

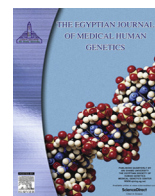
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Original article

A comparative study of mutation screening of sarcomeric genes (*MYBPC3*, *MYH7*, *TNNT2*) using single gene approach versus targeted gene panel next generation sequencing in a cohort of HCM patients in Egypt

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ABSTRACT

Background: NGS enables simultaneous sequencing of large numbers of associated genes in genetic heterogeneous disorders, in a more rapid and cost-effective manner than traditional technologies. However there have been limited direct comparisons between NGS and more established technologies to assess the sensitivity and false negative rates of this new approach. The scope of the present manuscript is to compare variants detected in *MYBPC3*, *MYH7* and *TNNT2* genes using the stepwise dHPLC/Sanger versus targeted NGS.

Methods: In this study, we have analysed a group of 150 samples of patients from the Bibliotheca Alexandrina-Aswan Heart Centre National HCM program. The genetic testing was simultaneously undertaken by high throughput denaturing high-performance liquid chromatography (dHPLC) followed by Sanger based sequencing and targeted next generation deep sequencing using panel of inherited cardiac genes (ICC). The panel included over 100 genes including the 3 sarcomeric genes. Analysis of the sequencing data of the 3 genes was undertaken in a double blinded strategy.

Results: NGS analysis detected all pathogenic and likely pathogenic variants identified by dHPLC (50 in total, some samples had double hits). There was a 0% false negative rate for NGS based analysis. Nineteen variants were missed by dHPLC and detected by NGS, thus increasing the diagnostic yield in this co-analysed cohort from 22.0% (33/150) to 31.3% (47/150).

Of interest to note that the mutation spectrum in this Egyptian HCM population revealed a high rate of homozygosity in *MYBPC3* and *MYH7* genes in comparison to other population studies (6/150, 4%). None of the homozygous samples were detected by dHPLC analysis.

Conclusion: NGS provides a useful and rapid tool to allow panoramic screening of several genes simultaneously with a high sensitivity rate amongst genes of known etiologic role allowing high throughput analysis of HCM patients and relevant control series in a less characterised population.

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Abbreviations: NGS, next generation sequencing; HCM, hypertrophic cardiomyopathy; MYBPC3, myosin binding protein C; MYH7, myosin heavy chain 7; TNNT2, cardiac troponin T2; MYF, Magdi Yacoub Heart Foundation; BA, bibliotheca alexandrina.

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1. Introduction

Hypertrophic cardiomyopathy (HCM) is a genetic cardiac disease characterised by left ventricular hypertrophy and myofibrillar disarray [1]. HCM is typically inherited in an autosomal dominant manner and, with an estimated worldwide reported prevalence of

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1:500, it is the most common inherited cardiac disease and a leading cause of premature sudden cardiac death (SCD) in the young [2].

Mutations in almost 50 genes have been implicated in HCM, including genes that encode for sarcomeric, Z-disc, calcium handling and mitochondrial proteins [3], although the evidence for many of these associations is doubtful [4]. Until recently however, genetic testing for HCM has focused on mutations in myosin heavy chain 7 (*MYH7*), myosin-binding protein C3 (*MYBPC3*) and cardiac troponin T (*TNNT2*), which account for between 35% and 60% of cases [5]. Traditionally such studies have been performed by single gene analysis, utilising either polymerase chain reaction (PCR) or denaturing high performance liquid chromatography (dHPLC) followed by direct Sanger sequencing. Using these approaches, HCM mutation profiles from many different population groups have been published over the last decade [6–18], including an Egyptian cohort of 192 HCM patients [19].

The recent advent of next generation sequencing (NGS) technologies has the power to revolutionise the genetic diagnosis of heterogeneous diseases, such as HCM. NGS can be used for either targeted gene panel or whole genome/exome sequencing [20], and enables rapid and cost-effective analysis of the three core HCM genes, as well as more comprehensive screening of all genes associated with HCM. However, until now there have been limited studies to assess the sensitivity of NGS and to compare its ability to detect variants with more established and traditional methods. In the present study, we have compared variant detection in the three commonly involved sarcomeric genes: *MYH7*, *MYBPC3* and *TNNT2* in a cohort of 150 Egyptian HCM patients using dHPLC/Sanger based analysis followed by NGS targeted deep sequencing assay.

