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THE GENETIC INVESTIGATION OF OLD TISSUE SAMPLES PARAFFIN-EMBEDDED AS SOURCE FOR MOLECULAR AUTOPSIES IN SUDDEN CARDIAC DEATH CASES

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ABSTRACT: Sudden cardiac death (SCD) is a major cause of death in young adults and children. Standard forensic autopsy procedures are often unsuccessful in explicating the causes of SCD. "Gene" or "molecular autopsy" has a great potential for identifying unknown causes of death. The aim of our study was to design a methodology of molecular analysis for investigating old tissue samples paraffin-embedded, collected from subjects with SCD. For molecular analysis we selected a Real Time PCR method with Taqman probes, the SCN5A gene which can be mutated in several channelopathies – Brugada syndrome, LQT, FAF and a frequent mutation of SCN5A gene - E1784K. 51 samples were investigated and the genotype was determined for 47 subjects. By this study we showed that the molecular analyze of genomic DNA extracted from formalin-fixed and paraffin-embedded tissues by Real Time with TaqMan probes method is an efficient and suitable approach for samples with old and fragmented DNA molecules, which can be used for the successful identification of normal alleles or specific pathogenic mutations. By molecular dissection, old cases with no identified cause of death can be deciphered and the positive molecular diagnosis will provide the basis for genetic counseling and treating surviving family members.

Keywords: sudden cardiac death, SCN5A gene, molecular autopsy, E1784K mutation

INTRODUCTION:

Sudden cardiac death (SCD) is in many developed countries a major cause of death in young adults and children. Standard forensic autopsy procedures are often unsuccessful in explicating the causes of SCD (Ackerman, 2009; Koplan *et al.* 2007).

In forensic field there is a great potential for the use of molecular testing to identify unknown causes of death, and a new investigatory tool called "gene or molecular autopsy" - for inherited arrhythmia syndromes and also for genetic predisposition to acquired arrhythmia has increasingly emerged (Oliva *et al.* 2011; Michaud *et al.*2008).

In these types of diseases gene defects alter the heart electrical activity predisposing the patient to fatal cardiac arrhythmias and the classic histopathological exam shows no morphologic changes in the myocardium (Ceauşu *et al.* 2013; Hostiuc *et al.* 2014; Dermengiu *at al.* 2010; Macarie *et al*. 2009).

Inherited arrhythmia syndromes are caused by genetic defects of heart ion channels genes. These types of diseases are sometimes referred to as cardiac channelopathies and include the long QT (LQT) syndrome, short QT syndrome (SQTS), Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT), and familial atrial fibrillation (FAF) (Ceauşu *et al.* 2008; Dermengiu *et al.* 2011).

The genes in which mutations could induce ion channels defects responsible for these diseases are numerous and code sodium, potassium, calcium channels or regulatory proteins. The LQT syndrome has 13 different types of disease and the most frequent mutated genes are: KCNQ1 (LQT1), KCNH2 (LQT2), and SCN5A (LQT3). In Brugada syndrome there are mutations in genes like SCN5A, CACNA1C, CACNB2 or SCN1B and in familial atrial fibrillation, defects in KCNQ1, KNCE2, KCNJ2, KCNE1L or SCN5A were found (Elliott *et al*. 2000; Priori *et al.* 2002; Wellens *et al.* 2014; Puranik *et al.* 2005; Campuzano *et al.* 2014).

The utility and the importance of postmortem molecular diagnosis or the molecular autopsy resides in the fact that a positive result impacts on surviving family members who could benefit of genetic counseling and can be included in clinical genetics programs with cardiology expertise in order to identify any other possible carriers of genetic defects and provide proper treatment or prevention strategies, possibly saving another family member's life (Sheikh *et al.* 2014; Tang *et al.* 2014; Michaud *et al.* 2009; Vavolizza *et al*. 2014).

