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Original Article

Setting up Multiplex Panels for Genetic Testing of Familial Hypertrophic Cardiomyopathy Based on Linkage Analysis

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Abstract

Background: Familial hypertrophic cardiomyopathy (HCM) is caused by mutations in genes encoding cardiac sarcomere proteins. Nowadays genetic testing of HCM plays an important role in clinical practice by contributing to the diagnosis, prognosis, and screening of high-risk individuals. The aim of this study was developing a reliable testing strategy for HCM based on linkage analysis and appropriate for Iranian population.

Methods: Six panels of four microsatellite markers surrounding MYH7, MYBPC3, TNNT2, TNNI3, TPM1, and MYL2 genes (24 markers in total) were selected for multiplex PCR and fragment length analysis. Characteristics of markers and informativeness of the panels were evaluated in 50 unrelated Iranians. The efficacy of the strategy was verified in a family with HCM.

Results: All markers were highly polymorphic. The panels were informative in 96-100% of samples. Multipoint linkage analysis excluded the linkage between the disease and all six genes by obtaining maximum LOD score ≤-2.

Conclusion: This study suggests a reliable genetic testing method based on linkage analysis between 6 sarcomere genes and familial HCM. It could be applied for diagnostic, predictive, or screening testing in clinical setting.

Keywords: Cardiomyopathy, Hypertrophic, Genetic linkage, Diagnosis

Introduction

Hypertrophic cardiomyopathy (HCM) is characterized by left ventricular hypertrophy (LVH) in the absence of predisposing conditions such as long-standing hypertension or valvular stenosis. It is the most common genetic cardiovascular disorder inherited in an autosomal dominant mode with a prevalence of at least 1:500 (1). HCM is the most common cause of sudden death in the youth and a major cause of morbidity in adults (2).

At least 27 genes have been proposed as HCM susceptibility genes (3). However, genes encoding protein components of the cardiac sarcomere (sarcomere genes) are accounted for the disease in most of HCM families (60-75%) (4-6). Up to 2012, more than 800 exonic and intronic disease associated mutations has been reported in sarcomeric genes (7). Locus and allelic heterogeneity make the direct mutation detection in HCM cases very difficult. In this situation, linkage analysis

can be very helpful by determining the causative locus in appropriate families before sequencing the gene for mutation detection.

In this study we proposed a set of 24 polymorphic microsatellite markers flanking 6 sarcomeric genes including beta-myosin heavy chain (*MYH7*), myosin binding protein C (*MYBPC3*), cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*) , alpha-tropomyosin (*TPM1*), regulatory myosin light chain (*MYL2*). The polymorphic indices of markers were evaluated in Iranian population. Multiplex PCR and fragment length analysis was set up to reduce time and money expenses. The feasibility of the method was checked in an Iranian HCM family.

Materials and Methods

Clinical investigations of a family with HCM

A 40 yr old Iranian woman with HCM was referred for genetic counseling to the Department of Medical Genetics at Tehran University of Medical Sciences in 2011. The clinical diagnosis of HCM had been made by the interventricular septal thickness of ≥13 mm, in the absence of other cardiac or systemic causes of LVH. Her available first-degree relatives were invited for clinical evaluation and blood sampling. The proband and her relatives underwent physical examination, 12-lead electrocardiography, transthoracic two-dimensional echocardiography, and Doppler studies in Shahid Rajaei Cardiovascular Medical Center, Tehran, Iran. The clinical diagnosis of HCM in relatives was established based on McKenna criteria (8). Proband's mother has died 22 years ago at the age of 51. No medical record was available regarding the cause of her death. However, Proband's explanation of her mother's clinical symptoms was consistent with HCM.

Sampling and DNA extraction

Blood samples were collected from all participants. DNA was extracted by phenol-chloroform method according to standard protocol, stored at 4 °C until analysis. Quality and quantity of the extracted DNA were assessed by Nano Drop spectrophotometer (ND-1000) as well as gel electrophoresis.

Sampling for population study

Fifty unrelated individuals referring to heart clinics of Shahid Rajaei Hospital for unrelated reasons were randomly recruited for the study. Blood sampling and DNA extractions were performed.

Ethical approval

This study was approved by the research Ethics Committee of Tehran University of Medical Sciences. Informed consent was obtained from all participants.

Marker Selection

Four microsatellite markers flanking every one of 6 sarcomere genes (*MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, *TPM1*, and *MYL2*) were selected from Marshfield genetic map (24 markers in total). Order of markers around each gene was defined by integrating information obtained from the Human Genome Sequencing database (NCBI build 37.3) and Marshfield genetic map (9). Localization of genes and flanking markers is shown in (Fig. 1). The nearest markers flanking upstream and downstream of each gene with mean heterozygosity (MH) more than 70 percent were assigned for the study. Exceptionally, due to the limitations for PCR multiplexing, 3 markers were selected with MH near 70% (Table 1). For *MYH7*, two intragenic markers (MYOI and MYOII) were successfully used in repeated gene tracking studies (10, 11). Therefore, these two markers were selected in our study.