2. Patients and methods

2.1. HCM patient population

The present study comprised 150 unrelated HCM index patients recruited from the different geographic regions of Egypt. Patients were diagnosed according to standard clinical evaluation by 2D echocardiography at Aswan Heart Centre and satellite HCM clinics as part of National BA HCM National Program. Demographic and clinical data of this cohort was described in our earlier paper [19]. The samples analysed were included based on availability of sufficient DNA for analysis by both assays.

The present study has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by Aswan Heart Centre Research Ethics Committee. Research subjects provided informed consent for participation in the study.

Genomic DNA was extracted from 4 to 6 ml of blood samples donated from index HCM patients and control subjects using Promega wizard genomic DNA purification Kit. Primers used for amplification of the coding exons and flanking intronic sequences of the three candidate sarcomeric genes: *MYH7* (NM_000257.2), *MYBPC3* (NM_000256.3), and *TNNT2* (NM_001001430.1) were previously described [9,21,22].

New primers set used for amplifying exon 5 were designed in the present study to bypass the intronic deletion polymorphism (c.506-12delC) commonly encountered among Egyptians (present at a frequency of 23%, as reported in our earlier study [19]) to enable screening of the full span of exon 5 (*MyBPC3* exon 5 new primers: F primer TTTGCAGGTGGCAGCAT; R primer GTCCCTCTCCCGTGTCTCC).

2.2. dHPLC/Sanger analysis

Initial screening for mutations in the three candidate sarcomeric genes: *MYH7* (NM_000257.2, 38 coding exons, 3rd to 40th

exons), *MYBPC3* (NM_000256.3, 34 exons, 1st to 34th), and *TNNT2* (NM_001001430.1, 16 coding exons, 2nd to 17th) was undertaken using heteroduplex analysis by dHPLC using WAVE™, Transgenomics, DNA Fragment Analysis System. The conditions for dHPLC were developed on the basis of amplicon-specific melting profiles predicted by the NAVIGATOR software (Transgenomics, San Jose, California, USA).

Samples showing a variant profile (different from the wild type pattern) in any of the amplicons were subjected to bidirectional sequencing using automated dye terminator cycle-capillary electrophoresis (ABI 3500 Applied Biosystems, Foster City, California, USA) to determine the nature of the sequence change, as shown in Fig 1.

2.3. NGS targeted resequencing

The samples were re-sequenced blindly using a custom capture assay (Agilent SureSelect), with an Illumina HiSeq2500 system which targets commonly involved sarcomeric genes (*MYBPC3*, *MYH7*, *TNNT2*) implicated in hypertrophic cardiomyopathy. Reads were demultiplexed (allowing zero mismatches) with HiSeq Control software and quality were checked in FastQC v.0.10.1. Low quality (<20) bases were trimmed using PrinSeq v0.20.4 [23] and the reads were aligned to hg19 reference using BWA v0.7.5 [24].

Marking duplicate reads, local realignment around indels and base quality score recalibration process were done in Picard v1.109 and GATK v2.8-1[25]. Alignment summary metrics and callability and coverage reports were calculated using Picard, Samtools v0.1.18 [26], Bedtools v2.11.2 [27] and in house perl scripts.

A subset file (ontarget) was created, based on reads mapping quality >8. This “ontarget” file was used to make consistent variant calls with GATK HaplotypeCaller and UnifiedGenotyper. Bases covered by at least 10 reads with a mapping quality ≥ 10 and base quality ≥ 20 were denoted as “callable”, i.e. adequately covered for variant calling with recommended GATK parameters [28]. Variants were annotated using the Ensembl API v75_37 [29] and Human Gene Mutation Database (HGMD) Professional version 2013.4 [30].

