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Analysis of gene mutations encoding sarcomeric proteins in sudden death cases caused by cardiomyopathy

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Objective: Comprehensive screening for 7 genes encoding sarcomeric proteins were performed in consented autopsy cases diagnosed as cardiomyopathies (CMs), and the possibility of the genetic based diagnosis by screening for these genes was also examined.
Methods: Blood samples and left ventricular samples were obtained from 37 sudden cardiac death cases caused by CMs and 200 blood samples were used as controls. Primers covering all coding exons were designed. DNA samples were amplified by polymerase chain reaction (PCR), and PCR products were sequenced by direct sequencing with the ABI 3130 genetic analyzer.
Results: Twenty-two mutations were detected in only the CM cases, including 4 new mutations. Forty-eight single nucleotide polymorphisms (SNPs) were also detected. Significant differences in allele frequencies between hypertrophic cardiomyopathy (HCM) and controls, and dilated cardiomyopathy (DCM) and controls were found in c.68-5delC and c.348 C>T in *TNNT2*, and c.25-8T>A in *TNNI3* and c.1927+89C>G in *MYBPC3*, respectively. It was indicated that at least 3 SNPs (c.68-5delC, c.348C>T and c.25-8T>A) and 2 SNPs (c.348C>T and c.1927+89C>G) were important to genetic based diagnosis of HCM and DCM, respectively.
Conclusions: Our data clearly suggested that genetic analysis of disease causing genes encoding sarcomeric proteins was useful to decide the diagnosis of CMs for forensic autopsy cases and to help prevent further deaths caused by CMs in their families.

Key words: hypertrophic cardiomyopathy, dilated cardiomyopathy, gene mutations, sarcomeric proteins, SNPs, single nucleotide polymorphisms

Introduction

Sudden cardiac death is one of the most common causes of death in developed countries, appearing as a major health problem.[1] The most frequent internal cause of sudden death in Japan, approximately 50% of deaths, is ischemic heart disease, with various forms of cardiomyopathies (CMs) being the second most common cause.[2] CMs are defined as diseases of the myocardium associated with cardiac dysfunction and can be complicated by heart failure, arrhythmias, and sudden death. The World Health Organization (WHO)/ International Society and Federation of Cardiology (ISFC) task force recommended that CMs be classified into two main groups, primary and specific. Primary CMs are diseases intrinsic to the myocardium itself and are classified into: dilated CM (DCM), hypertrophic CM (HCM), restrictive CM (RCM), arrhythmogenic right ventricular CM (ARVC), and unclassified CM. Specific CM is now used to describe myocardial diseases that are associated with specific cardiac or systemic disorders.[3]

DCM is characterized by cardiac dilatation and contractile dysfunction of the left and/or right ventricles. DCM produces a prominent increase in chamber volumes, showing systolic dysfunction, often leading to heart failure and later requiring cardiac transplantation. DCM can be caused from a diverse variety of etiologies that leads to cardiomyocyte dysfunction or injury. It is assumed that approximately 20%－50% of DCMs have a genetic basis.[4]

HCM is a myocardial disease defined by an unexplained left and/or right ventricular septum. About two-thirds of individuals with HCM have asymmetric septal hypertrophy. Twenty-five percent of patients also

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show a left ventricular outflow tract obstruction, which corresponds to the gravity of clinical manifestation and a worse prognosis. HCM is a clinically heterogeneous but relatively common autosomal dominant genetic disease. It is the most prevalent genetic cardiovascular disease, and more importantly is the most common cause of sudden cardiac death in the young, including trained athletes. To date, familial HCM is known to be caused by mutations in 1 of 23 genes encoding different proteins, most of them sarcomeric proteins. At least 18 genes encoding various sarcomeric proteins contribute to the largest number resulting in the term "sarcomeropathy," a synonym for HCM.[4]

A subset of patients with HCM has been reported to progress into the dilated phase of HCM (D-HCM, end-stage phase of HCM), characterized by left ventricular systolic dysfunction and cavity dilatation; and it was confirmed previously that D-HCM patients had more atrial fibrillation, sustained ventricular tachycardia, and/or fibrillation, and poor prognoses.[5]

ARVC is characterized by progressive fibrofatty replacement of the right ventricular myocardium, initially with typical regional and later global right and some left ventricular involvement, with relative sparing of the septum. Presentation with arrhythmias and sudden death is common, particularly in young individuals and competitive athletes.[6]

