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ORIGINAL ARTICLE

Denaturing high performance liquid chromatography: high throughput mutation screening in familial hypertrophic cardiomyopathy and SNP genotyping in motor neurone disease

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Aims: To evaluate the usefulness of denaturing high performance liquid chromatography (DHPLC) as a high throughput tool in: (1) DNA mutation detection in familial hypertrophic cardiomyopathy (FHC), and (2) single nucleotide polymorphism (SNP) discovery and validation in sporadic motor neurone disease (MND).

Methods: The coding sequence and intron–exon boundaries of the cardiac β myosin heavy chain gene (MYH7) were screened by DHPLC for mutation identification in 150 unrelated patients diagnosed with FHC. One hundred and forty patients with sporadic MND were genotyped for the A67T SNP in the poliovirus receptor gene. All DHPLC positive signals were confirmed by conventional methods.

Results: Mutation screening of MYH7 covered 10 kb with a total of 5700 amplicons, and more than 6750 DHPLC injections were completed within 35 days. The causative mutation was identified in 14% of FHC cases, including seven novel missense mutations (L227V, E328G, K351E, V411I, M435T, E894G, and E927K). Genotyping of the A67T SNP was performed at two different temperatures both in MND cases and 280 controls. This coding SNP was found more frequently in MND cases (13.6%) than in controls (6.8%). Furthermore, 19 and two SNPs were identified in MYH7 and the poliovirus receptor gene, respectively, during DHPLC screening.

Conclusions: DHPLC is a high throughput, sensitive, specific, and robust platform for the detection of DNA variants, such as disease causing mutations or SNPs. It enables rapid and accurate screening of large genomic regions.

The ability to screen for DNA sequence variants is fundamental to understanding conventional Mendelian disorders and individual susceptibility to common complex diseases. Numerous DNA screening techniques have been developed.[1] Denaturing gradient gel electrophoresis and chemical cleavage have high detection sensitivity, but they are formatted for manual use, involve toxic chemicals, and are technically challenging. Single stranded conformation polymorphism and heteroduplex analyses are simple and inexpensive, although they are labour intensive and are associated with low sensitivity (~ 80%). Immobilised DNA hybridisation arrays and enzymatic mismatch cleavage are promising techniques, but produce high numbers of false positive signals and are expensive.[1,2] In comparison, DHPLC can screen for DNA variants in a high throughput mode and with a superior detection rate (> 95%).[1,3–5]

> "The ability to screen for DNA sequence variants is fundamental to understanding conventional Mendelian disorders and individual susceptibility to common complex diseases"

Despite the discovery of hundreds of causative genes in rare genetic conditions through positional cloning (http://archive.uwcm.ac.uk/uwcm/mg/docs/hahaha.html), detection of underlying sequence changes in candidate or known target genes remains a challenge, both in research and diagnostic settings. To illustrate this, we have chosen to investigate familial hypertrophic cardiomyopathy (FHC), an autosomal dominant disorder (OMIM 192600) caused by at least nine genes encoding sarcomere proteins (http://www.angis.org.au/Databases/Heart/). The cardiac β myosin heavy chain gene (MYH7; OMIM 160760) is often involved in FHC, with 108 mutations.[6] However, most mutations are rare and family specific, and are widely distributed over the 40 exons of the gene.

Another area of immense effort involves the mapping of susceptibility genes for diseases that have complex gene–gene and gene–environment interactions.[7] The candidate disease related genes are usually present within large genomic intervals. Linkage disequilibrium mapping is one approach to narrowing this interval, but requires high density DNA markers, such as single nucleotide polymorphisms (SNPs).[7] This makes a robust experimental SNP discovery method highly desirable. Although in silico mining of SNPs from publicly available databases is inexpensive, this method has a low sensitivity of 27%.[8] The usefulness of DHPLC in SNP discovery and validation is illustrated by the neurodegenerative disorder sporadic motor neurone disease (MND).[9] Although 5–10% of MND cases are familial, more than 90% of cases are sporadic, and genetic contributing factors to these cases remain unknown. Viruses such as polio can be one environmental factor involved in sporadic MND (for

Abbreviations: DHPLC, denaturing high performance liquid chromatography; FHC, familial hypertrophic cardiomyopathy; MND, motor neurone disease; MYH7, cardiac β-myosin heavy chain gene; PCR, polymerase chain reaction; PVR, poliovirus receptor gene; SNP, single nucleotide polymorphism

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Table 1 MYH7 primers, amplicon sizes, and DHPLC analysis conditions

