Dealing With Unspecific Clinical Phenotypes in Molecular Autopsy - HPO-Driven Whole Exome Sequencing Analysis Versus Gene Panel Testing

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Abstract

Background: Molecular autopsy represents an efficient tool to save the diagnosis in up to one-third of sudden unexplained death (SUD). A defined gene panel is usually used for the examination. Alternatively, it is possible to carry out a comprehensive genetic assessment (Whole Exome Sequencing, WES), which also identifies rare, previously unknown variants. The disadvantage is that a dramatic number of variants must be assessed to identify the causal variant. To improve the evaluation of WES, the Human Phenotype Ontology (HPO) annotation is used internationally for deep phenotyping in the field of rare disease. However, a HPO-based evaluation of WES in SUD has not been described before.

Methods: We performed WES in tissue samples from 16 people after SUD. Instead of a fixed gene panel, we defined a set of HPO terms and thus created a flexible “virtual gene panel”, with the advantage, that recently identified genes are automatically associated by HPO terms in the HPO database.

Results: We obtained a median value of 68,947 variants per sample. Stringent filtering ended up in a median value of 276 variants per sample. Using the HPO-driven virtual gene panel we developed an algorithm that prioritized 1.4% of the variants. Variant interpretation resulted in eleven potentially causative variants in 16 individuals.

Conclusion: Our data introduce an effective diagnostic procedure in molecular autopsy of SUD with a non-specific clinical phenotype.

Introduction

Sudden death (SD) of apparently healthy individuals is amongst the most challenging scenarios in clinical medicine. Sudden cardiac death (SCD) is the predominant cause of SD, with structural cardiovascular abnormalities often evident at autopsy. 10–30% of SD remain unexplained by conventional forensic autopsy procedures (the so-called sudden unexplained death, SUD). One-fourth of these autopsy-negative SUD cases harbored an underlying pathogenic variant. Over 100 SD-predisposing cardiac channelopathy-, cardiomyopathy-, and metabolic disorder-susceptibility genes have been identified. Thus, molecular autopsy (post-mortem genetic testing) by high-throughput sequencing (HTS) technology represents an efficient tool to assess these diagnosis (1–3). The importance of molecular autopsy lies in its ability to identify pathogenic variants and thereby enabling risk prediction of asymptomatic relatives. However, identification of a causative variant in an individual who did not present with a specific clinical phenotype before the SUD is still challenging.

Since most of the reported likely causal variants were found in genes associated with cardiac disease a fixed panel-based approach with a limited number and clinically well-defined genes is commonly used for identifying the genetic causes of SUD (4–7). Nevertheless, the overall diagnostic yield of a fixed gene panel is limited. In comparison, whole exome sequencing (WES) has diagnostic power to identify potentially pathogenic variants also in rare causative genes, which have not been associated with SUD before and thereby elucidating novel pathomechanisms (8). For this reason, WES is increasingly used in clinical settings and represents the primary alternative to gene panel testing. However, data interpretation remains challenging because of a high incidence of variants of unknown significance (VUS) and the possible false assignment of variant pathogenicity. As both WES and targeted panel sequencing yield accurate genetic diagnoses, clinicians are faced with the challenge of deciding which method to use. To improve variant interpretation in WES, the Human Phenotype Ontology (HPO) was developed as a semantically computable international standardized vocabulary to capture phenotypic abnormalities in human (9). Although, the number of HPO terms has grown substantially since the clinical integration and use of this ontology was established (10). Another obvious advantage of phenotype-driven filtering is that recently identified genes are automatically associated by the HPO term in the HPO database. In comparison, the design of each targeted gene panel needs to be curated over time. Groza and coworkers developed a concept-recognition procedure that analyzes the frequencies of HPO disease annotations as identified in over five million PubMed abstracts by employing an interactive procedure to optimize precision and recall of the identified terms (11).

