Genetic diagnosis of hypertrophic cardiomyopathy using mass spectrometry DNA arrays and high resolution melting [1]

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ABSTRACT

Introduction: Hypertrophic cardiomyopathy (HCM), a complex myocardial disorder with an autosomal dominant genetic pattern and prevalence of 1:500, is the most frequent cause of sudden death in apparently healthy young people. The benefits of gene-based diagnosis of HCME for both basic research and clinical medicine are limited by the considerable costs of current genetic testing due to the large number of genes and mutations involved in this pathology. However, coupling two high-throughput techniques – mass spectrometry genotyping (MSG) and high resolution melting (HRM) – is an encouraging new strategy for HCM diagnosis. Our aim was to evaluate the diagnostic efficacy of both techniques in this pathology by studying 13 individuals with a clinical phenotype of HCM. *Methods*: Peripheral blood samples were collected from: (i) seven subjects with a clinical diagnosis of HCM,

Diagnóstico genético de miocardiopatia hipertrófica com recurso a espectrometria de massa com *arrays* de ADN e desnaturação de alta resolução

RESUMO

A Miocardiopatia Hipertrófica (MH), uma doença genética complexa do miocárdio com um padrão de transmissão autossómica dominante e prevalência de 1:500, é a forma mais frequente de morte súbita em jovens aparentemente saudáveis. Os benefícios de um diagnóstico baseado nos genes associados a MH, adequado à investigação básica e à prática clínica, estão, no entanto, limitados pelos consideráveis custos das estratégias actuais de diagnóstico genético, baseadas em sequenciação automática, devido ao elevado número de genes e mutações envolvidas nesta doença. Todavia,

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all bearing known mutations previously identified by dideoxy sequencing and thus being used as blinded samples (sample type 1); (ii) one individual with a clinical diagnosis of HCM negative for mutations after dideoxy sequencing of the five most common HCM genes, *MYH7*, *MYBPC3*, *TNNI3*, *TNNT2* and *MYL2* (sample type 2); and (iii) five individuals individual with a clinical diagnosis of HCM who had not previously been genetically studied (sample type 3). *Results*: The 13 samples were analyzed by MSG for 534 known mutations in 32 genes associated with HCM phenotypes and for all coding regions and exon-intron boundaries of the same HCM genes by HRM. The 32 studied genes include the most frequent HCM-associated sarcomere genes, as well as 27 genes with lower reported HCM phenotype association. This coupled genotyping strategy enabled us to identify a c.128delC (p.A43Vfs165) frame-shift mutation in the *CSRP3* gene, a gene not usually studied in current HCM genetics. The heterozygous *CSRP3* mutation was found in two patients (sample types 2 and 3) aged 50 and 52 years, respectively, both with diffuse left ventricular hypertrophy. Furthermore, this coupled strategy enabled us to find a novel mutation, c.817C>T (p.Arg273Cys), in *MYBPC3* in an individual from sample type 3, subsequently confirmed by dideoxy sequencing. This novel mutation in *MYBPC3*, not present in 200 chromosomes from 200 healthy individuals, affects a codon known to harbor an HCM-causing mutation – p.Arg253His. *Conclusion*: In conclusion, in the cohort used in this work coupling two technologies, MSG and HRM, with high sensitivity and low false positive results, enabled rapid, innovative and low-cost genotyping of HCM patients, which may in the short term be suitable for accurate genetic diagnosis of HCM.

consideramos que a conjugação de duas técnicas de elevada resolução – Genotipagem por Espectrometria de Massa (GEM) e Desnaturação de Alta Resolução (DAR) – tem demonstrado ser uma estratégia encorajante para o diagnóstico de MH. Foi nosso objectivo avaliar a eficácia diagnóstica destas duas técnicas no âmbito desta patologia, estudando 13 indivíduos com fenótipo de MH. Neste sentido, foram colhidas amostras de sangue periférico de: (i) sete indivíduos clinicamente diagnosticados com MH, todos com mutações previamente identificadas por sequenciação automática, e actuando como amostras *blinded* ("amostra tipo 1"); (ii) um indivíduo clinicamente diagnosticado com MH sem mutações identificadas após sequenciação dos 5 principais genes associados a MH, *MYH7*, *MYBPC3*, *TNNI3*, *TNNT2* e *MYL2* ("amostra tipo 2"); e (iii) cinco indivíduos clinicamente diagnosticados com MH sem estudo genético prévio ("amostra tipo 3"). As 13 amostras foram analisadas por GEM para 534 mutações conhecidas em 32 genes associados a um fenótipo de MH e para todas as regiões codificantes e fronteiras exão/intrão dos mesmos genes por DAR. Estes 32 genes estudados incluem os 5 genes mais frequentemente associados a MH e outros 27 genes reportados como associados a MH numa menor frequência. Esta estratégia permitiu-nos identificar uma mutação c.128delC (p. A43Vfs165) que origina uma alteração na grelha de leitura da proteína codificada pelo gene *CSRP3*, um gene usualmente não analisado pela estratégia corrente de diagnóstico genético. Esta mutação em heterozigotia no gene *CSRP3* foi encontrada em dois doentes (amostra tipo 2 e 3) com 50 e 52 anos respectivamente, e que apresentavam hipertrofia ventricular esquerda difusa. Esta estratégia permitiu-nos ainda identificar uma nova alteração c. C>T817 (p.Arg273Cys) no gene *MYBPC3* num indivíduo da amostra tipo 3 que foi posteriormente confirmada por sequenciação. Esta nova alteração no gene