Multiplexing

Selected Markers were assigned in two sets of 12 markers each (SET1 and SET2). Size fractionation of all 12 markers in each group was made possible by capillary electrophoresis using combination of fluorescently labeled primers. Three different fluorophores including FAM, HEX, and NED were used for labeling primers. Markers in SET1 cover three genomic loci containing *MYH7*, *MYBPC3*, and *TNNT2*. Markers in SET2 span the regions containing other 3 genes (*TNNI3*, *TPM1*, and *MYL2*). Possibility of multiplexing of size fractionation by capillary electrophoresis was also considered in selection of suitable markers.

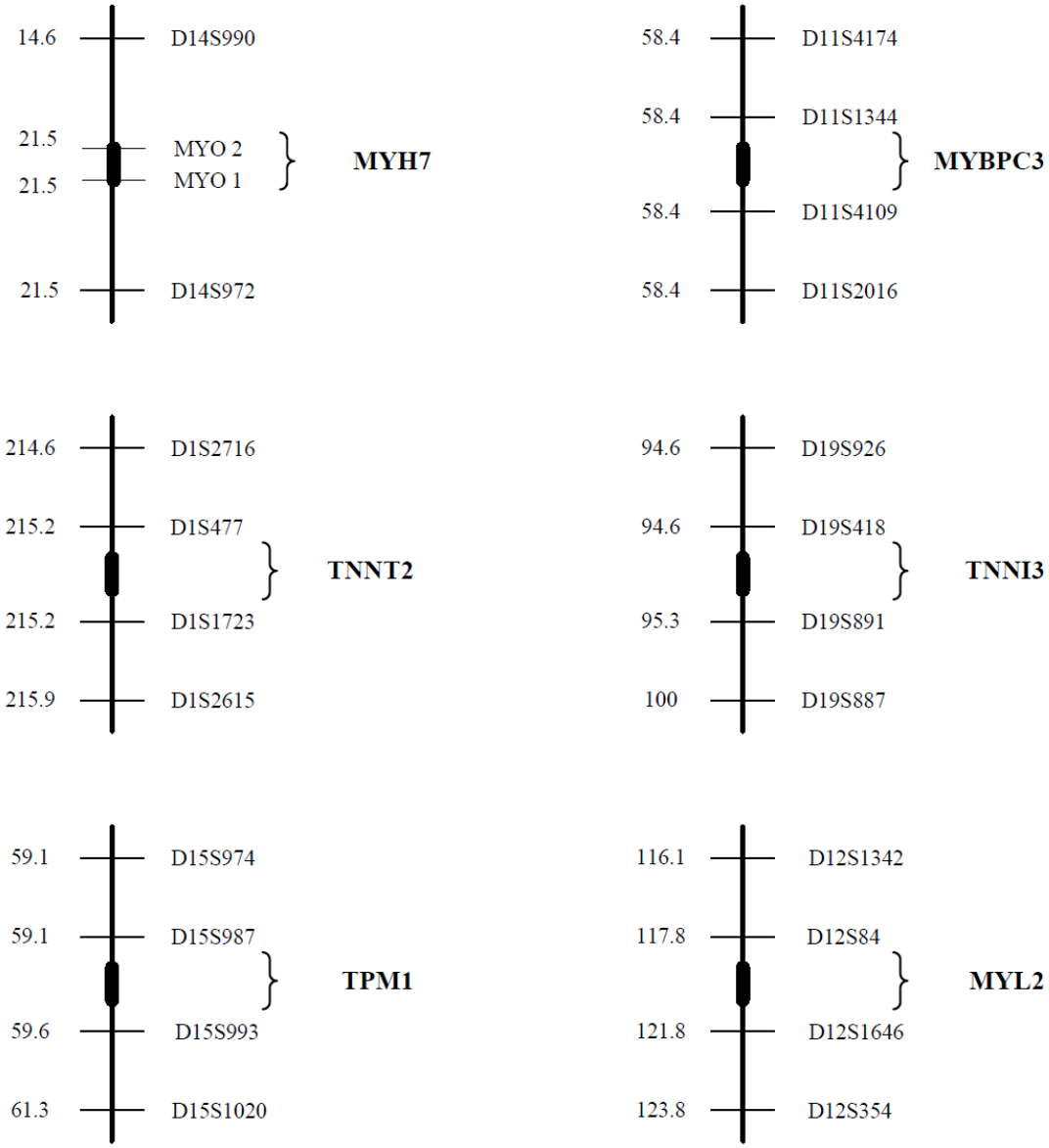

Fig. 1: Localization of microsatellite markers flanking sarcomere genes. The sex-averaged genetic distances were shown in centiMorgan from the p telomeric end of the chromosome. Data obtained from Marshfield genetic map