In most of the cases, gene mutations leading to HCM are detected at the heterozygous state and transmitted as an autosomal dominant inheritance. Since the discovery of the first mutation in the β myosin heavy chain gene (*MYH7*) in a large multiplex family in 1990, a large number of mutations on different genes encoding sarcomeric proteins has been found. Screening for mutations in other sarcomere genes resulted in the identification of more than 450 disease-causing mutations in 11 genes encoding proteins contributing to the structure and/or function of the sarcomere: *MYH7*, cardiac troponin T gene (*TNNT2*), cardiac troponin I gene (*TNNI3*), cardiac troponin C gene (*TNNC1*), cardiac myosin-binding protein C gene (*MYBPC3*), regulatory myosin light chain gene (*MYL2*), essential myosin light chain gene (*MYL3*), α myosin heavy chain (*MYH6*), α cardiac actin (ACTC), α tropomyosin (*TPM1*), and the gint sarcomere protein titin (*TTN*).[4]

In this study, comprehensive screening of sarcomere constitution protein genes, *MYH7*, *TNNT2*, *TNNI3*, *TNNC1*, *MYBPC3*, *MYL2*, and *MYL3* were preformed in consented autopsy cases diagnosed as CMs, in order to evaluate the prevalence of gene mutations in sudden death caused by CMs. The possibility of the genetic based diagnosis with these sarcomere genes was also examined.

Materials and Methods

DNA samples

Blood samples and left ventricular samples were obtained from 37 sudden cardiac death cases caused by CMs, 19 cases of DCM, 15 cases of HCM, and 3 cases of ARVC, in Kanagawa prefecture with informed consent of their family members. Profiles of these cases are shown Table 1. For the diagnosis of CMs, we referred to the American Heart Association Scientific Statement and the European Society of Cardiology Report.[7,8]

HCM was defined by the presence of ventricular hypertrophy with increased wall thickness accompanied by myofibrillar disarrays and interstitial fibrosis. DCM was defined by the presence of left ventricular dilatation, left ventricular systolic dysfunction and/or the clinical histories attributable to DCM shown in Table 1. ARVC is characterized by progressive fibrofatty replacement of the right ventricular myocardium, initially with typical regional and later global right and some left ventricular involvement, with relative sparing of the septum. Two hundred preserved samples that presented with no particular diseases were used as controls. DNA was extracted using QuickGene-800 (FUJIFILM, Tokyo) and stored at 4℃ until use.

Polymerase chain reaction (PCR) and electrophoresis conditions

Based on the sequences, which were already reported in NCBI, primers including exon were designed for *MYH7*, *TNNT2*, *TNNI3*, *TNNC1*, *MYBPC3*, *MYL2*, and *MYL3*.

DNA samples were amplified by polymerase chain reaction (PCR) as follows. Amplification was carried out using 1.0 μl of 5 to 10 ng DNA as a template and adding 5.0 μl of AmpliTaq Gold 360 Master Mix (Applied Biosystems; CA, USA), 1.0 μl of 10 μM forward Primer, 1.0 μl of 10 μM reverse Primer, and adjusting the total volume to 10.0 μl with sterile deionized water. The Veriti Thermal Cycler (Applied Biosystems) was used with an intial step of 95℃ for 10 minutes, then 35 cycles of denaturation at 95℃ for 30 seconds, annealing at 58－65℃ for 30 seconds, and extension at 72℃ for 1 minute, with a final step of 72℃ for 7 minutes. PCR products were sequenced by direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification using the BigDye XTerminator Purification Kit (Applied Biosystems), PCR products were electrophoresed using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Sequencing data were analyzed using SeqScape Software Ver.2.5.0 (Applied Biosystems). The resulting sequence data were compared with the reference sequence available on the NCBI database (*MYH7*: NM_000257.2, *TNNT2*: NM_000364.2, *TNNI3*: NM_000363.4, *TNNC1*: NM_003280.2, *MYBPC3*: NM_000256.3, *MYL2*: NM_000432.3, and *MYL3*: NM_000258.2).

This study has obtained the examination and recognition of Kitasato University School of Medicine and Hospital Ethics Committee (B 01-24).