MYBPC3, ausente em 200 cromossomas de indivíduos saudáveis, afecta um codão cuja mutação foi já associada a MH – p.Arg253His. Em conclusão, a conjugação de duas tecnologias, GEM e DAR, de elevada sensibilidade e baixo nível de resultados falsos positivos permitiu, na amostragem utilizada neste trabalho, uma genotipagem rápida, inovadora e de baixo custo em doentes com MH, podendo vir a tornar-se a curto prazo numa metodologia apropriada para o diagnóstico genético de MH.

Palavras-Chave:
Miocardiopatia hipertrófica; Diagnóstico genético; Espectrometria de Massa por Arrays; Desnaturação de Alta Resolução; Proteínas sarcoméricas; gene *CSRP3*; Proteínas do disco Z

Key words
Hypertrophic cardiomyopathy; Gene-based diagnosis; Array-based mass spectrometry; High resolution melting; Sarcomere proteins; *CSRP3* gene; Z-disc proteins

INTRODUCTION

Cardiomyopathies are associated with myocardial dysfunction which varies from an asymptomatic course to heart failure (HF) [1, 2, 3]. Hypertrophic cardiomyopathy (HCM), an autosomal dominant genetic disease which may afflict as many as 1 in 500 subjects and is an important risk factor for sudden death (SD) or HF disability at any age, is characterized by a hypertrophied, non-dilated left ventricle (LV), myocyte disarray and interstitial fibrosis [4]. HCM is the most frequent cause of SD in otherwise healthy young people; it may in fact be the only clinical manifestation of the disease. Genetic diagnosis is important in all patients with suspected HCM for several reasons: counseling on professional and/or leisure activities (particularly in young people with a family history or familial SD); for genetic counseling (in the belief that certain mutations may be more dangerous than others); and, in general, to facilitate genetic diagnosis in family members once a mutation has been identified in the index patient[1, 2, 3].

Mutations in genes coding for myofilament contractile proteins of the cardiac sarcomere, including MYBPC3, MYH7, TNNT2, TNNI3 and MYL2, represent the most common genetic subtype of HCM, with 30% to 65% prevalence in various cohort studies, and HCM has accordingly been defined as a disease of the sarcomere (*Figure 1*)[5]. Age dependency and absence of evident and unequivocal symptoms lead to a high probability of the condition being detected only after a sudden death (SD) event. Due to the large number of HCM-associated genes and the allelic heterogeneity of this disease, genetic screening techniques such as single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and denaturing high pressure liquid chromatography (dHPLC) are expensive and time-consuming. Automated dideoxy sequencing (AS) of the most frequent mutated HCM genes (*Table I* – bold), the current gold standard genetic diagnosis test for HCM in Portugal, has low throughput, high cost (€350/gene) and poor sensitivity for detecting copy number variations (CNVs) or insertions/deletions (indels) and does not include genes that code for other proteins related to myocardium contraction such as the Z-band (*Figure 1*). The Z band is critical for

cytoarchitecture and is involved in mechanosensory cardiomyocyte signaling[7]. It is composed of titin (TTN), teletonin (TCAP) and muscle Z protein (CSRP3). Remarkably, mutations in all of these genes have been found to cause HCM.

The fact that in more than one third of proband analysis no mutation is found[8] further reduces the cost-effectiveness of the current genetic test, and underlines the need to seek novel genes/mutations that enable reliable genetic diagnosis. The existence of more

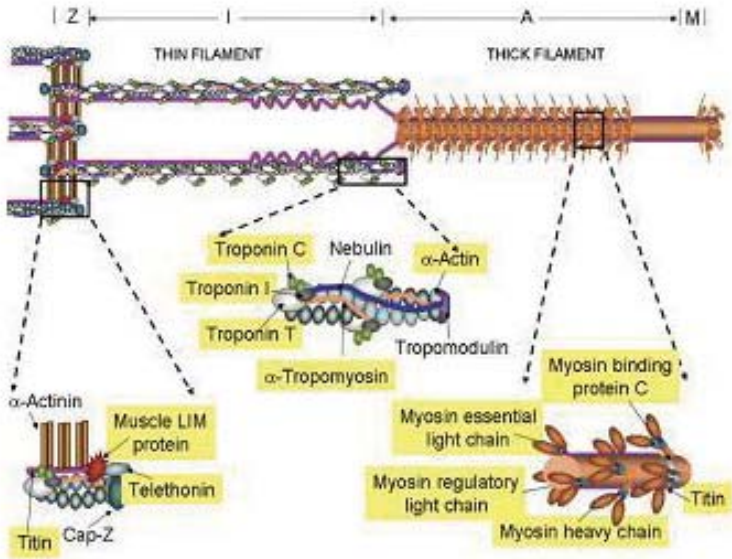

Figure 1. Sarcomere-related structures[6].

