The role of genetic testing in cardiac deaths under suspicion of hypertrophic cardiomyopathy: validating a low-cost method and presenting preliminary data of an Italian retrospective study

Camilla Tettamanti¹, Alessandro Bonsignore¹*, Simonetta Verdiани¹, Lucia Casarino¹, Francesco De Stefano¹, Francesco Ventura¹

Abstract: Background. Hypertrophic cardiomyopathy (HCM) is one of the cause of sudden cardiac death and it is characterized by phenotypic and genotypic heterogeneity. The majority of HCM is caused by mutations in genes encoding components of the cardiac tissue. MYH7 (encoding beta-myosin heavy chain), TNNT2 (encoding cardiac Troponin T) and TNNI3 (encoding cardiac Troponin I) genes account for 25% to 35% of all the above mentioned mutations and they are easily investigated together by using Sanger Method of DNA sequencing.

Materials and methods. The authors focus on the DNA sequence of those genes starting from different frozen tissues collected during forensic autopsies performed in a 5-year period (2011-2015) at the University of Genova, Italy. The analysis involved 9 cases of sudden cardiac death under macroscopically and histologically presenting as HCM.

Discussion. The presented retrospective study allowed the authors to validate a method of DNA extraction and sequencing from different frozen samples – collected in a wide range of years – which could be applicable also in small forensic centres thanks to the limited cost. As a matter of fact, being able to reach a certain post-mortem diagnosis of HCM is a crucial step to perform a proper counselling between the deceased's family.

Key Words: hypertrophic cardiomyopathy, sudden death, genetic counselling, low-cost analysis.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM), occurring with a prevalence of 1/500, is the most common cause of sudden cardiac death in young athletes and one of the most frequent in adult [1], and it is characterized by phenotypic and genotypic heterogeneity.

Particularly, its onset can be completely asymptomatic as well as it can reveal arrhythmias, cardiovascular dysfunctions and also sudden deaths also depending to the particular genotype of the mutation [2, 3].

Genetics is having an important impact in forensic pathology and one of the most important subjects of this application is the investigation of sudden cardiac death [4] and several hundreds of mutations among at least 27 different genes have been identified in determining HCM so far.

MATERIALS AND METHODS

Forensic laboratories use standard protocols but these could be different according to the type and the size of the sample, the clinical information, the time of sampling (i.e. routine vs. out-of-hours specimens), and circumstances and logistics. As a result, it is rarely possible to estimate the expected quality of extracted nucleic acids on the basis of the available information for an autoptic

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sample. Instead, standard methods of purification from tissue should be adopted and the extracted nucleic acids should be validated to decide if the material is of sufficient quality and quantity to be used for molecular studies.

The preliminary analysis involved 9 cases of sudden cardiac death.

Genomic DNA was extracted starting from different frozen tissues collected during forensic autopsies of cases of sudden cardiac death, performed in a 5-year period (2011-2015) at the University of Genova, Italy, in particular one case from myocardium, two cases from spleen and in six cases from blood. Particularly, one blood sample collected in 2011, one spleen sample in 2012, two in 2013 (one myocardium and one spleen), one blood sample in 2014 and two blood samples and one spleen in 2015.

The analysis started in March 2016. Proteins and harmful enzymes such as nucleases were subsequently digested by proteinase K using Puregene DNA purification kit. This kit is specially designed for purifying DNA from tissue sections and uses special lysis conditions to release DNA from tissue sections and to overcome inhibitory effects. The use of spin columns allows to concentrate high-quality DNA in small volumes.

Initial DNA purifications from different amount of autopic tissues were performed in order to identify optimal extraction method: tissue was found to be relatively in good state of conservation, although there was a tendency for degradation to increase with storage time.

Hundreds of mutations among at least 27 different genes [2] have been identified in determining HCM so far and the majority of HCM is caused by mutations in genes encoding components of the cardiac tissue. MYH7 (encoding beta-myosin heavy chain) [5], TNNT2 (encoding cardiac Troponin T) [6] and TNNI3 (encoding cardiac Troponin I) [7] genes account for 25% to 35% of all the above mentioned mutations.

The authors focus on the DNA sequence of eight exons (5, 9, 13, 19, 35 and 36 of MYH7, exon 9 of TNNT2, exon 8 of TNNI3 gene), reported in the reviewed literature between those with a high prevalence of mutations [8].

Particularly, standard conditions for buffer and primers concentration were tested. In all cases no significant variation were found in the signal intensity relative of each exon. The optimal deoxynucleotide triphohate (dNTP) concentration, directly related to magnesium concentration within the PCR reaction, was 1mM.

Although the sequences of the primers for the amplification of the 8 selected genes were partially available in the network or in the literature, after a preliminary investigation, they were not considered appropriate to our application. For this reason, the sequences of all the primers necessary for the screening of mutations were redesigned (Table 1).

Single exons and splice junctions of genes were amplified efficiently at annealing temperature of 58°C for 30 cycles and non-specific low molecular weight PCR products were observed.

Bioinformatics analysis was performed using ClustalW2, a program designed to align multiple sequences.

Genomic DNA was isolated in one case from myocardium, in two cases from spleen and in six cases from blood using Puregene DNA purification kit.