PCR Setup and Fragment length analysis

Primer sequences for genotyping of all 24-microsatellite markers were obtained from NCBI UniSTS databank (12). All Forward primers were labeled at the 5'end by a fluorophores (FAM, HEX, or NED). Detailed information regarding primer sequences, modifications and characteristics of markers is presented in (Table 1). PCR conditions were optimized for amplification of all 24 markers separately. Touchdown PCR, reducing cycles and shortening of extension time were used to decrease the stutter fragments of microsatellites.

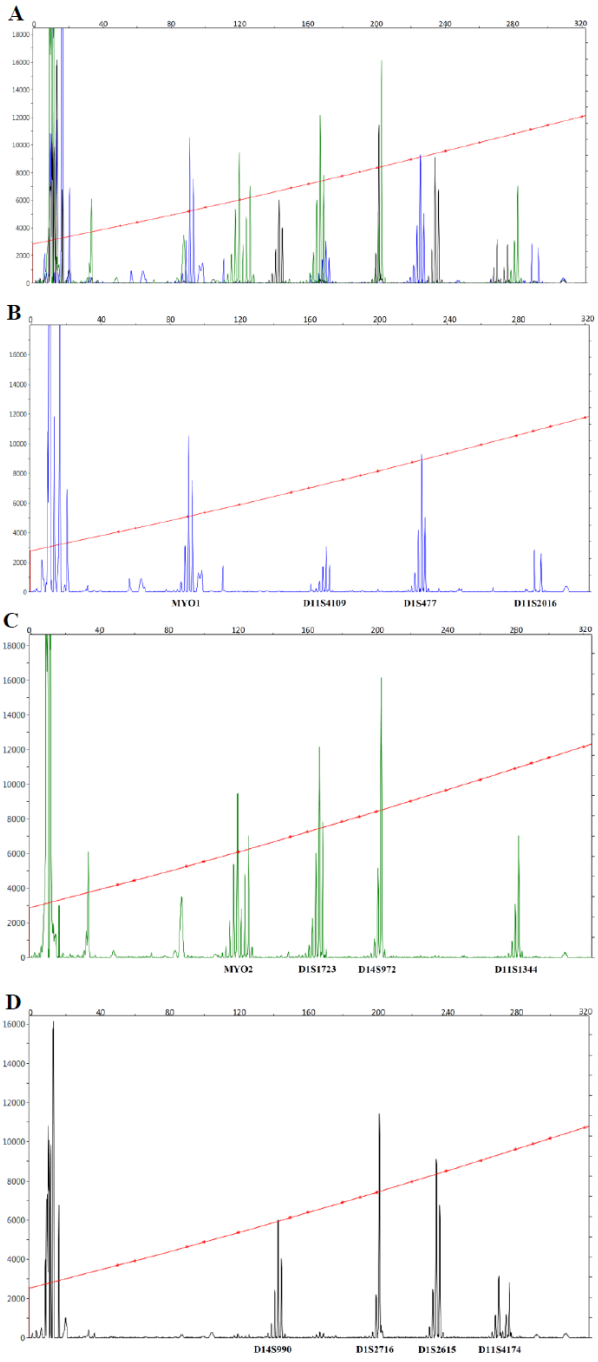

Fig. 2: (A) GeneScan results of multiplex fragment length analysis of SET1 including 12 markers analyzed with peakscanner software v1.0. Every 4 markers labeled by a fluorophore are shown separately: (B) FAM, (C) HEX, (D) NED. The fragment size was determine by comparison to the GeneScan™ 400HD ROX™ Size Standard, shown on the upper edge of each diagram.SET2 was not shown

In addition, multiplexing of PCR reactions were performed by defining appropriate conditions covering 12 markers of SET1 and 11 markers of SET2 (Table 1). Multiplexing of PCR for one marker in SET2 (D19S891) was not successful. The PCR product of this marker was mixed with multiplex PCR product of other 11 markers of SET2 before fragment analysis. Fragment length analysis was done for SET1 and SET2 separately. It was performed by using the Applied Biosystems 3130 Genetic Analyzers and Gene Scan® Analysis Software version 3.7. Gene Scan results were analyzed with peak scanner software v1.0 (Fig. 2). Identified genotypes were entered in a databank for statistical analysis.

Statistical analysis

Easy LINKAGE plus v5.05 (13) was used for linkage analysis. Testing for Mendelian errors was performed by using Merlin v1.0.1. Gene Hunter v2.1r5 software was used for haplotyping and multipoint parametric linkage analysis.

