Int J Legal Med (2012) 126:649–657
DOI 10.1007/s00414-012-0688-4

TECHNICAL NOTE

Detection of genetic variation in KCNQ1 gene by high-resolution melting analysis in a prospective-based series of postmortem negative sudden death: comparison of results obtained in fresh frozen and formalin-fixed paraffin-embedded tissues

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Received: 2 November 2011 / Accepted: 23 February 2012 / Published online: 9 March 2012

Abstract High-resolution melting (HRM) analysis is a recently developed molecular technique proved to be applicable for detection of genetic variation, notably in sudden cardiac death. In certain circumstances, especially in postmortem genetic investigations, the formalin-fixed and paraffin-embedded (FFPE) tissues are the only DNA source available. The present study aimed to develop HRM assays, optimized for the analysis of FFPE tissues, to detect sequence variations in KCNQ1 exons in a prospective population-based series of postmortem negative sudden death and to compare the results between the paired freshly frozen and FFPE tissue samples simultaneously obtained from the same case. The analyses were conducted in each case of sudden death involving cases younger than 35 years with no significant morphological anomalies particularly with no cardiac structural disease and with negatives toxicological investigations. HRM analysis was successfully optimized for 13 of the 16 exons of the KCNQ1 gene. All mutated samples were correctly identified by HRM whatever the type of tissue tested. However, for FFPE samples, HRM indicated more positive samples than classical sequencing, used in parallel, due to the degradation of DNA by formalin fixation. This is the first postmortem study of KCNQ1 mutation detection with HRM on DNA extracted from FFPE samples with adapted protocol. Despite the false-positive detection, we concluded that the use of HRM as a screening method with FFPE samples to analyze KCNQ1 mutations can reduce the number of sequencing reactions and, thus, results in substantial time and cost savings.

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Keywords High-resolution melting · Sudden cardiac death · KCNQ1 · Formalin-fixed and paraffin-embedded tissue

Introduction

In developed countries, sudden cardiac death (SCD) is considered as one of the most common cause of death, representing a major health problem [1, 2]. Although the majority of cardiac death victims are elderly, many children and young adults under the age of 35 years die each year due to various cardiac pathologies. Structural cardiovascular abnormalities are often evident at autopsy, including hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, myocarditis, or congenital coronary artery anomalies [3, 4]. However, it has been described that the cause of death remains unknown in 10–30 % of cases of sudden death in young adults and children [3, 5]. Potentially lethal and heritable ion channel disorders or "channelopathies" such as long QT syndromes (LQTS), catecholaminergic polymorphic ventricular tachycardia (CPTV) or Brugada syndrome may be responsible for a portion of those cases. In the last years, many of the mutated genes implicated in these channelopathies have been identified and their role in the development of cardiac arrhythmias has been confirmed [6].

To date, different technologies and genetic approaches have been developed for the diagnosis of these cardiac disorders. These technologies enable the single nucleotide mutation screening (with notably the iPLEX Gold technology from Sequenom [7] or the SNaPshot technology [8]), the scanning of known genes or the searching for new genes [9]. Among the molecular techniques, which aimed to detect novel and/or known DNA sequence variant,

the high-resolution melting (HRM) analysis is a recently developed methodology that has an enormous potential [10]. The HRM is a simple, semi-automated and cost-effective approach to identify single-base substitutions and small insertions/deletions with nearly 100 % detection [11, 12]. The HRM assay is based on a real-time polymerase chain reaction (PCR) amplification of genomic DNA with target specific primers in the presence of a fluorescent intercalating DNA dye followed by a fluorescent melting curves of PCR amplicons to discriminate between wild-type and mutant DNA. The HRM assay is particularly suited as a prescreening test to decide whether or not a specimen should be subjected to subsequent classical sequencing in order to identify nucleotide alteration(s). As recently reviewed, the list of genes analyzed by HRM is increasing [13]. Among the different cardiac channelopathy susceptibility genes implicated in sudden death with structurally normal heart (namely 12 genes in LQTS [14], seven genes in Brugada Syndrome [15], two genes in CPTV [16, 17]), three genes (KCNQ1, KCNH2, and SCN5A) have been studied by Millat et al. by HRM analysis [12, 18]. The KCNQ1, KCNH2, and SCN5A genes account for 70–75 % of definite congenital LQTS cases with a frequency in the affected people of 40–55, 35–45, and 2–8 %, respectively [14]. In the last study of Millat et al. [18], the HRM analysis was performed on DNA extracted from whole blood in a panel of LQTS-suspected patients.