Results

In this study, mutation analysis of *MYH7*, *TNNI3*, *TNNT2*, *TNNC1*, *MYBPC3*, *MYL2*, and *MYL3* were performed in 37 consented autopsy cases diagnosed as CMs and 200 control samples. A total of 22 mutations were detected in only the CM cases, and 48 single nucleotide

Table 1. Profiles of 37 sudden death cases diagnosed as cardiomyopathies

No.	Sex	Age (yrs) at death	Confirmed family history	Age (yrs) at first presentation	Situation	Clinical history
D01	F	43	No	23	na	DCM, CD, renal failure
D02	M	61	No	56	Bathing	DCM
D03	F	43	No	40	na	DCM
D04	M	73	No	49	Sleeping	DCM
D05	F	31	No	25	Exercising	DCM
D06	M	76	No	55	na	DCM
D07	M	58	No	48	Shopping	DCM
D08	M	59	No	54	Sleeping	DCM
D09	M	32	No	na	Exercising	Arrhythmia
D10	M	54	No	50	Working	DCM
D11	M	57	No	47	Sleeping	DCM
D12	M	55	No	52	na	DCM, DM, renal failure
D13	F	88	No	na	na	DM
D14	M	53	No	51	na	DCM
D15	M	69	No	59	Sleeping	DCM
D16	F	68	No	59	Sleeping	DCM
D17	F	77	No	69	Sleeping	DCM
D18	M	68	No	66	na	DCM
D19	M	58	No	na	Sleeping	DCM
H01	M	53	No	na	Working	na
H02	F	36	No	na	na	na
H03	F	58	No	na	Exercising	HT, hyperlipemia
H04	M	49	No	na	Sleeping	HT
H05	M	44	No	na	Sleeping	HT
H06	M	64	No	49	na	na
H07	M	55	No	na	Drinking	na
H08	F	49	No	na	Working	Af
H09	F	45	No	na	na	HCM, brain infarction, DM
H10	M	26	Yes	25	na	HCM
H11	M	51	No	31	Sleeping	HCM
H12	M	63	No	56	na	HCM
H13	M	60	No	na	Exercising	HT
H14	M	73	No	63	Sleeping	HCM
H15	M	53	No	51	Sleeping	HCM
A01	M	33	No	30	Working	Arrhythmia
A02	M	44	No	na	na	DM, schizophenia
A03	F	62	No	52	na	na

D, DCM; H, HCM; A, arrhythmogenic right ventricular CM; CD, collagen disease; DM, diabetes mellitus; Af, atrial fibrillation; HT, hypertension; na, not available

Table 2. Mutations located in the 7 genes detected in only the CM cases

Case	MYH7	TNNT2	TNNI3	TNNC1	MYBPC3	MYL2	MYL3
D01	c.639+31C>T				c.2671C>T (p.Arg891Trp)		
D02	c.732C>T (p.Phe244=) c.3337-3_-2insC				c.1777delT (p.Ser593fs:1)		
D03			c.109-17C>A				
D04			c.46C>A (p.Pro16Thr)				
D05	c.3853+27T>A						
D08	c.4239G>A (p.Ser1413=)	c.600+14A>T					
D10	c.732C>T (p.Phe244=) c.3337-3_-2insC c.3853+27T>A						
D11					c.3137C>T (p.Thr1046Met)		
D14	c.5793C>T (p.Gly1931=)						
D15	c.3337-3_-2insC c.3853+27T>A				c.706A>G (p.Ser236Gly)		
D16				c.24+9C>T			
H04						c.456C>T (p.Tyr152=)	
H06					c.75C>T (p.Ser25=) c.25+55T>C		
H07					c.3137C>T (p.Thr1046Met)		
H08		c.842+61A>G c.873C>T (p.Thr291=)					
H10							c.170C>G (p.Ala57Gly)
H11					c.1777delT (p.Ser593fs:1)		
H12					c.3218G>C (p.Arg1073Pro)		
H13					c.1777delT (p.Ser593fs:1)		
H14	c.732C>T (p.Phe244=) c.3853+27T>A						
A03					c.292+55C>T		

Table 3. SNPs located in the 7 genes detected in this study

Gene	Observed variants	Effect on protein
MYH7	1. c.-8-25G>T	
	2. c.189C>T	p.Thr63=
	3. c.895+17G>A	
	4. c.2163-56A>G	
	5. c.2967T>C	p.Ile989=
	6. c.3973-30A>G	
	7. c.5655+21C>T	
	8. c.*113G>A	
TNNT2	1. c.42-58G>A	
	2. c.53-11_53-7delCTTCT	
	3. c.68-71C>T	
	4. c.68-70G>A/T	
	5. c.68-5delC	
	6. c.97+48A>G	
	7. c.163+120T>C	
	8. c.164-50G>A	
	9. c.348C>T	p.Ile116=
	10. c.489+57G>C	
	11. c.601-404A>C	
	12. c.758A>G	p.Lys260Arg
	13. c.801+41C>G	
	14. c.802-122C>G	
	15. c.802-33C>T	
TNNI3	1. c.25-8T>A	
TNNC1	1. c.-171G>A	
MYBPC3	1. c.292+41G>C	
	2. c.506-12delC	
	3. c.1091-24C>T	
	4. c.1927+89C>G	
	5. c.2737+12C>T	
	6. c.3628-90G>A	
	7. c.3288G>A	p.Glu1096=
MYL2	1. c.132T>C	p.Ile44=
	2. c.274+20_274+21insGT	
	3. c.274+53G>A	
	4. c.275-58G>A	
	5. c.353+20delG	
	6. c.353+46_353+47insC	
	7. c.169+198delT	
	8. c.169+671T>G	
	9. c.170-384T>C	
	10. c.274+391T>G	
	11. c.275-311A>C	
MYL3	1. c.69C>T	p.Pro23=
	2. c.129+44G>T	
	3. c.307+37A>C	
	4. c.482-100C>T	
	5. c.*89G>A	