Exon	Forward primer 5′→3′	Reverse primer 5′→3′	Amplicon size (bp)	Temp (℃)	% Buffer B*
3	CCT CTT GAC TCT TGA GCA TGG	GTA CCC CTC TCT GTC ACC CA	392	61.9	58.9–68.9
4	TCC CTG TGA GAT CCT GGT TC	CAA GGA TGT TGG GAC GAG TT	398	62.5	59.0–69.0
5	AAC TCG TCC CAA CAT CCT TG	AGG TTA GGA GCT GCA CAG GA	443	61.5	59.8–69.8
6	ACC TTT CCC CCC CAC CCT CT	GGG CTG GAG GCT GGG ATC A	114	62.0	43.0–53.0
7	GGT CTC CAG TAG TAT TGT TCA	CAA GAA GGA GGC AGG TGA GAG	229	61.8	54.2–64.2
8	AGG GAG AAG AGC TCT CAC CTG	AAG TCC CAA GGC CAA GGT CAG	167	63.9	50.7–60.7
9	CCT TGG GAC TTG GAC TGG TG	AAA GAG GAG AAA AAC AGA GGG AGG	252	59.7	55.1–65.1
10	CCT TCT TCT CCC CAC CTG TTC	GTC TCA GTC GGT GGC TCT GAC	150	58.4	49.4–59.4
11	GCG AGC AGC CTC CAT GAG	CCC CTC ACT GCC AAT CCT C	216	62.0	53.6–63.6
12	CAT CAT ACT TCT TTT TTG GGG TCC	GCC CTC CAT GAC TTG ACA GC	232	64.0, 61.0	50.8–60.8
13	TTA CAG GCA TGA ACC ACA CAC C	GTG AAC TTG AAA ACT CTC ATC CC	267	62.1	55.7–65.7
14	AGA TGA TAA TGG GTG GGC AG	GAA ATA GCT GTT GAA TGT GGG AG	328	61.5	57.5–67.5
15	ACT CAC ACC CAC TTT CTG ACT	GAG GGG CTG CTA TTT TGT CTA	293	61.5	56.6–66.5
16	TTG ACC ATA GAG CAG AAT CCA TGT	GAC ATT GAA GTG GTG GGG TGT AG	475	61.0	60.2–70.2
17	TTC TTT TGT TGA CTC TCC TTC	GAT GGG GAG CCA AGT TGG CTG	134	61.7	48.0–58.0
18	TCT CTA TTG CAC TTT TTG GCC	TGG GTT GGC CTG AGT TTG TGG	140	60.6	48.6–58.6
19	CAG TCC AGT TCT CAC AGA CTC C	GGC TCC CCC TGT TCT ATG AG	206	62.0	53.1–63.1
20	GGA TCT GCA GGT GAC CCT GAA T	ACA ACA GGA AAA GCA TCA GAG G	283	59.6	56.2–66.2
21	ACT CCC CTC ATC CCA GCT CCA	CTT TTT TTC CTG ACA CTG CCC	342	61.2	57.8–67.8
22	AGG CTC AGC ACT CCT TTC AAT	AAG GCA GAG CAG GGT GGA AGA	335	61.3	59.7–69.7
23	CCC TCC TAT TTG AGT GAT GTG C	GGT CAG TAT GGT CTG AGA GTC C	404	61.0, 59.1	59.1–69.1
24	CCA CAG ATG GCA CCA AGC TG	TAT CTA GGC CCC ACA ACT CTC A	271	61.3	55.8–65.8
25	TGG GCC TGG GCT CCT TCT C	GGC AGC AGG GAG GGG ACA	197	62.9	52.6–62.6
26†	CGC CCG CCG CCG CCC TAC CTC ACC ATC CCT CCT TCC	AGC CCA GGG ACT CAG CAT C	160	63.4	52.7–62.7
27	GCG TGG GTC TGA GCC TTG TGT	GGG GAG GTG GGA GGA GGA AG	468	65.1, 62.2	60.1–70.1
28	TGC ACC TCT TAC ACC CCT TCA	GGG GAG ACT GTG GTG GGA AC	242	63.1, 61.1	54.7–64.7
29	GAG GAG GTG GGG ATA GAG AGG A	GTC AGT GTG CTC CTT GCT TGG	215	63.9	53.5–63.5
30	AGA AAG CTG AAC CCA CCT CCT	CCA GAA GTC AGG CTG CTC AGA	264	66.5, 64	55.6–65.6
31	CAT CCT CCC CAC CCT CTG C	GCT CTG GCC TCT CAC TGA ACC	293	63.8	56.5–66.5
32	CTT GGG GGC TGA AGA GTG AG	CCC CTC CCC AGC CTC TTG	234	62.7	54.4–64.4
33	TCA AAC CGA GTT ACC GTG TTC	TGA GAA CAG GGA CCA AAA GC	219	62.7	53.7–63.7
34	CCC TGA CTG TCT GCC TGC ATC	GGG GCA GGA GGA ATC TGG TG	436	62.8	59.7–69.7
35	GCT CAT GCC CAC TCT CCT GAT	TCA GGA ATC AGC AGG GGA GC	256	63.5	55.3–65.3
36	TCT ACC CAA CCC TCC CCC AAC	GGA TCG GGT CGG TGG AGT G	180	63.4, 61.4	48.6–58.6
37	CAG ACC ATG TGC CAC CTC TCT	GCA AAC TCT TCA TTC TCC TCA GC	388	64.9, 62.7	58.8–68.8
38	CAC CCC CTG CCT ACC CTC TGG	GGG AGG TGG GAG CAT GAG GTG	232	63.9	54.3–64.3
39	CTC ACC TCA TGC TCC CAC CT	TGA CTA GCA AAG CCC AAA AGAG	192	64.1	52.3–62.3
40	GAT CCT GGC TTT GTT TCC TTT CA	TGG GGC TTT GCT GGC ACC T	95	60.4	41.6–51.6

