A Novel Algorithm from Personal Genome to the Pathogenic Mutant Causing Mitochondrial Cardiomyopathy

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Abstract

Amazing progresses in both human genome analysis and bioinformatics *in silico* have made it possible to reach whole genome profiling in a short period with a reasonable cost and time. In this review, we have introduced the next step after reading the full genome sequence of both nuclear and mitochondrial genomes to identify the pathogenic site(s) in several cardiomyopathies. Considering ~3 million sites of single nucleotide polymorphism (SNP) per person, it is difficult to reach not a personal variant but a pathogenic site. The current algorithm might be promising for the identification of responsible gene, even in the case of polygenic nature.

Keywords

Electron microscopy • Genome • Heteroplasmy • Magnetic resonance spectroscopy • Mitochondrial cardiomyopathy • Mitochondriosis • Open reading frame (ORF) • Oxidative phosphorylation • Pathogenic mutant • Revised Cambridge resequencing system (rCRS) • Risk factor • transgene

Introduction

Human whole genome was reported just 10 years ago and the aim of coming decades is addressed to the clinical translation of personal genetic background of each patient, searching for the precise mechanism of pathogenicity, gene counseling, and/or tailored medicine to provide most suitable option for treatment [1]. For the assessment of genetic origin of heart failure and/or dilated cardiomyopathy (DCM), the mitochondrial (mt) genome represents one of the most informative and cost-effective researches, because of (1) the abundant rate of exons over introns, not like a nuclear genome, (2) short genome size to determine the whole DNA sequence [2] to profile the progression of various diseases [3], (3) repeating the beating throughout life with consuming and producing huge amount of adenosine triphosphate (ATP) in

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Table 1 Homology of mt-DNA sequence (8551–9300) to NUMT (Mitochondrial DNA-like sequences in the nucleus) of human genome. Also, note an accurate criticism raised by Yao et al. [39]

Sequences producing significant alignments

Accession	Description	E value	Max ident
NC 001807.4	Homo sapiens mitochondrion, complete genome	0	99%
NT 004350.19	Home sapiens chromosome 1 genomic contig, GRCh37 reference primary assembly	0	98%
NT 034772.6	Home sapiens chromosome 5 genomic contig, GRCh37 reference primary assembly	0	88%
NW 001838563.2	Homo sapiens chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence	0	98%
NT 022184.15	Homo sapiens chromosome 2 genomic contig, GRCh37 reference primary assembly	4.00E–91	96%
NT 167187.1	Homo sapiens chromosome 8 genomic contig, GRCh37 reference primary assembly	2.00E–55	85%
NW 001839126.2	Homo sapiens chromosome 8 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence	2.00E–55	85%
NW 923907.1	Homo sapiens chromosome 8 genomic contig, alternate assembly (based on Celera), whole genome shotgun sequence	2.00E–55	85%
NT 032977.9	Homo sapiens chromosome 1 genomic contig, GRCh37 reference primary assembly	2.00E–29	100%
NW 001838577.2	Homo sapiens chromosome 1 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence	2.00E–29	100%
NW 921351.1	Homo sapiens chromosome 1 genomic contig, alternate assembly (based on Celera), whole genome shotgun sequence	2.00E–29	100%
NT 007299.13	Homo sapiens chromosome 6 genomic contig, GRCh37 reference primary assembly	2.00E–24	86%
NW 001838987.1	Homo sapiens chromosome 6 genomic contig, alternate assembly (based on HuRef), whole genome shotgun sequence	2.00E–24	86%
NW 923184.1	Homo sapiens chromosome 6 genomic contig, alternate assembly (based on Celera), whole genome shotgun sequence	2.00E–24	86%

their own cells, and (4) continuous exposure to reactive oxygen species (ROS) produced in the oxidative phosphorylation with much fewer protective actions than nuclear genome.

The mt-genome includes abundant variants not related to the pathogenicity but reflecting the haplogroup or phylogeny to adopt extracellular environment [1]. Accordingly, mt-genome study is so meaningful and fascinating but it includes widespread problems, as follows: (1) ethical conflicts originated in a disclosure of patient's privacy [2], (2) methodological arguments to sample considerable amount of living human cardiomyocytes to evaluate the mutant's phenotype, (3) changes in the hetroplasmy rate during tissue culture, as is convenient for the analysis and the amplification [3], (4) environmental difference of cardiomyocytes in situ under mechanical and/or chemical stress(es) from cultured cells in vitro, and the resultant modification of the phenotype, (5) the case with no identical variant in rodent to prepare transgenic models [4], and (6) intrinsic problems to patents and licensing [5]. In this short review, we present a new scheme to overcome these dilemmas and to clarify the pathogenic mechanism of various mt-diseases, based on abundant sources of bioinformatics *in silico*. The homology of mt-DNA sequence with that of nuclear-DNA is shown in Table 1.