than 646 HCM phenotype-associated mutations in 32 different genes (*Table I*) makes full molecular testing by AS unacceptably slow and expensive, prompting DNA microarrays as an alternative technique[9-11]. Our group is currently validating iPLEX mass spectrometry genotyping (MSG) and high resolution melting (HRM) to improve HCM-gene based diagnosis. In contrast with AS genetic testing, high-throughput techniques such as MSG and HRM scanning allow rapid and cost-effective testing for a large number of different mutations simultaneously. MSG involves multiplex primary PCR using outer primers that flank HCM mutation sites followed by a homogeneous mass extend (hME) reaction with multiple single inner primers that together generate fragments of different mass specific for each genotype (iPLEX). To date, 534 mutations in 32 HCM-associated genes have been validated by our group (*Table I*) and a newly designed chip with 646 known mutations in 32 genes is being implemented. HRM is a high-throughput gene variation scanning technique that relies on the differential melting properties of sequences that vary in at least one nucleotide. For this reason it is used to identify novel mutations within samples. An advantage of MSG in comparison with other DNA microarray techniques[10] is its capacity for detecting indels[10]. Nevertheless, MSG is ineffective for detecting novel mutations[11], making our coupled strategy using both MSG and HRM more effective. The two techniques have overall costs of €50 and €350, respectively. We tested the validity of the MSG-HRM coupled strategy due to its potential for significantly increasing the throughput and speed of mutation detection, not to mention its promising value in association studies. This would apply to both known and unknown mutations in HCM patients or others, thus allowing analysis of genes specifically or randomly within a population or case study. Through this dual approach genes not previously studied by AS, such as those coding for Z-band proteins, will also be evaluated for mutations. The aim of this work was to develop new low-cost, accu-

rate, gene-based diagnosis that will contribute to the understanding of the genetic basis of disease in specific populations. This approach has the potential to significantly increase our understanding about HCM development and recognition, being a major support in the difficult clinical diagnosis of complex diseases.

METHODS

Peripheral blood was collected from 13 white patients (Portuguese) with a diagnosis of familial HCM, and from 100 healthy subjects (controls). Diagnosis was established on clinical, electrocardiographic (ECG) and echocardiographic (echo) grounds. The ECG/echo criteria have been previously discussed and published elsewhere[13]. Blood samples from HCM patients were divided into three groups: (i) seven blinded samples from patients with known mutations (detected previously by AS of the five most common HCM genes [*MYBPC3*, *MYH7*, *TNNT2*, *TNNI3* and *MYL2*] in a reference genetic diagnostic laboratory of reference)[12], (ii) a sample negative for mutations in the above HCM genes, and (iii) five samples not previously studied. The study was approved by the Ethics Committee of Faculdade de Medicina de Lisboa and all patients gave their written informed consent for genetic analysis.

DNA was extracted from the blood samples using a Blood DNA kit (Roche Diagnostics). All DNA samples were analyzed for 534 HCM-causing mutations (Human Genome Mutation Database http://www.hgmd.org; and the Harvard Sarcomere Protein Gene Mutation Database http://genepath.med.harvard.edu/~seidman/cg3/) in 32 HCM genes (*Table I*). Genotyping was performed using the iPLEX MassARRAY matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) method (Sequenom GmbH, Germany). The MSG system, based on the single-base extension of an extend primer into the region of DNA variation, allows the detection of insertions, deletions, and single-base substitutions in amplified DNA at multiplex levels of up to 36 DNA variants (*Figure 2*). The

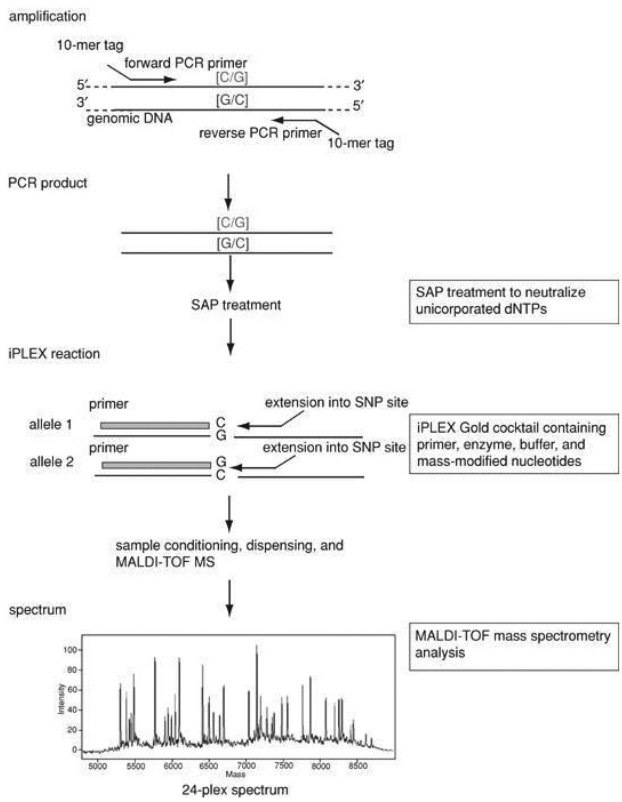

Figure 2. The MassEXTEND iPLEX reaction. An illustration of the genotype reaction of a C-to-G SNP[15].