The amplicon size includes the corresponding exon and intron–exon boundaries.
Temperature refers to the oven temperature(s) at which DHPLC analysis was performed.
*Range of elution buffer B that was determined by Navigator software based on amplicon sizes; †the underlining highlights the additional GC clamp.
DHPLC, denaturing high performance liquid chromatography.

review see Talbot[9]), and a key gene influencing the infectivity of polio would be that encoding the poliovirus receptor (PVR).

The purpose of our study was to evaluate the application of DHPLC as a high throughput tool in FHC mutation detection, and its applicability to SNP analysis in a sporadic MND association study.

METHODS

Study subjects

FHC

Probands came from 150 unrelated Australian families including six patients with known MYH7 mutations (who acted as positive controls).[10 11] The putative disease causing mutations in MYH7 were defined as rare missense changes with a population frequency of < 0.5%. Further functional and structural evaluation is beyond the scope of our present study. Controls (n = 100) were matched for ethnicity. Identified synonymous and non-synonymous coding SNPs with a population frequency > 1% might also represent cryptic exonic splicing signals, but this cannot be excluded.

MND

SNP genotyping was performed in 140 white patients with sporadic MND. The diagnostic criteria have been described previously.[12 13] The control group for MND comprised 280 unrelated subjects with no history of neurological disease. MND controls were matched with the patients with regard to ethnicity, age, and sex, and there was a control to case ratio of 2 : 1. Approval was obtained from the University of Sydney and the Royal Prince Alfred Hospital human research ethics committees.

DNA analysis

DNA was isolated from peripheral nucleated blood cells using a phenol chloroform method.[14] MYH7 primers for the coding regions were designed using Oligo 6 (Molecular Biology Insights, Cascade, Colorado, USA) and Navigator software (v1.5) (Transgenomic, Omaha, Nebraska, USA) based on the GenBank sequence (accession X52889) or adopted from the literature (table 1). Polymerase chain reaction (PCR) details are available on request. Primers to amplify the 465 bp amplicon from PVR exon 2 were designed using the same software based on reference sequence (accession AC068948).[13]

All amplicons were generated by PCR in a reaction volume of 50 µl using GenAmp 9700 (Applied Biosystems, Foster City, California, USA). After amplification, the amplicons underwent a denaturation step of 95℃ for five minutes, followed by ramping to ambient temperature over 45 minutes to encourage heteroduplex formation in samples heterozygous for a mutant allele. One representative sample from each positive profile group was sequenced using the ABI Prism 3700 DNA sequencer (Applied Biosystems). Mutations

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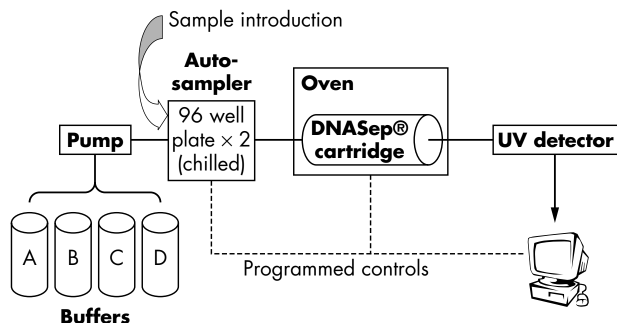

Figure 1 Diagram of the denaturing high performance liquid chromatography Wave system. Buffers (A, 0.1M triethylammonium acetate (TEAA); B, 0.1M TEAA with 25% acetonitrile; C, 8% acetonitrile for syringe wash; and D, 75% acetonitrile for cartridge cleanup) are pumped into the system individually or in a mixture through a multivalve mechanism. A sample is introduced via the autosampler, which holds 2 × 96 well chilled plates. The sample is eluted from the DNASep® Cartridge by linear gradient changes of buffers A and B (mobile phase) within an oven that has a precisely controlled temperature, and then travels through an ultraviolet (UV) detector. The amount of sample DNA and the elution time from the cartridge are automatically recorded in the computer.

in other samples with a similar elution profile were confirmed by restriction fragment length polymorphism analysis or amplification refractory mutation system.