Here, we performed WES in tissue samples of 16 individuals with SUD after autopsy and provided a practical guide for filtering and prioritizing genetic variants by a specific set of HPO terms (explaining a SUD), thus creating a “virtual panel” instead of using a fixed-panel approach.

Methods

Samples and preparation

Autopsies on 16 SUD cases (9 adults, 23-53 years and 7 infants, 4 weeks to 9 months) were performed by forensic pathologists including general autopsy investigations, toxicology and histology. Cases were included if no specific cause of SD could be established at the medicolegal investigation. DNA samples for WES were extracted from post-mortem liver and/or heart tissue. On request we have been informed by the ethics committee that a vote is not needed as all investigations were made after the release of confiscated tissues by the public prosecution office and complete anonymization. Due to anonymization, co-segregation analyses of the variants were not performed.

High throughput sequencing and bioinformatics pipeline

Next-generation sequencing analysis (NGS) of a custom capture kit (Agilent SureSelectXT) was carried out on an Illumina NextSeq 500 system (Illumina, San Diego, CA) using v2.0 SBS chemistry. Sequencing reads were aligned to the human reference genome (GRCh37/hg19) using BWA (v0.7. 13-r1126). SNV, CNV and INDEL calling on the genes was conducted using the varvis software platform (varvis™, Limbus Technologies GmbH, Rostock) subsequent coverage and quality dependent filter steps.

Human Phenotype Ontology (HPO) structure and selection of terms to create a virtual gene panel

The HPO currently contains over 13,000 terms. Most ontologies are structured as directed acyclic graphs, which are similar to hierarchies but differ in that a more specialized term can be related to more than one less specialized term. The HPO terms used for variant filtering in our study were selected with the goal of covering phenotypic abnormalities that explain an unexpected sudden natural death. We selected the HPO term "*arrhythmia"* (HP: 0011675, associated with 356 genes), which belongs to the subclass *abnormality of cardiovascular system electrophysiology. We added the HPO term "sudden cardiac death" (HP: 0001645, associated with 72 genes) for variant filtering, which belongs to the category "cardiac arrest". Since SUD is a fatal complication of seizures without recovery (12), we added the specific HPO term "status epilepticus*" (HP: 0002133, associated with 131 genes) which belongs to the category "*seizure"*. Since a lack of breathing may result in SD, we selected the HPO term "apnea" (HP: 0002104, associated with 266 genes) from the category "*Abnormal pattern of respiratory*". Taken together, all cases of the study were annotated with the following set of HPO terms: a*rrhythmia*, *sudden cardiac death,* status epilepticus and apnea. Overall, 672 different genes were associated with the selected HPO terms, thus creating a HPO-driven "virtual gene panel". HPO project data are available at http://www.human-phenotype-ontology.org. (Release: August 2020).

Nomenclature, interpretation and classification of genetic variants

The nomenclature guidelines of the Human Genome Variation Society (HGVS) were used to annotate DNA sequence variants (13). The functional consequence of missense variants was interpreted with the amino acid (AA) substitution effect prediction methods SIFT (Sorting Invariant from Tolerated; http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard. edu/pph2/), Mutation Taster, MAPP (http://mendel.stanford.edu/SidowLab/downloads/MAPP/index.html), GERP++ (http://mendel.stanford.edu/SidowLab/downloads/gerp/), Mutation Assessor (http://mutationassessor.org/r3/), FATHMM (http://fathmm.biocompute.org.uk/ fathmmMKL. htm), SiPhy (http://portals.broadinstitute.org/genome_bio/siphy/), PhyloP (https://ccg.epfl.ch/mga/hg19/phylop/phylop. html) and MetaLR (http://m.ensembl.org/info/genome/variation/prediction/protein_function.html#MetaLR). Splice-sites were predicted with MES (MaxEntScan; http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html) and SSF (SpliceSiteFinder; http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html). Population databases were used to assess the allele frequencies of the variants: Database of all known Single Nucleotide Polymorphisms (dbSNP153, https://www.ncbi.nlm.nih.gov/snp) and Genome Aggregation Database (gnomAD v2.2.1, https://gnomad.broadinstitute.org). The variants were classified according to the ACMG guidelines with the 5-tier classification system: class 5 (pathogenic), class 4 (likely pathogenic), class 3 (variants of unknown significance, VUS), class 2 (likely benign) and class 1 (benign) (14). The variant databases ClinVar and LOVD (Leiden Open-source Variant Database) were used.