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For DNA analysis, tissue samples of heart and spleen or EDTA blood are frozen and stored at -80°C. Tissue samples (heart only) can also be collected in RNA later solution and stored for several weeks at 4°C for postmortem mRNA expression analysis in different cardiac channelopathies (Erskine *et al.* 2014; Michaud *et al.* 2011).

In Romania the legal autopsy procedure doesn't include the collection of tissue samples for molecular analysis but only tissue samples formalin-fixation and paraffin embedding methodology which produces significant fragmentation of nucleic acids.

Consequently, formalin-fixed tissues are not suitable for Sanger sequencing-based testing methods which could allow us to investigate a whole gene by sequencing all its exons.

Next generation sequencing technologies (NGST) have shown promise on analyzing postmortem samples and may allow solving "cold cases" for which only formalin-fixed samples are available to be tested but these are expensive methods and one analysis could exhaust significant financial resources and could also depend on the quantity and quality of DNA extracted from this kind of samples. DNA extraction and isolation from paraffin embedded samples is a challenge and requires special extraction kits and experience in working with paraffin preserved tissues (Hertz *et al.* 2014; Deschênes *et al.* 2000).

The aim of our study was to design a methodology of molecular analysis for investigating old tissue samples paraffin-embedded which were collected from subjects with hypothetical SCD.

For molecular analysis we selected a Real Time PCR method with Taqman probes, which is suitable for FFPE tissue samples because it can identify short DNA fragments as found in fragmented DNA samples. The disadvantage of this method is that only one mutation can be identified with a pair of Taqman probes, the analysis of a large number of mutations being too expensive and time consuming.

Amongst the numerous genes responsible for SCD we selected the SCN5A gene which can be mutated in several channelopathies – Brugada syndrome, LQT and FAF. Several hundred mutations with pathological consequences were found in all gene exons, but the majority were rare, only a few having a higher frequency. From these mutations we selected the most frequent one - E1784K, a typical example of a cardiac sodium channel mutation with the capacity to present a mixed clinical phenotype of LQT3, Brugada syndrome 1, and conduction disorders (Uziębło-Życzkowska *et al.* 2014; Béziau *et al.* 204; Tester *et al.* 2014; Jiménez-Jáimez *et al.* 2013).

The E1784K mutation is a single base transition (G→A) at position 5350 in the 28th exon of SCN5A gene (AAG→GAG) which results in a replacement of a glutamic acid by a lysine in the 1784 position within the C-terminal domain of the gene product - Na channel (Thiene *et al.* 2014; Weber *et al.* 2010; La Grange *et al.* 2014).

MATERIALS AND METHODS:

We investigated 51 tissue samples from subjects in which the autopsy and the histopathological examination didn't find any clear cause of death. The samples were collected during a period of 5 years, from 2009 to 2013. The study group included 18 females and 33 males, 13 were children and 38 young adults. Sudden cardiac death consecutive to SCN5A mutations is found in children but also in young adults and we wanted to analyze subjects from all age groups.

The tissue samples collected in the course of autopsy from myocardium, lungs, brain, kidneys, liver, spleen, and pancreas were fixed in 10% buffered formalin and embedded in paraffin blocks for histopathological or immunohistochemical examinations.

For DNA isolation and purification we used a dedicated kit, QIAamp DNA FFPE Tissue. Up to 8 sections with a thickness of up to 10 µm were cut from each tissue sample and collected in a sterile tube. Paraffin was removed by dissolving the tissue sections in xylen, followed by lysis with proteinase K and formalin crosslinking removal by heating at 90°C. The next stages consisted of DNA binding in special columns, washing and eluting purified DNA.

The concentration and quality of isolated DNA were measured with NanoDrop ND-1000 (Thermo Scientific) and with an alternative method by Qubit fluorometric quantization using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Life Technologies Invitrogen).

