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Highlights

- Molecular autopsy should be implemented in forensic protocols
- Nearly 40% of sudden death young cases carry a cardiac potentially pathogenic variant
- It is crucial to undertake a careful genetic analysis in a clinical context
- Genetic analyses help to identify relatives at risk of sudden death

Post-mortem genetic analysis in juvenile cases of sudden cardiac death

Short Title: Genetic analysis in a forensic juvenile cohort

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Conflict of Interest

Dr Ramon Brugada is consultant of Ferrer-inCode. The other authors declare no conflicts of interest to disclose.

Abstract

Background. The reason behind a sudden death of a young individual remains unknown in up to 50% of postmortem cases. Pathogenic mutations in genes encoding heart proteins are known to cause sudden cardiac death. Objective. The aim of our study was to ascertain whether genetic alterations could provide an explanation for sudden cardiac death in a juvenile cohort with no-conclusive cause of death after comprehensive autopsy. Methods. Twenty-nine cases < 15 years showing no-conclusive cause of death after a complete autopsy were studied. Genetic analysis of 7 main genes associated with sudden cardiac death was performed using Sanger technology in low quality DNA cases, while in good quality cases the analysis of 55 genes associated with sudden cardiac death was performed using Next Generation Sequencing technology. Results. Thirty-five genetic variants were identified in 12 cases (41,37%). Ten genetic variants in genes encoding cardiac ion channels were identified in 8 cases (27,58%). We also identified 9 cases (31,03%) carrying 25 genetic variants in genes encoding structural cardiac proteins. Nine cases carried more than one genetic variation, 5 of them combining structural and non-structural genes. Conclusions. Our study supports the inclusion of molecular autopsy in forensic routine protocols when no conclusive cause of death is identified. Around 40% of sudden cardiac death young cases carry a genetic variant that could provide an explanation for the cause of death. Because relatives could be at risk of sudden cardiac death, our data reinforce their need of clinical assessment and, if indicated, of genetic analysis.

Keywords

Pediatric, Adolescent, Sudden Cardiac Death, Genetics, Next Generation Sequencing, Forensics

Introduction

Sudden death in people younger than 15 years old is a rare event, with an incidence between 1-5/100,000 individuals each year in developed countries (1). Despite this low prevalence, when a death occurs in this juvenile population, it carries a tremendous impact in both the family and community. Sudden death constitutes one of the most important unsolved challenges in the practice of forensic pathology. Several studies have reported that most part of sudden deaths in the young (< 40 years) are of cardiac origin (sudden cardiac death -SCD-), mainly caused by structural heart abnormalities identifiable at autopsy (cardiomyopathies) (2). However, in 10%-35% of these deaths, no structural alterations can be identified. In these cases a channelopathy, a genetic disease of the cardiac ion channels, is suspected (3-5). Both groups of cardiac alterations are due to inherited genetic defects, thus family members of the deceased individual are at risk of sudden death (6). This fact carries important implications in diagnosis and counselling of relatives. Though, the application of genetic testing in routine forensic investigation, to benefit diagnosis and possible family prevention, remains still very limited (7).

Currently, numerous genes have been associated with SCD but most part in low frequency (8, 9). However, in these last years, genetic research has focused on the identification of pathogenic mutations in seven main genes (*SCN5A, KCNQ1, KCNH2*, *KCNE1, KCNE2, KCNE3*, and *RyR2*) associated with channelopathies (like Brugada Syndrome-BrS-, Long QT Syndrome -LQTS-, Short QT Syndrome -SQTS-, and Catecholaminergic Polymorphic Ventricular Tachycardia -CPVT-) and 7 main genes (*MYBPC3, MYH7, PKP2, DSC2, DSP, DSC2,* and *LMNA*) associated with cardiomyopathies (like Hypertrophic Cardiomyopathy -HCM-, Arrhythmogenic Right Ventricular Cardiomyopathy -ARVC-, and Dilated Cardiomyopathy -DCM-).

Genetic analysis of these genes can help in the identification of the cause of death, even using mRNA (10), improving the evaluation of relatives at potential risk. Traditional Sanger sequencing is expensive to undertake this extensive analysis. However new genetic technologies (Next generation Sequencing -NGS-) has emerged as a cost-effective technology for broad genetic studies (11-13). The ability to perform analysis of large amount of genes at once has been brought to the clinical arena of several medical specialities, including cardiology. It is no secret though, that the

large amount of data generated is causing difficulties in clinical interpretation, especially when dealing with genetic variants of unknown significance (GVUS) or genetic variants in less common genes. In our study we analyzed a cohort of post-mortem cases, aged less than 15 years old, in order to investigate the role of genetics in death causality.

Methods

Forensics

A complete autopsy examination was performed according to current international regulations (14). Our inclusion criteria was: (a) age < 15 years (b) non-conclusive cause of death after complete autopsy (c) no signs of congenital heart alterations, cardiac infarct or other macroscopic anomalies (d) blood obtained < 48 hours after death. The study was approved by the ethics committee of our Hospital, and follows the Helsinki II declaration.

DNA sample

Genomic DNA was extracted with Chemagic MSM I from post-mortem whole blood (Chemagic human blood). DNA cases were checked in order to assure quality (Absorbance 260/280:260/230 should be a minimum 1,8:2,2 respectively), and quantify before processing to get the 3µg needed for the NGS strategy. Spectrophotometric measurements are performed to assess quality ratios of absorbance; DNA concentration is determined by fluorometry (Qubit, Life Technologies). DNA integrity was assessed on a 0,8% agarose gel.

DNA quality/integrity divided our cohort of 29 cases in two groups. The first group included 18 cases with low DNA quality/integrity and analyzed using Sanger sequencing (*SCN5A, KCNQ1, KCNH2, KCNE1, KCNE2, KCNE3* and *RyR2*). The second group included 11 cases analyzed using NGS technology (55 genes associated with SCD). Confirmation of variants identified in NGS analysis was performed using Sanger sequencing. As an internal control, two cases included in the second group were processed by both methods.