extend primer is designed to hybridize adjacent to the variant being assayed and is extended by one of four mass-modified terminator molecules into the site of the nucleotide variation (mutation). The primer extension products are analyzed using MALDI-TOF mass spectrometry and the genotypes differentiated on the basis on the mass of each allele (*Figure 2*). All the 534 HCM-causing mutations were analyzed using MassARRAY Assay Design 3.1 software. The design process produced 22 assay plexes. All polymerase chain reaction (PCR) and iPLEX reactions were performed under standard conditions[14] on a 384-well plate, allowing analysis of 17 samples against the 22 assay plexes on the one plate. The multiplex PCR was carried out using the Complete PCR Reagent Kit (3840 reactions, Sequenom) in a 5-ml volume on an ABI 7900 PCR system (Applied Biosystems). The data were analyzed with Typer 3.4 and 4.0 software (Sequenom).

Mutation scanning of all samples was performed using a high-resolution melting (HRM) technique in a real-time PCR thermal cycler (LightCycler 480, Roche Diagnostics). The primers were constructed according to the common HRM specifications [http://www.gene-quantification.de/LC480-Technical-Note-01-HRM.pdf], using the following online software and databases: UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway), Primer 3 (http://frodo.wi.mit.edu/), DINAMelt (http://dinamelt.bioinfo.rpi.edu/), Poland service (http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html) and Primer-BLAST (http://www.ncbi.nlm. nih.gov/tools/primer-blast). The HPLC grade primers were purchased from Metabion (Germany). The amplification, melting and fluorescence detection conditions were as indicated by the supplier (Roche Diagnostics). Briefly, after amplification (35 cycles of 95 ºC for 10 seconds, 60 ºC for 15 seconds and 72 ºC for 10 seconds), the PCR products were heated to 95 ºC and cooled to 40 ºC (for heteroduplex formation), and melting was monitored (by fluorescence emission) from 65 ºC to 95 ºC. Table I lists the exon-intron boundaries covered in the analysis (fragments include 100 bp 5' and 3' flanking intronic regions). Reference sequences of the genes were obtained from the Genome Browser of the University of California, Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgGateway). Results were analyzed independently and when a mutation or a new variant was found, the PCR product was purified and sequenced (as a paid service, Stab Vida, Portugal).

Whenever a mutation was confirmed in a proband, first- and second-degree relatives were tested for the identified variant in order to check the cosegregation with the disease in the family. Except for nonsense and frameshift mutations, due to short insertions or deletions and splice site mutations, a missense variant was considered as a mutation when: it cosegregated with the disease in the family; it affected an amino acid conserved among species and isoforms; and it was not detected in at least 200 chromosomes of healthy adult controls.

RESULTS

iPLEX results

In sample type 1, seven HCM patients were initially sequenced for the five most important HCM genes[12] and several mutations were found in two genes, *MYBPC3* and *MYH7*, as shown in Table II. Importantly, all these blinded mutations were also detected by MSG technology (*Table II*).

In sample type 3, comprising the five HCM patients not previously genetically studied, mutations were found by MSG in patients CMH016, CMH024, CMH030, CA02 (*Figures 3, 4,* and 5; *Table II*). In sample type 2, which includes patient CMH012, negative by AS for mutations in the five most common HCM genes, a mutation was detected by MSG (*Table II*; *Figures 3, 4,* and *5*). In patient CMH004 we were only able to detect a polymorphism by MSG (*Table II*). Two samples from healthy individuals (with no HCM disease) were used as controls in each MSG assay.

Finally, MSG analysis in patient CMH002 (sample type 3) failed to identify any known HCM mutation.

Table I. Exons and exon/intron boundaries included in the HRM analysis. In bold are the five main HCM-associated genes. The number of HCM-associated mutations known** and those analyzed in this work are also indicated.