An Algorithm to Reach the Pathogenic Mutant in mtCM

Necessity for Full Sequencing of the mt-Genome

In the outpatient section of the Tokyo University Hospital, we have followed ~80 cases with hypertrophic cardiomyopathy (HCM) and DCM, of which diagnosis was based on clinical and laboratory data including morphological, physiological, biochemical, serological, and, most importantly, pathological characteristics of endomyocardial biopsy samples [6]. For the conventional measurement of gene survey, we have employed gene polymorphism using PCR (polymerase chain reaction)-amplified SSCP (single-stranded conformation polymorphism) or RFLP (restriction-fragment-length polymorphism, Fig. 1) for ~15 years. In the recent 5 years, we have shifted to more time-saving and accurate modality, sequence-specific primer cycle elongation-fluorescence correlation spectroscopy (SSPCE-FCS), as described previously [7]. As candidate genes, we have selected several variants popular in Japan [8] and detected three pedigrees with identical mutations [9].

The classical methods to utilize PCR-based gene amplification often cause misreading of not the responsible, but the pseudogene(s) located in the other site. Particularly, nuclear genes preserve an incredible amount of pseudogenes with the same sequence as the ancient mtDNA [NUMT, Ref. 10] in part, even when the original mtDNA has already altered adapting to a new environment (Fig. 2). Consequently, whole mt-genome sequencing is preferable over the classical methods and would be essential in future to avoid misdiagnosis.

New Modality to Read the Whole mt-DNA Sequence

The whole mt-DNA sequences of all three probands and 10 Japanese volunteer patients without CM or heart failure as an internal standard were determined with GeneChip® Human Mitochondrial Resequencing Array 2.0 [11, Toyo-oka et al., submitted]. The DNA sequences different from the