Sanger sequencing

The genetic study included direct sequencing of *SCN5A* (NM_198056), *KCNQ1* (NM_000218), *KCNH2* (NM_000238), *KCNE1* (NM_000219), *KCNE2* (NM_172201), *KCNE3* (NM_005472), and *RyR2* (NM_001035). The exons and exon-intron boundaries of each gene were amplified (Verities PCR, Applied Biosystems, Austin, TX, USA), the PCR products were purified (Exosap-IT, Affymetrix, Inc. USB® Products, Cleveland, OH, USA) and they were directly sequenced in both directions (Big Dye Terminator v3.1 cycle sequencing kit and 3130XL Genetic Analyzer, both from Applied Biosystems) with posterior SeqScape Software v2.5 (Life Technologies) analysis comparing obtained results with the reference sequence from hg19. The identified variations were compared with DNA sequences from 300 healthy Spanish individuals (individuals not related to any patient and of the same ethnicity; 600 alleles), as control cases, and contrasted with Human Gene Mutation Database -HGMD- (http://www.hgmd.cf.ac.uk/ac/index.php), HapMap (http://hapmap.ncbi.nlm.nih.gov/), 1000 genomes project (http://www.1000genomes.org/), and Exome Variant Server -EVS- (http://evs.gs.washington.edu/EVS/). Sequence variants were described following the HGVS rules (http://www.hgvs.org/), and checked in Mutalyzer (https://mutalyzer.nl/).

NGS sample preparation

The DNA was fragmented by Bioruptor (Diagenode). Library preparation was performed according to the manufacturer's instructions (SureSelect XT Custom 0.5-2.9Mb library, Agilent Technologies, Inc). After capture, the indexed library was sequenced in a six-sample pool cartridge. Sequencing paired-end process was developed on MiSeq System (Illumina) using 2x150 bp reads length.

Custom Resequencing panel

We selected the most prevalent 55 genes involved in SCD-related pathologies, accordingly to available scientific literature (8, 9). The genomic coordinates corresponding to these 55 genes (Table 1) were designed using the tool eArray (Agilent Technologies, Inc.). All the isoforms described at the UCSC browser were included in the design. The biotinylated cRNA probe solution

was manufactured by Agilent Technologies and provided as capture probes. The final size was 432,512kbp of encoding regions and ´UTR boundaries. The coordinates of the sequence data is based on NCBI build 37 (UCSC hg19).

Bioinformatics

The secondary bioinformatic analysis of the data obtained included a first step trimming of the FAST-Q files with an in-house developed method. The trimmed reads are then mapped with GEM II and output is joined and sorted and uniquely and properly mapped read pairs were selected. Finally, variant call over the cleaned BAM file is performed with SAMtools v.1.19, GATK v2.7-4 together with an in house developed method to generate the first raw VCF files (see supplemental material). Variants were annotated with dbSNP IDs, Exome Variant Server and the 1000 Genomes browser, in-home database IDs and Ensembl information, if available.

Tertiary analysis was then performed. For each genetic variation identified, allelic frequency was consulted in EVS and 1000 genomes database. In addition, HGMD was also consulted to identify pathogenic mutations previously reported. *In silico* prediction of pathogenicity of novel genetic variations was assessed in CONDEL software (CONsensus DELeteriousness scores of missense SNVs) (http://bg.upf.edu/condel/), and PROVEAN (Protein Variation Effect Analyzer) (http://provean.jcvi.org/index.php). Alignment of DNA sequences for diffrent species was also performed for these novel variations using Uniprot database (http://www.uniprot.org/).

Confirmation of variants identified by NGS

Non-common (Minor Allele Frequency –MAF- < 1%) genetic variants were confirmed by Sanger method as mentioned before in “Sanger sequencing”.

Results

A total of 29 cases collected at Institut de Medicina Legal de Catalunya (IMLC), from April 2012 until June 2013, were included in our study. All cases included in our study were < 15 years old

(mean age 3.29 years old, with a wide range of death from 21 days to 14 years old) and complete autopsy concluded an undetermined cause of death. Toxicological results were negative in all cases. Macroscopic analysis did not showed any anomaly. Microscopic/histological analyses were also negative in all cases except for two which showed a slight fibrosis and patchy myocarditis. Body weight, body size, and heart weight was within appropriate limits for age (Table 2). Equally gender ratio was observed (Figure 1 and Table 2). We identified the highest mortality in individuals under one year of age (15 of the 29 cases -51.72%-), involving 5 males and 10 females (Figure 1 and Table 3). Also, the cases were classified by cause of death as: sudden death while sleeping -21 cases-, sudden death during emotion, stress or exercise -5 cases-, and sudden death by drowning -3 cases-. Death during sleeping was the most common scenario (21 of 29 cases -72.41%-), involving 8 males and 13 females. (Figure 1; Table 2 and 3).

The cases with low DNA quality/integrity were analyzed using Sanger sequencing (18 cases). The seven main SCD-related genes were analyzed (*SCN5A, KCNQ1, KCNH2, KCNE1, KCNE2, KCNE3* and *RyR2*). The second group included 11 cases with high DNA quality/integrity which allowed the genetic analysis using NGS technology (55 genes associated with SCD). Two cases were analyzed using both methods in order to perform and internal control of protocols and analysis. Both methods identified the same genetic variants (Table 4).