Gene	OMIM	Reference sequence *	Protein	Exons Analyzed /Total	Known mutations ** (analyzed in this paper)
MYBPC3	600958	NM_000256	Cardiac myosin-binding	32/32	241 (169)
MYH7	160760	NM_000257	β-cardiac myosin heavy protein chain	40/40	224 (224)
TNNT2	191045	NM_000364	Cardiac troponin T	16/16	37 (32)
TNNI3	191044	NM_000363	Cardiac troponin I	8/8	33 (26)
MYL2	160781	NM_000432	Regulatory myosin light chain	6/7	13 (10)
TPM1	191010	NM_0001018005	α-tropomyosin	9/10	11 (11)
ACTC1	102540	NM_005159	Cardiac actin	6/6	12 (8)
MYL3	160790	NM_000258	Essential myosin light chain	7/7	5 (5)
CSRP3	600824	NM_003476	Muscle LIM protein (MLP)	6/6	7 (7)
TCAP	604488	NM_003673	Telethonin	2/2	5 (5)
TTN	188840	NM_133378	Titin	2/45	9 (2)
TNNC1	191040	NM_003280	Troponin C	6/6	3 (1)
MYH6	160710	NM_002471	α-myosin heavy chain	13/39	5 (2)
MTCYB	516020	AC_000021.2	Cytochrome b of complex III	1/1	2 (2)
MTTG	590035	AC_000021.2	Mitochondrial RNA transfer, glycine	1/1	1 (1)
MTTI	590045	AC_000021.2	Mitochondrial RNA transfer, isoleucine	1/11 (1)	
OBSCN	608616	NM_052843	Obscurin	1/52	2 (1)
MYOZ2	605602	NM_016599	Myozenin 2	2/6	2 (2)
MYLK2	606566	NM_033118	Myosin light chain kinase 2	1/12	2 (2)
MYO6	600970	NM_004999	Myosin VI	1/35	1 (1)
DES	125660	NM_001927	Desmin	1/9	1 (1)
FXN	606829	NM_000144	Frataxin	1/5	1 (1)
PRKAG2	602743	NM_016203	y2 subunit of AMP-activated protein kinase	2/12	9 (7)
LAMP2	309060	NM_01399?	Lysosome-associated membrane protein-2	1/9	1 (1)
RAF1	164760	NM_002880	V-raf-1 murine leukemia viral oncogene homolog 1	1/17	1 (1)
CASQ2	114251	NM_001232	Calsequestrin 2	1/11	1 (1)
JPH2	605267	NM_020433	Junctophilin 2	6/6	4 (4)
VCL	193065	NM_014000	Vinculin	1/7	1 (1)
SLC25A4	103220	NM_001151	Solute carrier family 25 member 4	1/4	1 (1)
PLN	172405	NM_002887	Phospholamban	1/2	5 (1)
COX15	603646	NM_078476	Cytochrome C oxidase assembly protein 15	1/9	4 (2)
CAV3	601253	NM_033337	Caveolin 3	2/2	1 (1)

* obtained from UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway)
** Human Genome Mutation Database http://www.hgmd.org; and Harvard Sarcomere Gene Protein Mutation Database http://genepath.med.harvard.edu/~seidman/cg3/

Table II. Patients, genes and mutations detected by MSG.

Patient (sample type)	Gene (exon)	Mutation	Detected by MSG
CMH001 (1)	*MYBPC3* (25)	c.2693G>A, p.V896M,	Yes
CMH003 (1)	*MYH7* (23)	c.2770G>A, p.E924K	Yes
CMH004	*MYH7* (32)	c.4472C>G, p.Ser1491Cys (rs3729823)	Yes
CMH005 (1)	*MYBPC3* (25)	c.2824C>T, p.R943ter	Yes
CMH006 (1)	*MYBPC3* (25)	c.2824C>T, p.R943ter	Yes
CMH013 (1)	*MYH7* (9)	c.788T>C, p.I263T	Yes
CMH014 (1)	*MYBPC3* (16)	c.1727G>A, p.W576ter	Yes
CMH035 (1)	*MYBPC3* (15)	c.1502G>A, p.R501Q	Yes
CMH012 (2)	*CSRP3* (3)	c.128delC, p.A43Vfs165	Yes
CMH016 (3)	*TNNT2* (15)	c.833A>T; p.N278I	Yes
CMH024 (3)	*MYH7* (9)	c.788T>C; p.I263T	Yes
CMH030 (3)	*MYBPC3* (15)	c.1465G>A; p.R494Q	Yes
CA02 (3)	*CSRP3* (3)	c.128delC, p.A43Vfs165	Yes

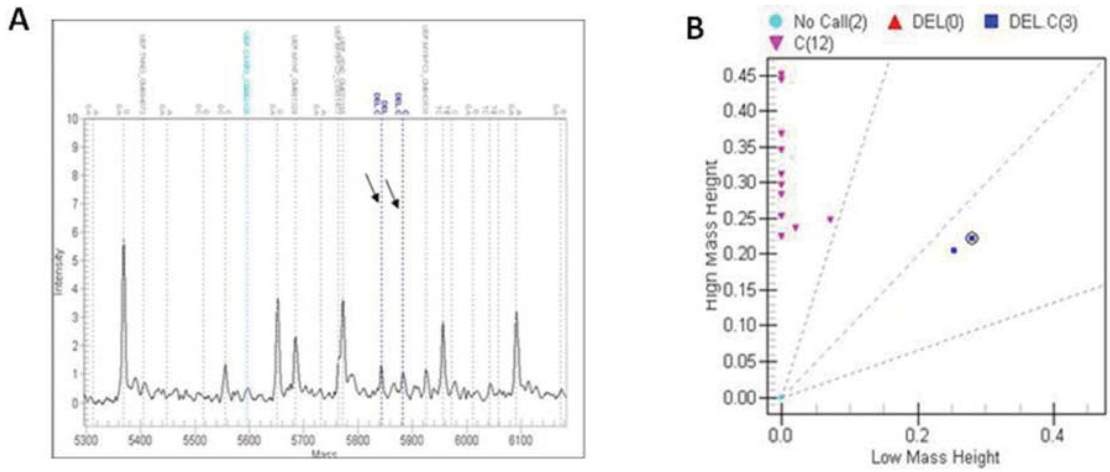

Figure 3. A. Graphical representation of MSG analysis illustrating the c.128delC mutation in the *CSRP3* gene. The presence of two peaks (arrows) indicates the mutation with a heterozygotic allelic pattern in which the masses correspond to the DEL and C alleles, respectively. B. Peak area plot corresponding to the wild-type allele (C) vs. the peak area of the mutated allele (DEL). The blue dots correspond to samples CMH012 and CA02, both heterozygotes for the c.128delC mutation in the CSRP3 gene. The pink dots correspond to negative samples referring to the same mutation.

