixed paraffin-embedded tissue; and WB, whole blood.
*Average [range].

on postmortem WB. An additional 172 benign and likely benign variants were detected via NGS. The pathogenic variant, *MYBPC3* c.2490dup (p.His831Serfs*2), has been reported previously in at least one other individual with HCM.[24] The family was counseled and referred for appropriate screening.

Case 3

A 25-year-old man presented to the emergency room with a few days history of shortness of breath and fever. He had been diagnosed with HCM as a child in the context of Noonan syndrome. His past surgical history included septal myectomy, mitral valve replacement, and tricuspid annuloplasty for regurgitant valvular disease. There was no definitive family history of cardiomyopathy or SCD.

Investigations revealed sepsis secondary to mechanical prosthetic mitral valve endocarditis with blood cultures positive for *Streptococcus agalactiae*. Despite initial improvement on antibiotics, he had a massive intracerebral hemorrhage in the context of anticoagulation for his mechanical mitral valve. He was deemed to be inoperable and subsequently died ≈3 weeks after presentation.

At autopsy, examination of the heart revealed findings in keeping with HCM including asymmetrical septal hypertrophy, marked biventricular gross and microscopic hypertrophy (632 g heart; expected mean 259 g), prominent dysplastic intramyocardial arterioles, and moderate to marked myocyte disarray. Other findings included craniofacial and skeletal abnormalities consistent with Noonan syndrome, as well as massive intracerebral hemorrhage, in keeping with the clinical impression.

FFPET NGS analysis revealed a substitution of guanine for cytosine at nucleotide 776 of *RAF1*, in addition to 11 benign and likely benign variants. The *RAF1* c.776C>G (p.Ser259Cys) variant was confirmed subsequently by Sanger sequencing on postmortem WB. This likely pathogenic variant has been reported independently in association with Noonan syndrome (http://www.ncbi.nlm.nih.gov/clinvar/RCV000159075.1/#clinical-assertions). In addition, a different variant at the same residue (p.Ser259Phe) has been reported previously in association with Noonan syndrome.[25] Both of these missense variants (Ser>Cys and Ser>Phe) result in a polar uncharged residue (serine) being replaced by a hydrophobic residue (cysteine or phenylalanine).

Case 4

A 57-year-old woman was admitted to the hospital for elective repair of a chronic thoracoabdominal aneurysm. She had a longstanding history of Marfan syndrome, having undergone prior aortic valve replacement for regurgitation and both ascending and descending aortic repair for aneurysm and type B dissection, respectively. Her family history is significant for Marfan syndrome in her mother, who died of heart disease in her 70s; however, molecular genetic evaluation was never performed.

She underwent uneventful thoracoabdominal aortic repair; however, she had a severe hypotensive episode after a dose of protamine in the immediate postoperative period.

Table 5. DNA Extraction From Formalin-Fixed Paraffin-Embedded Tissue Blocks Aged 6 Months to 15 Years

Case	Tissue Age (Years)	Postmortem Interval (Days)	DNA (ng/μL)	Volume (μL)
1	0.5	<2	83.0	100
2	0.5	<2	54.2	100
3	0.5	>2	41.0	100
4	0.5	>2	102.5	100
5	1	<2	13.7	50
6	2	>2	94.0	50
7	5	<2	108.5	50
8	5	<2	109.5	50
9	8	<2	38.8	50
10	8	<2	44.7	50
11	10	<2	77.1	50
12	10	<2	27.2	50
13	15	<2	44.1	50
14	15	<2	30.0	50

Echocardiogram demonstrated a massive intracardiac thrombus extending into the aorta. Surgical intervention and extracorporeal membrane oxygenation were deemed inappropriate, and the patient died after removal from cardiopulmonary bypass.

Autopsy confirmed massive thrombus involving all 4 cardiac chambers, marked biventricular hypertrophy and dilatation, and marked medial degeneration of remaining native aorta. Other significant findings included hypoxic encephalopathy and recent intradural hemorrhage.