The UV measure of DNA concentration with NanoDrop is not very accurate at low DNA concentrations and it is not able to differentiate between double stranded DNA of good quality, and degraded, fragmented DNA molecules. Fluorimetric DNA quantification with Qubit is more specific and allows detection of very low concentrations of DNA. The DNA concentration range measured by NanoDrop was placed between 2.5 ng/µl and 28.1 ng/µl, but when the DNA concentration of several samples was measured with Qubit, the values were 20 to 50 times lower, meaning that double stranded DNA molecules were fewer, the rest being fragmented DNA which couldn't be differentiated by NanoDrop from larger molecules (Table 2).

The Real Time amplification of DNA samples with Taqman probes was performed on a 7900HT Fast Real Time PCT System using a custom TaqMan Pre-designed SNP Genotyping Assay (Life Technologies, rs137854601).

The reaction mix was prepared from 12.5 µl TaqMan Genotyping Master Mix (with AmpliTaq Gold DNA Polymerase, deoxyribonucleotide triphosphates, ROX passive reference and buffer components), 0.63 µl TaqMan probes, a variable volume of DNA sample (which should contain 1 to 10 ng DNA) and nuclease free water up to a total volume of 25 µl. The amplification program consisted of an initial activation stage, 10 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C.

The TaqMan MGB Probes consist of target-specific oligonucleotides with a reporter dye at the 5′ end of each probe, a minor groove binder (MGB), which increases the melting temperature (Tm) and a nonfluorescent quencher (NFQ) at the 3′ end of the probe.

For the wild type allele of SCN5A gene with the normal nucleotide, G, in the 5350 position, the TaqMan probe was labeled with VIC dye and for the mutant allele, with A replacing G in the gene sequence, the specific TaqMan probe was labeled with 6FAM dye.

For that reason, a subject with homozygous genotype for the wild type variant (G/G) should present a fluorescence increase only for VIC dye, a homozygous genotype for the mutant allele (A/A) will be identified by a fluorescence increase only for 6FAM dye and a heterozygous genotype will have an increase of fluorescence for both dyes.

RESULTS AND DISCUSSIONS:

The analysis of fluorescence curves showed a wild type genotype (Table 2) for all subjects. Only the presence of the normal base, G, was detected in all investigated alleles by the fluorescence increase for VIC dye. There was no fluorescence increase for 6FAM dye specific for the mutant allele with the A base instead of the G base (Fig. 2 and Fig. 3).

In four cases there was no increase of fluorescence, even if DNA extraction and Real Time amplification was repeated for several times. Since we couldn't find a correlation between the lack of amplification and samples specifications – age or the quality of embedded tissue, our conclusion was that in biological samples with no amplification, DNA molecules were too denatured and very fragmented or it was too difficult to remove contaminants from samples (ex. formalin).

The cut off for fluorescence increase (the number of amplification cycles after which the fluorescence starts to increase) of positive control was significantly earlier, with 12 – 13 cycles before the samples' fluorescence increase, even if the calculated DNA quantity was same for positive control and samples – 1 ng (Fig. 2 and Fig. 3). This difference was determined by the poor quality of isolated genomic DNA, even if the concentration measured by NanoDrop method was considered to be appropriate.

However, we obtained amplification and a fluorescence increase in 47 samples (92 %) and we were able to determine the subjects' genotype even if we had a low DNA concentration in all samples, which proved that the method we chose is solid and suitable for molecular analysis of this type of samples.

Fluorimetric DNA quantification for several samples with Qubit showed values which were much lesser than the values obtained with NanoDrop method. However, a more accurate quantification didn't influence the amplification; some samples with a higher DNA concentration were negative for fluorescence increase and need it a new DNA extraction, which allowed us to identify the genotype in a new amplification reaction; other samples, even if the DNA concentration was very low were successfully amplified without DNA re-extraction. Therefore, we concluded that the quality of the biological samples is more important than the actual DNA concentration and all samples should be considered as having very low DNA concentrations (0.05 – 1 ng/µl) which are satisfactory for Real Time amplification.