Results

Specific selection of HPO terms added to variant assessment prioritizes 1.4% of the filtered variants

WES was performed in nine adults and seven infants with post autopsy unclear SD. In total, we obtained a median value of 68,947 per sample (Fig. 1). The first step involved filtering by quality, allele population frequency, functional impact (missense, stop, frameshift, splice variants, in frame insertions) and variants not classified as likely benign and benign in different variant databases (ClinVar, LOVD). These steps resulted in a mean value of 276 variants per sample. The second filter step with the HPO-driven virtual panel (arrhythmia, sudden cardiac death, status epilepticus and apnea), variant classification and mode of inheritance prioritized a mean value of four variants per sample (1.4% of 276 variants). Interpretation of the identified variants in context with the age of the individual at the SUD event resulted in eleven potentially causative variants in 16 individuals (Table 1). Four variants were associated with the HPO term "arrhythmia", seven with the HPO term "sudden cardiac death", two with the HPO term "status epilepticus" and one with the HPO term "apnea" (Table 1). Interestingly, the majority of potentially causative variants was identified in infants. Six of seven infants carried at least one potentially causative variant, and three of nine adults carried at least one potentially causative variant.

Table 1
List of identified variants after the described filter setting and HPO annotation

Age of SUD	Gene	*OMIM	NM_number	variant	AA change	variant type	HPO match	GnomAD	dbSNP	MetaLrR
8 months	DSG2	125671	NM_001943.4	c.81 + 1G > C	p.(?)	splice_donor	Sudden cardiac death			
3 months	UPB1	606673	NM_016327.2	c.917-1G > A	p.(?)	splice_acceptor	Status epilepticus	0.0017889	143493067	
4 weeks	SCN4A	603967	NM_000334.4	c.787G > A	p.(Val263Ile)	missense	Arrhythmia, Apnea	0.0000121		0.97394
3 months	RYR2	180902	NM_001035.2	c.1939C > T	p.(Arg647Cys)	missense	Sudden cardiac death	0.0001465	202040519	0.96504
	SCN8A	600702	NM_014191.3	c.5392G > A	p.(Asp1798Asn)	missense	Status epilepticus			0.92015
9 months	AKAP9	604001	NM_005751.4	c.7096A > G	p.(Ile2366Val)	missense	Sudden cardiac death	0.0000248	368823780	0.08032
5 weeks	SCN5A	600163	NM_198056.2	c.3520C > T	p.(Arg1174Trp)	missense	Arrhythmia, Sudden cardiac death	0.0000348	367906630	0.9016
28 years	RYR2	601239	NM_001390.4	c.1571G > A	p.(Arg524His)	missense	Sudden cardiac death	0.0000906	142108185	0.50319
32 years	RAF1	164760	NM_002880.3	c.1334T > G	p.(Leu445Arg)	missense	Arrhythmia	0.0000239		0.95649
23 years	SCN5A	600163	NM_198056.2	c.3152T > C	p.(Val1051Ala)	missense	Arrhythmia, Sudden cardiac death	0.000004		0.83793
	RBM20	613171	NM_001134363.2	c.215A > T	p.(Asn72Ile)	missense	Sudden cardiac death			0.80363

Whole exome sequencing (WES) was performed in 16 individuals with SUD. The age of the individuals at the SUD event and the eleven potentially causative v filtering process, name of the gene, OMIM, Reference Sequence (NM_number) of the gene, variant, amino acid (AA) change, variant type (splice_donor, splice_ Human Phenotype Ontology (HPO) match, population frequency (GnomAD), Single Nucleotide Polymorphism database number (dbSNP), MetaLR logistic reg score (MetaLrRank), ACMG criteria and classification are listed. Evidence of pathogenicity of each variant was shown. Pathogenic criteria: PVS1 (very strong) (strong), PP3, PP4 (supporting). Evidence of benign impact: BS1 (strong).