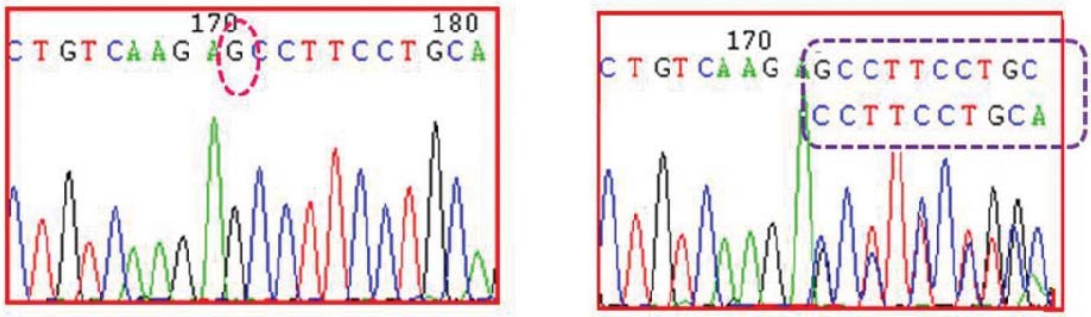

Figure 4. Chromatograms resulting from sequencing exon 3 of the *CSRP3* gene in a healthy control (left) and CMH012 and CA02 patients (right).

High-resolution melting analysis

The CMH002 sample with no gene alteration detected by MSG was subjected to HRM covering 32 genes, thee five main HCM-associated genes (*bold in Table I*) and the 27 other genes with low association with disease (*Table I*). HRM primers were designed to prevent the formation of nonspecific PCR products (primer dimers, other fragments), fragment folding, and multiple melting domains. The performance of the primer pairs was verified by analysis of the melting temperature peak (first derivative of the melting curve). Mutation scanning was only performed in fragments with a unique melting temperature peak. Of the tested primers, 100% were found suitable for mutation scanning. The same control samples used as negative samples for MSG were used for HRM. The detection of heteroduplexes was achieved by comparing the melting profiles, through difference plots (plots of differences between each point of the melting profile of the reference samples and the analyzed sample). If differences were judged significant, in that they fell outside the accepted range of variation of the wild-type samples, they were marked as variants. Fragments that included variations by this method were sequenced for confirmation and 4% were found to be false positives. Testing of fragments with known variants revealed no false negatives. HRM enabled us to discover a

novel variation within exon 7 of the MYBPC3 gene (NM_000256) (*Table III*; *Figure 5A*).

Automated sequencing of this exon revealed the presence of a novel mutation, c.817C>T/p.Arg273Cys, in the MYBPC3 gene in patient CMH002 (*Figure 5B*).

Despite the fact that this mutation is uncharacterized at the cellular level and has not been previously reported in the literature, we were unable to detect it in 200 chromosomes obtained from healthy control subjects (results not shown).

DISCUSSION AND CONCLUSIONS

There are several genetic causes for left ventricular hypertrophy, and differential diagnosis is important because treatment options and prognosis may differ. Among these, HCM is the most frequent cause of SD in otherwise healthy young people; it may in fact be the only clinical manifestation of the disease. Genetic diagnosis is important in all patients with suspected HCM for several reasons: counseling on professional and/or leisure activities (particularly in young people with a family history or familial SD); for genetic counseling (in the belief that certain mutations may be more dangerous than others); and, in general, to facilitate genetic diagnosis in family members once a mutation has been identified in the index patient.

In everyday practice, genetic diagnosis by standard procedures is difficult since these are technically complex, time-consuming and expensive. MSG and HRM have been shown to be a promising strategy, enabling low-cost, accurate and rapid diagnosis of HCM. In this study a novel mutation in the *MYBPC3* gene was identified by combining MSG and HRM. Using MSG, we were also able to detect a known mutation, c.128delC (p.Ala43ValfsX165)[7], in exon 3 of the *CSRP3* gene (NM_003476) (*Figures 3 and 4*), not detected by current HCM genetic diagnosis by AS of the five most common HCM genes. Within the analyzed group this mutation was detected in two samples from unrelated individuals (CMH012 and CA02) (*Figures 3 and 4*) and its presence was subsequently confirmed by AS (*Figure 4*). The CMH012 sample had previously been