CONCLUSIONS AND PERSPECTIVES:

By this study were identified the genotypes of 47 subjects using a new approach and an appropriate molecular method like Real Time with TaqMan probes. Even if the E1784K mutation in SCN5A gene is the most commonly identified mutation in both LQTS and BrS, being responsible for 5-10% of LQTS and associated with 20% of BrS [13, 14], only wild-type alleles and normal genotypes were identified, but this methodology can also identify specific pathogenic mutations in cases of SCD and, consequently, establish a positive diagnostic.

In order to determine the spectrum of SCN5A gene mutations specific to Romanian population it is necessary to study larger groups of subjects using powerful methods as molecular sequencing of the whole SCN5A gene for several hundred samples collected from a larger number of forensic departments. By such a collaborative multi-center study, all pathogenic mutations of SCN5A gene and their specific frequency can be determined.

If the spectrum of SCN5A mutations will be identified, then it will be possible to design TaqMan probes as in the present study for the most frequent mutations which account for 90% of cases and analyze all biological samples, new or old which are not "DNA friendly", being formalin fixed and paraffin-embedded.

The same procedure can be applied for the study of other genes with mutations that can cause the SCD syndrome, like the KCNQ1 or CACNA1C genes. Finally, a specific test for Romanian population with Taqman probes for the most frequent mutations of several genes causing SCD can be the result of a larger future study.

Using this new method for molecular autopsy, old cases with no identified cause of death can be deciphered and acquire a positive diagnostic which will provide the basis for genetic counseling and treating surviving family members, representing the practical clinical value of this approach.

This complex molecular diagnostic of familial cases is challenging because is difficult to establish a link between the molecular autopsy of a sudden death case and the cardiologic evaluation of their family members due to legal and ethical issues of postmortem genetic testing. Therefore it is required a close collaboration between specialists from different medical fields, like forensic specialists and general practitioners or medical genetics specialists.

Table 1.
Subjects' age and gender structure.

Sample No.	Year of Sample collection	Sex	Age (years)
1	2009	Female	38
2	2009	Female	42
3	2009	Male	46
4	2009	Male	36
5	2009	Female	24
6	2009	Female	29
7	2009	Male	40
8	2009	Female	38
9	2009	Male	46
10	2009	Female	0
11	2010	Female	7
12	2010	Male	31
13	2010	Male	24
14	2010	Female	5 months
15	2010	Male	43
16	2010	Male	8 months
17	2010	Male	42
18	2011	Male	41
19	2011	Male	37
20	2011	Male	27
21	2011	Male	43
22	2011	Male	3
23	2011	Male	42
24	2011	Female	3
25	2011	Male	23
26	2011	Female	17
27	2011	Male	4
28	2011	Female	45
29	2011	Male	45
30	2011	Female	39
31	2012	Male	45
32	2012	Male	35
33	2012	Male	2 months
34	2012	Male	10 month
35	2012	Female	3 months
36	2012	Male	42
37	2012	Female	0
38	2012	Male	40
39	2012	Male	26
40	2012	Male	41
41	2012	Male	44
42	2013	Female	45
43	2013	Male	24
44	2013	Male	3 months
45	2013	Male	40
46	2013	Female	2 months
47	2013	Male	38
48	2013	Female	27
49	2013	Male	28
50	2013	Female	9
51	2013	Male	41