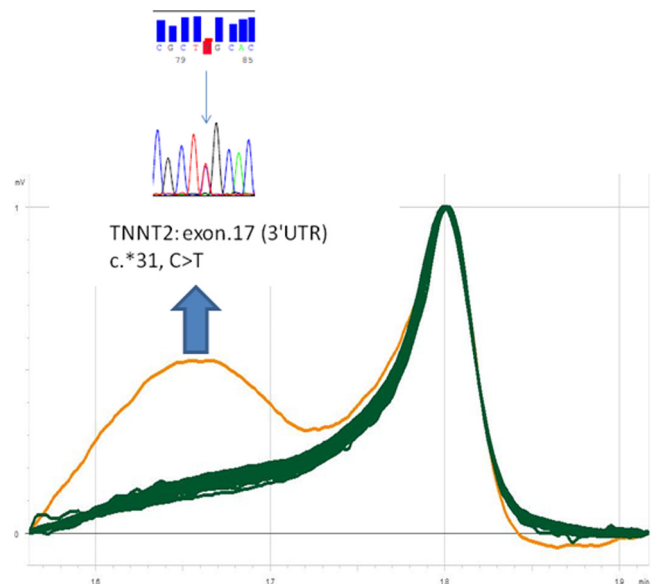


Fig. 1. Electropherogram of dHPLC screening of *TNNT2* exon 17, sample showing a variant profile (in orange) is subjected to Sanger based sequencing analysis to identify the nature of the sequence change.

3. Results

The current cohort were co-analysed by two approaches of complimentary dHPLC/Sanger and by targeted resequencing NGS techniques. Both analyses were undertaken independently and blindly.

Twenty-four likely pathogenic variants were detected in *MYH7*, *MYBPC3* and *TNNT2* by dHPLC/Sanger, in 35 patients (Table 1). Two patients had double hits, rendering a diagnostic yield using dHPLC/Sanger analysis of 22% (33/150) in this cohort.

The 150 samples were then analysed on an NGS targeted panel for deep sequencing of the three sarcomeric genes, with the average percentage of coding sequence considered callable as follows: *MYH7*: 97.5%, *MYBPC3*: 99.8%, *TNNT2*: 99.9%. All variants identified by dHPLC/Sanger were also detected using NGS, representing a 0% false negative rate for this technique in this study. In addition, a further 21 variants were not identified by dHPLC/Sanger and were detected only by NGS (Table 1).

All variants detected only by NGS were subsequently confirmed by Sanger based sequencing using capillary electrophoresis. NGS assay showed a 0% false positive rate for these three genes. In total, fourteen samples were classified as having putative pathogenic variant over the number of patients detected by dHPLC/Sanger. Hence, NGS contributed towards a higher positive diagnostic yield of 31.3% (47/150 patients) in comparison to the yield of dHPLC (22%, 33/150) in the same co-analysed cohort (Fig 2).

3.1. Discrepancies between dHPLC and NGS

The 19 individual variants that were detected only by NGS and missed by dHPLC were investigated to determine why the initial screening had failed to detect them.

The dHPLC profile was similar to the wild type profile for 12 variants. Fig 3 shows examples of samples with variants not picked by dHPLC as a different profile from the wild. This is a known limitation in sensitivity of dHPLC technique, with a reported sensitivity of 95% [31].

Detailed analysis of samples not detected by dHPLC showed six variants were present in a homozygous state (c.1227-2A>G, c.1321G>A, c.2458C>G, c.2618C>A, c.3676C>T in *MYBPC3* and c.1064C>T in *MYH7*). Homozygosity is not likely to be detectable by dHPLC as this method relies on detection of mismatched heteroduplexes which is only possible to be detected in DNA of heterozygous individuals (Fig 3B) and is considered a limitation in the dHPLC assay.

Additional issues included the initial primer design for *MYBPC3* exon 5 covered a region enclosing the commonly encountered SNP c.506-12delC (rs11570050), which occurs at a frequency of 0.23 in Egyptians, [19]. The presence of the allele with the deletion provided a technical limitation in reading exon 5 in full. In the patient sample, the variant of interest with relevance to pathogenicity, c.649A > G, occurred in cis with the c.506delC SNP. Sanger sequencing failed to identify the variant with the earlier described primer set [21]. However following detection of the variant by NGS, it was subsequently confirmed by Sanger sequencing with redesigned primers (described above in Materials and methods section).