After genetic analysis, we did not identify any previously reported pathogenic genetic variant nor novel/rare variant in 17 cases (16 analyzed by Sanger and 1 by NGS). Therefore, 12 cases (41.37%) carried at least one rare/novel or previously reported genetic variant located in 18 different genes (9 males –75%-). Out of these, 3 cases carried only one genetic variation, 3 cases carried two genetic variations, 1 case carried 3 genetic variations, 2 cases carried 4 genetic variations, and 3 cases carried 5 genetic variants (Figure 2). Hence, a total of 35 genetic variants (10 genetic variants in 8 genes encoding cardiac ion channel proteins –*SCN5A, KCNQ1, KCNH2, RyR2, GPD1L, CACNA1C, CACNB2* and *ANK2*- and 25 genetic variants located in 10 genes encoding structural proteins –*TTN, DSC2, DSP, MYBPC3, DMD, FBN1, MYH7, MYH6, TGFB3,* and *PKP2*-) were identified in these 12 cases (Figure 3 and Table 3). Of them, 27,58% of all cases carried at least one genetic variation in any of the arrhythmogenic genes studied. In addition, 31,03% of all cases

carried at least one genetic variation in any of the structural genes analyzed in our NGS panel (Figure 2). Regarding all 35 genetic variations identified, 31 were *missense* (88.57%) -14 novel-, one already known *nonsense* variation -*PKP2*_p.R413STOP- (2.85%), one novel deletion causing an *in-frame* deletion *CACNB2*_c.del47CGG> (2,85%), and two novel *intronic* variations (*MYH7_c*.5157+4A>G and *TGFB3*_c.755-5T>C) (5.71%) (Figure 3 and Table 4).

Sanger sequencing (18 cases)

In low quality DNA cases we identified 3 genetic variants (*KCNH2*_p.R892C, *SCN5A*_p.E1685D, *SCN5A*_p.E1685V) in 2 cases (Table 4). The *KCNH2*_p.R892C variant was previously reported (rs201627778) with very low MAF (0.0116/0.0227/0.0154) and *in silico* prediction classified it as damaging. Autopsy report described the cause of death being drowning. The two genetic variants identified in *SCN5A* were novel and were both predicted to be deleterious. Curiously, both genetic variants are in the same aminoacid, inducing a diverse combination of potential changes in the protein sequence -GAG (E,Glu) > GTG (V,Val)/GAT (D,Asp)/GTT (V,Val)-.

NGS sequencing (11 cases)

We identified 32 genetic variants in 10 cases. Two cases carried only one genetic variation (20%), 2 cases carried two genetic variations (20%), 1 case carried 3 genetic variations (20%), 2 cases carried 4 genetic variations (10%), and 3 cases carried 5 genetic variants each (30%)(Figure 2). Of these 32 genetic variants, 25 were identified in structural genes (78.12%), and 7 variants were identified in genes encoding for arrhythmogenic proteins (21.87%)(Figure 2). Sixteen of the 32 genetic variants were previously reported and 15 were novel. Specifically, four variants were classified as pathogenic in HGMD database and had been previously associated with cardiomyopathies (*DSC2*_p.L732V -CM0910201, ARVC-, *MYBPC3*_p.R896H -CM992932, HCM-, *DMD*_p.A2395T -CM072994, Muscular Dystrophy-, and *PKP2*_p.R413STOP -CM660431, ARVC-) (Table 4).

Specifically, the first case carried two genetic variants, one novel (*TTN*_p.G21091S) and other one (*DSC*2_p.L732V) classified as pathogenic (CM0910201-ARVC-). Autopsy reported no histological alterations and circumstance of death was sleeping. The second case carried a novel genetic

variation in *GPD1L*, predicted as potentially pathogenic. This gene is associated with BrS, causing death usually at rest. He died while sleeping. The third case carried 3 genetic variations (*CACNA1C*_p.R2021Q, *DSP*_p.A306T and *MYBPC3*_p.R896H). The latter was published as pathogenic (CM992932) and associated with HCM. The other two variants remained of uncertain significance after *in silico* prediction; one was located in an arrhythmogenic gene (*CACNA1C*) and the other one in a desmosomal gene (*DSP*). He died while sleeping, without histological alterations. The fourth case carried 5 genetic variations, two previously reported (*DSP*_p.M316V and *DMD*_p.A2395T) and 3 novels (*DSP*_p.R105Q, *CACNB2*_c.del47CGG> and *FBN1*_p.P392R). One of reported variations has been associated with Muscular Dystrophy (MD) (CM072994). All other variations were predicted as potentially pathogenic. This case carried an *in-frame* deletion (*CACNB2*_c.del47CGG>). The patient died due to drowning with no histological changes. The fifth case carried two novel genetic variations in TTN protein, predicted both as potentially pathogenic (*TTN*_p.R1859S and *TTN*_p.P29832T). However, no histological alterations were reported after autopsy and situation of death was at rest. The sixth case carried four novel genetic variations (*RyR2*_p.L73V, *MYBPC3*_p.G800R, *PKP2*_p.G5D and one intronic *MYH7*_c.5157+4A>G). Autopsy reported death under emotional stress with no histological changes. The seventh case carried 5 genetic variations (*KCNQ1*_p.G621S, *MYH6*_p.S1734L, *TTN*_p.E15868K, *TTN*_p.M8580I and *TTN*_p.A5671S), only one novel (*TTN*_p.A5671S) and all predicted as neutral except one missense variation in TTN (*TTN*_p.E15868K). No histological alterations were reported and autopsy data reported that the patient died while resting. The eighth case analyzed by NGS technology also carried 5 genetic variants (*DSC2*_p.V597F, *CACNA1C*_p.R1937C, *ANK2*_p.P2383L, *TGFB3*_c.755-5T>C and *DMD*_p.Q1443R), predicted as uncertain significance except one (*CACNA1C*_p.R1937C), predicted as damaging. Histological report showed no alterations, and death occurred during stress. The ninth case carried only one genetic variant (*TTN*_p.E25693D), predicted as unclear after *in silico* prediction. No histological alterations were identified during autopsy and died at rest. The last case carried four genetic variants, three classified uncertain after *in silico* prediction (*MYH7*_p.L1591Q, *TTN*_p.V31995A and *TTN*_p.A20255P) and one *nonsense* variation (*PKP2*_p.R413STOP), previously reported

associated with ARVC (CM660431). This patient died during exercise and post-mortem histological study identified myocarditis (Table 3 and 4; Figure 2 and 3).