polymorphisms (SNPs) were also detected (Tables 2,3).

Mutations detected in only the CM cases

MYH7: Six mutations, including 3 silent mutations, c.732C>T (p.Phe244=), c.4239G>A (p.Ser1413=), and c.5793C>T (p.Gly1931=), 2 single base substitutions (c.639+31C>T and c.3853+27T>A), and 1 insertion mutation (c.3337-3_-2insC), were detected in this study. Three mutations detected in DCM cases, c.639+31 C>T, c.4239G>A (p.Ser1413=), and c.5793C>T (p.Gly1931=), were unique.

TNNT2, *TNNI3*, and *TNNC1*: Six mutations, c.46C>T (p.Pro16Thr) and c.109-17C>A in *TNNI3*, c.873C>T (p.Thr291=), c.600+14A>T, c.842+61A>G in *TNNT2*, and c.24+9C>T in *TNNC1*, were detected in 3 genes encoding troponin complex. These were unique. c.600+14A>T and c.842+61A>G in *TNNT2*, c.46C>T (p.Pro16Thr) in *TNNI3*, and c.24+9C>T in *TNNC1* were new mutations detected in the present study.

MYBPC3: Eight mutations, including 4 missense c.706A>G (p.Ser236Gly), c.2671C>T (p.Arg891Trp), c.3137C>T (p.Thr1046Met), and c.3218G>C (p.Arg1073Pro), 1 silent c.75C>T (p.Ser25=), 1 frameshift c.1777delT (p.Ser593fs:1), and 2 single base substitutions c.25+55T>C and c.292+55C>T, were detected in this study.

MYL2 and *MYL3*: Two mutations, c.170C>G (p.Ala57Gly) in *MYL3* and c.456C>T (p.Tyr152=) in *MYL2*, were identified. The mutation c.170C>G (p.Ala57Gly) in *MYL3* was unique.

SNPs detected

Forty-eight SNPs were detected in the present study (Table 3). The correlation between CMs except 3 rare ARVC cases and control cases were analyzed. Allele frequencies of these SNPs and odds ratio among CMs and control cases were calculated. No significant differences between CM and control cases were observed in 44 SNPs except c.68-5delC and c.348C>T (p.Ile116=) in *TNNT2*, c.25-8T>A in *TNNI3*, and c.1927+89C>G in *MYBPC3* (data not shown). Significant differences in allele frequencies between HCM and control cases, and DCM and control cases, were found in the c.68-5delC in *TNNT2* (P = 0.010*) and the c.348C>T (p.Ile116=) in *TNNT2* (P = 0.026*), and the c.25-8T>A in *TNNI3* (P = 0.006*) and the c.1927+89C>G in *MYBPC3* (P = 0.02*), respectively. Correlation between mutant types and HCM or DCM cases were confirmed.

Discussion

Mutations detected in only the CM cases

MYH7: *MYH7* is 23 kb gene located on chromosome 14q11.2-q13, consists of 40 exons, 38 of which are coding exons, and encodes a protein of 1935 amino acids. The globular amino-terminal part corresponds to the motor domain that contains the ATP (adenosine-5'-triphosphate) binding site and the actin binding site.

All of the amino acid alterations detected in *MYH7* were silent mutations. The c.732C>T (p.Phe244=), c.4239G>A (p.Ser1413=), c.5793C>T (p.Gly1931=) were detected in only the CM cases. Silent mutations not leading to amino acid changes are generally considered to be normal variants and are thought to have no role in diseases.[9] However, a few studies described exonic silent mutations able to induce exon skipping in the genes associated with the corresponding diseases.[10,11] And recent reports have also provided experimental evidence that silent mutation can lead to changes in mRNA stability, protein folding, and function.[12,13] Further study is warranted to determine the extent to which silent mutations affect protein function and to elucidate the underlying mechanisms. The notion that these changes are silent, neutral, or unimportant is clearly too simplistic.