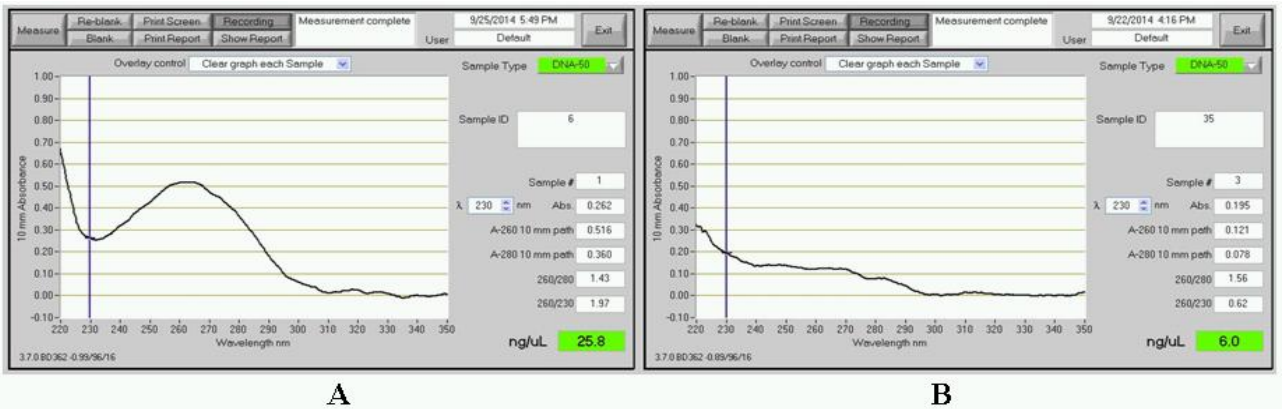

Fig 1. DNA concentration measured with NanoDrop ND-1000. A. Good concentration. B. Low concentration and poor quality.

Table 2.
DNA purity and concentration. The genotype of each sample.

Sample No.	DNA purity NanoDrop A260/A280	DNA concentration NanoDrop ng/µl	Qubit flurimetric quantitation ng/µl	Melting curve	Genotype SNC5A sequence
1	3.40	2.50	0.06	Present	Wild Type G/G
2	1.69	6.50	0.30	Present	Wild Type G/G
3	1.55	8.20	-	Present	Wild Type G/G
4	1.78	3.60	-	Present	Wild Type G/G
5	1.41	10.20	0.40	Present	Wild Type G/G
6	0.85	3.30	-	Present	Wild Type G/G
7	1.16	4.20	-	Present	Wild Type G/G
8	1.28	3.00	-	Present	Wild Type G/G
9	1.57	3.90	-	Present	Wild Type G/G
10	1.57	10.50	-	Present	Wild Type G/G
11	1.24	5.70	-	Present	Wild Type G/G
12	2.01	3.80	-	Present	Wild Type G/G
13	1.88	4.00	-	Present	Wild Type G/G
14	1.47	13.10	-	Present	Wild Type G/G
15	1.43	3.20	-	Present	Wild Type G/G
16	1.32	3.80	-	Present	Wild Type G/G
17	1.60	4.90	-	Present	Wild Type G/G
18	2.08	2.50	-	Present	Wild Type G/G
19	1.03	2.60	-	Present	Wild Type G/G
20	-	2.20	-	Present	Wild Type G/G
21	2.15	4.20	0.10	Present	Wild Type G/G
22	2.68	2.40	-	Present	Wild Type G/G
23	1.47	5.50	-	Present	Wild Type G/G
24	1.82	5.60	-	Present	Wild Type G/G
25	1.61	7.10	-	Present	Wild Type G/G
26	1.54	3.30	-	Present	Wild Type G/G
27	1.85	2.70	-	Present	Wild Type G/G
28	1.81	8.10	-	Present	Wild Type G/G
29	1.70	3.60	-	Present	Wild Type G/G
30	1.44	2.00	-	Present	Wild Type G/G
31	*1.44*	*5.00*	-	*No increase of fluorescence*	*Undetermined*
32	2.10	5.20	-	Present	Wild Type G/G
33	1.57	28.10	1.17	Present	Wild Type G/G
34	1.62	4.20	-	Present	Wild Type G/G
35	1.41	11.90	0.26	Present	Wild Type G/G
36	1.32	8.50	0.20	Present	Wild Type G/G
37	1.21	3.10	-	Present	Wild Type G/G
38	1.82	1.90	-	Present	Wild Type G/G
39	*1.49*	*2.40*	-	*No increase of fluorescence*	*Undetermined*
40	1.34	6.30	-	Present	Wild Type G/G
41	1.33	7.30	-	Present	Wild Type G/G
42	*1.76*	*1.50*	-	*No increase of fluorescence*	*Undetermined*
43	0.00	0.00	-	Present	Wild Type G/G
44	1.74	7.10	-	Present	Wild Type G/G
45	1.33	0.50	-	Present	Wild Type G/G
46	1.66	3.10	-	Present	Wild Type G/G
47	0.12	0.10	-	Present	Wild Type G/G
48	1.38	3.20	-	Present	Wild Type G/G
49	0.75	1.10	-	Present	Wild Type G/G
50	*0.89*	*1.10*	-	*No increase of fluorescence*	*Undetermined*
51	1.70	8.40	-	Present	Wild Type G/G