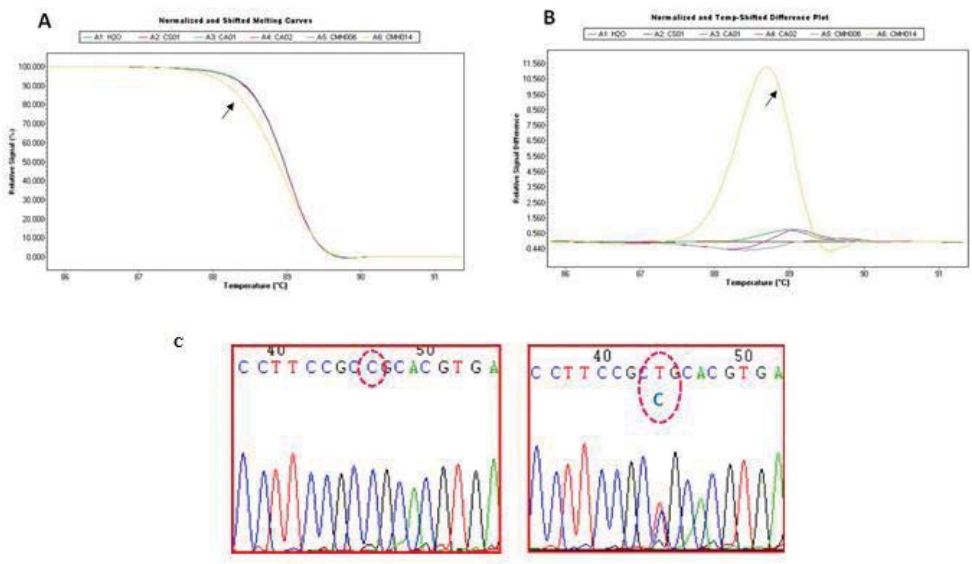

Figure 5. A. Melting curves of exon 7 of the *MYBPC3* gene (NM_000256). The arrow indicates the altered profile indicating a variation in sample CMH002. B. Difference plot of the melting curves. The arrow indicates a possible variation. Two healthy controls were used as a reference. C. Sequencing of exon 7 of the *MYBPC3* gene (NM_000256). Left – healthy control, Right – HCM patient (CMH002).

Table III. Patient, gene and mutation detected by HRM.

Patient (sample type)	Gene (exon)	Mutation	confirmed by sequencinge
CMH002 (3)	*MYBPC3* (7)	c.817C>T, p.Arg273Cys	Yes

* obtained from UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway)
** Human Genome Mutation Database http://www.hgmd.org; and Harvard Sarcomere Gene Protein Mutation Database http://genepath.med.harvard.edu/~seidman/cg3/

sequenced for *MYBPC3*, *MYH7*, *TNNT2*, *TNNI3* and *MYL2* mutations, with no relevant sequence alterations. The CA02 sample had not previously been studied.

Both patients (CMH012, female and CA02, male) presented sub-aortic obstruction at rest and septal thickness (measured by echo) was 25 mm and 23 mm respectively. The CMH012 patient was followed for 6 years. Additionally, she had angina pectoris, heart failure due to ventricular diastolic dysfunction and syncopal events unrelated to effort or dysrhythmia, similarly to the symptoms of the male patient reported elsewhere[7]. The CMH012 patient died from heart failure at the age of 50, not long after testing. Her sister is also clinically affected (mild phenotype with mild septal hypertrophy), while her daughter has no ECG or echo criteria for HCM. Despite our efforts we have not yet been able to genotype them.

We have shown that MSG enabled the detection of the c.128delC mutation in exon 3 of the *CSRP3* gene. The mutation was previously described as causing HCM in a 53-year-old male, diagnosed at 46 years of age[7]. This patient was reported to suffer from angina pectoris and dyspnea, and also presented presyncope on follow-up[7]. Also AS of the five main sarcomeric genes did not revealed any HCM-causing mutation for this patient[7]. The c.128delC mutation generates a frame shift starting at the 43rd codon (out of 195 in the normal protein), thereby changing the structure of muscle LIM protein. This changes conserved regions (figure 6A), including zinc fingers, which are considered important for protein-protein interaction. These facts are consistent with the hypothesis that this mutation is the cause of the HCM phenotype.

In the case of sample CMH002, in which no mutation was detected using MSG, HRM was used for mutation scanning in all 32 HCM genes. This procedure enabled detection of the c.817C>T/p.Arg273Cys mutation in the *MYBPC3* gene for this patient. To our knowledge, there has been no report of this mutation causing HCM. Its absence from the polymorphism databases (www.ensembl.org; www.ncbi.nlm.nih.gov) and from the 200 analyzed chromosomes excludes it from being a common variant (results not shown). The conservation of this amino acid across mammals (*Figure 6B*), the difference between the properties of the amino acids changed (arginine – polar, basic; cysteine – acid, non-polar), and reports of an HCM-causing mutation in the same codon (16), are all consistent with a disease-causing mutation. Patient CMH002 (47 years old) was first diagnosed with HCM at the age of 44 in the course of infectious endocarditis of the mitral valve. The ECG was apparently normal but he had major echo criteria with a septal thickness of 27 mm. His brother had HCM diagnosed at the age of 28 and there have been three sudden deaths in their mother's family (ages <50 years).