DHPLC platform and software

DHPLC analysis was undertaken on the Transgenomic Wave® Nucleic Acid Fragment Analysis System (Model 3500 HT; Transgenomic), controlled by Navigator software (fig 1). The autosampler can hold 192 samples and introduces 5–20 µl of each sample to the DNASep® Cartridge. The DNA fragments were eluted by linear gradient changes of buffers A and B (table 1), with ratios determined by Navigator software based on amplicon size. Eluted DNA fragments were detected by the system's ultraviolet detector. DNA sequence variant detection depends on heteroduplex formation between wild-type and mutant DNA single strands.[15 16] At elevated temperatures, the less thermostable heteroduplexes start to melt at the mismatched region, and as a result the DNA elutes earlier than corresponding homoduplexes. Thus, oven temperature prediction is crucial.

Navigator software integrates several activities including amplicon design, melting profile prediction, large scale analysis, normalisation, and automated mutation calling. A unique feature of the software is the superimposing and normalising of DHPLC profiles to increase the sensitivity, speed, and accuracy of data analysis. Multiple computer stations can work in a networked client/server mode so that data can be shared between collaborating laboratories.

RESULTS

FHC mutation screening in MYH7

Mutation screening of MYH7 covered 10 kb with 38 amplicons (table 1) and was performed on 150 patients. The high throughput HT3500 model allows analysis of one injection in only 2.5 minutes compared with eight minutes for earlier models. One hundred and ninety two injections can easily be completed in an overnight run. Analysis of 5700 amplicons with over 6750 injections can be completed within 35 days with a continuous supply of PCR products. The analysis conditions were predicted using Navigator software (table 1).

Screening of MYH7 revealed 17 disease related mutations (table 2) in 21 families, which accounted for 14% of all families tested. These included seven putative novel mutations (L227V, E328G, K351E, V411I, M435T, E894G, and E927K) that were absent in 200 normal control chromosomes. All six positive controls were verified. In addition, 19 SNPs were identified, including one non-synonymous, 12 synonymous coding SNPs, and six intronic polymorphisms (table 2).

The M435T, R453C, and R453H changes identified in the exon 14 amplicon had unique elution profiles (fig 2A). Even within the same codon, two point mutations one base pair apart—R453C (M2-9123, C>T) and R453H (M3-9124, G>A)—had distinct profiles. Similar observations were made for R403W–R403Q and R719W–R719Q, which are also one base pair apart. Moreover, the same point mutations had a consistent DHPLC profile—for example, the E894G mutation could be identified in three apparently unrelated families (fig 2B) and the R663C and R719Q changes were the same in two sets of two unrelated families. Combinations of multiple DNA variants altered the elution profiles and different combinations resulted in unique profiles (fig 2C).

To reduce the number of amplicons analysed by DHPLC, especially when screening for rare mutations, we tested a pooling strategy by mixing multiple DNA samples before PCR amplification. A sample containing a heterozygous polymorphism (MYH7 exon 38, 23485 G>A) was mixed with wild-type sample at a frequency of one variant/diploid up to one variant/40 chromosomes (fig 3). The polymorphism was successfully identified at a range of 2.5–50% chromosomes.

SNP discovery, validation, and genotyping in MND

In PVR exon 2, one sample with the A67T change (15952 G>A) was identified via direct sequencing.[13] To validate that this coding SNP was polymorphic in our target subjects, DHPLC was used for genotyping. Initial genotyping was performed at 63.4℃ based on Navigator software. However, the mutant DNA could not be differentiated at this temperature or even at 65.9℃ (fig 4A). The DHPLC profile change was seen when the temperature was increased to 66.4℃, and was optimal at 66.9℃ (fig 4B). The optimal temperature was then re-estimated using the δ helicity algorithm (http://www.mutationdiscovery.com/), and was found to be 67℃.