In cases of autopsy-negative sudden cardiac death in the young adult, postmortem genetic diagnosis of mutations in cardiac ion channel genes was performed from different sort of samples: DNA immediately extracted from blood or tissue taken during autopsy [19], DNA extracted from EDTA blood samples [20], DNA extracted from paraffin-embedded tissue [2, 21, 22], or DNA extracted from frozen tissue [23]. Concerning the problematic investigations on sudden cardiac death in France, the principal limitation to perform prospective or retrospective postmortem molecular study is the possibility of having access to the appropriate sampling and storage material [9, 24]. Because of the constraints that are required to collect frozen fresh tissues (such as the necessity to have dedicated spaces and specific equipment to preserve the samples), the FFPE samples are more frequently the unique source of DNA.

If HRM analysis was previously validated as a screening method for EGFR and KRAS mutation detection on degraded DNA from FFPE tissues in lung or colorectal cancer studies [25–29], no previous study has evaluated the use of HRM for mutation screening on FFPE samples on gene implicated in sudden cardiac death. The main objective of the present study was to develop HRM assays, optimized for the analysis of FFPE tissues, to detect sequence variations in KCNQ1 exons by using as control the freshly frozen specimens simultaneously obtained from the same case. The KCNQ1 gene was selected in a first approach because it represents a large percentage of the long QT syndrome mutations (40–55 % according to Hedley et al. [14]), and for its low number of small size exons.

Materials and methods

Process of investigations

During 3 years (2008–2010), 5 g of fresh tissue of heart and liver were retained and frozen at −80° at the initial autopsy of individuals 0–40 years of age where the cause of sudden death was not immediately apparent. After 4–6 weeks, time during which histopathology and toxicology results became available, we determined if genetic investigations should start within the judicial mandate which is to establish the cause of death. The inclusion criteria were: no organic abnormality, no macroscopical, or microscopical abnormalities of the heart and negatives toxicological analyses. All the histopathological examinations of the heart was realized by the same histopathologist specialized in cardiac disease according to the guidelines of Association for European Cardiovascular Pathology [1]. For each case, tissue samples (heart and liver) were processed under freshly frozen and FFPE conditions in parallel. The time of fixation in the buffered formaldehyde is specified in Table 1. We also included in the cohort four negatives controls (wild-type samples). Forensic investigations including genetic testing were carried out within the judicial mandate which is to determine the cause of death.

DNA extraction and quantification

The DNA extraction was adapted to the type of tissue. For the FFPE tissue, we used the protocol consisting in the association of phenol-chloroform and QIAamp DNA mini® Kit (Qiagen) as previously described [30]. The frozen samples were extracted and purified by using the QIAamp DNA Mini®

Table 1 Characteristics of the cohort

Case	Sex	Age	Time of formalin fixation (days)
1	F	4 Months	17
2	M	20 Years	7
3	M	2 Months	11
4	F	3 Years	16
5	M	20 Years	4
6	F	16 Years	11
7	F	8 Days	16
8	M	3 Months	6

F female, *M* male

Kit according to the manufacturer's instructions. Quantification of all DNA samples was made with the NanoDrop™ 8000 Spectrophotometer (Thermo Scientific®).

Design of HRM primers

Primers giving rise to short amplicons were designed as they are more likely to result in satisfactory amplification from degraded FFPE DNA. Each primer, designed with Primer3 software, was analyzed with FastPCR software (Institute of Biothechnology, University of Helsinki) for the detection of hairpin or dimer formations and was submitted to Basic Local Alignment Search Tool search to ensure they are specific for the target species and gene.

Identical primers pairs were used for HRM analysis and sequencing of KCNQ1 exons. Primers sequence is listed in Table 2. Two types of primers, high-performance liquid chromatography (HPLC) and non-HPLC were tested.

PCR and HRM conditions

Real-time PCR amplification and HRM analysis were performed on Light Cycler®480 Real-Time PCR Systems (Roche Diagnostics) by using the Light Cycler®480 High Resolution Melting Master kit (Roche Applied Science) according to the manufacturer's instructions.