Eleven potentially causative variants were identified in 16 individuals with post autopsy unclear sudden death

Stringent filtering in combination with HPO annotation ended up in eleven candidate variants, three of them have not been identified before (Table 1). Nine variants were missense and two splice variants. Both splice variants were classified as likely pathogenic. The splice variant c.81 + 1G > C was found heterozygous in *DSG2* which was annotated by the HPO term “sudden cardiac death”. The variant was identified in an eight months old infant and is not listed in population-specific databases, but at the same position a nucleotide change from G to T is listed in gnomAD (rs1237620145, gnomAD MAF: 0.003%). The rare truncating variant is located within the second exon of *DSG2* and is predicted to cause a splice donor malfunction. The variant was classified as likely pathogenic. The other splice variant c.917-1G > A was found homozygous in a three months old infant in *UPB1*, annotated by the HPO term “status epilepticus”. The variant is listed in population-specific databases (rs143493067, gnomAD MAF: 0.18%), and is classified as likely pathogenic/pathogenic in LOVD and with “conflicting interpretation” of pathogenicity in ClinVar.

The nine missense variants were classified as VUS (Table 1). Four variants in adults and four variants in children were located in genes that previously have been reported to be associated with cardiac channelopathies and cardiomyopathies, respectively. One variant was identified in *SCN4A* in a four weeks old infant and another in *SCN8A* in a three months old infant. Two individuals carried two VUS in different genes (Table 1).

Discussion

A key challenge in using WES in molecular autopsy is finding the true causal variant among hundreds of rare variants. By filtering genes known to be associated with a particular HPO term, we shift the analysis focus from the entire exome to that part of the exome that is clinically interpretable in a diagnostic setting. Instead of using a fixed panel-based approach, we designed a HPO-driven virtual gene panel, with the advantage, that recently identified genes are automatically associated by HPO terms in the HPO database and developed an algorithm that prioritized 1.4% of the variants by several filtering steps.

The two likely pathogenic variants found in our study, were detected in children (< 12 months). Genetic studies in SIDS cohorts collectively suggest that up to 15–20% of SIDS cases might be explained by inherited cardiac diseases not detectable during conventional forensic autopsy investigations (15–17). However, our data further highlight, that interpretation of putative pathogenic variants in SIDS is challenging.

The homozygous variant in *UPB1* was annotated by the HPO term “status epilepticus” and has been recently published to trigger seizures due to ß-ureidopropionase (UPB) deficiency in a recessive mode of inheritance (18). Assmann et al. reported the same variant also homozygous in a four months old boy with an acute life threatening event (ALTE) with febrile status epilepticus (19). The extent of the reduction in enzyme activity caused by a particular *UPB1* variant, along with other genetic and environmental factors may determine whether people with UPB deficiency develop neurological problems and the severity of these problems. Therefore, in many affected individuals with absent or mild neurological problems, the condition may never be diagnosed, and may thus explain that the here identified variant has been found homozygous in one of 141,426 genomes from unrelated individuals. Importantly, epileptic seizures can induce malignant arrhythmias, possibly due to seizure-related effects on the autonomic nervous system (20). However, the homozygous likely pathogenic variant in *UPB1*, recently associated to status epilepticus, has not been linked to SD before. Thus, a fixed gene panel-based approach consisting well-known genes linked to SD would have missed the variant in *UPB1*. In comparison, the HPO-dirven virtual panel is a flexible system that does not have to be adjusted over time as new genes are added.