Results

Genotyping of all 24 markers was performed for 50 unrelated people. Table 2 shows characteristics of different alleles for each marker including repeat number and frequency. Selected markers had 5 to 15 alleles.

Mean heterozygosity (MH) and polymorphic information content (PIC) of each marker are presented in Table 3. All markers showed PIC above 50%. Mean heterozygosity was above 70% in majority of markers. Only three markers had a mean heterozygosity of 60-70%.

Table 4 shows the frequency of combined markers heterozygosity in the panel of markers used around each gene locus. The panel of markers around each locus was considered informative in each sample if at least one of 4 markers was heterozygous. Having this, *MYBPC3*, *TNNI3*, and *MYL2* panels showed 100% informativeness (informative in all 50 evaluated samples). *MYH7*, *TNNT2*, and *TPM1* panels were informative in

96%, 98%, and 98% of samples respectively. Over 70% of cases were heterozygous for 3 to 4 markers flanking each gene.

Case study

Nine members of proband's family were evaluated. In addition to the proband herself, three other members of pedigree were diagnosed with HCM. Simulation of 1000 replicates by Slink resulted in a maximum expected two-point LOD score of 2.10 at θ=0.00 (average=1.33±0.83 standard deviation).

Haplotype analysis for the six genes under study was performed. Marker D12S1646 was removed from the analysis due to the observed Mendelian error, possibly caused by genotyping error. The structure of haplotypes in each locus is shown in (Fig. 3).

Multipoint linkage analysis excluded the linkage between the disease and all six genes by obtaining maximum LOD score ≤-2 (Fig. 4). The analysis was performed under the assumption that the proband's mother had HCM, although her diagnosis cannot be confirmed due to lack of medical documents. Therefore, analysis was repeated by assigning unknown status for her. Again, multipoint linkage analysis showed negative LOD score, suggesting linkage exclusion in all 6 genes.