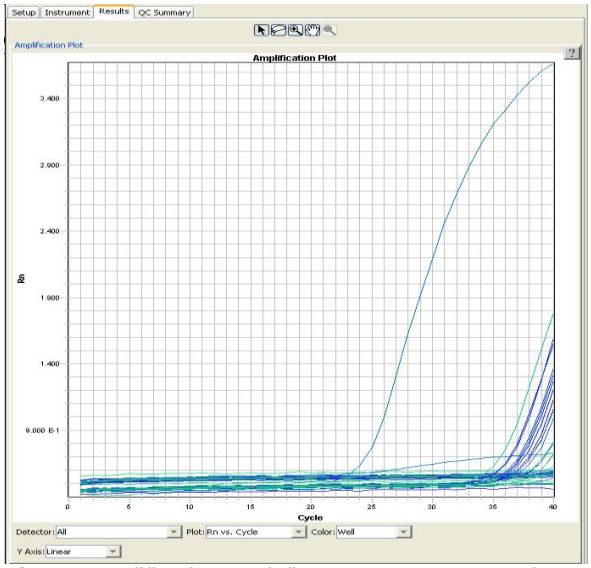

Fig 2. Amplification and fluorescence curves of DNA samples and positive control. Cut off (C_T) value for the positive control (with 1 ng DNA) starts at the 23rd amplification cycle. For investigated DNA samples the C_T values start at the 35th cycle to the 39th cycle. Only the wild–type alleles showed (sequence with the normal nucleotide, G) fluorescence increase.

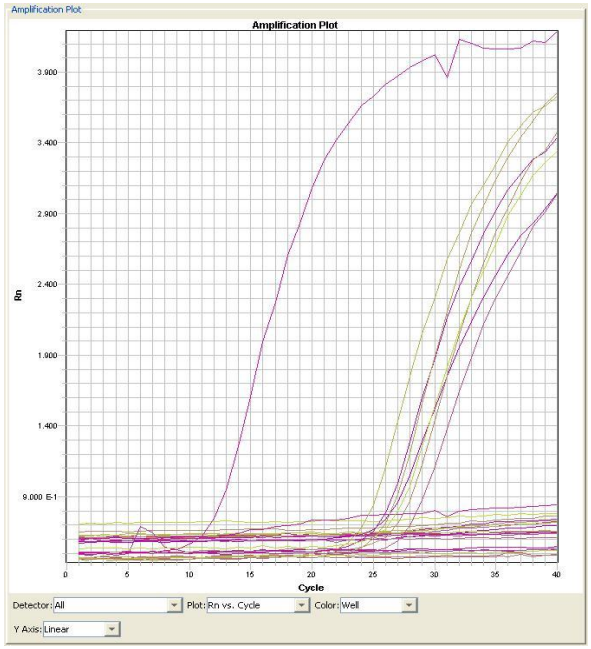

Fig 3. Amplification and fluorescence curves of DNA samples and positive control. Cut off (C_T) value for the positive control (with 1 ng DNA) starts at the 11th amplification cycle. For investigated DNA samples the C_T values start at the 24th cycle. Only the wild–type alleles showed (sequence with the normal nucleotide, G) fluorescence increase.

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