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Genetic analysis in post-mortem samples with micro-ischemic alterations

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ABSTRACT

Sudden cardiac arrest is a leading cause of death worldwide. Most cardiac arrests happen in patients who have previously suffered a myocardial infarct. The risk of sudden death after infarction may increase in people who carry a pathogenic genetic alteration in cardiac ion channels. We hypothesized that micro-ischemia could trigger lethal arrhythmogenesis, thus we sought to identify genetic alterations in cardiac ion channels in patients with micro-ischemic disease. We studied a cohort of 56 post-mortem samples. Autopsy studies identified myocardial infarction as the cause of death in each case. We used both Sanger sequencing and next-generation sequencing to screen candidate genes associated with sudden cardiac death. We identified six rare *missense* genetic variations in five unrelated patients. Two variants have been previously reported; one is associated with atrial fibrillation (*SCN5A*_p.H445D), and the other is predicted to be benign (*ANK2*_p.T2059M). The novel variants were predicted *in silico* as benign, except for one (*RyR2*_p.M4019T), which was classified as deleterious. Our post-mortem, micro-infarction cohort displayed a rate of nearly 10% non-common genetic variants. However, the clinical significance of most of the identified variants remains unknown due to lack of family assessment. Further analyses should be performed in large cohorts to clarify the role of ion-channel gene analysis in samples showing microscopic ischemic alterations.

1. Introduction

Sudden cardiac death (SCD) can result from several cardiovascular diseases and is one of the leading causes of death in the world [1]. SCD can occur at any age; overall, coronary artery disease and acute myocardial infarction (MI) are primarily responsible for SCD in people over 35 years old [2]. Cardiac arrest is the most common cause of death in patients who have suffered a previous MI, and the risk increases within the first month after MI [3]. The scar related to the MI provides the underlying substrate, and cardiac conduction abnormalities and repolarization deficiencies play a key role in the development of lethal arrhythmias and ventricular fibrillation (VF) after MI. Recent reports suggest that deaths in the first month after MI are more likely to be mechanical; several months after MI, the risk of arrhythmic death increases [3].

MI is thought to result from a combination of genetic variants and environmental factors [4]. The genetic risk of MI is thought to be compounded by common genetic variants in different genes; however, much of the heritability of MI remains unexplained. In recent years, uncommon single nucleotide variations in arrhythmogenic genes have been reported as risk factors for arrhythmia leading to SCD during acute ischemia [5,6] and in the early weeks

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after MI [7]. For example, recently, 4.3% of patients showing signs of MI were found to carry rare variants in the sodium channel-related gene *SCN5A* that could be involved electric dysfunction and SCD [8]. These data are relevant to post-mortem assessments in determining both the genetic cause of death and for genetic screening in family members. Rare pathogenic variants in these arrhythmogenic genes have been associated with some disorders such as Long QT Syndrome (LQTS) and Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT). However, the role of uncommon genetic variants in cardiac ion channel genes and their relationship to previous MI remains poorly understood.

We sought to perform a genetic analysis of the major cardiac ion channels in a group of patients with micro-injuries in the myocardium associated with coronary alterations. We hypothesized that rare genetic variants could predispose patients to lethal arrhythmogenesis and increase the risk of SCD, while likely triggering VF and SCD.

2. Material and methods

2.1. Forensics

Whole blood samples from complete autopsy examinations [9] performed between February 2012 and February 2015 were included in our study if the following criteria were met: (a) age <50 years; (b) no macroscopic signs of congenital heart disease, cardiac infarction, or other anomalies that could explain the death; (c) negative toxicological analysis; (d) microscopic study showing coronary disease/ischemia; and (e) blood samples obtained <48 h after death. This study was approved by the ethics committee of our hospital and follows the Helsinki II declaration.

2.2. DNA samples

Genomic DNA was extracted with Chemagic MSM I from post-mortem whole blood. DNA was checked for quality and quantified before processing to obtain 3 μg for NGS. Spectrophotometric measurements were performed to assess quality ratios of absorbance (260/280:260/230 was a minimum of 1.8:2.2); DNA concentration was determined by fluorometry (Qubit, Life Technologies). DNA integrity was assessed on a 0.8% agarose gel. DNA quality/integrity divided our cohort in two groups. The first group included samples with low DNA quality/integrity, which were analyzed using Sanger sequencing. The second group included samples with high DNA quality/integrity, which were analyzed using NGS technology. Confirmation of the variants identified by NGS analysis was performed using Sanger sequencing. As an internal control, some cases were analyzed by both methods.

