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International Journal of Forensic Sciences
ISSN: 2573-1734

Retrospective Molecular Analysis in Five Cases Probably Associated with Sudden Death the National Institute of Legal Medicine and Forensic Sciences of Colombia

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Research Article

Volume 8 Issue 2

Received Date: March 09, 2023

Published Date: May 11, 2023

DOI: 10.23880/ijfsc-16000304

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Abstract

In recent years, significant advances have been made in understanding the genetic factors that predispose to sudden cardiac death, finding multiple affected genes that cause arrhythmic disorders, which could trigger sudden death in structurally normal hearts, to determine these genetic variants. It has an important role as a complement in autopsies of deaths to be determined. Cases and controls were analyzed through the study of the exome by next-generation sequencing. The variants were filtered following international recommendations, different software was used to determine the possible variants associated with cardiomyopathies and cardiac channelopathies. Twelve structural variants were found in six genes associated with different types of cardiomyopathies. Seven variants were found in six genes associated with cardiac channelopathies, and additionally, twenty-one variants were found in twelve genes of uncertain significance, four variants may be of clinical relevance. In deaths whose causes remain to be determined after performing the autopsy and considering negative toxicological, virologic, and microscopy results, it is extremely important to carry out a molecular analysis because the cause of death is possibly due to a channelopathy or an arrhythmia that is difficult to detect during the autopsy.

Keywords: SCD; Myocardiopathies; Channelopathies; NGS

- **Highlights**

In cases of sudden unexplained death, molecular studies can be extremely important to discern the possible cause of death.

When there are no cosegregation studies, in silico prediction studies of protein stability are very important.

Each of these variants alone may not have contributed significantly to the death, but variants taken together may partially contribute to a general genetic predisposition.

Introduction

The from a forensic point of view, sudden death is mainly defined as a quick, unexpected and natural death. When confronted with the study of a case of sudden cardiac death in an adult or in a child over one year of age, the pathologist generally places the case in one of the following three categories in order of frequency: 1) ischemic heart disease, 2) conditions or diseases grossly associated with sudden death, and 3) hearts that are normal at least on gross examination [1].

Heart disease is the most frequent cause of sudden death and, in turn, of these ischemic heart diseases in adults. Non-ischemic causes are, for example, respiratory diseases (pulmonary embolism and asthma), neurological diseases (cerebral hemorrhage and epilepsy). Even so, no cause of death is determined after a thorough post-mortem examination in approximately 5% of cases [2].

The genetic diagnosis of SCD is very complex, since it is still not easy to identify the causal pathogenic variant, since there are many genes involved, as in the case of channelopathies that present a Mendelian pattern of inheritance with different degrees of penetrance and variable expressivity of the disease [3], turning them into complex diseases, favoring the advancement of molecular tests that can understand the breadth and depth of heart diseases. These discoveries have directly affected the approach to snapshot autopsies by pathologists [4].

Molecular diagnosis has become especially useful in the investigation of SCD, not only to elucidate the cause of death, but also to identify risk factors because this type of disease has a family basis, of genetic origin. That can be monogenic and autosomal dominant, recessive, sex-linked, etc., and the autopsy can be the only possibility that a family can be referred to a cardiology hospital to receive adequate genetic counseling [5-7].

Studies to date have shown that ion channels can function as part of large complexes of macromolecules that play crucial roles in the transcription, translation, post-translational modification, degradation of all cardiac ion channels, among others [8-10].

Therefore, understanding the structure and dynamic signaling of multiprotein assemblies is vital to understanding heart function during disease processes [11], and exome analysis can help elucidating some of these aspects that can modify domains in proteins can cause certain pathogenicity.

For this, the hypothesis will be handled: Given the high genetic addition that the Colombian population presents, it is possible to find new gene switches of the ion channels and that may be associated with the events of MSC. Our objective was to identify groups of genes that are possibly associated with sudden cardiac death, through the analysis of five people who died due to sudden death with a negative toxicological result, for which the TruSight One panel (clinical exome) was obtained

Materials and Methods

Subjects

Five (5) cases associated with sudden cardiac death and five (5) controls were selected. The corresponding review of the autopsy protocols was carried out, considering the circumstances of death, clinical history, macro and microscopic studies, toxicology and virology analysis as appropriate, during the autopsy.