The first step was to determine the optimal $MgCl_2$ concentration for each exon with the four negatives controls. MgCl2 concentrations between 1.2 and 4 mM were evaluated with a touchdown PCR program (annealing temperature from +65 to 53°C). The second step was to test two different PCR touchdown program (annealing temperature from +65 to 53°C and 68 to 59°C). The DNA concentration was adapted depending on the Cp value. PCR amplifications were carried out in a final volume of 20 μL containing 10 μL of Master mix, 0.25 μM of each primer, DNA, and $MgCl_2$ at indicated concentration in Table 2.

The PCR conditions were: pre-incubation step at 95°C for 10 min for activation of the polymerase, followed by 35 cycles of 95°C for 10 s, a touchdown for 15 s (1°C/s) with annealing temperature from 65 to 53°C or 68 to 59°C (depending on the exon at indicated in Table 2) and extension step at 72°C for 20 s. After the amplification, the PCR products were denatured at 95°C for 1 min and cooled down to 40°C. The final HRM step was performed from 70 to 95° C with increase of 1°C per second with 25 acquisitions per degree. As HRM could induce some false positives, all samples were tested in duplicate with four negative controls

Table 2 Specific primers used and HRM conditions for HRM analyses

Exon		Primers (5′→3′)		PCR conditions				HRM conditions
		Forward	Reverse	Amplicon Size (bp)	T°a (°C)	DNA (ng)	MgCl2 (mM)	
1	1a	CGG CAG GCC CTC CTC GTT	TCC AGC GAG AAG GGG CAT TTT	134	68–59	30	2.5	DS
	1b	GAA AGG AAG CGC TGG GGT TG	GGC CAA GGT CGG AAG CAA CT	211	68–59	30	2.5	
	1c	CCT TCT CGC TGG AAC TGG	CCG GTG GCG ATA CTC AC	300	65–53	30	2.5	
2		ACT GCC GTG TCC CTG TCT T	TAT CAG GGC AGG ACC AAT GT	248	65–53	30	2.5	
3		AAA CAG GTT GCA GGG TCT GAA G	TCC TTC CTG GTC TGG AAA CCT G	254	65–53	30	2.5	
4		CCC TCT CCT GCA CTC CAC	GAG CTT GTG GCA CAG ACG	120	68–59	30	2.5	
5		AGC CCC ACA CCA TCT CCT T	AGG TTG GGG ACA GGA CGG A	171	68–59	30	3	
6		AGC CCG ACA CTG TGT GTT TT	GCC TGG AAG TTT CCG ACT TA	191	65–53	30	2.5	
7		TGG GTT TGG GTT AGG CAG TTG	AAG GAG CCA GGG AAA ACG CA	223	65–53	30	2.5	
8		GAG CCT CCT GTC CAT TCC TT	GGC TGG ATG CAA CAA TAA CA	183	68–59	30	2.5	
9		GAA CAG GGA GGG GGA GCT GT	TGG TGG CAG GTG GGC TAC TC	231	68–59	45	2.5	
10		TAG GGC CTG GCA GAC GAT GT	CCC AAC TGC CTG AGG GGT TC	222	68–59	30	2.5	
11		TGT CCC CAC ACT TTC TCC TC	TTC ACG CAC ATG TAG GCA CT	166	65–53	30	2.5	
12		TGG CCA CTC ACA ATC TCC T	GCC TTG ACA CCC TCC ACT A	222	68–59	30	2.5	
13		AGG AGA AGT GAT GCG TGT CTT TTT G	AGA GGC AAG AAC TCA GGG TCT CAG C	244	68–59	45	2.5	DS
14		GTG TGA ACT GGT GTC TGT GTC CTT	GTT TCT GTG TCA GTT ACT CTG GGC	107	65–53	30	2.5	
15		CCC AGC ACT TGG CCC TGA TT	ACG CAG ACC ACA GGG AGG TG	178	65–53	30	2.5	
16		CAC CAC TGA CTC TCT CGT CTG C	CCA TCC CCC AGC CCC ATC	297	65–53	30	2.5	DS

DS direct sequencing

for each exon in order to decrease significantly the number of false positive calls.

HRM analysis

The HRM curve analysis was performed on the Light Cycler® 480 Gene Scanning Software (Version 1.5). The efficiency and sensibility of the amplification was deduced from the amplification curves. The specificity of amplification was assessed using melting peaks analysis and agarose gel electrophoresis. Gene scanning information was derived from melting curves analysis: the melting curves were normalized, temperature-adjusted and, finally, a difference plot was generated. The four wild-type controls were selected as the baseline for comparison. Samples were considered mutated when significant difference of fluorescence level for all duplicate fell outside of the range of variation detected for the wild-type controls.