TNNT2, *TNNI3*, and *TNNC1*: The thin filament associated regulatory proteins, troponin and tropomyosin subunits, are responsible for regulating the contractile force of the cardiac myofibrils.[14] Troponin is a complex of three different proteins, troponin C (Ca^{2+}-binding component), troponin I (inhibitory component), and troponin T (tropomyosin-binding component). Troponin I has been shown to be in relative proximity to actin in the relaxed state, and an increase in free intracellular Ca^{2+} and binding of Ca^{2+} with troponin C triggers the contraction mechanism.[15]

New missense mutation p.Pro16Thr in *TNNI3* detected in a DCM case was located in the N-terminal region. This region contains serine residues that are phosphorylated by PKA. It was indicated that the phosphorylation of *TNNI3* may have a unique and important regulatory role in controlling cardiac function. Alterations of cardiac troponin I phosphorylation levels are important in the development of CM. Threonine residue is also phosphorylated by regulatory kinases. The phosphorylation of this mutation may be an important factor in the transition of a failing heart to a dilated phenotype.

The splice mutations, c.600+14A>T in *TNNT2*, c.109-17C>A in *TNNI3*, and c.24+9C>T in *TNNC1*, were detected in the DCM cases. The introns have a functional role in transcription efficiency, activity, and regulating gene expression.[16,17] These splice mutations may be an important factor in the development of CM.

A silent mutation (p.Thr291= in *TNNT2*) and a splice mutation (c.842+61A>G in *TNNT2*) were detected in an HCM case. It was reported that patients with double mutations generally exhibit a more severe form of HCM than do patients with single gene defects. This is especially true for homozygous patients.[18,19]

MYBPC3: *MYBPC3* is one of the genes that account for most known mutations and is located on chromosome 11p11.2 consisting of 35 exons. Myosin binding protein C constructs the normal form of the thick filament by binding to myosin and plays a role in regulating cardiac contractility by phosphorylation.[20-24]

The missense mutation c.2671C>T (p.Arg891Trp) in *MYBPC3* was accompanied with a change of the charge of the altered amino acid. The missense mutation with change of the charge of the altered amino acid detected in *MYH7* has a clinically poor prognosis to cause structural change of the protein.[25-29] On the other hand, mutations in *MYBPC3* can lead to a smaller degree of hypertrophy, often developing later in life.[30,31] However, in the CM case that has the missense mutation with change of the charge of the altered amino acid detected in this study, age at sudden death was 43 years old for a DCM case. Therefore, the CM cases that have the *MYBPC3* missense mutation with change of the charge of the altered amino acid also had a clinically poor prognosis. The p.Arg891Trp mutation was located in the fibronectin type III region. The p.Ser593fs:1, p.Thr1046Met, and p.Arg1073Pro mutations were located in the immunoglobulin region. Furthermore, p.Thr1046Met and p.Arg1073Pro occurred on the site binding to titin and myosin. These results indicated that sarcomere caused structural changes by these mutations, and therefore CM developed.

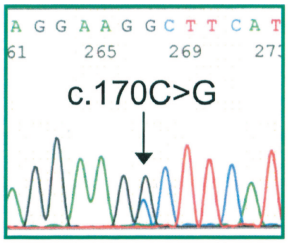
HCM is an autosomal dominant disease and molecular gengetic studies of familial HCM have demonstrated that about half of the patients have mutations in the genes encoding sarcomeric proteins. Three genes, *MYH7*, *TNNT2*, and *MYBPC3*, account for most of the known mutations. However, the pathogenesis of inherited forms of DCM is not well defined. The frequencies of the causative gene abnormalities of *MYBPC3* in familial HCM and DCM were calculated as 40% and 21%, respectively. Our results suggested that genetic analysis of *MYBPC3* was useful to decide the diagnosis of CM for forensic autopsy cases.

MYL2 and MYL3

The regulatory myosin light chain (RLC) and the essencial

myosin light chain (ELC) were described to be associated with the myosin heavy chain, which contains specific binding sites to them. Functionally, they are thought to modulate and regulate the actin-myosin interaction. Two distinct genes, *MYL2* and *MYL3* encode RLC and ELC, respectively.

MYL2 and *MYL3* are located on chromosomes 12q23-q24.3 and 3p21.2-p21.3, consisting of 7 exons and 6 exons, respectively, and encode polypeptides of 166 amino acids and 195 amino acids, respectively.[32] Six putative functional domains have been characterized: an actin binding site, a proline-rich region, and 4 EF-hand domains. The protein belongs to the superfamily of EF-hand domains, which includes calmodulin and troponin C.