Inclusion and Exclusion Criteria

Negative cases in structural coronary heart disease, with microscopically normal cardiomyocytes, negative for toxicology and virology, under 40 years of age. Controls whose death was caused by violent death, excluding those by suicide.

DNA Extraction

Total DNA extraction was performed from blood that was in the evidence center of the National Institute of Legal Medicine and Forensic Sciences, using the QIAamp® DNA Blood Midi/Maxi kit, following the manufacturer's recommendations.

Preparation of Libraries

Sample library preparation was performed with the TruSight One Sequencing Panel Series (Illumina), which includes 4834 clinically relevant genes, using the Nextera XT Kit (Illumina). The quantification of the libraries was carried out using the Quantitating dsDNA Kit using the Quantus™

Fluorometer Instrument and following the manufacturer's recommendations.

NGS Sequencing

Paired-end NGS sequencing of 2x150 bp with the MiSeq kit (Illumina Inc., San Diego, CA. USA) using MiSeq Reagent V3 (150 cycles), according to the protocol of the commercial house, was performed. The alignment of DNA readings was performed against the reference genome GRCH37/hg19.

Variant Annotation

The detected variants were annotated using the bioinformatic tools SnpEff [12-14], which integrate the population databases: dbSNP [15], of the 1000 genomes project (1000 Genomes Project Consortium), the NHLBI Exome Sequencing Project (ESP) [16], database [17], and the gnomAD and exomAD databases (http://gnomad.broadinstitute.org). Variants with a MAF of 0.01 were filtered out. Information related to the association between human phenotype and causative genes was added with ClinVar [18,19]. In silico predictive algorithms were included: SIFT [20], PolyPhen-2 [21], MutationTaster [22], LRT [23], Mutation Assesor [24], FATHMM (Functional Analysis Through Hidden Markov Models) [25], MetaSVM [26], RadialSVM, LR, VEST3, CADD, GERP++ [27-29], to assess the pathogenicity of the identified variants. Determining the minor allele frequency (MAF) to filter variants. The norms and guidelines for the interpretation of sequence variants suggested by the American College of Medical Genetics and Genomics (ACMG) were followed for the classification of causality of each of the variants [30].

Prediction of SNP Impact on Protein Stability

I-Mutant3.0 tools were used to predict the stability of a protein-based on the presence and type of microvariant. For MuPro structural analysis allows the calculation of protein stability variations at arbitrary SNPs.

For the final predictive results of the I-Mutant 3.0 and Mupro tools, the results of the two previous bioinformatics tools were integrated, considering that if the prediction for the SNP is that stability decreases in two tools, the SNP would be considered as a high-risk ARX pathogen.

For the analysis of the effect of the SNP on the 3D structure of proteins and physicochemical properties, the HOPE server was used, which searches for 3D structures of proteins by collecting structural information from a series of sources, including calculations in the 3D coordinates of the protein. UniProt base sequence annotations and predictions from DAS services, are available at http://www.cmbi.ru.nl/hope/.

Results

Clinical exome sequencing was performed in five unrelated SUD cases, although the data provided covered sequence variants in 4834 genes, the analysis focused on 184 genes associated with heart disease or sudden death. Bioinformatics filtering was performed considering the quality of the variants, population frequency, information provided by the various databases and in silico prediction and following the recommendations of the ACMG/AMP group. Once the different filters were made, relevant variants were found in ten genes that are presented in (Table 1).

Cases	Gene	rsID	Func	Genetic Variant	Change AA	zygosity	MAF			
							ExAC	1000g	gnomAD exome	gnomAD genome
I	TRDN	rs372169818	exon2	c.G196A	p.V66I	het	0,04971	0.00019968	0,06909	Absent
	TTN	rs746749916	exon154	c.G48780T	p.W16260C	het	0,008302	Absent	0,01633	Absent
	TTN	.	exon186	c.T73979C	p.I24660T	het	Absent	Absent	0,004069	3.23E-05
	CAV3	rs753990961	exon2	c.A185G	p.Y62C	hom	0,008264	Absent	0,004063	Absent
I, II	DSP	rs78652302	exon24	c.A3701T	p.E1234V	het	0.0095	0.00399361	0.0095	0.0112
II	TTN	rs776534823	exon27	c.G5905A	p.A1969T	het	0,008243	Absent	0,008139	3.23E-05
	TTN	rs181189778	exon64	c.T16495A	p.S5499T	het	0.0003	0.00019968	0.0003	0.0003
II, V	DSG2	rs142841727	exon15	c.T2759G	p.V920G	het	0.0032	0.00319489	0.0036	0.0033