Discussion

Our study identifies genetic alterations associated with SCD that provide a cause of death in a juvenile post-mortem cohort. Our cohort showed a global gender ratio 1:1, in divergence with published data about higher incidence of sudden death in male gender, mainly at young ages (15). Specifically, in individuals younger than 1 year old, the number of females studied was higher (3:1). This fact could be a spurious result due to reduced number of cases. Regarding the cause of death, it has been previously reported that the majority of SCDs in the very young (0 to 5 years) did not occur while doing sport or moderate to vigorous physical activity, but most occurred at home, while sleeping (16-18). In addition, as the age increases (10–19 years) there is a higher likelihood of an event to occur during physical activity or while doping sport (24%) (19). Our cohort is in harmony with these studies, showing most part of deaths while sleeping at the early ages and during emotion, stress and exercise after age 10.

Regarding genetic alterations, we identified 41,37% of cases carrying at least one pathogenic and/or potentially pathogenic genetic variation that could be responsible of the death. This percentage is higher than observed in other postmortem studies probably because we analyzed, for the first time, arrhythmogenic and structural genes together, while previous reports focused on arrhythmogenic genes only. Concerning arrhythmogenic genes, 27,58% of our cases carried at least one pathogenic and/or potentially pathogenic genetic variation. These results are in concordance with previous studies of similar cohorts (20). Regarding cases less than one year of age, we identified a plausible genetic cause of death in 3 out of 15 cases (20%), two in arrhythmogenic genes, and one in a structural gene, in agreement with previous reports (3, 21-24), which identified a pathogenic variation associated with a cardiac channelopathy in 10%-15% of infants and nearly 20% of non-infant cases. In contrast, other studies have reported low rates of genetic carriers but their cohorts included cases in whom the cause of death was completely unknown (1, 5, 25-27).

Genetic screening using Sanger sequencing identified 3 genetic variants in 2 cases out of 18 (11.11%). The percentage of identified variants with this technology is similar to previous reports -11% (28), 13.5% (3), 15% (22)-. To our knowledge only in one report the percentage was higher (26%) (20). It may be due to a wider range of ages (1-69 years old) included in the cohort analyzed. In contrast, in another report the percentage was lower (4%) (29) but the cohort analyzed included just SIDS cases. Regarding our subgroup of SIDS, we identified 3/15 (20%) cases carrying a potentially pathogenic variation. Our percentage is superior because Evans et al. only analyzed the *HCN2* and *HCN4* genes. In some of our SIDS cases analyzed, different genetic variants were identified in structural genes. It is already reported several cases of SIDS carrying genetic variations in genes encoding structural proteins associated with HCM (30). As discussed in the mentioned report, SIDS cases had a completely normal heart suggesting that HCM was so incipient that it may not be diagnosed even during autopsy. Despite that it cannot be proven that these are causative variations, because the autopsy showed a structurally normal heart, they could certainly behave like modulating variants of the phenotype.

In cases analysed using NGS technology, a total of 10 cases carried at least one genetic variation (90.9%). The higher percentage of genetic variations identified by NGS in comparison to Sanger is essentially due to the major number of genes screened by NGS. Four cases carried one genetic variation classified as pathogenic. All other genetic variations were predicted potentially pathogenic or GVUS after *in silico* prediction. One of the current challenges in clinical interpretation of genetic data is the definition of pathogenicity. To date, no clear description is established, and most part of genetic variant remains as GVUS. We believe that several items should be included in the definition of pathogenicity such as *in silico* prediction, *in vitro* and *in vivo* studies, as mentioned recently (31). Without doubt, family segregation plays a key role in defining causality. Unfortunately, sudden death families may be small, and there is important variable expressivity in the phenotypes. It has been reported that young people carrying more than one genetic variant potentially associated with any SCD-disease often manifest earlier onset or more severe presentation of both channelopathies (32), and cardiomyopathies (33).

At our point of view, two interesting cases are included in our cohort. The first case showed patchy inflammatory infiltrates concluding myocarditis, and carried different genetic variations associated with ARVC or DCM. The role of inflammation in structural cardiac diseases has been largely suggested (34, 35) but no concluding data has been published, so far. It could be that myocarditis act as a trigger of a lethal arrhythmia previous to structural changes in a heart genetically predisposed. However, we believe that further genetic studies should be performed, including both clinical assessment and genetic analysis of family members. It occur the same in the second case showing slight fibrosis but no genetic variation was identified. Despite no genetic alteration identified, it is well known that structural cardiac diseases are progressive pathologies, and a reduced fibrosis in a situation of emotion/stress could be a trigger of a lethal arrhythmia. Except these two cases showing myocarditis and slight fibrosis, all cases included in our study showed no histological changes and no previous clinical symptoms of disease. This fact agrees with incomplete penetrance, the most dangerous hallmark of all SCD-diseases, often implying that sudden may be the first manifestation of the pathology.

Limitations

Our study has limitations that should be mentioned. The first one is the small number of cases. We believe that a larger cohort should be studied using both genetic technologies, referring to the same and other ethnicities. However, the results obtained in our study are in concordance with previous studies. The second limitation is the poor or absent clinical information of the cases available while they were alive which could have helped elucidate the cause of death. In addition, and as mentioned before, genetic and clinical analysis of relatives could be critical to elucidate the role of GVUS identified in our study. Therefore, the genetic results should be cautiously interpreted before translation into clinical practice. Third, a digenic/compound phenomenon occurs in most part of cases, and we cannot discard association of genetic alterations as the cause of death. Fourth, cases without genetic variation identified could carry a genetic defect in another gene not included in our NGS custom-panel, or could be caused by copy number variations (CNV), already reported as associated with arrhythmogenic syndromes leading to SCD, such as LQTS (36) and ARVC (37).