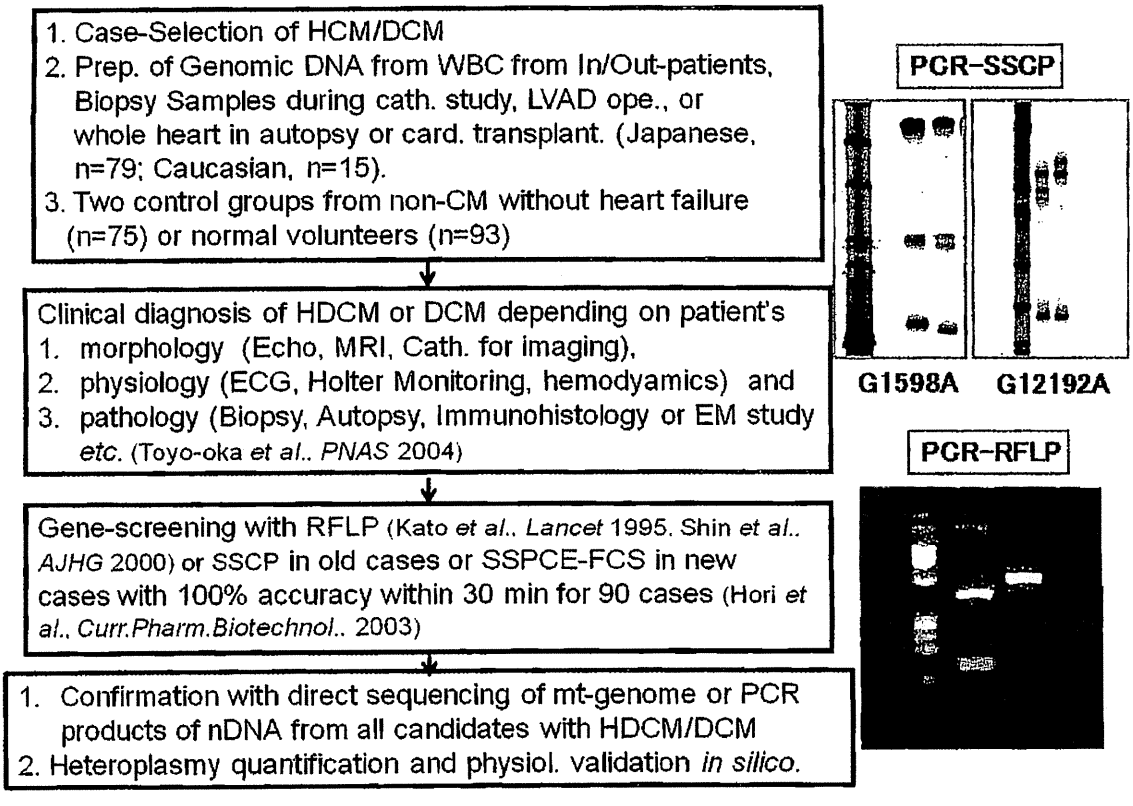

Fig. 1 Classical screening steps of wide variety of DCM/HCM in nuclear and mitochondrial genomes

a

tRNAT	species	15888 95	97	15901 7	10	11	16 23	28	32	37	40	46 53
Primates	Homo sapiens	GTCCTTG TA	GTAT	AAACTA	ATAC A	CCAGT	CTTGTAA	ACCGG AGAT	GAAAA	CCT	TTTTC	CAAGGACA
	Gorilla gorilla	GCCCTTG TA	GTAC	AGACCA	ATAC A	CCAGT	CTTGTAA	ACCGG AAAC	GAAGA	CCT	CCTTC	CAAGGGCA
	Pan troglodytes	GCCCTTG TA	GTAT	AAACTA	ATAC A	CCGGT	CTTGTAA	ACCGG AAAC	GAAAA	CTT	TCTTC	CAAGGACA
	Hylobates lar	GCCCTTG TA	GTAT	AAGCCA	ATAC A	CCGGT	CTTGTAA	GCCGG AACT	GAAAT	CTT	CCTTC	CAAGGACA
	Pongo pygmaeus	GCCCCTGTA	GTAC	AAATAA	GTAC G	CCAGC	CTTGTAA	CCTGA AAAT	GAAGC	CCC	CCTTC	CACGGGCA
	Papio hamadryas	GCCCTTG TA	GTAC	AAACTA	ATAC A	CTGGT	CTTGTAA	ACCAG AAAT	GGAGC	A	CCTCC	CCAGGGTA
	Bos taurus	GTCTTTG TA	GTAC	ATCTA	ATAT A	CTGGT	CTTGTAA	ACCAG AGAA	GGAGA	ACAACTAA	CCTCC	CTAAGACT
	Cebous albifrons	GTCCTTG TA	GTAT	ATCCAA	TTAC C	CCGGC	CTTGTAA	ACCGG AAAA	GGAGG	CACGGTA	ACTCC	CCAGGACA
	Lemur catta	GCCCTTG TA	GTAT	AACTTA	ATAC C	CTGGT	CTTGTAA	ACCAG ACAT	GGAGA	ACCCCCT	CCTCC	CAAGGACA
	Macaca mulatta	GCCCTCGTA	GTAT	AAATTA	GTAC A	CTGGC	CTTGTAA	ACCAG AAAT	GAACA	C	TCTTC	CTAGGGCA
	Tarsius bancanus	GTCCTCG TA	GTAT	AACCA	TTAC C	TTGGT	CTTGTAA	ACCAA AAAT	GAAGG	AACCCAA	CCTCC	CTAGGACC
Dermoptera	Cynocephalus variegatus	GTCCCTG TA	GTAT	AATAA	TTAC T	CTAGT	CTTGTAA	ACCAG AAAT	GGAGG	GAGCAC	CCTCC	CCAGGACA
Oryctero-podidae	Oryctolagus cuni	GTCCTTG TA	GTAT	AAACTA	TTAC C	ATGGT	CTTGTAA	ACCAT AAAT	GGATC	TAAC	CCTCC	CCAGGACA
Cetartio-dactyla	Balaenoptera acutorostrata	GTCTTTG TA	GTAT	AACTAA	TTAC C	CCGGT	CTTGTAA	ACCGG AAAA	GGAGA	GCGAACCACACCTCC		CTAAGACT