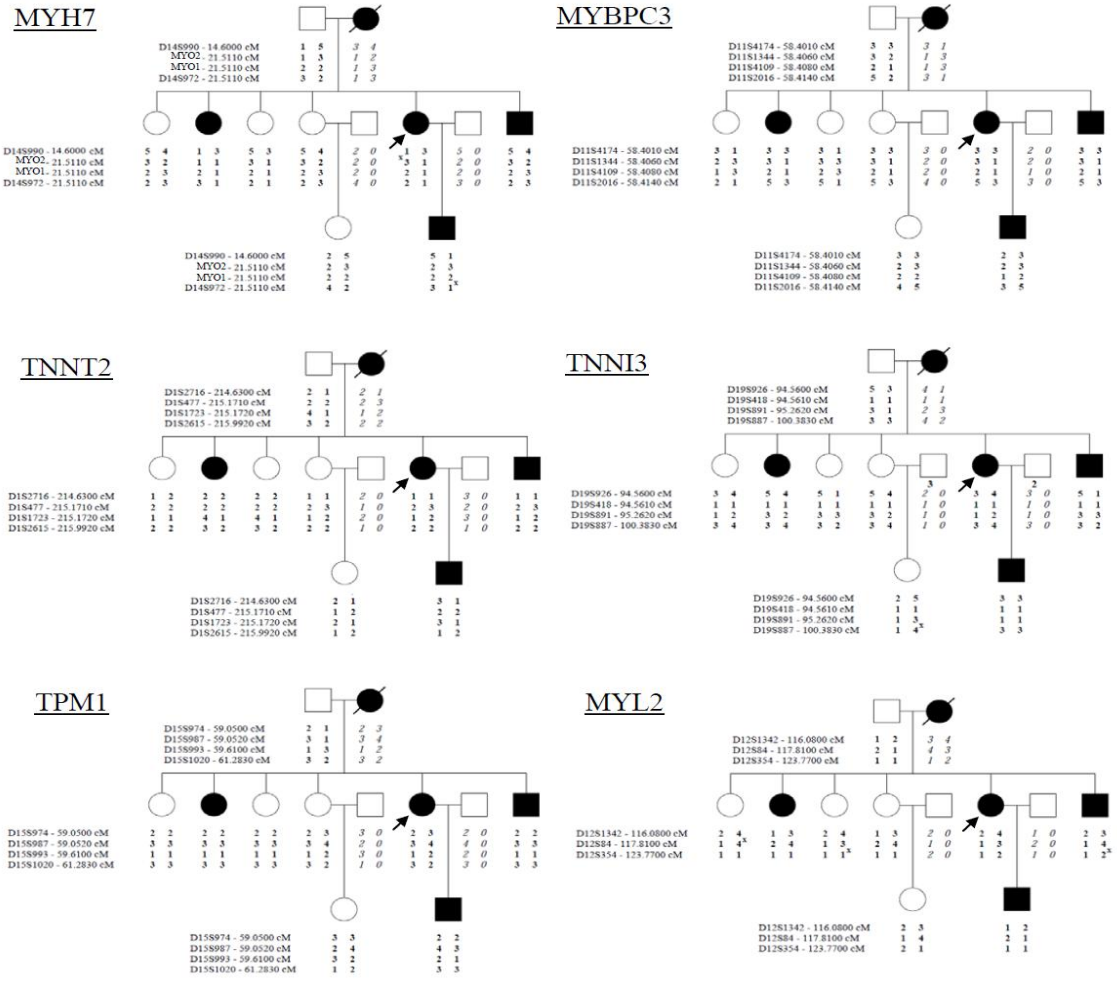

Fig. 3: The structure of selected markers haplotypes flanking each sarcomere gene in a family with HCM. The proband indicated with an arrow. Cross-overs are shown by "x". The sex-averaged genetic distance of each marker in centiMorgans (cM) from p telomere of chromosomes is noted

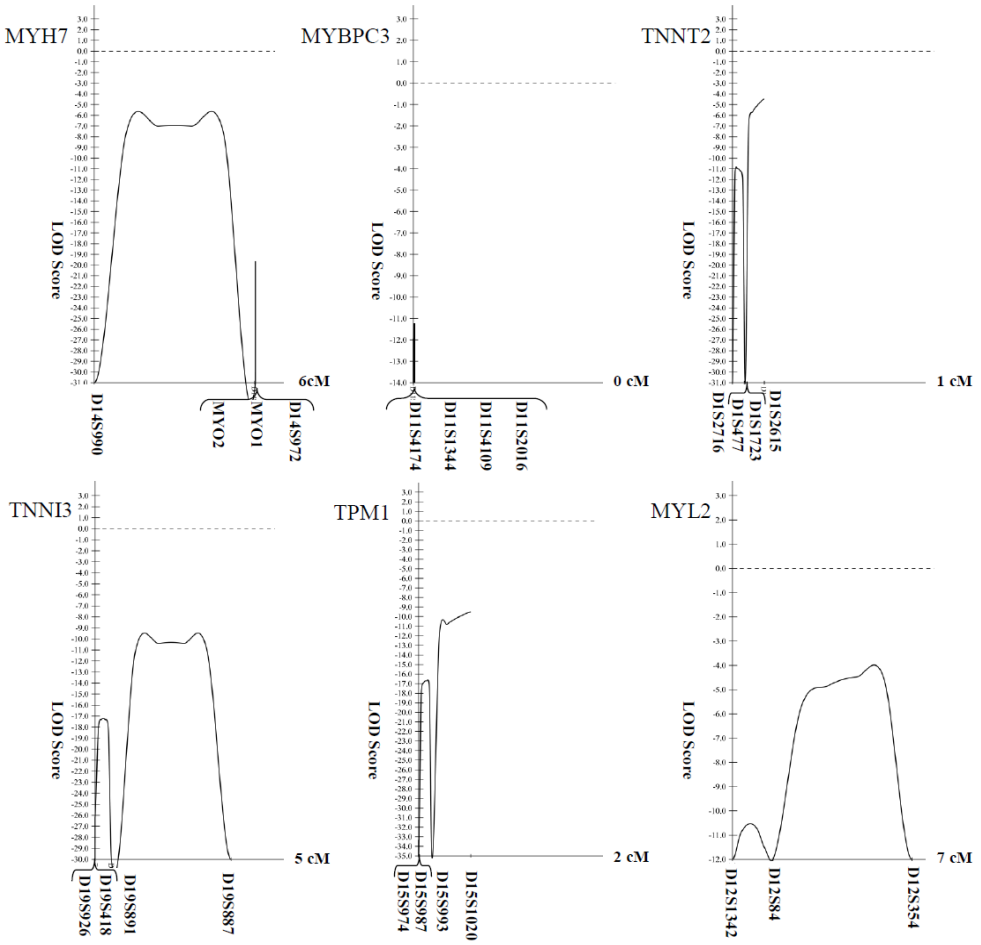

Fig.4: The LOD plot of Multipoint linkage analysis between the disease and six sarcomere genes. The relative localization of each marker and the genetic distance between them are shown on X axis in centiMorgans (cM)

Discussion

Profound locus and allele heterogeneity, large sarcomere genes with numerous exons and intronic disease causing mutations make the direct mutation detection for HCM time and money consuming. Though new methods such as next generation sequencing/ exome sequencing are helpful (14-16), they are demanding in terms of cost, equipment, and expertise. Such methods are available just in few countries around the world. Therefore applying alternative methods for HCM genetic analysis is a necessity in many countries.