FFPET NGS analysis of the genes in capture panel 2 revealed that guanine at nucleotide 4505 of *FBN1* was mutated to adenine, which was confirmed subsequently by Sanger sequencing on postmortem WB. An additional 43 benign and likely benign variants were detected via NGS. The likely pathogenic *FBN1* c.4505G>A (p.Cys1502Tyr) variant has been identified in 1 additional patient with Marfan syndrome in the Mayo Clinic database and has been reported independently in association with Marfan syndrome.[26] This variant occurs in an EGF-like calcium-binding domain of fibrillin-1 which contains 6 highly conserved cysteine (Cys) residues. Thus, this variant removes a cysteine residue within the domain and may cause protein misfolding by loss or gain of disulfide bonds. *FBN1* missense variants affecting cysteine residues are considered strongly predictive of pathogenicity according to the revised Ghent nosology for the Marfan syndrome.[27]

Discussion

To our knowledge, this study is the first of its kind to demonstrate the feasibility of using FFPET-derived DNA for the detection of pathogenic genomic variants in heritable CV disease. Through a variety of standard quality metrics in the context of NGS, we have shown that FFPET provides equivalent quantity and quality of DNA to the current de facto gold standard in postmortem genetic testing (WB). The results also demonstrate that FFPET can be used to reliably identify pathogenic variants in phenotype-positive/genotype unknown autopsy cases and is a stable repository for genetic query for at least 15 years.

Using the custom panel of 101 genes, NGS seems to provide a robust and accurate screening and diagnostic identification of HCVDs. It is worth noting, however, that, at least with respect to the platform used in this study, it may not entirely replace Sanger sequencing in some circumstances. In particular, certain exons consistently demonstrated lower NGS coverage with all 3 sample types (FFPET, DBS, and WB). Furthermore, with older or more degraded FFPET samples, in some cases, it may not be possible to reliably interrogate certain genomic regions. FFPET has a disadvantage to blood DNA in that FFPET DNA is usually not a robust specimen for large deletion/duplication analyses. Thus, in cases with a high clinical/phenotypic pretest probability and a genotype-negative NGS screen, additional investigation using traditional sequencing methods may be required (but may not be ideal if FFPET is the only specimen source). This type of scenario, however, is not unique to FFPET, as supplemental Sanger sequencing is often used in targeted NGS assays using WB samples as well.

Postmortem genetic testing is increasingly recognized as a valuable tool for adjudicating cause and manner of death.[28] Arguably, it is most useful in cases of phenotype-positive SCD, where it can be used for complete characterization and subsequent targeted kindred screening. For example, a death in a young adult due to a ruptured ascending aortic aneurysm in the absence of chronic hypertension or stimulant abuse, or a sudden death of an athlete with findings consistent with HCM. In addition, it can be used in several other clinical situations, including identification of diseases that traditional gross and histological examination fails to disclose (often referred to as autopsy-negative cases) and adjudication of subtle or forme fruste cases that have worrisome, but not diagnostic, findings of a disease. It is, however, important to recognize that although clarity may be derived from identification of a known pathogenic mutation, a negative or ambiguous (variant of undetermined significance [VUS]) result may not be clinically helpful.[7] Nevertheless, collection and analysis of these data may allow for more comprehensive understanding of these diseases and, ultimately, better interpretation of genetic findings.

Despite these potential benefits to clinical care, the practical considerations of cost and specimen retention do come into play, particularly when involving ME/C offices that have already constrained resources. The most ubiquitous way that tissue is archived currently, FFPET blocks, does not readily lend themselves to classical (Sanger) sequencing methods. The advent of NGS has not only allowed for development of broad panel-based assays but also provided a platform to reconsider what specimen types might be amenable to genomic interrogation. To date, development of NGS assays using FFPET has been minimal in scope and applicable primarily in the oncology setting where the tumor is the tissue source and the NGS analysis is often limited to hotspot genetic regions. Herein, we describe the clinical verification of a novel application of NGS FFPET analysis for germline variants in entire coding regions of genes for detection of HCVDs, which is especially beneficial in the exploration of sudden death cases.