2.3. Sanger sequencing

The genetic study involved the direct sequencing of main gens associated with arrhythmogenic disorders [10]: *SCN5A* (NM_001099404.1), *KCNQ1* (NM_000218.2), *KCNH2* (NM_000238.3), *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 were directly sequenced in both directions (Big Dye Terminator v3.1 cycle sequencing kit and 3130XL Genetic Analyzer, both from Applied Biosystems). Posterior SeqScape Software v2.5 (Life Technologies) analysis was used to compare the results with the reference sequence from hg19. The identified variations were compared to DNA sequences from 300 healthy Spanish individuals (individuals not related to any patient and of the same ethnicity; 600 alleles) as control cases, and compared to the 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/), The Exome Aggregation Consortium (ExAC) (exac.broadinstitute.org), and the Exome Variant Server (EVS) (http://evs.gs.washington.edu/EVS/). Sequence variants were described following the Human Genome Variation Society rules (HGVS) (http://www.hgvs.org/) and checked in Mutalyzer (https://mutalyzer.nl/).

2.4. Next Generation Sequencing analysis

DNA was fragmented by Bioruptor (Diagenode). Library preparation was performed according to the manufacturer's instructions (SureSelect XT Custom 0.5–2.9 Mb library, Agilent Technologies, Inc). After capture, the indexed library was sequenced in a ten-sample pool cartridge. The sequencing of paired-ends was performed on a MiSeq System (Illumina, CA, USA) using a 2 × 150 bp reading length. We selected the 15 most prevalent genes involved in SCD-related primary electric pathologies (*ANK2, CACNA1C, CACNB2, CASQ2, CAV3, GPD1L, HCN4, KCNE1, KCNE2, KCNH2, KCNJ2, KCNQ1, RYR2, SCN4B,* and *SCN5A*), according to available scientific literature [11]. Probes corresponding to these 15 genes were designed using eArray (Agilent Technologies, Inc.). All gene isoforms described in Ensembl 75 (http://www.ensembl.org/) that have been linked to either a RefSeq code (http://www.ncbi.nlm.nih.gov/refseq/) or CCDS (https://www.ncbi.nlm.nih.gov/CCDS/) were included in the design. The biotinylated cRNA probe solution was manufactured by Agilent Technologies and provided as capture probes. The coordinates of the sequence data were based on NCBI build 37 (UCSC hg19) [12].

2.5. Confirmation of variants identified by Next Generation Sequencing

Non-common (Minor Allele Frequency – MAF – <1%) genetic variants were confirmed by the Sanger method as mentioned above.

3. Results

Our cohort included a total of 56 post-mortem samples. All samples were from individuals <50 years old (ranging from age 24 to 50; mean age: 42.4 years). Samples were collected from 5 females (8.9%) and 51 males (91.1%). Cases were classified as sudden death while sleeping (37 cases; 67.1%) or sudden death while experiencing emotion, stress, or exercise (19 cases; 33.9%) (Table 1).

Complete autopsy concluded an undetermined cause of death after macroscopic analysis. Body weight, body size, and heart weights were within appropriate limits for age, except in 18 individuals who were overweight [body mass index (BMI) 25.0–29.9] [13]. All of these cases were males; 13 died while sleeping (Table 2). Toxicological results were negative in all cases. After microscopic/histological analysis, cardiac ischemia/coronary thrombosis was identified in all cases (Figs. 1 and 2).

Seven SCD-related genes were analyzed by Sanger sequencing (*SCN5A, KCNQ1, KCNH2, KCNE1, KCNE2, KCNE3,* and *RyR2*) in cases with low DNA quality/integrity (40 cases). NGS technology was used to sequence the 15 main genes associated with SCD in the second group of 16 samples with high DNA quality. Seven cases were analyzed using both methods to perform an internal control of protocols and analysis. Both methods identified the same genetic variants. A total of six *missense* rare variations (four of which were novel) were identified in five (8.9%) of the samples