Tracking mutated genes by using surrounding DNA markers through linkage analysis is valuable for familial HCM, especially for predictive and screening purposes. Linkage analysis can also be used as the first step in mutation detection. It can reduce the number of candidate genes for direct sequencing through exclusion mapping. In addition, it can identify the most likely disease gene even in families with small size.

We proposed panels of microsatellite markers flanking 6 genes which cause 60 to 75% of familial HCM. Our study showed marker characteristics in Iranian population not studied before. All of 24 markers were highly polymorphic (mean heterozygosity more than 70% except 3 markers with mean heterozygosity from 60% to 70%). All markers are also highly informative represented by Polymorphic Information Content (PIC) more than 0.5. Informativeness of panels also was eva-

luated. One hundred percent of samples had at least one heterozygous marker in panels of each 3 genes (*MYBPC3*, *TNNI3*, and *MYL2*). For the other 3 genes (*MYH7*, *TNNT2*, and *TPM1*) 96-98% of samples had at least one heterozygote marker in related panels. Two samples showed homozygosity for all 4 markers around one gene. One sample showed homozygosity for all markers around two genes (*MYH7* and *TNNT2*). Looking to their pedigrees, it was realized that these samples had consanguineous parents (first cousin). Offsprings of first cousins have autozygosity in 1/16 of their loci. Therefore finding homozygous haplotypes in these people is expected. The above findings were obviously independent from the Informativeness of the markers used. Therefore, we confidently suggest using these panels for future gene tracking experiments in our population.

In addition, multiplexing of PCRs for all 24 markers in only 3 reactions and 2 runs of capillary electrophoresis make our strategy very cost-effective. Not forgetting that the whole experimental process can be performed in only one day, this is a very short time in comparison with other available testing methods.

Using linkage strategy for mutation detection in familial HCM was performed before (6, 17-19). However, multiplexing was reported in only one study. Mogensen et al. reported multiplexing of 28 markers surrounding 9 genes in 10 reactions and 10 runs of capillary electrophoresis for fragment length analysis (20).

Applying the panel for the presented family with HCM showed the power of our strategy in exclusion of these 6 common HCM genes. This strategy was also successfully applied for gene tracking in a number of pedigrees with familial HCM (data was not shown.)

Conclusion

Our study presented a fast, cost-effective and reliable method for diagnostic, predictive, or screening testing in familial HCM. It should not be forgotten that gene tracking is useful only for the familial forms of diseases. Suggested strategy cannot be used for genetic diagnosis in sporadic cases.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

The authors declare that there is no conflict of interests.

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Table 1: Characteristics of microsatellite markers flanking each sarcomere gene and their primer sequences and modifications. * MH: Mean Heterozygosity. ** SET1; Initial denaturation: 95°C for 3 min, 10 cycles of touch down PCR with denaturation: 95°C for 30 sec, annealing: 65-60°C for 30 sec (decreases 0.5°C per cycle) with no extension step, 20 cycles of normal PCR with denaturation: 95°C for 30 sec, annealing: 60°C for 30 sec with no extension step, final extension: 72°C for 2 min. Final concentration of MgCl2 was 0.3 mM. SET2; PCR program was the same as SET1. Final concentration of MgCl2 was 0.5 mM. S#; Initial denaturation: 95°C for 3min, 30 cycles of normal PCR with denaturation: 95°C for 30 sec, annealing: 55°C for 30 sec with no extension step, final extension: 72°C for 2 min. Final concentration of mgCl2 was 1.2 mm