3.2. Classification of *MYH7*, *MYBPC3* and *TNNT2* variants

In total, 50 variants were detected in 61 patient samples by NGS, 35 were scored as putative pathogenic, 12 were scored as variants of uncertain significance (VUS) and 3 were scored as likely benign. The variants detected in this study were assessed for their likely pathogenic effect based on variant type, previous reports

linking them to HCM, and variant frequencies reported in web based publically accessed databases [32]. In addition to testing for segregation in affected families whenever possible as illustrated in pedigrees of Fig 4.

Truncating variants (nonsense, frameshift or essential splice site) or missense variants not present at a frequency >0.01% in ExAC [33] and absent from 200 population matched controls were considered as putatively pathogenic. Any variant present at a frequency of >0.1% in ExAC and not previously linked to disease was considered to be likely benign (Table 1). All others were classified as variants of unknown significance (VUS), which included two variants, *MYBPC3*:c.148A>G in sample PM48/PA45 since it did not co-segregate in affected family members of PM48, and also *MYBPC3*:c.3800G>A a second hit in sample PA4 which was non co-segregating in two affected family members in addition to the segregating pathogenic mutation p.R719Q detected in all affected family members (Fig 4).

MYBPC3 contributed for almost half of the variants detected in the present cohort. The likely pathogenic variants detected included 10 missense and 5 truncating variants including 2 non sense mutations, an essential splice site variant c.1227-2A>G (in a homozygous state) and two frame shift mutations (A179QfsX59, D506TfsX7). The latter two variants were each detected in 3 Egyptian HCM patients in addition to a nonsense mutation, p.W1098X, was detected in 4 unrelated index patients.

There were 18 putative pathogenic variants detected in *MYH7* in 23 patients, and PA8 had double hits. There were 16 missense and two truncating variants, one is an essential splice variant c.5791-1G>A and another is frame shift mutation, p.S1924AfsX9. The latter mutation was detected in 5 patients in the current cohort (5/150, 3%) and was absent in population matched controls and other population databases. Most of the missense variants were detected in the head motor region of *MYH7* (14/16, 87.5%) which is a recognised mutation hotspot [33]. In *TNNT2*, 2 likely pathogenic missense variants were detected in 3 patients (Table 1).

Of the 35 distinct pathogenic variants, 21 have previously been linked to cardiomyopathy and 14 are novel and described only in the Egyptian HCM patients. Several patients had more than a single variant, in addition to the six homozygous patients (highlighted by an asterisk in Table 1). Three patients were double heterozygotes. One patient (PA26) had missense variants in *MYBPC3* (c.2311G>A, p.V771M) and also in *MYH7* (c.799C>G, p.L267V), the second (PM43) had a frameshift variant in *MYH7* (c.5769delG) and a missense variant in *MYBPC3* (c.2470G>A, p.D824N), the third (PA8) had 2 hits in *MYH7* (c.925G>A, p.D309N & c.5769delG). These double hits provides a possible explanation for the phenotype heterogeneity commonly described in HCM.

4. Discussion

Next generation sequencing is an immensely powerful approach to genetic analysis with the potential to transform diagnosis of genetically heterogeneous diseases like HCM. Targeted resequencing of a panel of known cardiomyopathy genes allows for a comprehensive genetic screening in a relatively quick and inexpensive manner. NGS approaches are now being used in diagnostic laboratories, though most continue to validate any findings with targeted mutation Sanger sequencing. However the sensitivity of NGS in a diagnostic setting, and an evaluation of the false negative rate of this technology, remains difficult to assess as traditional sequencing approaches and NGS are rarely run in parallel.

In this study, we have compared the performance of NGS with a well characterised and clinically established sequencing method (dHPLC/Sanger) in detecting variants in the three major HCM genes in a cohort of Egyptian HCM patients. NGS detected all 50 variants

Table 1
Details of the variants detected in this study by both dHPLC/Sanger and NGS and by NGS alone in the three genes analysed.