III	TRPM4	rs138603244	exon17	c.A2365G	p.S789G	het	0.0009	0.00319489	0.0005	0.002
	AKAP9	rs2230768	exon18	c.G4841A	p.R1614Q	het	0.0023	0.00898562	0.0018	0.0084
	ANK3	rs41274676	exon37	c.C4465T	p.P1489S	het	0.0019	0.00079872	0.0017	0.0019
	ANK3	rs201547988	exon37	c.C11159T	p.T3720M	het	0.0002	0.00019968	0.0002	0.0002
	TTN	rs759415579	exon168	c.C67100T	p.P22367L	het	0,008287	Absent	0,03257	Absent
IV	ANK2	rs61734478	exon38	c.G6634A	p.G2212S	hom	0.0047	0.0219649	0.0037	0.014
	CACNA1C	rs185788586	exon43	c.C5689T	p.R1897C	het	0.0075	0.00858626	0.0101	0.0018
V	TTN	rs75686037	exon27	c.C5093T	p.P1698L	het	0.0063	0.00698882	0.0072	0.0026

Table 1: Variants Selected for Analysis According to Population Frequencies with A MAF ≤ 0.05.

Of the sixteen variants detected, nine variants are of uncertain significance, two present conflicting interpretations of pathogenicity, the rest is benign or slightly benign. Ten variants associated with Catecholaminergic polymorphic ventricular tachycardia, Arrhythmogenic right ventricular cardiomyopathy, Dilated cardiomyopathy 1G, Progressive familial heart block type 1B, Long QT syndrome, Romano-Ward syndrome, Brugada syndrome, Primary dilated cardiomyopathy and Hypertrophic cardiomyopathy, (Table 2).

Cases	Change AA	CLNDN	CLNSIG
I	p.V66I	Catecholaminergic polymorphic ventricular tachycardia	Uncertain significance
	p.W16260C	---	Uncertain significance
	p.I24660T	---	Uncertain significance
	p.Y62C	---	Uncertain significance
I, II	p.E1234V	Arrhythmogenic right ventricular cardiomyopathy	Conflicting interpretations of pathogenicity
II	p.A1969T	Dilated cardiomyopathy 1G	Uncertain significance
	p.S5499T	Dilated cardiomyopathy 1G	Uncertain significance
II, V	p.V920G	Arrhythmogenic right ventricular cardiomyopathy	Conflicting interpretations of pathogenicity
III	p.S789G	Progressive familial heart block type 1B	Benign
	p.R1614Q	Long QT syndrome, Romano-Ward syndrome	Benign, Likely benign
	p.P1489S	---	Uncertain significance
	p.T3720M	---	Uncertain significance
	p.P22367L	---	Uncertain significance
IV	p.G2212S	Long_QT syndrome	Benign
	p.R1897C	Long QT syndrome, Brugada syndrome	Benign, Likely benign
V	p.P1698L	Hypertrophic cardiomyopathy, Dilated cardiomyopathy 1G	Benign, Likely benign

Table 2: Clinical Significance of the Variants Found in Five Cases of Unexplained Death.

In silico pathogenic predictors, pathogenic (D), probably pathogenic (Pp), and benign (T) variants were determined. A cutoff point of 20 were used for the CADD algorithm. Prediction of conservation of the sequence used a cutoff point greater than 4.4. Results are shown in (Table 3).