In addition to verifying an NGS-based panel assay for FFPET analysis, we explored the clinical use of this analysis in selected (phenotype positive) postmortem cases. We detected putative pathogenic/likely pathogenic variants in all 4 cases described; 3 of the cases with cardiomyopathy and 1 case with thoracoabdominal aneurysm. Although the individual with the thoracoabdominal aneurysm was diagnosed clinically with Marfan syndrome and had a family history of such, the other 3 HCM cases had no definitive family history of cardiomyopathy or SCD (one of the HCM cases had clinically diagnosed Noonan syndrome albeit with no prior genetic testing). In all cases presented, the families were given post-test genetic counseling, which provided them the opportunity to discuss follow-up targeted genetic testing in at-risk family members.

Notably, providing a genetic diagnosis to explain cause of death is helpful on its own. Further extending this finding to help rule-in and rule-out disease in at-risk family members provides a great deal of value added. In family members who test positive, appropriate measures can be taken to provide

them with timely follow-up testing, management, and interventions. For family members who test negative, they can be presumed to have a much lower risk for developing the condition (ie, population risk), and costly tests, imaging, and management can be avoided.

It is anticipated that the cost of genetic testing (especially in genotype-positive cases) outweighs the cost of continuing care, imaging, assessments, etc. for family members in the absence of genetic testing. We have calculated the cost for FFPET DNA extraction and NGS reagents at $272 per sample. Labor and overhead costs for the analysis averages out to ≈$1030 per sample but is highly dependent on variables such as sample multiplexing and number and types of variants detected per sample. In addition, costs of genetic testing continue to trend downward with decreasing costs of the technology and general advances.

This model of testing takes the guesswork out of knowing who in the family is at risk for a genetic disorder, thereby streamlining and optimizing care. Of course, this applies only to cases where a genetic cause can be demonstrated definitively. For example, out of the 4 cases presented here, 1 variant of uncertain significance was detected in each of the 2 HCM cases. Had these VUSs been the sole findings in these patients (ie, no pathogenic or likely pathogenic variants), predictive testing of the VUS in family members would not be applicable. Family testing might be beneficial to help assign more definitive pathogenicity to the VUS, but testing the VUS for predictive purposes on its own would not be advised. Thus, there are challenges to interpreting molecular genetic testing results because we have an incomplete understanding of the spectrum of genomic variability and which areas of proteins are critical for normal function. This has resulted in a sort of genetic purgatory where the significance of some detected variants may be largely unknown.[7] This is especially true when genes of uncertain significance are included in the testing panel.

In light of this, interrogation of FFPET samples could prove tremendously beneficial given the new ability to evaluate archived cases, usually housed at academic medical centers, and perform detailed genotype-phenotype analyses. Such studies will help to better appreciate the genetic heterogeneity of the disorders and may eventually allow for new diagnostic standards to be generated.

Disclosures

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

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CLINICAL PERSPECTIVE

Genetic testing for cardiovascular disease has become increasingly commonplace in today's clinical setting. Fueled by our ever-expanding knowledge of the genetic basis for many forms of cardiovascular disease, new laboratory techniques and more comprehensive panels are being rapidly developed and incorporated into clinical practice. Despite remarkable advances, there are still profound limitations to this testing. Variants and mutations unique to families present interpretive challenges, particularly in cardiomyopathies, arrhythmia syndromes, and connective tissue diseases where these private variants are relatively commonplace, resulting in a litany of variants of undetermined significance. Elucidating the significance of these findings is imperative, particularly when a family member has died of one of these conditions. Decedent tissue testing is incredibly useful in interpreting results in the surviving relatives and even in determining the role of genetic testing in a given family. However, such testing is hampered by the ubiquitous use of formalin fixation in pathology practices. Although formalin provides remarkable tissue preservation for light microscopy and an assortment of ancillary tests, its effect on DNA has been greatly limiting. Next-generation sequencing technology, by virtue of its biochemistry, now presents a means of sequencing in spite of the DNA fragmentation that formalin fixation begets. Herein, this study demonstrates the efficacy of next-generation sequencing in the interrogation of formalin-fixed tissues for heritable cardiovascular diseases. This advancement opens the door for comprehensive testing of tissue stored in pathology/autopsy practices, while also providing an avenue on which archived material can be studied for academic purposes.

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Technical Advances for the Clinical Genomic Evaluation of Sudden Cardiac Death: Verification of Next-Generation Sequencing Panels for Hereditary Cardiovascular Conditions Using Formalin-Fixed Paraffin-Embedded Tissues and Dried Blood Spots

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