Finally, another limitation is the lack of *in vitro* studies of all GVUS in order to clarify their potentially pathogenic role. Although several bioinformatic tools were used, *in vitro* analysis could also help to elucidate the role of the GVUS in cardiac physiology.

Clinical Implications

It is crucial to undertake a careful genetic analysis in a clinical context, taking into account the medical information, situation surrounding the death, and also family information and their clinical investigation. The genetic data, while of limited value, especially in structural variations in a normal heart, should become another piece of information to help risk stratify the patients and family members, to adopt preventive and therapeutic strategies.

Conclusion

We have identified a potentially pathogenic genetic variation in 41% of SCD young cases (of which nearly 30% in arrhythmogenic genes), supporting the implementation of the molecular autopsy in forensic protocols. Despite present lack of knowledge in pathogenicity classification of ambiguous genetic variants, identification of pathogenic or potentially pathogenic genetic variations in cases of unexplained sudden death enables the undertaking of clinical assessment, genetic counselling and preventive measures of relatives at risk, with the aim to prevent new cases of sudden death in their families.

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Tables

DISEASE	GENES
Brugada Syndrome	*CACNA1C, CACNB2, GPD1L, HCN4, SCN5A*
Long QT Syndrome	*ANK2, CACNA1C, CAV3, KCNE1, KCNE2, KCNH2, KCNJ2, KCNQ1, RYR2, SCN4B, SCN5A*
Short QT Syndrome	*CACNA1C, CACNB2, KCNH2, KCNJ2, KCNQ1*
Catecholaminergic Polymorphic Ventricular Tachycardia	*CASQ2, KCNJ2, RYR2*
Hypertrophic Cardiomyopathy	*ACTC1, ACTN2, CAV3, CSRP3, GLA, JPH2, LAMP2, LDB3, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOZ2, PDLIM3, PLN, PRKAG2, RYR2, TCAP, TNNC1, TNNI3, TNNT2, TPM1, TTN, VCL*
Dilated Cardiomyopathy	*ACTC1, ACTN2, CAV3, CRYAB, CSRP3, DES, DMD, DSC2, DSG2, DSP, EMD, LAMP2, LDB3, LMNA, MYBPC3, MYH6, MYH7, PKP2, PLN, SCN5A, SGCD, TAZ, TCAP, TNNC1, TNNI3, TNNT2, TPM1, TTN, VCL*
Arrhythmogenic Right Ventricular Cardiomyopathy	*DES, DSC2, DSG2, DSP, JUP, LMNA, PKP2, PLN, TGFB3, TTN*

Table 1.- List of the 55 SCD-related genes included in our panel and its association with the disease. Some genes are associated with more than one disease.

Case	Age	Gender	Situation of death	Histology	Toxicology	Body Weight (Kg)	Body Size (cm)	Heart Weight (gr)	Gene_Aminoacid	Gene Reference
1	47 d	female	Sleeping	negative	negative	4.725	52	25	-	-
2	10 y	male	Drowning	negative	negative	50	140	215	*KCNH2_p.R892C*	NM_000238.3
3	1 m	female	Sleeping	negative	negative	3.5	51	24	-	-
4	3 m	male	Sleeping	negative	negative	4.8	53	25	*TTN_p.G21091S* *DSC2_p.L732V*	NM_133378.4 NM_024422.3
5	8 m	female	Sleeping	negative	negative	9	66.5	40	-	-
6	12 y	male	Sleeping	negative	negative	51	160	264	*GPD1L_p.V337F*	NM_015141.3
7	18 m	female	Sleeping	negative	negative	12	78	54	*CACNA1C_p.R2021Q* *DSP_p.A306T* *MYBPC3_p.R896H*	NM_199460.2 NM_004415.2 NM_000256.3
8	2 m	female	Sleeping	negative	negative	4.8	54	36	-	-
9	3 y	male	Drowning	negative	negative	15	97	61	*DSP_p.R105Q* *DSP_p.M316V* *CACNB2_c.del47CGG>_* *DMD_p.A2395T* *FBN1_p.P392R*	NM_004415.2 NM_004415.2 NM_201596.2 NM_004006.2 NM_000138.4
10	2 y	male	Drowning	negative	negative	14	83	56.6	-	-
11	2 m	female	Sleeping	negative	negative	6.5	58	33	-	-
12	1 m	female	Sleeping	negative	negative	4	43	27	*SCN5A_p.E1685D* *SCN5A_p.E1685V*	NM_001099404.1 NM_001099404.1
13	3 y	male	Sleeping	negative	negative	16	96	84.4	*TTN_p.R1859S* *TTN_p.P29832T*	
14	12 y	male	Emotion, stress, exercise	negative	negative	53	154	230	*RYR2_p.L73V* *MYBPC3_p.G800R* *PKP2_p.G5D* *MYH7_c.5157+4A>G*	NM_001035 NM_000256.3 NM_004572.3 NM_000257.2
15	21 d	female	Sleeping	negative	negative	4.1	50	24	-	-
16	11 m	male	Sleeping	negative	negative	12,3	74	56	*KCNQ1_p.G621S* *MYH6_p.S1734L* *TTN_p.E15868K* *TTN_p.M8580I* *TTN_p.A5671S*	NM_000218.2 NM_002471.3 NM_133378.4 NM_133378.4 NM_133378.4