Subsequently, genotyping was performed at 64.1℃ (manually adjusted from 63.4℃) and 66.9℃ in 140 MND cases and 280 controls. In total, 840 injections were completed within a week. The frequency of the A67T change in MND cases (13.6%) was significantly higher than in controls (6.8%; $p = 0.03$; odds ratio, 2.16; 95% confidence interval, 1.10 to 4.22). All genotyping results were confirmed by CfoI restriction enzyme digestion. Two novel coding SNPs (E116E, 16101 G>A and R133R, 16152 G>A) were identified by DHPLC analysis (fig 5). The E116E SNP was present at both temperatures (fig 5A, B), whereas a second SNP (R133R) was seen only at 64.1℃, and not at 66.9℃ (fig 5C, D).

DISCUSSION

Mutation detection and SNP discovery in large genomic areas require automated high throughput technologies. Ideally, there should be minimal modification of PCR components and post PCR steps. Although considered the gold standard, direct DNA sequencing to detect variants has drawbacks, including two post PCR purifications, false positive signals as a result of data derivation from non-variant sequence background, and a low signal to background noise ratio.[1] Data analysis can be effort intensive and error prone, especially when screening large numbers of samples.

DHPLC analysis meets most of the above requirements for high throughput detection of DNA variants, and has become

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Table 2 Identified DNA variants in MYH7

FHC no.	Location of nucleotide change	Amino acid change	Note
644	Exon 8, **6475 C>G**	**L227V†**	**Novel** missense
274	Exon 11, **7550 A>G**	**E328G†**	**Novel** missense
739	Exon 12, **8266 A>G**	**K351E†**	**Novel** missense
125*	Exon 13, 8847 C>T	R403W	Reported missense
113*	Exon 13, 8848 G>A	R403Q	Reported missense
400	Exon 13, **8871G>A**	**V411I†**	**Novel** missense
1022	Exon 14, **9070 T>C**	**M435T†**	**Novel** missense
59*	Exon 14, 9123 C>T	R453C	Reported missense
1227	Exon 14, 9124 G>A	R453H	Reported missense
676*	Exon 16, 10457 G>A	V606M	Reported missense
484	Exon 18, 11281 C>T	R663C	Reported missense
1203	Exon 18, 11281 C>T	R663C	Reported missense
97*	Exon 19, 12147 C>T	R719W	Reported missense
473	Exon 19, 12148 G>A	R719Q	Reported missense
1232	Exon 19, 12148 G>A	R719Q	Reported missense
6	Exon 21, 12765 G>A	R787H	Reported missense
114	Exon 23, **13968 A>G**	**E894G†**	**Novel** missense
280	Exon 23, **13968 A>G**	**E894G†**	**Novel** missense
743	Exon 23, **13968 A>G**	**E894G†**	**Novel** missense
707*	Exon 23, 14009 C>G	L908V	Reported missense
1275	Exon 23, **14066 G>A**	**E927K†**	**Novel** missense
968	Exon 32, **17153 C>G**	**S1519C‡**	**Novel** coding SNP
22	Exon 3, 4582 C>T	T63T	Reported coding SNP
976	Exon 7, 6311 A>G	A199A	Reported coding SNP
1053	Exon 8, 6528 T>C	F244F	Reported coding SNP
895	Exon 11, 7542 C>T	D325D	Reported coding SNP
1101	Exon 12, 8310 G>A+8343 C>T	K365K+D376D	Reported coding SNPs
8	Exon 12, 8310 G>A	K365K	Reported coding SNP
11	Exon 12, 8310 G>A+8277 C>T	K365K+G354G	Reported coding SNPs
1204	Exon 12, 8277 C>T+8343 C>T	G354G+D376D	Reported coding SNPs
1146	Exon 12, 8277 C>T	G354G	Reported coding SNP
1199	Exon 12, 8343 C>T	D376D	Reported coding SNP
739	Exon 12, **8266 A>G**+8310 G>A	K351E+K365K	**Novel** and reported coding SNPs
256	Exon 24, 14438 T>C	I989I	Reported coding SNP
579	Exon 24, 14438 T>C+**14507 C>T**	I989I+A1022A	Reported and **novel** coding SNPs
482	Exon 25, **15807 G>A**	A1061A	**Novel** coding SNP
451	Exon 27, **17852 G>A**	Q1127Q	**Novel** coding SNP
1199	Intron 2, **4360 G>T**+ Exon 3, 4582 C>T	Non-coding and T63T	**Novel** non-coding and reported coding SNPs
256	Intron 2, **4360 G>T**	Non-coding	**Novel** non-coding SNP
674	Intron 4, **5217 G>A**	Non-coding	**Novel** non-coding SNP
2079	Intron 19, **12245 A>G**	Non-coding	**Novel** non-coding SNP
973	Intron 26, **17833insC**	Non-coding	**Novel** non-coding SNP
1199	Intron 29, **18910 C>T**	Non-coding	**Novel** non-coding SNP
929	Intron 38, **23485 G>A**	Non-coding	**Novel** non-coding SNP