The second identified likely pathogenic variant was detected in *DSG2* in an eight months old girl. Pathogenic variants in *DSG2* are associated with arrhythmogenic right ventricle cardiomyopathy (ARVC), a disease that importantly predominantly affects adults in the 4th or 5th decade of life. If ARVC is diagnosed in the infantile stage, there should be clearly identifiable morphologic changes of the heart (fibrosis, dilation, fatty infiltration) before death occurs. Nevertheless, another study identified variants in *DSG2* associated with SUD in infants (21), indicating that interpretation of variants in context with the age of the individual at the SUD event is challenging.

Beside the two likely pathogenic variants, we identified nine VUS. The majority of the VUS has been identified in genes previously having been reported to be associated with cardiac channelopathies (*SCN5A, AKAP9, RYR2*) and cardiomyopathies (*RBM20, RAF1*) (2, 22–24). One variant was identified in *SCN4A* in a four weeks old infant. *SCN4A* variants are described as cause of autosomal-dominant myotonia and periodic paralysis (25). Affected members developed in utero- or neonatal-onset muscle weakness of variable severity. In seven cases, severe muscle weakness resulted in death during the third trimester or shortly after birth (26). Interestingly, variants in *SCN4A* have also been reported in patients with clinical diagnosis of Brugada syndrome, a primary arrhythmia syndrome (27). Another potentially causative variant was identified in *SCN8A* in a three months old infant. Pathogenic variants in *SCN8A* have been associated with a wide spectrum of epilepsy phenotypes, ranging from benign familial infantile seizures to epileptic encephalopathies with variable severity (28). Now, there are no forensic guidelines on the management and interpretation of VUS. Grassi et al recently discussed the main elements and issues that differentiate the forensic management of cases in which VUS are found (29). Our data highlight that HPO-based filtering could be used as complementary approach in particular to prioritize VUS by HPO-matches. Before one of the candidate variants can be defined as “causative variant” further investigations (f.e. co-segregation analyses, functional studies) are needed. To date, many studies that used HTS identified putatively pathogenic variants in molecular autopsy but only a small number performed co-segregation analysis. Due to complete anonymization, co-segregation analyses of the variants cannot be performed. Campuzano et al demonstrated the value of co-segregation in SUD (30). The presence of rare variants in asymptomatic family members aided the exclusion of some variants as being causative of the SUD. Glengarry and co-workers reported that co-segregation studies are challenging to perform especially if the proband is an infant, due to difficulties in tracking families once a pathogenic variant which explains SD is found (31).

Our data further highlight that phenotype and genotype data should be used in conjunction to prioritize variants for further evaluation and may thus increase the overall solve rate especially in cases without specific clinical phenotypes like SD. In particular, HPO provides a structured, comprehensive and an international standard that could be used for developing algorithms and computational tools for clinical differential diagnostic in SUD.

Conclusion

Molecular autopsy should be included in forensic protocols when no conclusive cause of death is identified. Prioritization of variants by a specific set of HPO terms could be used as a complement approach to perform a diagnosis in molecular autopsy. Identification of causative variants in molecular autopsy of SUD can allow prevention of SD in relatives.

Abbreviations

ARVC Arrhythmogenic right ventricle cardiomyopathy

GnomAD Genome Aggregation Database

HPO Human Phenotype Ontology

HTS High-Throughput Sequencing

LOVD Leiden Open-source Variant Database

MAF Minor Allel Frequency

SD Sudden Death

SCD Sudden Cardiac Death

SUD Sudden Unexplained Death

SIDS Sudden Infant Death Syndrome

UPB ß-Ureidopropionase

VUS Variant of Unknown Significance

WES Whole Exome Sequencing

Declarations

Ethics approval and consent to participate: On request we have been informed by the ethics committee that a vote is not needed as all investigations were made after the release of confiscated tissues by the public prosecution office and complete anonymization. Due to anonymization, co-segregation analyses of the variants were not performed.

Consent for publication: Not applicable" in this section.

