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Final Research Report

2018-DU-BX-0204

Solving Cases of Sudden Unexpected Natural Death in the Young through Comprehensive Postmortem Genetic Testing

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This Final Research Report summarizes what we accomplished for this project, entitled "Solving Cases of Sudden Unexpected Natural Death in the Young through Comprehensive Postmortem Genetic Testing" (award 2018-DU-BX-0204).

Please note that study-level data are in several manuscripts currently in preparation for peer-reviewed journals. This final report will be updated to incorporate the data and changes in narrative made during peer-review process. I would like to request not to distribute this repot in its current form to public website. It takes months for an article to be polished for submission and several weeks for peer-reviews prior to final publications. I appreciate your patience and understanding.

The list of manuscripts in preparation to publish study-level data is as below:

1) Molecular Genetic Characterization of Sudden Deaths Due to Thoracic Aortic Dissection or Rupture -under review by Cardiovascular Pathology (see attached)
2) Cardiac Genes Analysis and Phenotype Characterizations of a Large Sudden Deaths Cohort – in preparation, intended to submit to Journal of the American College of Cardiology (JACC)
3) Molecular Analysis in Sudden Unexplained Deaths in Epilepsy (SUDEP) – in preparation
4) Thrombophilia Genes Analysis in Fatal Pulmonary Embolism – in preparation

1. Summary of the project

1.1 Major goals and objectives

Sudden Unexpected Natural Death (SUND) in the Young ($\leqslant$ 50 years old) presents vexing challenges for forensic pathologists when comprehensive forensic investigation (e.g. scene, autopsy, toxicology, microbiology testing, and metabolic screening) offer no clues toward a cause of death. Cardiac arrhythmia diseases and sudden unexpected death in epilepsy have been reported as possible mechanisms of death in autopsy-negative cases. As clinical evaluation of the decedent cannot be performed in the postmortem setting, testing disease-associated genes becomes important for establishing a diagnosis of the cause of SUND. Furthermore, it is imperative to identify the underlying etiology and any genetic causes behind positive findings (e.g. massive pulmonary embolus; hypertrophied or dilated heart; dissected and ruptured thoracic aorta), as the testing results not only explain the pathology and the cause of death, but also inform lifesaving clinical care for high-risk surviving family members.

The **primary goals** of this project are twofold. First, we aim to identify and test a large number of SUND cases and provide answers to the medical examiners and the families. Second, we aim to evaluate the utility of molecular testing in postmortem investigations and to inform forensic practice standards and guidelines.

To accomplish the goals, **two objectives** are established. First, at the case level, we plan to complete the molecular analysis of genes associated with conditions for sudden death in over 1000 SUND cases in the Young and provide answers to the medical examiners through our

testing results and genetic counseling to families of the deceased (Some of the decedents died over decade ago and weren't tested due to lack of technological capacity in the past). Second, at the cohort level, we aim to ascertain the diagnostic yield of molecular testing in SUND by demographics (age, gender, and ethnicity) and pathological findings and through publications to inform forensic practice standards.

The testing was performed by our in-house laboratory, which is accredited by the College of American Pathologists (CAP). Molecular Genetics Laboratory in the New York City Office of Medical Examiner has several validated molecular testing panels for SUND (a cardiac-focused 132-genes panel, an epilepsy-focused 159-genes panel, a thoracic-aortic-dissection-and-rupture focused 20-genes panel, a thrombophilia focused 5-genes panel, and sickle cell disease molecular analysis) using the massive parallel sequencing technology. Sequence variants are interpreted using ACMG/AMP guidelines. Diagnostic reports were issued by board-certified laboratory director and board-certified genetic counselor who also provides genetic counseling to the families.