Bold fonts are novel DNA variants based on the literature and dbSNP (Build 121, http://www.ncbi.nlm.nih.gov/SNP). The base numbers are based on the reference sequence (GenBank accession X52889).
*Known MYH7 mutations that have been reported previously[10,11]; †novel missense mutations were identified in patients with FHC, but absent from 200 normal control chromosomes; ‡this DNA variant was identified in an FHC proband; however, it was also present in 200 normal control chromosomes, with a population frequency of 3%.
FHC, familial hypertrophic cardiomyopathy; SNP, single nucleotide polymorphism.

the method of choice.[1,16] It is considerably cheaper and simpler than sequencing, and enables sequencing to be limited to a few abnormal amplicons identified by DHPLC. In our current study, we demonstrated the high throughput capacity of DHPLC by screening 150 FHC families for disease causing mutations in a large multi-exon gene (MYH7). PVR A67T SNP was also efficiently genotyped in 420 subjects. Twenty one SNPs were identified from analysis of the MYH7 and PVR genes. Our results were consistent with previous evaluations, which concluded that DHPLC is an accurate, reliable, and cost effective method for mutation screening and SNP discovery.[1,16–19]

Several conditions are important to ensure optimal results in high throughput DHPLC analysis. First, specific and efficient PCR amplification is crucial to avoid false positives because group analysis is essential in high throughput work. It is hard to correct samples manually one by one for their intensity. Normalisation of DHPLC signals below 2 mV is prone to false positives.

Second, to optimise resolution in the rapid analysis option, the elution window and times must be adjusted so that heteroduplex and homoduplex peaks are separated by at least 1.5 minutes from the injection peak and 0.4 minutes from the wash peak (fig 4).

Next, prediction of the melting temperature is essential. Regions that differ by > 10℃ in the same amplicon should be avoided. If this is not possible, the addition of a GC clamp may be necessary, as is seen in the MYH7 exon 27 amplicon (table 1), to reduce the number of temperatures required to screen an amplicon. However, its use should be minimised because PCR yields may fall.[20] In our current study, temperatures predicted by Navigator software usually allowed the successful detection of most DNA variants, regardless of their number and type. However, the estimated temperature of 63.4℃ failed to identify the PVR A67T change (fig 4A). The correct higher temperature was predicted by the δ helicity algorithm, so the use of different algorithms can be helpful in predicting melting temperature.

Finally, to maintain continued efficiency and reproducibility over large sample sizes, known mutation samples with defined profiles should be checked regularly for quality control. The DNASep cartridge showed satisfactory reproducibility, which extended over 6000 injections in our experience. However, the profile for some variants differed slightly

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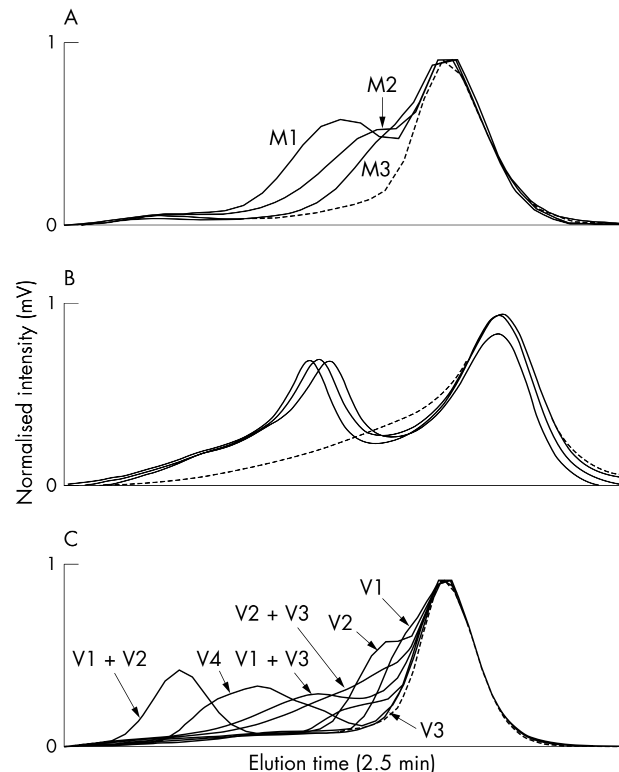