17	14 y	female	Emotion, stress, exercise	negative	negative	55	160	230	*DSC2_p.V597F* *CACNA1C_p.R1937C* *ANK2_p.P2383L* *TGFB3_c.755-5T>C* *DMD_p.Q1443R*	NM_024422.3 NM_199460.2 NM_001148.4 NM_003239.2 NM_004006.2
18	8 y	female	Sleeping	negative	negative	48	120	195	-	-
19	11 y	male	Emotion, stress, exercise	negative	negative	51	165	244	-	-
20	3 y	male	Sleeping	negative	negative	13	96.5	85	*TTN_p.E25693D*	NM_133378.4
21	1 m	male	Sleeping	negative	negative	3.8	42	23	-	-
22	4 m	female	Sleeping	negative	negative	7.4	60	35	-	-
23	10 y	male	Emotion, stress, exercise	myocarditis	negative	35	138	247	*MYH7_p.L1591Q* *PKP2_p.R413STOP* *TTN_p.V31995A* *TTN_p.A20255P*	NM_000257.2 NM_004572.3 NM_133378.4 NM_133378.4
24	56 d	female	Sleeping	negative	negative	4.3	50	26	-	-
25	2 m	male	Sleeping	negative	negative	5.1	58	39	-	-
26	2 m	female	Emotion, stress, exercise	Slight fibrosis	negative	4.8	58	75.4	-	-
27	13 m	female	Sleeping	negative	negative	11	75	60	-	-
28	41 d	male	Sleeping	negative	negative	3.1	51	15	-	-
29	18 m	female	Sleeping	negative	negative	7.8	79	73	-	-

Table 2.- Main autopsy data about cases and genetic variations identified. Y, years, M, months, D, days. ARVC, Arrhythmogenic Right Ventricular Cardiomyopathy. HCM, Hypertrophic Cardiomyopathy. MD, Muscular Dystrophy.

	Cause of death			
Age	Sleeping	Drowning	Emotion, stress, exercise	
0 – 1	5:9	0:0	0:1	5:10 (15)
2 – 5	2:3	2:0	0:0	4:3 (7)
6 – 9	0:1	0:0	0:0	0:1 (1)
10 - 14	1:0	1:0	3:1	5:1 (6)
	8:13 (21)	3:0 (3)	3:2 (5)	14:15 (29)

Table 3.- Age represents range of ages in years. Relation Male:Female. Between parentheses represents the total. Fifteen cases showed less than 1 year of age old. Twenty-one cases died at rest/sleeping.

Case	Technology	Gene/Aminoacid	dbSNP	MAF (%)	HGMD	PolyPhen2	CONDEL	PROVEAN	Mutation Taster
1	sanger	-	-	-	-	-	-	-	-
2	sanger	*KCNH2_p.R892C*	rs201627778	0.0116/0.0227/0.0154	-	-	Deleterious	Deleterious	Disease causing
3	sanger	-	-	-	-	-	-	-	-
4	sanger/NGS	*TTN_p.G21091S*	Novel	-	-	Benign	-	Deleterious	Disease causing
		DSC2_p.L732V	rs151024019	0.1279/0.0681/0.1076	CM0910201 (ARVC)	-	Neutral	Neutral	Polymorphism
5	sanger	-	-	-	-	-	-	-	-
6	NGS	*GPD1L_p.V337F*	Novel	-	-	-	Deleterious	Deleterious	Disease causing
7	NGS	*CACNA1C_p.R2021Q*	rs112414325	0,2183/0,0518/0,1652	-	Benign	-	Neutral	Disease causing
		DSP_p.A306T	rs368193211	0.0116/0.0/0.0077	-	-	Neutral	Neutral	Disease causing
		MYBPC3_p.R896H	rs35078470	0,4818/0,1511/0,3748	CM992932 (HCM)	-	Neutral	Neutral	Polymorphism
8	sanger	-	-	-	-	-	-	-	-
9	sanger/NGS	*DSP_p.R105Q*	Novel	-	-	-	Neutral	Neutral	Disease causing
		DSP_p.M316V	rs201672777	0.0116/0.0/0.0077	-	-	Neutral	Deleterious	Disease causing
		CACNB2_c.del47CGG>_	Novel	-	-	-	-	-	-
		DMD_p.A2395T	rs72466590	0,2229/0/0,142	CM072994 (MD)	-	Neutral	Neutral	Polymorphism
		FBN1_p.P392R	Novel	-	-	-	Neutral	Deleterious	Disease causing
10	sanger	-	-	-	-	-	-	-	-
11	sanger	-	-	-	-	-	-	-	-
12	sanger	*SCN5A_p.E1685D*	Novel	-	-	-	Deleterious	Deleterious	Disease causing
		SCN5A_p.E1685V	Novel	-	-	-	Deleterious	Deleterious	Disease causing
13	NGS	*TTN_p.R1859S*	Novel	-	-	-	-	Deleterious	Disease causing
		TTN_p.P29832T	Novel	-	-	-	-	Deleterious	Polymorphism
14	NGS	*RYR2_p.L73V*	Novel	-	-	Damaging	-	Neutral	Disease causing
		MYBPC3_p.G800R	Novel	-	-	Damaging	-	Deleterious	Disease causing
		PKP2_p.G5D	Novel	-	-	-	Damaging	Neutral	Disease causing
		MYH7_c.5157+4A>G	Novel	-	-	-	-	-	-
15	sanger	-	-	-	-	-	-	-	-
16	NGS	*KCNQ1_p.G621S*	rs199472820	-	-	-	Neutral	Neutral	Polymorphism
		MYH6_p.S1734L	rs151324358	0.0116/0.0908/0.0384	-	-	Neutral	Neutral	Polymorphism
		TTN_p.E15868K	rs201510986	0/0.0549/0.0169	-	Damaging	-	Neutral	Disease causing
		TTN_p.M8580I	rs201728165	0/0.1571/0.0497	-	Benign	-	Neutral	Polymorphism
		TTN_p.A5671S	Novel	-	-	Benign	-	Neutral	Polymorphism