Gene	Variant nomenclature at coding DNA level	Variant nomenclature at protein level	Variant type	Num. Samples (IDs)		ExAC count (freq.)	Classification of pathogenicity	Novelty of putative pathogenic variants
				Both	NGS			
MYBPC3	c.355G>A	p.E119K	Missense	1 (PM64)		0	Putative pathogenic	No
MYBPC3	c.416C>G	p.S139X	Nonsense	1 (PA15)		0	Putative pathogenic	Yes (this cohort only)
MYBPC3	c.534_541del	p.A179QfsX59	Frameshift	3 (PA25, PA46, PA52)		0	Putative pathogenic	Yes (this cohort only)
MYBPC3	c.772G>A	p.E258K	Missense	1 (PA59)		3 (0.00003903)	Putative pathogenic	No
MYBPC3	c.956A>C	p.E319A	Missense	1 (PA33)		6 (0.00007891)	Putative pathogenic	No
MYBPC3	c.1227-2A>G		Essential Splice Site		1* (P90)	0	Putative pathogenic	No
MYBPC3	c.1516delG	p.D506TfsX7	Frameshift	3 (P63, P70, P62)		0	Putative pathogenic	Yes (this cohort only)
MYBPC3	c.2308G>A	p.D770N	Missense		1 (P88)	1 (0.00000830)	Putative pathogenic	No
MYBPC3	c.2311G>A	p.V771M	Missense	1 (PA26)		1 (0.00005661)	Putative pathogenic	No
MYBPC3	c.2458C>G	p.R820G	Missense		1* (PA20)	0	Putative pathogenic	No
MYBPC3	c.2470G>A	p.D824N	Missense	1 (PM43)		1 (0.00000829)	Putative pathogenic	No
MYBPC3	c.2977C>T	p.L993F	Missense	1 (PA16)		0	Putative pathogenic	Yes (this cohort only)
MYBPC3	c.3293G>A	p.W1098X	Nonsense	4 (PA6, PA42, PA56, PA93)		0	Putative pathogenic	Yes
MYBPC3	c.3412C>T	p.R1138C	Missense	1 (PU11)		3 (0.00003719)	Putative pathogenic	No
MYBPC3	c.3676C>T	p.R1226C	Missense		1* (PM34)	7 (0.00005805)	Putative pathogenic	No
MYH7	c.632C>T	p.P211L	Missense		1 (PA27)	3 (0.00002471)	Putative pathogenic	No
MYH7	c.665A>G	p.Q222R	Missense		1 (P3)	0	Putative pathogenic	No
MYH7	c.746G>A	p.R249Q	Missense	1 (PA55)		0	Putative pathogenic	No
MYH7	c.799C>G	p.L267V	Missense	1 (PA26)		0	Putative pathogenic	Yes (this cohort only)
MYH7	c.925G>A	p.D309N	Missense	1 (PA8)	1 (PA40)	3 (0.00002486)	Putative pathogenic	No
MYH7	c.1064C>T	p.A355V	Missense		1* (P80)	0	Putative pathogenic	No
MYH7	c.1182C>A	p.D394E	Missense	1 (PA54)		0	Putative pathogenic	Yes (this cohort only)
MYH7	c.1816G>A	p.V606M	Missense		1 (P35)	0	Putative pathogenic	Yes
MYH7	c.2147G>C	p.G716A	Missense	1 (PA30)		0	Putative pathogenic	Yes (this cohort only)
MYH7	c.2155C>T	p.R719W	Missense	1 (PM60)		0	Putative pathogenic	No
MYH7	c.2156G>A	p.R719Q	Missense	2 (PA4, PM68)		0	Putative pathogenic	No
MYH7	c.2389G>A	p.A797T	Missense	1 (P16)		4 (0.00003296)	Putative pathogenic	No
MYH7	c.2609G>A	p.R870H	Missense		1 (P77)	0	Putative pathogenic	No
MYH7	c.2779G>A	p.E927K	Missense	1 (PA12)		0	Putative pathogenic	No
MYH7	c.4145G>A	p.R1382Q	Missense	1 (PM65)		0	Putative pathogenic	No
MYH7	c.4258C>T	p.R1420W	Missense		1 (PM24)	1 (0.00000824)	Putative pathogenic	No
MYH7	c.5769delG	p.S1924AfsX9	Frameshift	3 (PA24, PM3, PM43)	2 (PA11, PA8)	0	Putative pathogenic	Yes (this cohort only)
MYH7	c.5791-1G>A		Essential Splice Site		1 (P33)	0	Putative pathogenic	No
TNNT2	c.221T>C	p.L74S	Missense	2 (PM1, PM12)		0	Putative pathogenic	No
TNNT2	c.857G>A	p.R286H	Missense	1 (P102)		7 (0.00007882)	Putative pathogenic	No
MYBPC3	c.148A>G	p.S50G	Missense	1 (PM48)	1 (PA45)	3 (0.00003887)	VUS ¹	NA
MYBPC3	c.530G>A	p.R177H	Missense	1 (P62)		92 (0.00094933)	VUS	NA
MYBPC3	c.1321G>A	p.E441K	Missense	4 (PA38, PA123, P12, P101)	1* (P65)	18 (0.00016221)	VUS	NA
MYBPC3	c.1458-17C>G		Splice Region	1 (P17)		0	VUS	NA
MYBPC3	c.1458-7C>A		Splice Region	1 (P89)		0	VUS	NA
MYBPC3	c.1564G>A	p.A522T	Missense		1 (PA26)	47 (0.00039070)	VUS	NA
MYBPC3	c.2149-8C>T		Splice Region	1 (P100)		2 (0.00002204)	VUS	NA
MYBPC3	c.2618C>A	p.P873H	Missense		1* (P20)	13 (0.00020272)	VUS	NA