Amino acid sequence

Homo sapiens	E	E	F	K	E	A	F	M	L	F	D
Rattus	E	E	F	K	E	A	F	Q	L	F	D
Mus musculus	E	E	F	K	E	A	F	L	L	F	D
Pongo abelii	E	E	F	K	E	A	F	M	L	F	D
Bos taurus	E	E	F	K	E	A	F	T	L	F	D

Figure 1. Sequence analysis of the missense mutation c.170C>G (p.Ala57Gly) located in highly conserved domains of gene coding for *MYL3* detected in HCM cases

c.170C>G (Ala57Gly) in *MYL3* was detected in the HCM case of a 26-year-old man (H10 shown in Table 1). He was diagnosed as having HCM at 25 years old. An autopsy was performed, and the HCM diagnosis was confirmed. His brother also died of HCM.

The Ala57 amino acid was located in highly conserved domains across species[33,34] (Figure 1), and it was located in the EF-hand domain of the calcium binding site, which was important in the structure or function of proteins in the cardiac sarcomere. ELC plays an important role in the regulation of the contractile cardiac system, therefore, the decreasing force generation was a primary mechanism for the pathogenesis of HCM associated with c.170C>G (Ala57Gly). Also, there were no mutations in other sarcomeric genes investigated in this study. In the present study, 22 mutations were detected in only the CM cases. The phenotype in inherited CM is probably a complex interplay of both genetic and environmental factors. The role of genetic factors in modifying a disease phenotype, increasing susceptibility to secondary causes of disease, and influencing responses to therapy, needs to be studied further.[4] Further studies are also warranted to determine the functional role of these mutations.

SNPs

Significant differences in allele frequencies between HCM and control cases, and DCM and control cases, were found in 4 SNPs c.68-5delC and c.348C>T (p.Ile116=) in *TNNT2*, and c.25-8T>A in *TNNI3* and c.1927+89C>G in *MYBPC3*, respectively. Correlation between mutant types and HCM or DCM cases were

Table 4. Distributions of groups and their frequencies in cardiomyopathies and controls

	Groups											
	A*	B*	D	E	G	I	J	K*	M	N	O	P
All CM	3 (21.4)	1 (50.0)	1 (25.0)	17 (14.7)	0 (0)	0 (0)	1 (3.7)	11 (19.6)	0 (0)	0 (0)	2 (13.3)	0 (0)
DCM	0 (0)	0 (0)	0 (0)	8 (6.9)	0 (0)	0 (0)	1 (3.7)	8 (14.3)	0 (0)	0 (0)	1 (6.7)	0 (0)
HCM	3 (21.4)	1 (50.0)	1 (25.0)	7 (8.0)	0 (0)	0 (0)	0 (0)	3 (5.4)	0 (0)	0 (0)	0 (0)	0 (0)
ARVC	0 (0)	0 (0)	0 (0)	2 (1.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.7)	0 (0)
Control	8 (57.2)	0 (0)	2 (50.0)	82 (70.7)	3 (100)	8 (100)	25 (92.6)	34 (60.7)	4 (100)	8 (100)	11 (73.3)	6 (100)

Parentheses indicate %.

①, c.68-5delC; ②, c.25-8 T>A; ③, c.348 C>T; ④, c.1927+89 C>G

A: ①, ②, ③, ④. B: ①, ②, ③. C: ①, ②, ④. D: ①, ③, ④. E: ②, ③, ④. F: ①, ②. G: ①, ③. H: ①, ④. I: ②, ③. J: ②, ④. K: ③, ④. L: ①. M: ②. N: ③. O: ④. P: no mutation. C, F, H, L: no observed groups were omitted.

*P = 0.008; OR = 0.17, 95% CI = 0.04-0.75. A(HCM) vs. other groups.

*P = 0.0003; B(HCM) vs. other groups.

*P = 0.007; OR = 0.30, 95% CI = 0.11-0.80. K(DCM) vs. other groups.

confirmed. The silent mutation c.348C>T (p.Ile116=) in *TNNT2* detected in HCM is a region involved in binding to the tropomyosin. Introns have a functional role in transcription efficiency, activity, and regulating genetic expression.