Figure 2 Detection of unknown DNA variants. All denaturing high performance liquid chromatography (DHPLC) profiles are detected at the optimal temperatures and are shown after normalisation by Navigator software. The wild-type control is indicated by the dashed line. (A) Different mutations have unique profiles. The M435T, R453C, and R453H changes were found in the MYH7 exon 14 amplicon (M1 = 9070 T>C, M2 = 9123 C>T, and M3 = 9124 G>A, respectively). M2 and M3 at the same codon of R453 are two point mutations one base pair apart, but they have distinct profiles. (B) The same changes have consistent profiles, especially obvious after normalisation. A missense change (MYH7 exon 23, 13968 A>G, E894G) was found in three different unrelated patients. (C) Combinations of different variants have unique DHPLC profiles. Multiple DNA variants were found in the MYH7 exon 12 amplicon: V1 = 8310 G>A, K365K; V2 = 8277 C>T, G354G; V3 = 8343 C>T, N376N; and V4 = 8266 A>G+8310 G>A; K351E+K365K.

between and within columns over time. This reinforces the importance of the positive controls during each run when genotyping.

> "The identification of DNA variants with low level mosaicism is an additional feature of denaturing high performance liquid chromatography analysis"

For all samples tested, DHPLC was found to be reproducible and unique for a particular variant (fig 2). With experience, specificity for detecting DNA variants can approach 100% because all positives for MYH7 mutation detection or PVR SNP genotyping were confirmed by direct sequencing or restriction fragment length polymorphism analysis/amplification refractory mutation system analysis. All six FHC families with known MYH7 mutations were identified using the estimated optimal temperatures, suggesting high sensitivity, although this cannot be determined accurately because we sought unknown mutations. Nevertheless, the sensitivity in our study was probably close to 100% because false negatives were not found in PVR genotyping of 420 individuals when compared with the genotypes obtained from restriction fragment length poly-

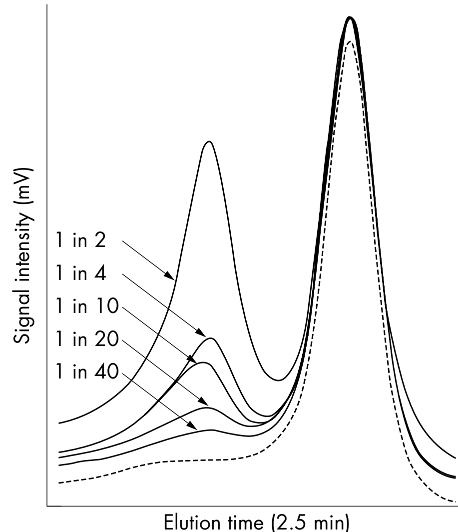

Figure 3 Sensitivity of pooling DNA samples. A heterozygous single nucleotide polymorphism was identified in MYH7 intron 38 (23485 G>A) in a sample. Various amounts of known wild-type sample (10 ng/sample) were mixed with the above heterozygous sample before polymerase chain reaction amplification. Different profiles after normalisation by Navigator software are shown (one variant in 2–40 copies of normal alleles). The homozygous wild-type control is indicated by a dashed line.

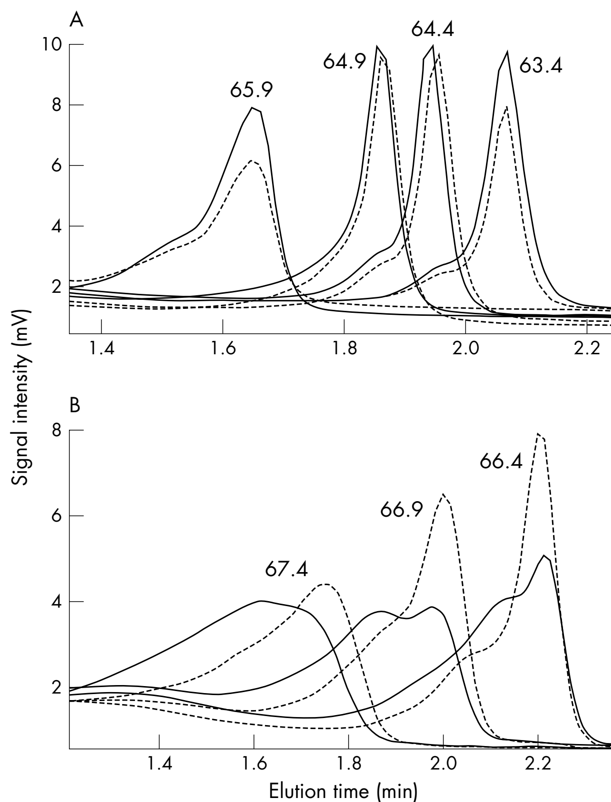

Figure 4 Denaturing high performance liquid chromatography (DHPLC) analysis at different temperatures without normalisation. The wild-type control is indicated by the dashed line. (A) Samples with and without a heterozygous A67T single nucleotide polymorphism in PVR exon 2 run at 63.4, 64.4, 64.9, and 65.9°C. No significant difference was seen between the A67T heterozygote and the wild type. (B) Samples with and without the heterozygous A67T allele run at 66.4, 66.9, and 67.4°C with a timeshift of 0.8 minutes. Significant changes in DHPLC profiles were seen compared with the wild type at these three temperatures, with the most obvious one at 66.9°C.