Gene	Marker	Forward Primer	Reverse Primer	MH *(%)	Length (bp)	5' Modification	Primer Concentration (pmol/µl)	PCR Condition**
MYH7	MYO 1	CTGCATCTGAGCATATGGGA	CATTCAGACTATGCAGGCTT	66	90-102	FAM	0.2	SET1
	MYO 2	ATGCCATGTCTATCTGTGCC	AACATCCTCTAACCCTACCCC	81	108-132	HEX	0.2	SET1
	D14S990	GTCCACTTGGTCATGGAAAC	AAGTTGCACTGTGACTGGG	85	135-161	NED	0.2	SET1
	D14S972	TTAACGCATAACAGCCAAGA	TCTGACTGCCTCCATGA	74	201-211	HEX	0.4	SET1
MYBPC3	D11S1344	CCCTGAACTTCTGCATTCAC	GCGCCTGGCTTGTACATATA	82	273-293	HEX	0.2	SET1
	D11S4109	CTGGGAGTTAGGAGACCTGG	TTGAAGAGCCCTCACAGAC	85	155-185	FAM	0.2	SET1
	D11S4174	GATTAAATGCCCACTATGTAGC	GATAGCTTTCCCAGATGGTT	73	277-295	NED	0.2	SET1
	D11S2016	TGCGGCATTATTCATAATCA	ATTTTTTTGGATGAAGTAATACTGG	77	281-301	FAM	0.4	SET1
TNNT2	D1S477	CAGTACAGGTCAACCAAGACGTATG	TCTACAAGGGGCCACTCAG	68	216-230	FAM	0.2	SET1
	D1S1723	AACTGTGTCCAGCAGCAACT	TATGTGCCTGTTGTGTGCAT	83	167-181	HEX	0.2	SET1
	D1S2716	GGCTGCCAAGTCCACTG	GGGTCCTAAAGATAGAAAAATGTCC	66	196-206	NED	0.2	SET1
	D1S2615	ACAGCGCCTGGCTATAA	GACAATGTTGTAGTGCCTGG	78	232-243	NED	0.2	SET1
TNNI3	D19S418	ACCAGGCATCCAGTGTTT	CAACTATCCCGCCTTTGT	67	81-93	FAM	0.2	SET2
	D19S891	AAATTCAACAGCCATTATGG	CGTACCCCTTATCTGATGA	76	99-117	NED	0.5	S#
	D19S926	TCTGGTGAGAATTCCTAAGTAGTTC	GGCCTTATGCGTGAGTAGTT	80	95-113	HEX	0.3	SET2
	D19S887	TATCCAATGCCACAGAAAA	AAGGTTTGCTTGTTTGGGT	74	246-262	NED	0.3	SET2
TPM1	D15S987	ACAGTCCTGCCCTTAGAAA	TAGAACGCTGCCCTCAC	74	162-179	HEX	0.2	SET2
	D15S993	AGAAACCCAGGCTGACTT	GCACTGTTGTGGTCTAATCC	82	177-189	NED	0.2	SET2
	D15S974	TCATAGAATCAGCCAGCCA	AGGGTCAGGAATGGGTC	88	115-146	FAM	0.2	SET2
	D15S1020	TGCACAATGGATACTAAACAGC	CGATAGAGCAAGACTGTCTCAA	86	211-231	NED	0.3	SET2
MYL2	D12S84	GCTTACAGTAGGTGCTTAATAAATG	TGTCTCTAGGCTAATGGCTT	84	198-219	HEX	0.2	SET2
	D12S1646	ACCACTCCATTGCTGGC	GCTGGGTAAGAACCTCTGC	72	247-259	FAM	0.2	SET2
	D12S1342	AGTTTGACCCCCCAGA	GCAGAAGATGAGGGCA	83	266-288	HEX	0.2	SET2
	D12S354	GGTGGTTCTGGGTCAGAT	GGTTTCCTAATTTCAAGTCAA	73	187-205	FAM	0.3	SET2

Table 2: Characteristics of different alleles for selected markers including repeat numbers and frequency in 50 unrelated samples. Markers indicated with * had half-repeat units (alleles with sizes greater than the typical allele size by one base) represented by 0.5 unit

MYH7					MYBPC3				TNNT2		
MYO1	**MYO2**	**D14S990**	**D14S972**	**D11S1344**	**D11S4109**	**D11S4174**	**D11S2016**	**D1S477**	**D1S1723**	**D1S2716**	**D1S2615***
14 (0.15)	18 (0.01)	12 (0.01)	12 (0.10)	18 (0.12)	11 (0.11)	18 (0.02)	4 (0.01)	12 (0.08)	16 (0.01)	9 (0.24)	14 (0.01)
15 (0.46)	22 (0.01)	15 (0.07)	13 (0.44)	19 (0.14)	15 (0.01)	19 (0.02)	10 (0.05)	16 (0.13)	17 (0.01)	11 (0.03)	14.5 (0.06)
16 (0.29)	23 (0.05)	16 (0.07)	14 (0.11)	20 (0.03)	16 (0.02)	20 (0.03)	11 (0.05)	18 (0.60)	18 (0.18)	12 (0.48)	15 (0.02)
17 (0.09)	24 (0.31)	17 (0.25)	15 (0.21)	21 (0.02)	17 (0.02)	21 (0.21)	12 (0.12)	19 (0.11)	19 (0.05)	13 (0.22)	15.5 (0.31)
18 (0.01)	25 (0.30)	18 (0.07)	16 (0.13)	22 (0.14)	18 (0.21)	22 (0.41)	13 (0.45)	20 (0.01)	20 (0.32)	14 (0.3)	16 (0.12)
	26 (0.14)	19 (0.16)	17 (0.01)	23 (0.32)	19 (0.14)	23 (0.13)	14 (0.23)	21 (0.05)	21 (0.19)		16.5 (0.04)
	27 (0.07)	20 (0.19)		24 (0.15)	20 (0.16)	24 (0.13)	15 (0.06)	23 (0.01)	22 (0.05)		17 (0.34)
	28 (0.05)	21 (0.13)		25 (0.03)	21 (0.16)	25 (0.04)	16 (0.03)	24 (0.01)	23 (0.06)		18 (0.10)
	29 (0.01)	22 (0.04)		26 (0.02)	22 (0.06)	26 (0.01)			24 (0.06)		
	30 (0.02)	23 (0.01)		27 (0.03)	23 (0.05)				25 (0.03)		
	31 (0.01)				24 (0.01)				26 (0.02)		
	32 (0.01)				25 (0.01)				27 (0.02)		
	34 (0.01)				26 (0.01)						
					28 (0.03)						