It was examined whether screening of these 4 SNPs could provide for genetic based diagnosis of CM. The association between these 4 SNPs in the CM cases and controls were analyzed. Thirty-seven cases diagnosed as CM and 191 controls were classified into 16 groups indicated in Table 4. Distribution of these frequencies was also indicated. Our data clearly revealed that no significant differences between these groups of CM cases and controls were observed excepted groups A and B in HCM cases, and group K in DCM cases. These groups were significantly more frequent in HCM and DCM cases compared to those in controls, respectively (A, P = 0.008; B, P = 0.0003 in HCM; K, P = 0.007 in DCM). Groups A, B, and K were characterized by 4 SNPs (c.68-5delC, c.348C>T (p.Ile116=), c.25-8T>A and c.1927+89C>G), 3SNPs (c.68-5delC, c.348C>T (p.Ile116=), c.25-8T>A) and 2 SNPs (c.348C>T (p.Ile116=) and c.1927+89C>G), respectively. These results suggested that at least 3 SNPs (c.68-5delC and c.348C>T (p.Ile116=) in *TNNT2* and c.25-8T>A in *TNNI3*) and 2 SNPs (c.348C>T (p.Ile116=) in *TNNT2* and c.1927+89C>G in *MYBPC3*) were important to the genetic based diagnoses of HCM and DCM, respectively.

In most cases of sudden death, cause and manner of death can be established, with many attributable to cardiac abnormalities evident at autopsy. A significant number of sudden cardiac deaths, however, particularly in young people, remains unexplained following a comprehensive medicolegal investigation, including autopsy and laboratory tests. The most important unsolved challenge in the practice of forensic pathology is the failure to determine a cause of death, particularly in a previously healthy young person who has died suddenly and unexpectedly.[1] Diagnosis of the genetic causes of sudden death is important because close relatives are also at potential risk of having a fatal cardiac condition. A comprehensive postmortem investigation is vital to determine the cause of death and provides the opportunity to assess the potential risk to the family after appropriate genetic counseling. Effective evaluation of relatives, guided by genetic testing, can therefore prevent further deaths in the family.

We performed molecular screening of *MYH7*, *TNNT2*, *TNNI3*, *TNNC1*, *MYBPC3*, *MYL2*, and *MYL3* in consented autopsy cases diagnosed as CM. Understanding the pathogenesis of CM could provide for genetic based diagnoses, risk stratification, treatment, and prevention of cardiac phenotypes. It would be desirable to survey other disease-causing genes to determine a more accurate prevalence of gene mutations in sudden death caused by CM in Japan.

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References

1. Rodriguez-Calvo MS, Brion M, Allegue C, et al. Molecular genetics of sudden cardiac death. *Forensic Sci Int* 2008; 182: 1-12.
2. Tokyo Medical Examiner's Office Annual Report 2012. Available at: http://www.fukushihoken.metro.tokyo.jp/kansatsu/database/24toukeihyou-toukeizu.html. Accessed 1 September, 2013.
3. Richardson P, McKenna W, Bristow M, et al. Report of the 1995 World Health Organization/International Society and Federation of Cardiology task force on the Definition and Classification of cardiomyopathies. *Circulation* 1996; 93: 841-2.
4. Friedrich FW, Carrier L. Genetics of hypertrophic and dilated cardiomyopathy. *Curr Pharm Biotechnol* 2012; 13: 2467-76.
5. McKenna WJ, Thiene G, Nava A, et al. Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Task Force of the Working Grop Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopaathies of the International society and Federation of Cardiology. *Br Heart J* 1994; 71: 215-8.
6. Hamada T, Kubo T, Kitaoka H, et al. Clinical features of the dilated phase of hypertrophic cardiomyopathy in comparison with those of dilated cardiomyopathy. *Clin Cardiol* 2010; 33: E24-8.
7. Maron BJ, Towbin JA, Thiene G, et al. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. *Circulation* 2006; 113: 1807-16.