1.2 Research questions

Through testing a large number of SUND, we aim to ascertain the diagnostic yield of molecular testing in SUND by demographics (age, gender, and ethnicity) and pathological findings. The research questions we ask are:

1) What portion of deaths ⩽50 years of age, due to undetermined cause, can be attributed to pathogenic variants in genes associated with cardiac channelopathy, cardiomyopathy, or epilepsy? How is it different from or similar to the clinical data?
2) What portion of deaths ⩽50 years of age, due to aortic dissection, or ruptured saccular aneurysm, can be attributed to pathogenic variants in genes associated with aortopathy? How is it different from or similar to the clinical data?
3) What portion of deaths ⩽50 years of age, due to seizure or epilepsy, can be attributed to pathogenic variants in genes associated with epilepsy or cardiac channelopathy? How is it different from or similar to the clinical data?
4) What portion of deaths ⩽50 years of age, due to cardiomyopathies, can be attributed to pathogenic variants in genes associated with hypertrophic cardiomyopathy, dilated cardiomyopathy, or arrhythmogenic cardiomyopathy? How is it different from or similar to the clinical data?
5) What portion of deaths ⩽50 years of age, due to hypertensive cardiovascular disease, can be attributed to pathogenic variants in genes associated with cardiac channelopathy or cardiomyopathy? How is it different from or similar to the clinical data?
6) What portion of deaths ⩽50 years of age, due to pulmonary embolism, can be attributed to pathogenic variants in anti-coagulant genes? How is it different from or similar to the clinical data?

1.3 Research design, methods, analytical and data analysis techniques

<u>Case Selection Criteria and Suitable Samples for Molecular Testing</u>

The case inclusion/exclusion criteria as the following: 1) include cases whose age-at-death is 50 years or younger; 2) include cases whose manner of death is natural or undetermined;

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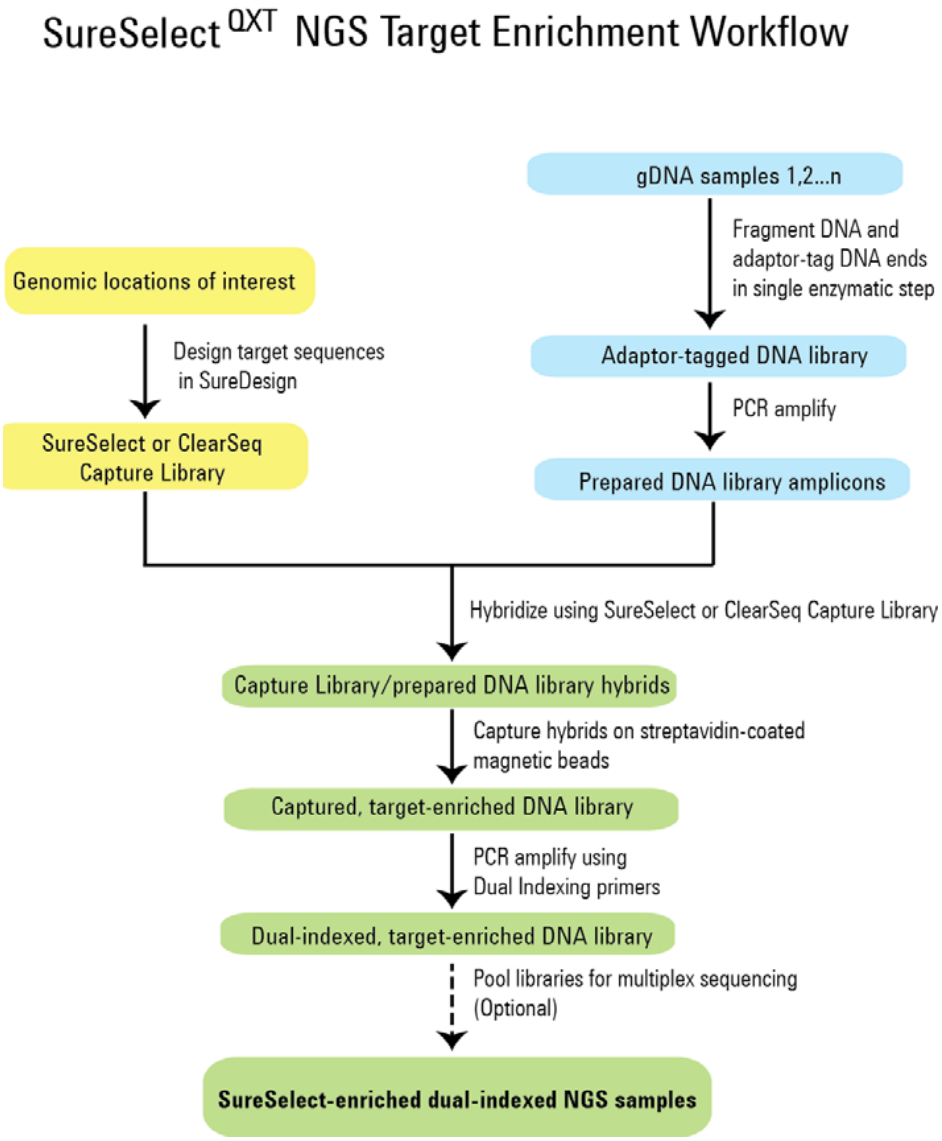
exclude cases whose manner of death is accident, homicide, suicide or therapeutic complications; 3) include cases whose cause of death is due to thoracic aortic dissection, ruptured saccular aneurysm, cardiac arrhythmia of uncertain etiology, various cardiomyopathies, epilepsy or seizure (non-traumatic), pulmonary embolism, or hypertensive cardiovascular disease.