Cases	Change AA	SIFT pred	Polyphen2 HDIV pred	Polyphen2 HVAR pred	LRT pred	Mutation Taster pred	Mutation Assessor pred	FATHMM pred	Radial SVM pred	LR pred	CADD phred	GERP++ RS
I	p.V66I	T	D	D	D	D	L	T	T	T	19.57	5.82
	p.W16260C	D	D	D	.	D	H	D	D	D	12.74	5.87
	p.I24660T	D	P	P	.	D	L	T	T	T	14.53	5.59
	p.Y62C	D	D	D	D	D	M	D	D	D	17.36	3.44
I, II	p.E1234V	T	D	D	D	N	L	T	T	T	20.4	5.2
II	p.A1969T	D	D	D	.	D	L	T	T	T	12.34	5.12
II	p.S5499T	D	D	D	.	D	M	T	T	T	14.29	6.03
II, V	p.V920G	T	P	B	N	N	L	T	T	T	4378	2.5
III	p.S789G	T	D	P	D	D	M	T	T	T	26.5	5.09
	p.R1614Q	T	P	B	N	N	N	T	T	T	8925	1.43
	p.P1489S	D	D	D	D	D	M	T	D	D	17.55	5.69
	p.T3720M	D	D	P	N	D	L	T	T	T	16.37	5.3
	p.P22367L	D	D	D	.	D	H	T	D	D	19.08	6.03
IV	p.G2212S	.	D	B	N	D	L	T	T	T	13.77	3.85
	p.R1897C	T	D	P	N	N	N	T	T	T	8708	4.26
V	p.P1698L	D	D	D	.	D	M	T	T	T	11.85	5.05

Table 3: Prediction of pathogenicity of SNPs in silico for SIFT, PolyPhen2, Mutation Taster, LRT, Mutation Assesor, FATHMM, MetaSVM, RadialSVM, LR, CADD and GERP++.

Prediction of SNP Impact on Protein Stability

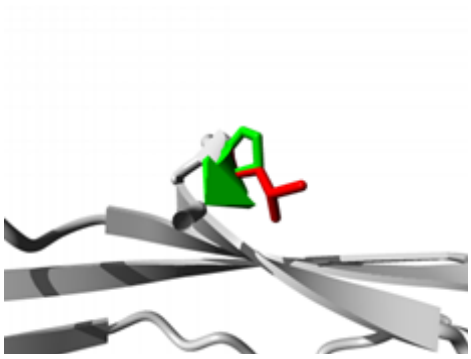
Changes in the protein stability of variants were examined using I-Mutant 2.0 and MUpro software (Table 4). The results predicted either an increase or a decrease in the free energy upon amino acid substitutions. I-Mutant, it predicts the stability by examining the Gibbs free energy by the ΔΔG value = ΔG (New protein) - ΔG (Wild type) in kcal/mol, which is calculated at pH 7 and 25 °C. Scores <0 are predicted by the algorithm to indicate decreased stability, whereas scores >0 are considered to indicate increased stability. The DDG (ΔΔG) prediction by I-Mutant 2.0 showed that the 17 (72.3%) nsSNPs had a decreased stability value with DDG < 0 whereas −10 (22.7%) nsSNPs had an increased stability value with DDG > 0. With regard to MUpro reported that 1 (4.5%) substitutions increased the stability protein structure while 21 (95.5%) substitutions decreased it.

Gen	rsID	AA Change	I-Mutant 3.0 Prediction	RI	DDG score prediction	MuPro Prediction	MuPro Score
TRDN	rs372169818	p.V66I	Decrease	6	-0.45	Decrease	-0.76372025
TTN	rs746749916	p.W16260C	Decrease	8	-1.64	Decrease	-0.3799672
TTN	-------	p.24660T	Decrease	7	-2.01	Decrease	-1.8524381
CAV3	rs753990961	p.Y62C	increase	2	-1.07	Decrease	-0.94970534
DSP	rs78652302	p.E1234V	Increase	2	0.09	Decrease	-0.57600156
TTN	rs776534823	p.A1969T	Decrease	3	-0.6	Decrease	-1.0005569
TTN	rs181189778	p.S5499T	Decrease	0	-32	Decrease	-0.65558429
DSG2	rs142841727	p.V920G	Decrease	9	-2.02	Decrease	-2.2447524
TRPM4	rs138603244	p.S789G	Decrease	9	-1.12	Decrease	-1.3670468