### Table 1. Primer sets used to amplify MYH7, TNNT2 and TNNI3 exons for mutations analysis

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>MYH7</td>
<td>5</td>
<td>AACTCCCAAAATCACCAGCC</td>
<td>GCCCTATCCCGAGTTCCCTTC</td>
</tr>
<tr>
<td>MYH7</td>
<td>9</td>
<td>GACAAGCTGCTCCGGGAGTTGAG</td>
<td>AACAGAGGGGGAGGGGAGAG</td>
</tr>
<tr>
<td>MYH7</td>
<td>13</td>
<td>ACCTGGCCAGCAGTCTCATC</td>
<td>AACTCTCATCCCCACCATGCC</td>
</tr>
<tr>
<td>MYH7</td>
<td>19</td>
<td>AAGGCAAATCTGACAAGACAACA</td>
<td>CTGGCTCCCCCTGGTCTATG</td>
</tr>
<tr>
<td>MYH7</td>
<td>35</td>
<td>TTGCTCCCTGCCCCTAGGTT</td>
<td>AGCAGGAAAAAGATGGAGA</td>
</tr>
<tr>
<td>MYH7</td>
<td>36</td>
<td>TGCTCAATGCTTCTTCTGTC</td>
<td>GCCACAGTAATCGCTGAAGAAT</td>
</tr>
<tr>
<td>TNNT2</td>
<td>9</td>
<td>CAGAGGTCTTTTGCACTC</td>
<td>GCCCTCAAAAAGAGATGGAG</td>
</tr>
<tr>
<td>TNNI3</td>
<td>8</td>
<td>CTCTTCTCCTGCCACTC</td>
<td>GGATAGGAGAAGGTAGGG</td>
</tr>
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### Table 2. PCR conditions

<table>
<thead>
<tr>
<th>Buffer 10x</th>
<th>dNTPs 1mM</th>
<th>MgCl2 25mM</th>
<th>Taq 5U/ul</th>
<th>Primers F+R 100uM</th>
<th>H2O to 25ul</th>
</tr>
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<tbody>
<tr>
<td></td>
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PCR conditions are summarized in Table 2. In order to remove excess of dNTPs and primers not used during amplification 5 ul of PCR products were purified using a kit (PCR Product Pre-Sequencing Kit, Amersham Pharmacia Biotech), which includes SAP enzymes (Alkaline Shrimo Phosphatase) and EXO (Exonuclease I). The samples were incubated at 37°C for 30 minutes and then 95°C for 5 minutes then stores at -20°C until needed. The purified PCR products were analyzed by direct sequencing on a 16 capillaries automated sequencer, ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Bioinformatics analysis was performed using ClustalW2, a program designed to align multiple sequences.
exons have been amplified by PCR with specifically designed primers. Direct sequencing has carried out the screening of mutations.

RESULTS AND DISCUSSION

In all cases it was possible to obtain a complete DNA sequence.

None of the cases revealed evidence of HCM sequence variants; instead, in three subjects the c.gcG/gcA synonymous variant (rs 3729830) in the exon 35 MYH7 was found (Fig. 1).

The introduction of a new protocol in forensic field requires a preliminary validation.

For this, the authors have studied the conditions on the efficiency and reproducibility of a low-cost amplification system of eight exons (5, 9, 13, 19, 35 and 36 of MYH7, exon 9 of TNNT2, exon 8 of TNNI3 gene), reported in the reviewed literature between those with a high prevalence of mutations and validated a method of DNA extraction and sequencing from different timing and tissue frozen samples collected.

The possibility to perform DNA molecular analyses as routine test on tissues that had been stored prior to the study, confirmed the robustness of the protocol. This in turn emphasizes the potential role of this strategy in opening archives to high through put sequencing for use in both retrospective and prospective clinical studies. The data are from few samples, so they should obviously be considered as preliminary.

Specialized companies almost routinely offer this procedure made by direct sequencing. However, this system may not be practicable in laboratories that analyse a small number of cases.

Molecular genotyping has important biomedical and forensic applications. However, limiting amounts of human biological material often yield genomic DNA of insufficient quantity and poor quality for a reliable analysis. Although the modern Genomic techniques require from nanogram to microgram quantities of genomic DNA, but insufficient amount often interferes with some classical molecular analyses requiring high quality nucleic acids.

Moreover, the technologies of Next-generation DNA sequencing are still too expensive for a broadcast in all Labs of Forensic Institutes, so it is important to find easily analysable target with Sangar Method, able to cover a number of the up-to-date known mutations.

CONCLUSION

The presented retrospective study allowed the authors to validate a low-cost method of DNA extraction and sequencing from different frozen samples collected in a wide range of years.

The described approach could be considered a good strategy for time and resources saving for an initial screening of mutations from autoptic old frozen for both prospective and retrospective archive-based studies.

According to this, further analysis would be performed in order to reveal the real incidence of HCM occurring in sudden unexplained death; as a matter of fact, being able to reach a certain post-mortem diagnosis of HCM is a crucial step to perform a proper counselling between the deceased’s family. Moreover, after a genetic diagnosis of HCM, the first-degree relatives may undergo a possible screening to prevent other sudden deaths allowing timely therapeutic measures and a mandatory follow-up in order to develop preventive strategies as implantable cardioverter defibrillator [2], which have clearly been shown to reduce the risk of sudden cardiac death [9].

Conflict of interest. The authors declare that they have no conflict of interest.

References