Suitable samples for molecular testing are postmortem bloodstain cards or non-formalin-fixed tissue samples in RNAlater®.

Testing methodologies

Next-generation sequencing target enrichment will be conducted using the SureSelect^{QXT} NGS target Enrichment kit from Agilent Technologist and the workflow is shown in Fig. 1, which is summarized below:

A. Sample Preparation: Step 1. Prepare the genomic DNA samples; Step 2. Fragment and adaptor-tag the genomic DNA samples in a single reaction followed by purification; Step 3. Amplify adaptor-ligated libraries; Step 4. Purify amplified DNA using magnetic beads; Step 5. Assess Library DNA quantity and quality.
B. Hybridization: Step 1. Normalize prepped DNA samples for hybridization; Step 2. Hybridize the gDNA library; Step 3. Capture the hybridized DNA.
C. Indexing and Sample Processing for Multiplexed Sequencing: Step 1. Amplify the captured libraries to add index tags; Step 2. Purify the amplified indexed libraries using magnetic beads; Step 3. Assess indexed DNA quality; Step 4. Pool samples for multiplexed sequencing.



We used NextSeq500 from Illumina, an existing instrument in the laboratory, for the sequencing runs and data collection. This instrument has two output modes: mid-output flow cells allows testing for 48 samples with 95% of targets having over 100X coverage; the high-output flow cells allows for more than 96 samples to be tested with high coverage. Based on the availability of on-going cases and archived cases for testing, we designed a workflow that is cost and time effective. MG lab has two robotic tools for liquid handling (Freedom EVO NGS (TECAN) workstations) — one in pre-amplification lab and another in post-amplification lab — to automate the testing process (See picture in Section 4.1). Sanger sequencing will be used to confirm reportable variants.

Data Analysis and Variant interpretation

Primary NGS data analysis (generating quality sequencing reads and de-multiplexing) was performed using NextSeq 500 Local Run Manager (Illumina). Secondary NGS data analysis (sequencing quality filtering, reads alignment, and variant annotation) was performed using NextGENe v2.4.2.3 (SOFTGENETICS®). Tertiary NGS data analysis (applying preferred transcript for each gene and classification of variants) was performed in Geneticist Assistant v1.8.3 (SOFTGENETICS®). The genomic reference coordinate used was GRCh37/hg19. The

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preferred transcript for each gene is consistent with that from Human Gene Mutation Database (HGMD®). The regions of interest for each gene tested include the coding regions and ± 10 bp introns flanking exon/intron junctions. Sanger sequencing is used to 1) confirm reportable variants with low confidence (the coverage is below 100x, allele frequency is less than 25%, or reads balance is less than 0.25), and 2) to provide sequencing data for clinically significant regions of a gene that has no NGS data coverage. Sanger sequencing data analysis is performed in Mutation Surveyor v5.1.2 (SOFTGENETICS®).

The clinical relevance of sequence variants was evaluated in accordance with the standards and guidelines for the interpretation of sequencing variants established by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP). Variants are classified into five categories: pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, or benign. A variant identified as a secondary finding was reported in accordance with the policy statement of ACMG SF v.3.17.

Reporting

Molecular diagnostic reports contain testing results of pathogenic variants, likely pathogenic variants, and variants of uncertain significance, alongside evidence for variant classification and their impacts on family, are reported back to ordering medical examiners. Each report is uploaded to Case Management System (CMS) in NYC-OCME.