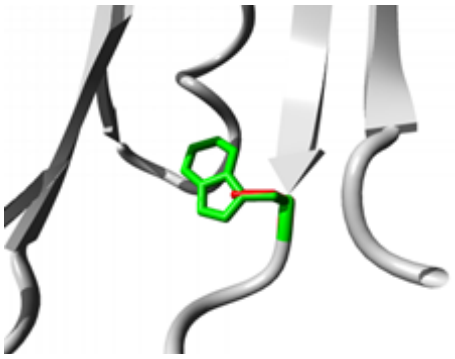
AKAP9	rs2230768	p.R1614Q	Decrease	4	-0.33	Decrease	-0.59876134
ANK3	rs41274676	p.P1489S	Decrease	9	-1.9	Decrease	-1.4299976
ANK3	rs201547988	p.T3720M	Decrease	4	-0.39	Decrease	-0.37011007
TTN	rs759415579	p.P22367L	Increase	2	-0.3	Decrease	-0.38920806
ANK2	rs61734478	p.G2212S	Decrease	4	-1.01	Decrease	-0.40436484
CACNA1C	rs185788586	p.R1897C	Decrease	6	-1.08	Decrease	-0.67510594
TTN	rs75686037	p.P1698L	Decrease	4	-0.44	Increase	0.83737709

Table 4: Protein Stability of the Snps by I-Mutant 3.0 and Mupro: Mupro: Predicts that the Mutation Could Relatively Destabilize Protein or not. Score Smaller than Zero, Means that the Substitution Decreases the Protein Stability. Conversely, the Score >0 Means that the Mutation Increases Protein Stability. I-Mutant: Predicts Whether A Point Mutation Stabilizes Or Destabilizes The Native Protein Structure Based On The Free Energy Change (ΔΔG).

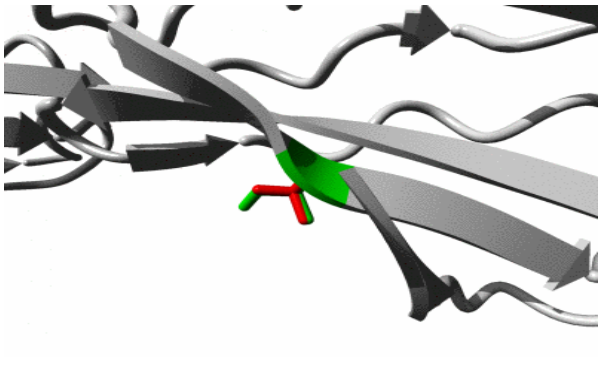
The analysis 3D structure prediction, visualization and physiochemical changes of substitutions: In order to study the biophysical properties of these mutations, Project HOPE server was used to serve this purpose, RaptorX was used to predict a 3D structure model for TTN, TRPM4 and KCND3 protein (Figure 1).



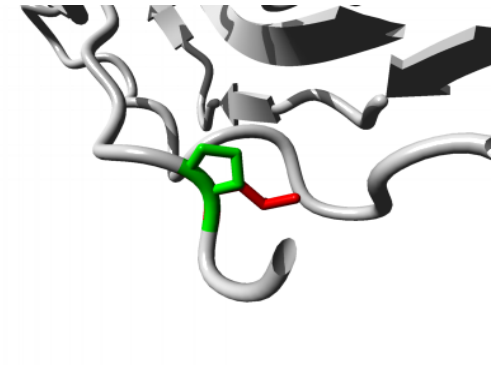
a-1) p.P1698L : Proline a Leucine TTN



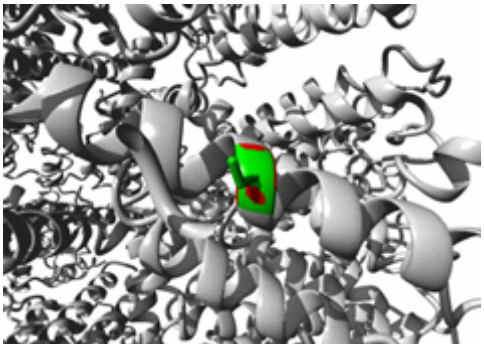
a-2)p.W16260C: Tryptophan a Cysteine TTN