Sequence analysis

PCR products showing aberrant melting curves were purified by using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations and directly analyzed by capillary electrophoresis on ABI Prism 3500 genetic analyzer (Applied Biosystems) using the BigDye® Terminator. The four negatives controls were also sequenced. All putative KCNQ1-associated mutations and other variants were denoted using known and accepted nomenclature [31]. The reference sequence used is the NM_000218.2.

Results

Samples studied

DNA was stored at the time of autopsy from 23 unrelated sudden unexplained deaths in the young victims. In 15 cases, laboratory test revealed a diagnosis such that molecular autopsy was not required. These included four cardiac diagnoses such as acute myocarditis (one), acute myocardial infarction (two) or endocardial fibroelastosis (one), and 11 various noncardiac diagnoses including respiratory infections (two), and narcotic or drug intoxication (nine). Eight cases were referred for channelopathies gene screening. Among these eight cases, we distinguish children and young adults aged between 1 and 35 years old and infants aged less than 1 year (Table 1).

Optimisation of HRM conditions

Despite the use of different primers pairs (carefully selected with Primer3 software) and different amplification protocol (modifications of DNA concentration, MgCl2 concentrations, annealing temperature), PCR optimisation of three exons did not allow to obtain a sufficient amplicon quality for subsequent HRM analysis. Most of them present abnormal PCR characteristics such as presence of nonspecific products or high Cp value. Consequently, these three exons (1, 13, and 16) were analyzed by direct sequencing (Table 2).

For two successfully optimized exons, we tested the same primers pairs with or without purification with HPLC. No differences were observed in the melting curves (results not shown). Therefore, we decided to use only non-HPLC primers for economic reason.

Mutation scanning and sequencing results

For eight exons (3, 4, 7, 8, 9, 10, 11, and 15), the melting curves analysis showed only one population whatever the type of storage and the type of tissue, indicating that no variant were detected. In such case and in order to monitor false negatives, we used a random control strategy to sequence heart or liver samples for each case. No mutation was detected in random samples (Fig. 1a).

For three exons (2, 6, 12), five cases were identified to be "mutant" in frozen and FFPE tissue-derived DNA. The HRM profiles on exon 12 of frozen tissue samples and their matched FFPE samples is represented in Fig. 1b. The direct sequencing confirmed the HRM results and found three genomic variant, c.858 C>T in exon 6 for the case 5, c.1590+14 T>C in intron 12 for cases 6 and 8, c.477+96 del GG in the intron 2 for cases 1 and 4.

For exon 14, the melting curves showed two distinct population corresponding to frozen and FFPE tissue-derived DNA (Fig. 1c). We used a random control strategy to sequence heart or liver samples for each case. No mutation was detected in random samples.

For exon 5, we observed a discordant results between the paired frozen and FFPE samples. Indeed, no variant were identified in frozen samples while three distinct populations were identified in FFPE tissue-derived DNA (Fig. 1d). All samples identified as "mutant" by HRM were sequenced and no mutation was found. Consequently, theses samples were in fact wild-type.

The direct sequencing of the three non-optimized exons (exons 1, 13, and 16) detected a substitution c.1638 G>A in exon 13 for two cases (Table 3).

Discussion and conclusions

The HRM analysis is an attractive screening method that works with a great precision in detecting single nucleotide substitutions that produce missense, nonsense, and splice site mutations or small insertion/deletions. However large