1.4 Expected applicability of the research

The project has several impact values:

1) First, it has direct impact on how the medical examiners in NYC-OCME certify sudden death, and how at-risk relatives are counseled and referred for proper medical care to avoid a recurrent death in the family.
2) As we evaluate efficiency and utility of the expanded genetic analysis, we have gained a better understanding of the tested diseases in SUND in the young, which guides future test development. By expanding our testing beyond the arrhythmogenic cardiac genes, we seek to fill in knowledge gaps regarding mechanisms of SUND beyond strictly cardiac etiologies, and become better able to certify cause of death, which currently challenges the forensics community. The advantage of the expanded panel will be an inclusion of new conditions not widely tested for in current postmortem analysis. In addition, our project expanded our existing partnerships with clinical and research programs in cardiogenetics by including experts in epilepsy and aortopathy. These partnerships include clinical referrals for familial testing and care for at-risk family members to clinics.
3) Finally, through our peer-reviewed publications, the results of the project will help inform further research and policy decisions, impact public health and safety, as well as the medicolegal field. In addition to publications, we have presented the data in national scientific meetings so the novelty and important findings from this paper will be disseminated to a broader audience in the forensic and medical fields alike.

In summary, we believe that this project will have a significant impact in justice through improved approaches to death certification and family counseling, providing evidence to shift the current paradigm in postmortem genetic testing, and enhance death prevention in at-risk family members through new diagnostic and therapeutic methods.

2. Participants and other collaborating organizations

2.1 What individuals have worked on the project?

The overall project oversight is the responsibility of the Principal Investigator, Dr. Yingying Tang at OCME. Molecular Genetics Laboratory members of scientists are participated in case review and selections, analytical testing, quality management of reagents, supplies and instrumentation, as well as report drafting and reviews. The entire work is conducted in NYC OCME.

3. Outcomes

3.1 Results and findings

Study-level data are in several manuscripts being prepared for peer-reviewed journals (listed below). Links to those publications will be added to this final report.

1) Molecular Genetic Characterization of Sudden Deaths Due to Thoracic Aortic Dissection or Rupture -under review by Cardiovascular Pathology (see attached)
2) Cardiac Genes Analysis and Phenotype Characterizations of a Large Sudden Deaths Cohort – in preparation, intended to submit to Journal of the American College of Cardiology (JACC).
3) Epilepsy Genes Analysis in Sudden Unexplained Deaths in Epilepsy (SUDEP) – in preparation
4) Thrombophilia Genes Analysis in Fatal Pulmonary Embolism – in preparation

4. Artifacts

4.1 Dissemination activities (publications, conference papers, etc)

1. Joshua E Motelow, Natalie C Lippa, Joseph Hostyk, Evin Feldman, Matthew Nelligan, Zhong Ren, Anna Alkelai, Joshua D Milner, Ali G Gharavi, **Yingying Tang,** David B Goldstein, Steven G Kernie Children with Critical Illness Carry Risk Variants Despite Non-Diagnostic Whole Exome Sequencing Publication date 2022/1/1 Journal medRxiv Publisher Cold Spring Harbor Laboratory Press **doi:** https://doi.org/10.1101/2022.05.01.22274445
2. Angela Baldwin, Greg Dickinson, Zarrin Hossein-Zadeh, Avneesh Gupta, Yvonne Milewski, Michelle Stram, **Yingying Tang,** Rebecca Folkerth Neuropathology of Microangiopathy in Sickle Cell Trait American Association of Neuropathologists, Inc. Abstracts of the 98th Annual Meeting June 9-12, 2022 Bonita Springs, Florida; **PMID: 35595840 DOI: 10.1093/jnen/nlac027**
3. Molecular Genetic Analysis of Sudden Unexpected Death in the Forensic Setting **Yingying Tang,** American Association of Neuropathologists, Inc. Abstracts of the 98th Annual Meeting June 9-12, 2022 Bonita Springs, Florida; Special course lecture
4. 2022 National Institute of Justice Forensic Science Research and Development Symposium. Solving Cases of Sudden Unexpected Natural Death in the Young Through Comprehensive Postmortem Genetic Testing, NIJ award# 2018-DU_BX-0202; **Yingying Tang** **DOI: 10.3768/rtipress.2022.cp.0015.2204**

5. Attachment

Molecular Genetic Characterization of Sudden Deaths Due to Thoracic Aortic Dissection or Rupture -under review by Cardiovascular Pathology