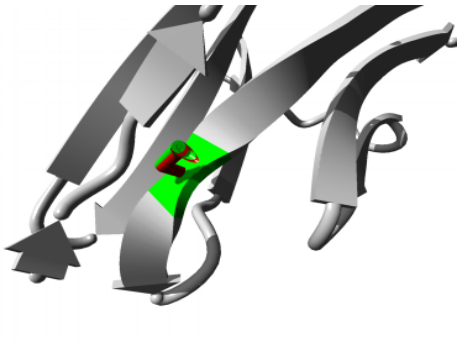
a-3) p.I24660T: Isoleucine to Threonine TTN



a-4) p.P22367L: Proline to Leucine TTN



b) p.S789G: Serine to GlycineTRPM4



c) p.R571H: Arginine to Histidine KCND3

Figure 1: Close-up of the pathogenic variant TTN, TRPM4 and KCND3 genes. The protein is grey, and the side chains of the wild type and mutant residues are shown and colored green and red, respectively. Variant: a-1) p.P1698L, a-2) p.W16260C, a-3) p.I24660T, and a-4) p.P22367L; b) p.S789G, and c) p.R571H.

For Figure 1:

TTN gen: a-1) (p.P1698L): the amino acid Proline changes to Leucine at position 1698. Wild-type and mutant amino acids differ in size. The mutant residue is larger, this could lead to bumps, the hydrophobicity of the wild-type and mutant residue differs. Hydrophobic interactions, either in the core of the protein or on the surface, will be lost.

a-2) p.W16260C: Wild-type and mutant amino acids differ in size, the mutated residue is smaller than the wild-type residue, the mutation will cause an empty space in the core of the protein. The mutated residue is located in a domain that is important for the binding of other molecules and in contact with residues in a domain that is also important for binding. The mutation could disturb the interaction between these two domains and, as such, affect the function of the protein.

a-3) p.I24660T: Wild-type and mutant amino acids differ in size, the mutated residue is smaller than the wild-type residue, the mutation will cause an empty space in the core of the protein, the hydrophobicity of the wild-type and mutant residue differs. The mutation will cause the loss of hydrophobic interactions in the core of the protein.

a-4) p.P22367L: Wild-type and mutant amino acids differ in size. The mutated residue is larger than the wild-type residue, the wild-type residue was buried in the core of the protein. The mutant residue is larger and probably won't fit.

TRPM4: b) p.S789G: Wild-type and mutant amino acids differ in size, the mutated residue is smaller than the wild-type residue. The mutation will cause an empty space in the core of the protein.

KCND3: c) p.R571H: There is a difference in charge between the wild-type and the mutated amino acid, The charge of the wild-type residue will be lost, which can lead to loss of interactions with other molecules or residues, Wild-type and mutant amino acids differ in size. The mutated residue is located in a domain that is important for the main activity of the protein. Mutation of the residue could alter this function.

The analysis of the clinical exome in case I identified six variants: one in the DSP gene (rs78652302) heterozygous, associated with arrhythmogenic right ventricular cardiomyopathy (ARVC), however, in clinical significance it appears conflicting pathogenicity interpretation; a heterozygous variant in the TRDN gene (rs372169818), of moderate impact, associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), of uncertain significance and four variants of uncertain significance in the TTN genes (rs746749916 and p.I24660T), in the gene CAV3 (rs753990961) and in the DSPP gene (p.D1143E). All four variants are heterozygous, nonsense, and of moderate impact.

In case II, four variants were identified: two in the TTN gene (rs776534823 and rs181189778) heterozygous, nonsense of moderate impact, of uncertain significance, associated with Limb-gerdle muscular dystrophy, type 2 diabetic cardiomyopathy and dilated cardiomyopathy 1G; one in the DSP gene (rs78652302) also reported for case 1; and one in the DSG2 gene (rs14841727) heterozygous, nonsense, of moderate impact, associated with ARVC, type 2 diabetic myopathy and cardiovascular phenotype, presents conflict of interpretation of pathogenic.