Table 1 (continued)

Gene	Variant nomenclature at coding DNA level	Variant nomenclature at protein level	Variant type	Num. Samples (IDs)		ExAC count (freq.)	Classification of pathogenicity	Novelty of putative pathogenic variants
				Both	NGS			
MYBPC3	c.3800G>A	p.R1267H	Missense		1 (PA4)	5 (0.00004239)	VUS ¹	NA
MYH7	c.4520-3C>T		Splice Region	1 (PA1)		11 (0.00009061)	VUS ²	NA
TNNT2	c.690-4G>T		Splice Region	1 (P32)		19 (0.00015650)	VUS	NA
TNNT2	c.832C>T	p.R278C	Missense	1 (PM68)		40 (0.00042906)	VUS	NA
MYBPC3	c.472G>A	p.V158M	Missense	2 (P14, P16)		2348 (0.09043291)	Likely benign	NA
MYBPC3	c.649A>G	p.S217G	Missense		2 (PM46, PA70)	248 (0.00226757)	Likely benign	NA
MYBPC3	c.2149-5C>T		Splice Region	1 (P87)		118 (0.00127947)	Likely benign	NA

* – homozygous. 1 – variant does not segregate with disease in affected families. 2 – present at a frequency of 0.005 in Egyptian population control samples.

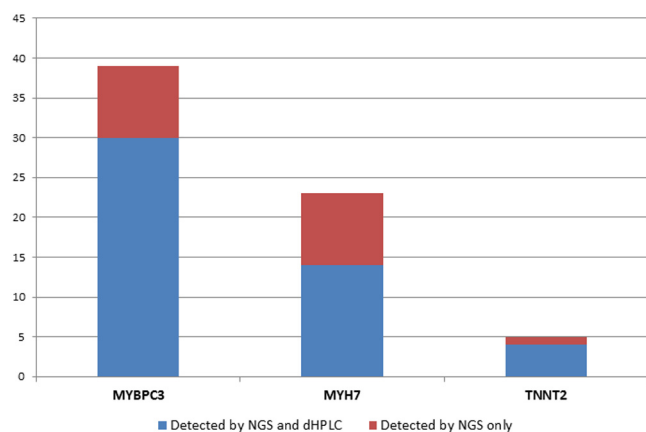


Fig. 2. Number of all variants (pathogenic, VUS and benign) detected in each gene by both dHPLC/Sanger and targeted NGS, or by targeted NGS only and confirmed by Sanger.

identified by dHPLC, giving it a zero false negative rate in this study and indicating that this technology is sufficiently sensitive for use in diagnostic laboratories.