17	NGS	*DSC2_p.V597F* *CACNA1C_p.R1937C* *ANK2_p.P2383L* *TGFB3_c.755-5T>C* *DMD_p.Q1443R*	rs143040393 rs185788586 rs35960628 Novel Novel	0,0116/0,2497/0,0923 0,1437/0,1257/0,1379 0/0,0908/0,0308 - -	- - - - -	- Damaging - - -	Neutral - Neutral - Neutral	Deleterious Neutral Neutral - Neutral	Polymorphism Disease causing Polymorphism - Disease causing
18	sanger	-	-	-	-	-	-	-	-
19	sanger	-	-	-	-	-	-	-	-
20	NGS	*TTN_p.E25693D*	Novel	-	-	Benign	-	Neutral	Disease causing
21	NGS	-	-	-	-	-	-	-	-
22	sanger	-	-	-	-	-	-	-	-
23	NGS	*MYH7_p.L1591Q* *PKP2_p.R413STOP* *TTN_p.V31995A* *TTN_p.A20255P*	rs61737004 rs372827156 rs555945684 rs72646880	- 0.0/0.0227/0.0077 0.0833/0.0/0.056 0.315/0.0264/0.2242	- CM660431 (ARVC) - -	- - Damaging Damaging	Neutral - - -	Neutral - Deleterious Deleterious	Disease causing - Disease causing Disease causing
24	sanger	-	-	-	-	-	-	-	-
25	sanger	-	-	-	-	-	-	-	-
26	sanger	-	-	-	-	-	-	-	-
27	sanger	-	-	-	-	-	-	-	-
28	sanger	-	-	-	-	-	-	-	-
29	sanger	-	-	-	-	-	-	-	-

Table 4.- Genetic data of variations identified. MAF (Minor Allele Frequency) expressed as EA/AA/ALL respectively. EA (European-American)/AA (African-American)/ALL. Y, years, M, months, D, days. NGS, Next Generation Sequencing. ARVC, Arrhythmogenic Right Ventricular Cardiomyopathy. HCM, Hypertrophic Cardiomyopathy. MD, Muscular Dystrophy.

Figure legends

Figure 1.- Distribution by ages and cause of death. Black colour represents Male. Grey colour represents Female. A.- Number of cases by age. Range of ages is shown in years (0-1, 2-9, and 10-14). B.- Cause of death. Most part of our cases were females died sleeping during the first year of life.

Figure 2.- Genetic variation. Black colour represents Sanger sequencing. Grey colour represents NGS technology. A.- Identification of genetic variations in arrhythmogenic or structural genes. A total of 35 genetic variations were identified (3 cases using Sanger). Twenty-five genetic variations were identified in structural genes (all using NGS technology). Ten genetic variations were identified in arrhythmogenic genes (3 using Sanger and 7 using NGS). B.- Number of cases carrying from 1 to 5 genetic variations. Twelve cases carry a total of 35 genetic variations (2 cases using Sanger). Three cases were identified carrying 1 (one using Sanger and 2 using NGS), 2 (one using Sanger and 2 using NGS) and 5 genetic variations (all using NGS). Two cases carry 4 variants (NGS) and only one case carries 3 genetic variations (NGS).

Figure 3.- Distribution of rare genetic variations using NGS analysis. A.- Gene and number of genetic variations identified in arrhythmogenic genes. B.- Gene and number of genetic variations identified in structural genes.