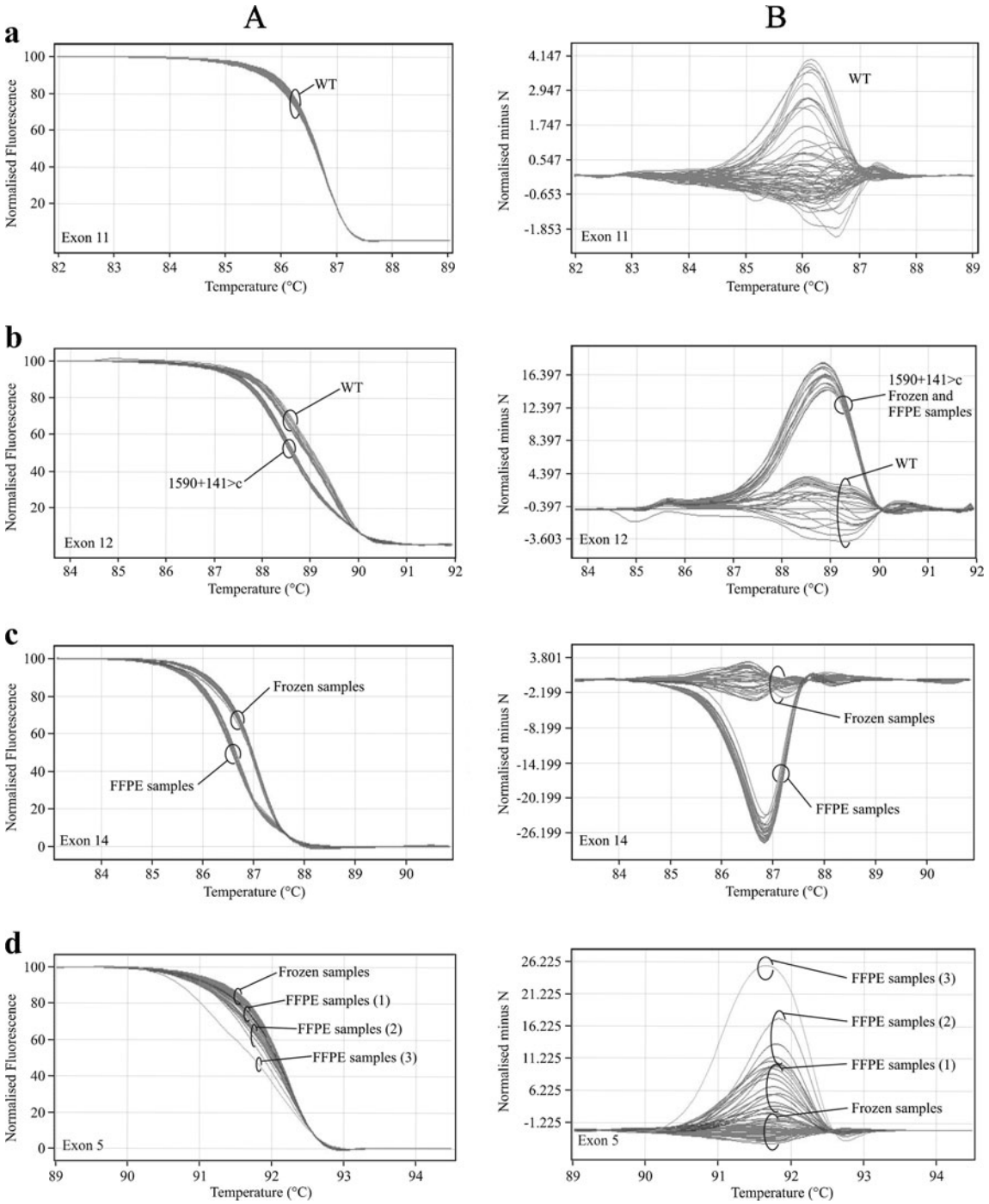

Fig. 1 Normalized plot (**a**) and difference plot (**b**) of on example of each case observed: *a* one population whatever the type of storage (frozen or FFPE) and the type of tissue indicating that no variant were detected, *b* two populations whatever the type of storage and the type of tissue corresponding to the wild-type and two variants, *c* two distinct populations corresponding to the frozen samples and the FFPE samples, and *d* no variant observed in frozen samples and different populations observed in FFPE samples

whole gene, multiple exon, or single exon deletions or duplications elude detection by this approach. According to the publicly available website (http://www.fsm.it/cardmoc) maintained by priori (last update in December 2010) among over 300 mutations listed on KCNQ1, the majority (80 %) are single nucleotide substitutions and the remaining 20 % are small in frame insertion or deletions. Consequently, the HRM could theoretically detect the majority of the KCNQ1 mutations actually described, excepted the rare copy number variations that explain around 3 % of LQTS in patients with no point mutation in the KCNQ1 and KCNH2 genes [32, 33].