8. Elliott P, Andersson B, Arbustini E, et al. Classification of the cardiomyopathies: a position statement from the European Society Of Cardiology Working Group on Myocardial and Pericardial Diseases. *Eur Heart J* 2008; 29: 270-6.
9. Kimchi-Sarfaty C, Oh JM, Kim IW, et al. A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science* 2007; 315: 525-8.
10. Chao HK, Hsiao KJ, Su TS. A silent mutation induces exon skipping in the phenylalanine hydroxylase gene in phenylketonuria. *Hum Genet* 2001; 108: 14-9.
11. Liu W, Qian C, Francke U. Silent mutation induces exon skipping of fibrillin-1 gene in Marfan syndrome. *Nat Genet* 1997; 16: 328-9.
12. Montera M, Piaggio F, Marchese C, et al. A silent mutation in exon 14 of the APC gene is associated with exon skipping in a FAP family. *J Med Genet* 2001; 38: 863-7.
13. Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV, et al. The sounds of silence: synonymous mutations affect function. *Pharmacogenomics* 2007; 8: 527-32.
14. Fatkin D, Graham RM. Molecular mechanisms of inherited cardiomyopathies. *Physiol Rev* 2002; 82: 945-80.
15. Takeda S, Yamashita A, Maeda K, et al. Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form. *Nature* 2003; 424: 35-41.
16. Robberson BL, Cote GJ, Berget SM. Exon definition may facilitate splice site selection in RNAs with multiple exons. *Mol Cell Biol* 1990; 10: 84-94.
17. Sterner DA, Berget SM. In vivo recognition of a vertebrate mini-exon as an exon-intron-exon unit. *Mol Cell Biol* 1993; 13: 2677-87.
18. Richard P, Charron P, Carrier L, et al. Hypertrophic cardiomyopathy: distribution of disease genes, spectrum of mutations, and implications for a molecular diagnosis strategy. *Circulation* 2003; 107: 2227-32.
19. Richard P, Isnard R, Carrier L, et al. Double hetrozygosity for mutations in the beta-myosin heavy chain and in the cardiac myosin binding protein C genes in a family with hypertrophic cardiomyopathy. *J Med Genet* 1999; 36: 542-5.
20. Flashman E, Redwood C, Moolman-Smook J, et al. Cardiac myosin binding protein C: its role in physiology and disease. *Circ Res* 2004; 94: 1279-89.
21. Winegrad S. Cardiac myosin binding protein C. *Circ Res* 1999; 84: 1117-26.
22. Carrier L, Bonne G, Bahrend E, et al. Organization and sequence of human cardiac myosin binding protein C gene (MYBPC3) and identification of mutations predicted to produce truncated proteins in familial hypertrophic cardiomyopathy. *Circ Res* 1997; 80: 427-34.
23. Oakley CE, Chamoun J, Brown JL, et al. Myosin binding protein-C: enigmatic regulator of cardiac contraction. *Int J Biochem Cell Biol* 2007; 39: 2161-6.
24. Bahrudin U, Morisaki H, Morisaki T, et al. Ubiquintin-proteasome system impairment caused by a missense cardiac myosin-binding protein C mutation and associated with cardiac dysfunction in hypertrophic cardiomyopathy. *J Mol Biol* 2008; 384: 896-907.
25. Watkins H, Rosenzweig A, Hwang DS, et al. Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic Cardiomyopathy. *N Engl J Med* 1992; 326: 1108-14.
26. Anan R, Greve G, Thierfelder L, et al. Pronostic implications of novel beta cardiac myosin heavy chain gene mutations that cause familial hypertrophic cardiomyopathy. *J Clin Invest* 1994; 93: 280-5.
27. Komamura K, Iwai N, Kokame K, et al. The role of a common TNNT2 polymorphism in cardiac hypertrophy. *J Hum Genet* 2004; 49: 129-33.
28. Konno T, Shimizu M, Ino H, et al. A novel missense mutation in the myosin binding protein-C gene is responsible for hypertrophic cardiomyopthy with left ventricular dysfunction and dilation in elderly patients. *J Am Coll Cardiol* 2003; 41: 781-6.
29. Konno T, Shimizu M, Ino H, et al. A novel mutation in the cardiac myosin-binding protein C gene is responsible for hypertrophic cardiomyopathy with severe ventricular hypertrophy and sudden death. *Clin Sci (Lond)* 2006; 110: 125-31.
30. Erdmann J, Raible J, Maki-Abadi J, et al. Spectrum of clinical phenotypes and gene variants in cardiac myosin-binding protein C mutation carriers with hypertrophic cardiomyopathy. *J Am Coll Cardiol* 2001; 38: 322-30.
31. Charron P, Dubourg O, Desnos M, et al. Clinical features and prognostic implications of familial hypertrophic cardiomyopathy related to the cardiac myosin-binding protein C gene. *Circulation* 1998; 97: 2230-6.
32. Fodor WL, Darras B, Seharaseyon J, et al. Human ventricular/slow twitch myosin alkali light chain gene: characterization, sequence, and chromosomal location. *J Biol Chem* 1989; 264: 2143-9.
33. Morano I. Tuning the human heart molecular motors by myosin light chains. *J Mol Med (Berl)* 1999; 77: 544-55.
34. Lee W, Hwang TH, Kimura A, et al. Different expressivity of a ventricular essential myosin light chain gene Ala57Gly mutation in familial hypertrophic cardiomyopathy. *Am Heart J* 2001; 141: 184-9.