TNNI3				TPM1				MYL2			
D19S418	**D19S891**	**D19S926**	**D19S887**	**D15S987***	**D15S993**	**D15S974**	**D15S1020**	**D12S84**	**D12S1646**	**D12S1342***	**D12S354**
11 (0.05)	12 (0.18)	12 (0.46)	16 (0.04)	22 (0.30)	17 (0.03)	20 (0.01)	15 (0.02)	16 (0.09)	15 (0.03)	16.5 (0.18)	11 (0.18)
12 (0.06)	13 (0.03)	14 (0.03)	17 (0.01)	23 (0.13)	18 (0.05)	21 (0.02)	16 (0.02)	17 (0.06)	16 (0.14)	17.5 (0.15)	13 (0.31)
13 (0.39)	14 (0.06)	15 (0.21)	18 (0.07)	23.5 (0.05)	19 (0.15)	22 (0.12)	18 (0.17)	18 (0.05)	17 (0.10)	18 (0.01)	14 (0.08)
14 (0.27)	15 (0.12)	16 (0.09)	19 (0.24)	24 (0.24)	20 (0.27)	23 (0.03)	19 (0.20)	20 (0.01)	18 (0.28)	18.5 (0.24)	16 (0.36)
15 (0.11)	16 (0.06)	17 (0.06)	20 (0.40)	24.5 (0.25)	21 (0.18)	24 (0.06)	20 (0.15)	21 (0.10)	19 (0.15)	19.5(0.05)	17 (0.05)
16 (0.10)	17 (0.12)	18 (0.09)	21 (0.19)	25 (0.03)	22 (0.23)	25 (0.04)	21 (0.12)	22 (0.17)	20 (0.25)	20 (0.01)	18 (0.01)
17 (0.02)	18 (0.09)	19 (0.06)	22 (0.03)		23 (0.08)	26 (0.05)	22 (0.22)	23 (0.28)	21 (0.05)	20.5 (0.04)	21 (0.01)
	19 (0.27)		23 (0.02)		24 (0.01)	27 (0.05)	23 (0.07)	24 (0.14)		21 (0.21)	
	20 (0.07)					28 (0.03)	24 (0.03)	25 (0.05)		21.5 (0.06)	
						29 (0.08)		26 (0.04)		22 (0.05)	
						30 (0.29)		27 (0.01)			
						31 (0.13)					
						32 (0.05)					
						33 (0.03)					
						34 (0.01)					

Table 3: Mean heterozygosity (MH) and polymorphic information content (PIC) of selected markers in 50 unrelated samples

Gene	Marker	MH (%)	PIC
MYH7	MYO 1	67	0.61
	MYO 2	78	0.75
	D14S990	84	0.82
	D14S972	72	0.68
MYBPC3	D11S1344	81	0.80
	D11S4109	86	0.85
	D11S4174	75	0.71
	D11S2016	72	0.68
TNNT2	D1S477	60	0.57
	D1S1723	79	0.79
	D1S2716	66	0.60
	D1S2615	75	0.72
TNNI3	D19S418	74	0.71
	D19S891	84	0.82
	D19S926	72	0.68
	D19S887	73	0.70
TPM1	D15S987	77	0.73
	D15S993	81	0.78
	D15S974	86	0.85
	D15S1020	84	0.81
MYL2	D12S84	84	0.82
	D12S1646	80	0.77
	D12S1342	83	0.81
	D12S354	73	0.68

Table 4: Frequency of combined markers heterozygosity in the panel of markers used around each sarcomere gene

	Proportion of individuals showing combined markers heterozygosity in markers panel related to each gene					
	MYH7	MYBPC3	TNNT2	TNNI3	TPM1	MYL2
4-marker heterozygosity	38	32	20	30	32	28
3-marker heterozygosity	36	40	54	44	44	43
2-marker heterozygosity	20	26	12	18	18	18
1-marker heterozygosity	2	2	12	8	4	11
No heterozygote marker	4	0	2	0	2	0