Table 3 Details of KCNQ1 variations identified by HRM and confirmed with sequencing for eight cases of the cohort studied

Nucleotide numbering starts from the ATG start codon

Nucleotide change (NM_000218.2)	Amino acid change (NP_000209.2)	Region	Case number	Previous report (if any)
c.477+96 del GG	Intronic variant	Intron 2	1, 4	
c.858 C>T	p.D286D	Exon 6	5	
c.1590+14 T>C	Intronic variant	Intron 12	6, 8	[34, 35]
c.1638 G>A	p.S546S	Exon 13	1, 5	[34–38]

In the present study, whatever the type of storage condition (frozen or FFPE), not all the KCNQ1 exons can be studied by the HRM which was successfully optimized for 13 of the 16 KCNQ1 exons. Despite the use of different primer pairs and different amplification protocols, we did not obtain a sufficient amplicon quality for three exons: the exons 1, 13, and 16. Millat et al. meet similar difficulty in their study performed on DNA extracted from whole blood in a panel of patients with a suspicion of LQTS. Indeed, despite the use of different kits (LightCycler® 480 High Resolution Melting Master kit and SYTO-9 dye combined with other DNA polymerases), the HRM was not optimized for two of the 16 KCNQ1 exons (exons 1 and 16) and for 12 of the 15 KCNH2 exons [18]. One of the hypotheses that might explain the difference of results concerning the exon 13 between our study and the Millat's publication is the use of a different instrument (Light Cycler®480 Real-Time PCR Systems from Roche Diagnostics versus Rotor-Gene 6000 analyser from QIAGEN) with a different software and a different PCR kit (LightCycler® 480 High Resolution Melting Master kit versus LightCycler® 480 Probes Master kit and SYTO-9 dye).

Consequently, the HRM analyses could not be performed, in our study, on these three exons which had to be explored with direct sequencing to get the whole genetic information of the KCNQ1 gene. Despite the different time of formalin fixation of the samples varying from 4 to 17 days, the melting curve behavior was identical whatever the type of storage condition for 11 of the 13 exons which were screened by HRM analysis. In our cohort of eight cases, among the sequence variants detected by HRM and confirmed by sequencing, we distinguished: one deletion of two nucleotides in the intron 2 (c.2477+96 del GG) not previously described, a single nucleotide substitution on exon 6 (c.858 C>T) not previously described which was synonymous (p.D286D) and a single nucleotide substitution in intron 12 (c.1590+14 T>C) which was previously described [34, 35]. The direct sequencing of exon 13 revealed a single nucleotide substitution (c.1638 G>A) that were previously described as synonymous polymorphism [35–38]. Consequently, no mutation with amino acid change and therefore with functional effect on ion channel was detected in our cohort. No conclusion concerning the cause of death in our prospective-based series of postmortem negative sudden death could be emitted and complementary investigations on other genes involved in channelopathies must be obviously performed.

If we compare the low yield of genetic testing in our cohort with the data of the 10 years of published studies of molecular autopsies of sudden death in the young, we observed a KCNQ1 mutation detection rate extremely variable according to the publications. For example, the KCNQ1 mutation detection rate was 0 % in a cohort of 12 cases [22] and 59 cases [3], 2.5–3 % in a cohort of 33 cases [19] and 35 cases [8], 7 % in two cohorts of 14 cases [7, 39], 10 % in a cohort of 49 cases [40], and 20 % in a cohort of 10 cases [41]. Consequently, considering the small size of our study population and the variability of the KCNQ1 mutation detection rate, the low yield of genetic testing observed in our study does not contradict the data of the literature.

Table 4 Cost analysis of direct sequencing (DS) versus HRM/targeting direct sequencing (TDS) of all exons in the KCNQ1gene for eight samples according to the type of samples (frozen or FFPE)

Parameter	DS of 16 exons	HRM/TDS of 13 exons couples with DS of 3 exons							
Type of samples	FFPE or frozen	FFPE				Frozen			
		HRM	TDS	DS of 3 exons	Subtotal	HRM	TDS	DS of 3 exons	Subtotal
Featuring disposable cost	600 €	110 €	80 €	110 €	300 €	110 €	25 €	110 €	245 €
Chemical costs	2,100 €	830 €	270 €	400 €	1,500 €	830 €	80 €	400 €	1,310 €
Total working costs in Euros (if unitary working costs is set to 23 € h^{-1})	506 €	161 €	115 €	115 €	391 €	161 €	23 €	115 €	299 €
Hands on time and time of results interpretation	22 h	7 h	5 h	5 h	17 h	7 h	1 h	5 h	13 h
Total cost analysis	3,206 €				2,191 €				1,854 €

However, this result highlights the fact that the low number of cases included in our prospective series of postmortem negative sudden death constitutes the principal limitation of the present study. It can be explained by the very strict inclusion criteria used and the low number of autopsy performed each year at the Institute of Legal Medicine of Strasbourg, France (200 autopsy annually). To increase the number of cases, a national or multinational multicenter study might be a potential solution with the risk of losing sample quality monitoring. Indeed, in the present study, we had the possibility to control rigorously each processing step from tissue sample to the genetic analyses allowing a very good monitoring of the samples.