Availability of data and materials: The variants classified during the current study are available at https://databases.lovd.nl/shared/variants.

Conflicts of interest/Competing interests: The authors declare no conflict of interest/competing interests.

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Author´s contribution: US: Conceptualization, **Formal analysis, Methodology, Investigation, Validation, Data curation, Software, Review & Editing. AH: Methodology, Validation. SK: Data curation, Review & Editing. FS: Software, Review & Editing. AL: Validation, Review & Editing. ABP: Review & Editing. EHF: Funding acquisition, Resources,** Conceptualization, **Review & Editing. OP: Resources, Review & Editing. ID: Project administration, Supervision, Conceptualization, Formal analysis, Data curation, Visualization, Roles/Writing- original draft preparation.**

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Figures

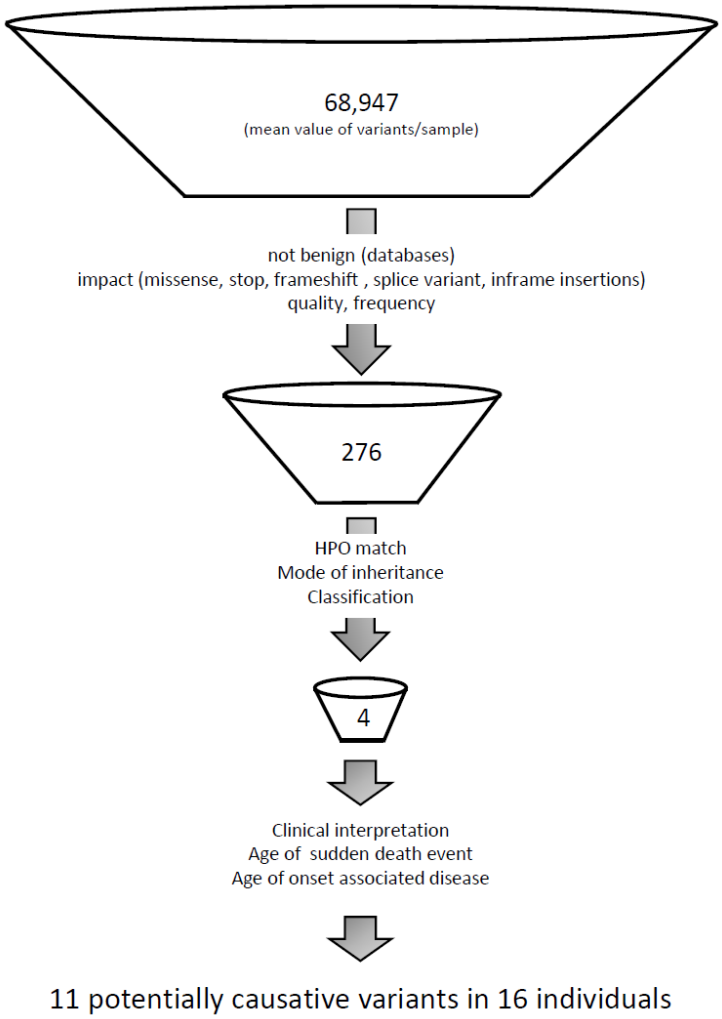

Figure 1

Flow chart of variant filtering for identification of potentially causative variants in SUD Whole exome sequencing (WES) was performed in samples of 16 individuals who died suddenly and cause of death was not conclusive after a complete autopsy. Flow chart of variant filtering and the median value of variants identified are shown. Overall, a mean value of 68,947 variants per sample were identified. The first step involved filtering by quality, population frequency, functional impact, LOVD and ClinVar classification to discard variants classified as likely benign/benign. This filter step resulted in a mean value of 276 variants per sample. Filtering by HPO matches (sudden cardiac death, arrhythmia, status epilepticus or apnea), mode of inheritance and variant classification, ended up in a mean value of four variants per sample. Clinical interpretation including the age of death resulted in eleven potentially causative variants.