In this work we were able to detect all the blinded mutations in the tested samples and also to detect a mutation, c.128delC, in exon 3 of *CSRP3*, a gene not currently analyzed by standard HCM genetic testing, in two unrelated HCM patients, and a novel mutation, c.817C>T/p.Arg273Cys, in exon 7 of *MYBPC3* in another HCM patient. These mutations are the probable cause of these patients' HCM phenotype. Nevertheless, to further characterize the biological effect of these mutations in both genes, family members will be screened (first- and second-degree relatives with or without HCM symptoms) and functional studies using cardiomyocyte cultures harboring these mutations are currently been performed. Taken together, these results highlight the

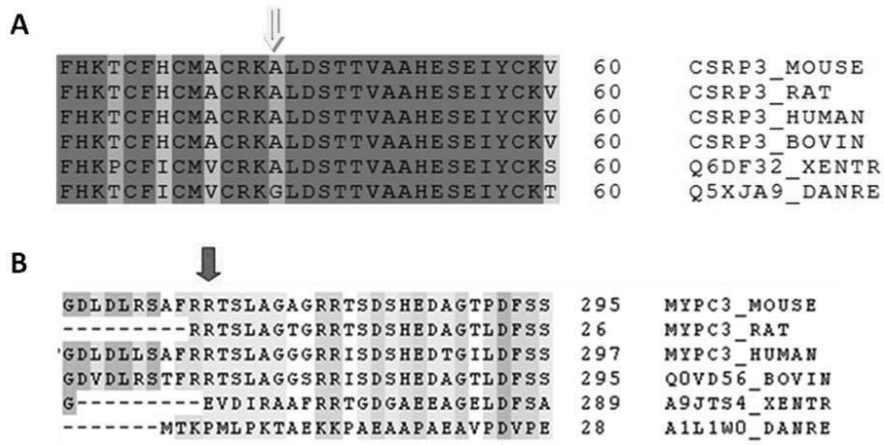

Figure 6. A. Conservation across species of the region between the 40th and 60th amino acid positions of muscle LIM protein (source: http://www.uniprot.org/). The arrow indicates the first amino acid changed by the *CSRP3* c.128delC mutation. (BOVIN – Bos taurus; DANRE – Danio rerio [zebrafish]; HUMAN – Homo sapiens; MOUSE – Mus musculus; RAT – Rattus norvegicus; XENTR - Silurana tropicalis [western clawed frog]). B. Conservation across species of a region of myosin-binding protein C (source: http://www.uniprot.org/). The arrow indicates the first amino acid changed by the mutation (legend as for figure 6A).

multiplex capacity of the MSG-based array, with which we were able to screen for 534 known mutations (in 32 genes) in at least 15 HCM patients, and also highlight the ability of HRM to rapidly scan all coding regions and intron-exon boundaries of the 32 HCM-associated genes to find new variations and probable disease-causing mutations. High-throughput coupled MSG-HRM constitutes a strong candidate tool for fast and accurate diagnosis in multigenic diseases such as HCM. However, MSG-based array genotyping is much less expensive and has a much lower false positive rate (1%) than HRM, making it a promising technique when the mutation status of a population and the genes involved in the disease are fully known.

In this work we aimed to improve our gene-based diagnostic tools by using two new high-throughput genotyping technologies. In this regard, understanding the genetic basis of HCM in the Portuguese population provides the opportunity for gene-based diagnosis [17]. The benefits of gene-based diagnosis of HCM for basic research and for clinical medicine are limited by the considerable costs of current genetic testing strategies and an incomplete knowledge of all disease genes. Recent genetic studies demonstrate variability in the types of HCM mutations among different populations. For example, 4% of the South Asian (Indian) population carries a common sarcomere protein gene mutation in MYBPC3 that causes HCM and increases the risk for heart failure >6-fold (18). In the Netherlands, a single founding mutation also accounts for a substantial percentage of HCM, while in the US, there is marked genetic heterogeneity and most HCM patients have a unique pathogenic mutation [17, 19]. The genetic basis for HCM in Portugal has not yet been extensively studied (1, 13, 20, our work). Because most HCM mutations were defined in many different populations (http://www.hgmd.org), its suitability for detecting HCM mutations in Portuguese population is still unknown, even though we were able to identify all the blinded mutations. In this regard, and due to the small number of samples analyzed in this work, we are currently genotyping more than 100 clinically characterized HCM patients from different regions of Portugal with an extended version of our MSG array that will be able to detect 646 mutations in the 32 HCM-associated genes. Moreover, the number of known HCM mutations continued to expand; 250 HCM mutations have recently been found by our collaborators at the Harvard Medical School in US patients (C.E Seidman and J. Seidman, personal communication). These

mutations and the new mutation detected in this work will also be added to our MSG-based array, so as to optimize detection of HCM mutations in Portuguese patients.

In conclusion, in this work we have fulfilled our initial objectives and demonstrated the diagnostic efficacy of MSG and HRM in this pathology by studying 13 HCM patients. We were able to detect all the blinded mutations in the tested samples, a mutation in a gene *(CSRP3)* not currently analyzed by standard HCM genetic testing, and a novel mutation in the *MYBPC3* gene. These methodologies are currently being applied by our group to a cohort of more than 100 Portuguese HCM patients.

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