If the melting curve behavior in HRM analyses were equivalent whatever the type of storage for the majority of the exons, for two exons (numbers 5 and 14) however, the results observed between the paired frozen and FFPE samples were discordant (Figs. 1c–d): all cases were grouped as wild-type in frozen samples and as variant in FFPE samples with no sequence variation detected by sequencing of all FFPE samples. The detection of false positives by HRM could be explained by the PCR artifacts induced either from Taq polymerase error or from errors attributed to chemical reactions of formalin on DNA. The cumulative effects of these two phenomena influence the melting profile of the amplicon depending on the degree of DNA damages [42–44]. The same phenomenon of false positive was previously described in a study performed on FFPE samples for other genes [25, 28]. For example, Do et al. reported a level of 12.5 % of false positive with 200 FFPE samples tested for the detection of somatic EGFR and KRAS mutations [25]. The level of false positive could not be calculated in our study owing to the low number of cases included in the cohort.

Given the existence of false positive and the necessity to sequence directly the three exons with non-optimal amplification conditions, we can wonder why it would not be more efficient and more economical to sequence directly the 16 exons of KCNQ1? To respond to this question, we compared, for the eight cases of our cohort, the cost of the HRM coupled with targeted direct sequencing (HRM/TDS) and the direct sequencing (DS) of the 16 exons of KCNQ1, without considering the cost of the equipments. We evaluated the relative featuring disposable cost, chemical cost, hands-on time, and time of results interpretation by the two techniques (HRM/TDS versus DS) with the two types of samples by taking into account the necessity to sequence the false positives observed with FFPE samples. The results, presented in Table 4, showed that the cost of direct sequencing of the 16 exons, whatever the type of samples, is 3,206 Euros, the cost of the HRM analysis (realized with samples in duplicate) coupled with targeted sequencing on variant detected, with direct sequencing of three exons (1, 13, and 16) and with the sequencing of false positives only with FFPE samples is 2,191 Euros for FFPE samples and 1,854 Euros for frozen samples. Therefore, with frozen samples, the cost reduction of HRM/TDS versus DS is more than 40 %. Moreover, even with false positive results, the mutation screening with HRM/TDS on FFPE samples allowed a cost reduction of more than 30 % compared with direct sequencing of all exons in the gene of interest. The principal reason of such results is the reduction of sequencing reactions. Indeed, in our study, the screening of mutations with HRM allowed to eliminate up to 70 % of the sequencing reactions for FFPE samples. The same results were described in previous study of Do et al. in which it was estimated that up to 80 % of sequencing reactions can be eliminated for the KRAS and EGFR genes if samples are screened by HRM [25].

In conclusion, the realization of postmortem genetic diagnosis of mutations in cardiac ion channel genes in France is limited by the quality of the samples more frequently collected by the forensic pathologists, namely FFPE samples. When only FFPE samples are available, our initial evaluation provided evidence that the HRM method coupled with targeted sequencing is applicable on FFPE samples. Even with false positives, we have estimated that the HRM allowed a reduction of sequencing reactions number by up to 70 % and thus result in substantial time and cost savings allowing a routine use. The validation of HRM method as a screening method of mutations on KCNQ1 gene on FFPE samples requires at least his verification in a large number of samples. Moreover, although KCNQ1 represents a large percentage of the long QT syndrome it must be develop further investigation on other genes implicated in sudden cardiac death.

While it is true that the starting material in retrospective genetic studies is DNA extracted from FFPE samples, it should be a priority to establish a protocol for frozen samples collection in autopsy for potential prospective studies in France.

Acknowledgments We gratefully thank the Genetic Diagnostic Laboratory of the University Hospital of Strasbourg, directed by Professor Mandel, for the free access to the HRM platform. We also thank Aurélie Marchet and Daniel Montagnon for their technical help.

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