In Case III, five variants were identified: one in the TRPM4 gene (rs138603244) heterozygous, nonsense of moderate impact, associated with progressive familial heart block type 1B, cardiovascular phenotype, benign; one in the AKAP9 gene (rs2230768) heterozygous, nonsense, of moderate impact, associated with LQT syndrome, Romano-Ward syndrome, cardiovascular phenotype, considered benign/probably benign; three variants of uncertain significance in the TTN genes (rs759415579) and in the ANK3 gene (rs201547988 and rs41274676) the three heterozygous, nonsense and moderate impact variants.

In case IV, two variants were identified: one in the ANK2 gene (rs61734478) homozygous, nonsense, of moderate impact, associated with LQT syndrome, cardiovascular phenotype, benign; one in the CACNA1C gene (rs185788586) heterozygous, nonsense, moderate impact associated with LQT syndrome, Brugada syndrome, Timothy syndrome, cardiovascular phenotype, benign/probably benign.

In case V, two variants were identified: one in the TTN gene (rs75686037) heterozygous, nonsense, of moderate impact, associated with hypertrophic cardiomyopathy, Limb-gerdle muscular dystrophy, type 2 dilated myopathy, Markesbery-Griggs type distal myopathy, cardiomyopathy dilated 1G, hereditary myopathy with early respiratory failure, early-onset diabetic myopathy, diabetic myopathy with fatal cardiomyopathy, dominant diabetic myopathy, recessive diabetic myopathy, benign/probably benign

Discussion

Authors of this study, the use of NGS allowed the identification of possible variants associated with cardiac disease in five victims of sudden cardiac death (Table 1).

In the case of the infants, while each of these variants alone may not have contributed significantly to death, it may be plausible that each of the variants observed in these cases partially contributed to an overall genetic predisposition, in an infant going through critical stages in its development, it is possible that these factors together may have contributed to the death of infants, which is known as "polygenic risk score", an additive effect caused by multiple low-risk variants, each with a low pathogenic effect, but which collectively can produce sub threshold functioning of physiological pathways of interaction [28,29]. However, more tests should be done.

In all five cases the deaths occurred during sleep. Cardiac diseases responsible for sudden death are characterized by autosomal inheritance, locus heterogeneity, and variable expressivity [30], which is consistent with our results.

The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published guidance for interpretation of sequence variants based on in silico predictions, population frequencies and functional analysis, pathogenic and likely pathogenic variants have additional implications for sudden death diagnostic purposes [31].

We found three variants with pathogenicity interpretation conflict in the DSP, DSG2 and MYBPC3 genes and seven variants of unknown significance and based on ACMG/AMP, which is very complicated in the forensic context.

Which were limited in our study, in addition, the antemortem clinical history of the deceased persons was not available.

The proper interpretation of the variants found is very important, since the interpretation of pathogenicity cannot be underestimated or overestimated, since it could bring consequences of uncertainty for families, therefore the analysis must be multidisciplinary and use as many of analysis tools to determine if a variant is pathogenic.

Conclusion

This Clinical exome analysis of five unrelated SUD cases revealed 15 protein-altering variants (MAF < 0.05), some of them clinically relevant, and the need arises to contact relatives for more detailed studies. The detection of variants of uncertain significance leads to the need to develop specific forensic guidelines that allow an adequate interpretation of rare genetic variants, together with a multidisciplinary team.

The variants found in the TRDN, TTN, CAV3, DSP, DSG2, TRPM4, AKAP9, ANK3, ANK2, and CACNA1C genes alone would not explain the cause of death, however, considering the frequencies population, the results of pathogenicity predictors, the presence of two or more variants in the same gene or different genes could lead to sudden cardiac death.

Sudden unexpected death of infants or adults can be attributed to a myriad of different diseases, only one of which is a hereditary heart disease, and being able to explore the possibilities of other diseases in a deceased person brings many challenges, especially when not accounted for. With the medical history.

Acknowledgments

The financial support was provided by the University of Magdalena and the University of Applied Sciences and Environmental Sciences UDCA, within the agreement they have with the National Institute of Legal Medicine and Forensic Sciences.

Ethical Approval

Ethical approval for this study principles contained in the updated Declaration of Helsinki were followed, as well as Laws, Decrees, and Resolutions related to viscerotomies and the use of forensic samples for research and teaching at the National Institute of Legal Medicine and Forensic Sciences.

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