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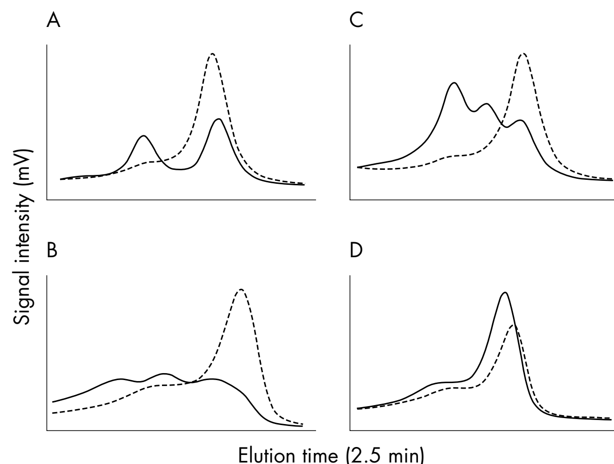

Figure 5 Denaturing high performance liquid chromatography (DHPLC) profiles of DNA variants at two different temperatures without normalisation. A sample with a heterozygous change in PVR exon 2 (E116E, 16101 G>A) shows significant differences in DHPLC profile compared with the wild-type control (dashed line) at both 64.1˚C (A) and 66.9˚C (B). A second heterozygous change in PVR exon 2, (R133R, 16152 G>A) shows a significant difference at 64.1˚C (C) only, and not at 66.9˚C (D).

morphism analysis. Although the prevalence of MYH7 mutations in our study population (14%) is lower than some earlier estimations, it is not inconsistent with more recently reported mutation frequencies (12–13%) for this gene.[21 22]

The identification of DNA variants with low level mosaicism is an additional feature of DHPLC analysis.[19 23 24] We found that DHPLC could detect as few as 2.5% variants (fig 3) when a sample was pooled with up to 20 normal samples, as reported previously.[23] However, care should be taken because the DHPLC positive signal diminished rapidly with increasing numbers of normal chromosomes. This function is superior to direct sequencing and can be used for the detection of somatic mosaicism, loss of heterozygozity in tumour DNA, and heteroplasmy with mitochondrial DNA.[1 18 23 25]

With the software provided, objective comparisons using DHPLC analysis were more sensitive and less time consuming than subjective assessment using single stranded conformational polymorphism or heteroduplex analysis. In most cases, heterozygous profiles consisted of multiple peaks that were easily distinguishable from the wild-type profile. In others, heterozygotes were only distinguishable from the wild-type by a shoulder in the peak, or by a wider peak (for example, M2 and M3, respectively, in fig 2A). In these circumstances, the ability to overlay and normalise chromatographic profiles with the wild-type profile enhanced identification.

DHPLC is distinct from other DNA variant detection techniques because of its purification capacity in conjunction with a fragment collector. DHPLC requires only 8–16 μl of PCR product for screening, so the remaining product from a 50 μl reaction can be used for confirmatory procedures, such as sequencing or cloning. The ability to screen only for heterozygous variants is a limitation of DHPLC analysis. However, this can be overcome by a mixing strategy—a homozygous mutant can be mixed with a ''wild-type'' sample at a 1 : 1 ratio before denaturing to generate the heteroduplex population.

In summary, we found that DHPLC offers a high throughput, automated technique that enabled rapid screening of a large gene (MYH7) in many patients with FHC. With the estimation of melting temperature using two different algorithms, in addition to empirical testing with a few additional temperatures, we found this technique to be highly sensitive and specific for DNA variant detection. It also offers considerable advantages for efficient SNP validation, as shown in our MND study.

Take home messages

- We found denaturing high performance liquid chromatography (DHPLC) to be a high throughput, automated technique that enabled rapid screening of a large gene (MYH7) in patients with familial hypertrophic cardiomyopathy
- The estimation of melting temperature using two different algorithms, in addition to empirical testing with a few additional temperatures, made this technique highly sensitive and specific for DNA variant detection
- DHPLC also offered considerable advantages for efficient single nucleotide polymorphism validation, as assessed by analysis of the poliovirus receptor gene in motor neurone disease

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