In addition to detecting all of the variants identified by dHPLC/Sanger, targeted NGS assay found an additional 19 variants in this cohort, increasing the genetic diagnostic yield for this cohort from 22% (33/150 patients) in comparison to 31.3% (47/150). This has highlighted the limitations in the sensitivity of dHPLC, both in detecting variants with a profile too similar to wild-type and in its intrinsic inability to detect homozygous variants. All variants identified solely by NGS were subsequently verified by Sanger sequencing, giving an overall false positive rate of 0% for NGS in the three genes analysed.

The issue of homozygous mutations is likely to be of particular importance in certain disease conditions and population groups. The consanguinity rate among Egyptians has been reported to be between 30% and 40% [34], indicating that homozygous variants are likely to be more prevalent among Egyptians than in other populations. Indeed in this study, six homozygous patients were identified (five in MYBPC3, one in MYH7). While there have been some reports of homozygous mutations linked to HCM in these three genes [6,13,18], the proportion of homozygosity in the Egyptian cohort (4%, 6/150) is significantly higher than in the reported studies. Accordingly, the mutation screening using dHPLC which detect variants through formation of heterduplexes could detect variants

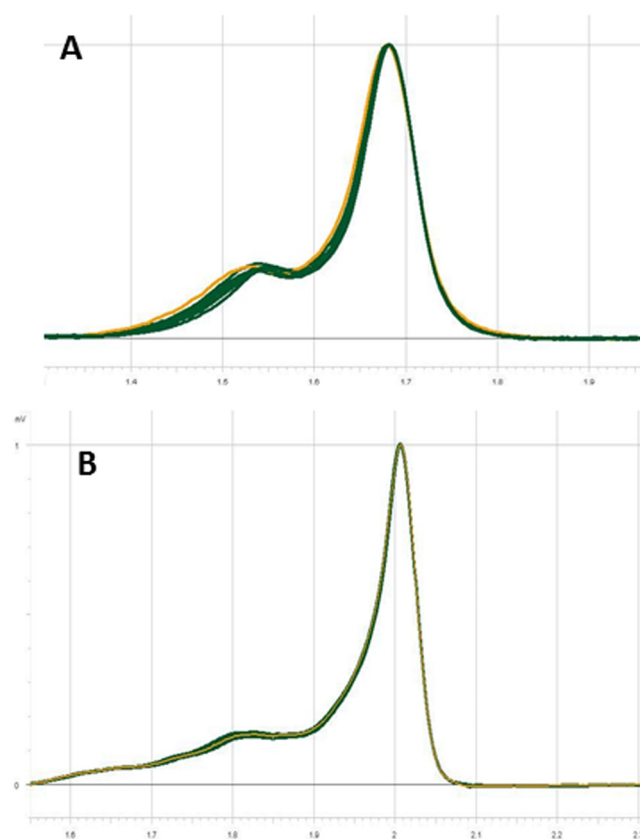


Fig. 3. dHPLC WAVE™ Analysis showing electropherogram of wild profile samples in GREEN and missed sample for variant detection in ORANGE. [A] MYH7 exon 8: P3 (c.665A>G; p.Q222R), heterozygous. [B] MYBPC3 exon 15: P65 (c.1321G>A; p.E441K, homozygous).

in heterozygosity including double and compound heterozygote samples. However, such assay would have a limitation in detecting variants in homozygous status and would be of lower sensitivity in such populations with expected higher rate of homozygosity due to high prevalence of consanguinity. Utilising sequencing technologies, like NGS, that are able to detect these variants in comparison to primary mutation screening by dHPLC is critical for accurate genetic diagnosis in such highly consanguineous populations. The effect of homozygosity and mutation dosage on HCM

Conflict of interest

Professor Stuart Cook occasionally consults for Illumina Inc. The other authors have no conflict of interest to declare.

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