Figures

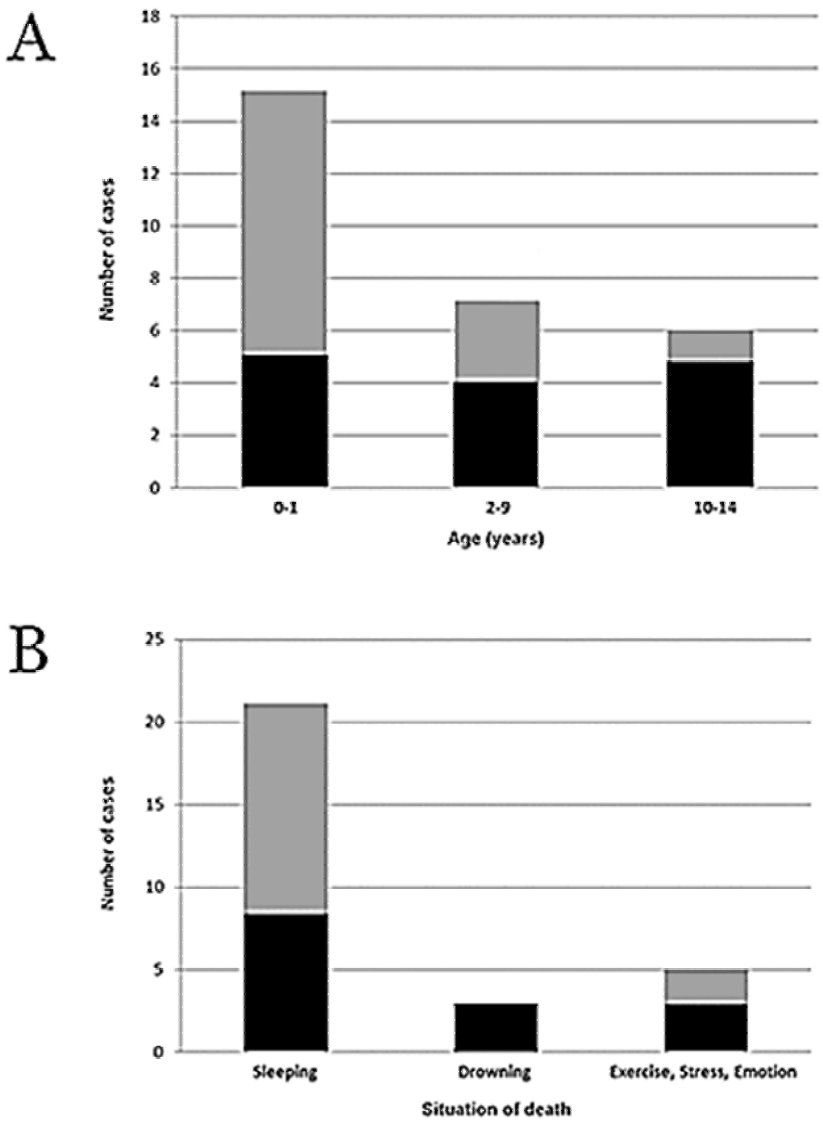

Figure 1.-

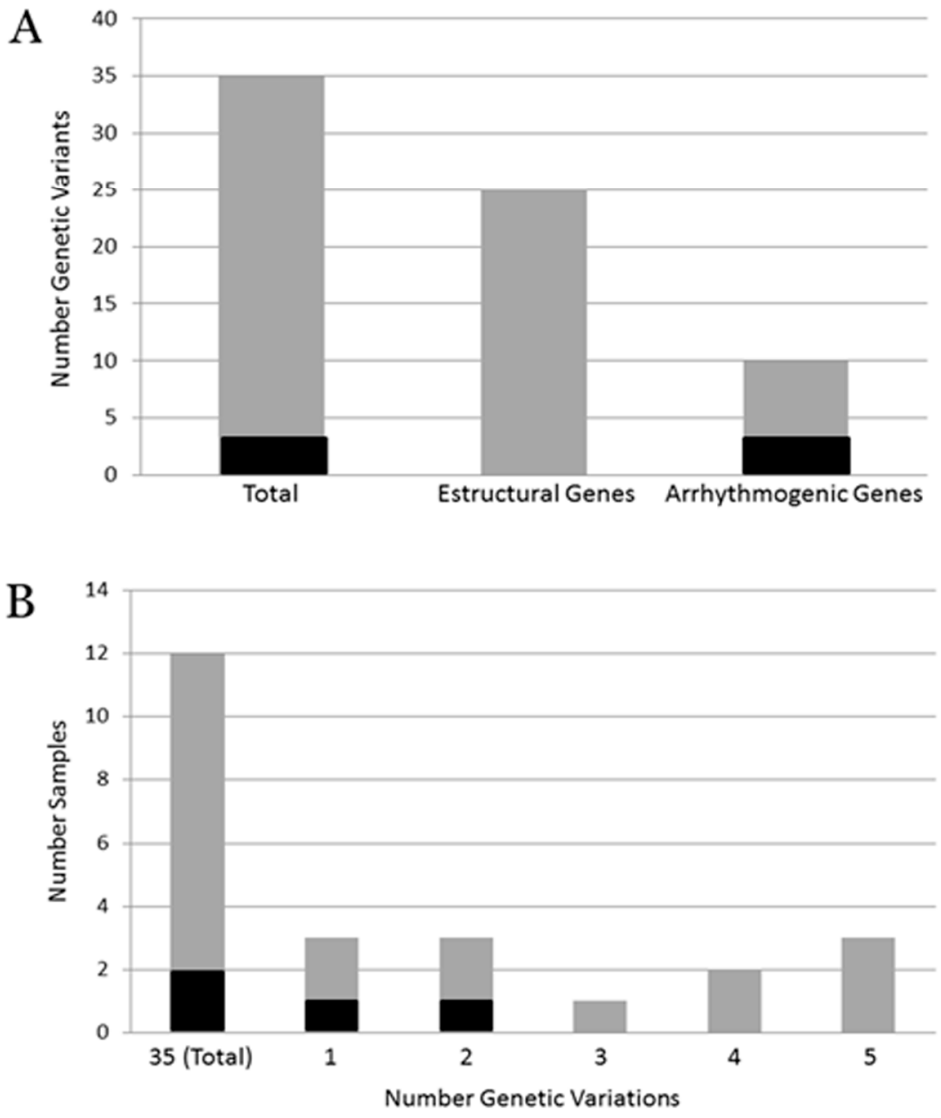

Figure 2.-

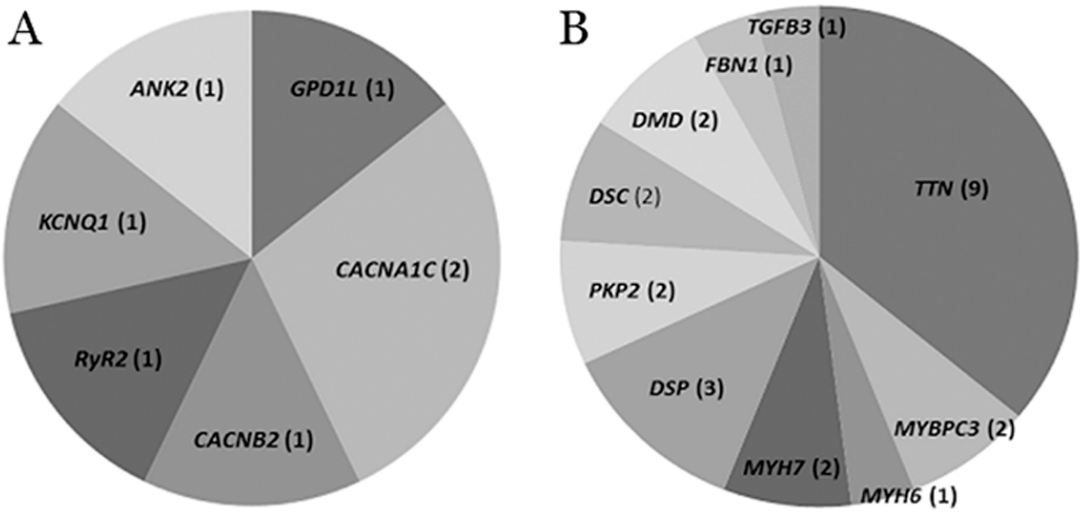

Figure 3.-