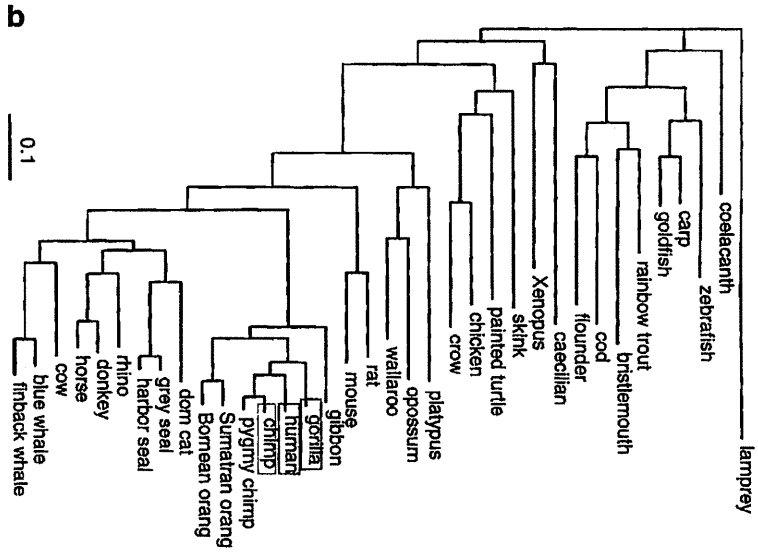

Fig. 2 (**a**) The maximum likelihood estimate of $tRNA^{Thr}$ in vertebrate phylogeny, focusing on primates. Red-, green-, and blue-colored nucleotides indicate a modifier gene of the current HDCM or DCM, several neurodegenerative disease, and modulator gene of LHON (Leber's hereditary optic neuropathy), respectively. (**b**) The estimation of mtDNA in vertebrate phylogeny (Cited from, Broughton et al. [40])

world-standard rCRS were confirmed with the classical dye-terminator method (Sanger sequencing) equipped with size separation in capillary using mitoSEQ™ Resequencing System for the Human Mitochondrial Genome.

Identification of Pathogenic Mutant in the ORF of the mt-Genome

For the evaluation of physiological significance of open reading frame (ORF), it would be reasonable to assume that the synonymous mutation has no or less meaning in the pathogenesis, except the modification of codon usage in nuclear or mitochondrial genome [12–14]. The mutation within ORF would directly cause the conformational change in the encoded protein (transgene) and accordingly result in the functional modification, if any. For the integration of all 13 polypeptides coded by the mt-gene into the inner membrane, most of mt-proteins abundantly include hydrophobic domains and are buried in the phospholipid bilayer. Among the mt-proteins, ATPase 6 is the most hydrophobic peptide and mutation of the current case from alanine to threonine

occurred in the midst of the hydrophobic rigid structure (Toyo-oka et al., submitted). Thus, it would be conceivable to assume that the present mutation causes a serious alteration in oxidative phosphorylation at the final step to synthesize ATP. Another mutation to cause NARP (neuropathy, ataxia, and retinitis pigmentosa) confirmed the scheme described above in the same *ATP6* gene [15].

The other mutations in ORF constitute the main source of the mitochondrial gene-related diseases and the predicted structure of the transgene, that is, LHON in *ND1*, *ND4*, or *ND6* [16–19] or KSS with the large 5 kb deletion spanning from *ATP8* to *ND5* of [20, 21] a wait a more fine analysis like an ionic charge of the constituent amino acids, modulation of helical structure, and intragenic suppressor action in LHON *ND1* gene [22]. For the functional prediction, the higher-ordered structure of the ND6 gene [23] or gene interference between nuclear and mt-genomes might be much more informative to estimate the mutant function (Toyo-oka et al., submitted).

Pathogenic Mutation in tRNA of the mt-Genome

The tRNA is another large source of mt-gene mutations, because tRNA is situated at the critical step of protein synthesis and the defect will cause a serious problem in the production rate of each component protein in mitochondria. Several mutations have been reported on MELAS 3,243 in $tRNA^{Leu(UUR)}$ [24, 25] and MERRF 8,344 in $tRNA^{Lys}$ [26, 27] or dilated cardiomyopathy (DCM) in $tRNA^{Thr}$ (Toyo-oka et al., submitted). McFarland et al. raised five criteria [28]:

1. ~Three-fourths of mutation sites in stem regions of the secondary structure
2. Pathogenic hot spots in both the acceptor and anticodon stems
3. Disruption of Watson–Crick base pairs
4. More common pathogenicity in C-G base pairing than A-T pairing secondary to the lower thermodynamic energy
5. Preferential pathogenicity in loop structure with unusual number of nucleotides that may affect the tertial structure. To these criteria, we add here the following three items more for pathogenicity:
6. Medical records describing the identical mutation in other mitochondrion-related diseases, especially in energy-consuming tissues, like neurodegenerative diseases in brain, inner ear, or retina; skeletal or cardiac muscles, like myopathy, HCM, or DCM; and endocrine organs, like diabetes mellitus with or without angiopathy
7. Conservation of the wild-type sequence in nonhuman primates, suggesting the biological significance
8. Pathological features of mitochondriosis in the electron microscopy of biopsy samples

When each criterion is precisely inspected, each item is not independent, but some overlap among these stratifications. Furthermore, each item may require scoring for the more exact prediction in future. Particularly, the morphological observation using fresh sample to avoid the postmortem degeneration is critical to proceed to an advanced step of an accurate diagnosis for the genetic diseases.

The endomyocardial biopsy samples provide several characteristic findings in mitochondria, involving accumulation of a huge number of bizarre-formed mitochondria, that is, mitochondriosis (Toyo-oka et al., submitted), concentric cristae [24], hypertrophic mitochondria within myocytes, and vessel walls with or without paracrystalline mitochondrial inclusions [25, 26].

Other Comments on Gene Analysis

The DNA sequence in rRNA is meaningful for exact and efficient protein synthesis but the clinical significance of the mutant is still obscure, except the rare case of A1555G mutation in 12S-rRNA with sensory hearing loss or DEAF gene [29, 30].

As the initial step of assignment of the pathogenic mutations coding a mitochondrial gene, the nuclear gene should be separately or independently examined not to be mixed with each other. Then the combination of two analyses would yield unexpected results that show double or sometimes triple mutation, and pathogenesis

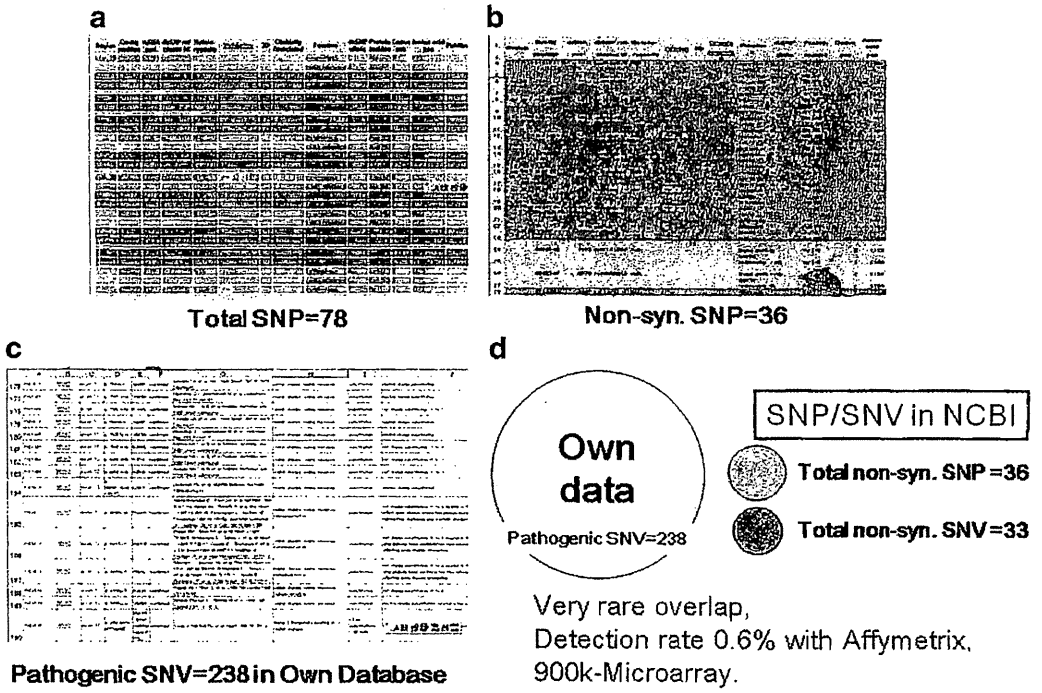

Fig. 3 Less agreement of the microarray commercially available now, comparing NIH SNP/SNV data to our handmade database of *MYH7* gene. Note that the overlap was very rare among these databases and that the detection rate between the two databases was 0.6% with Affymetrix, 900k-Microarray

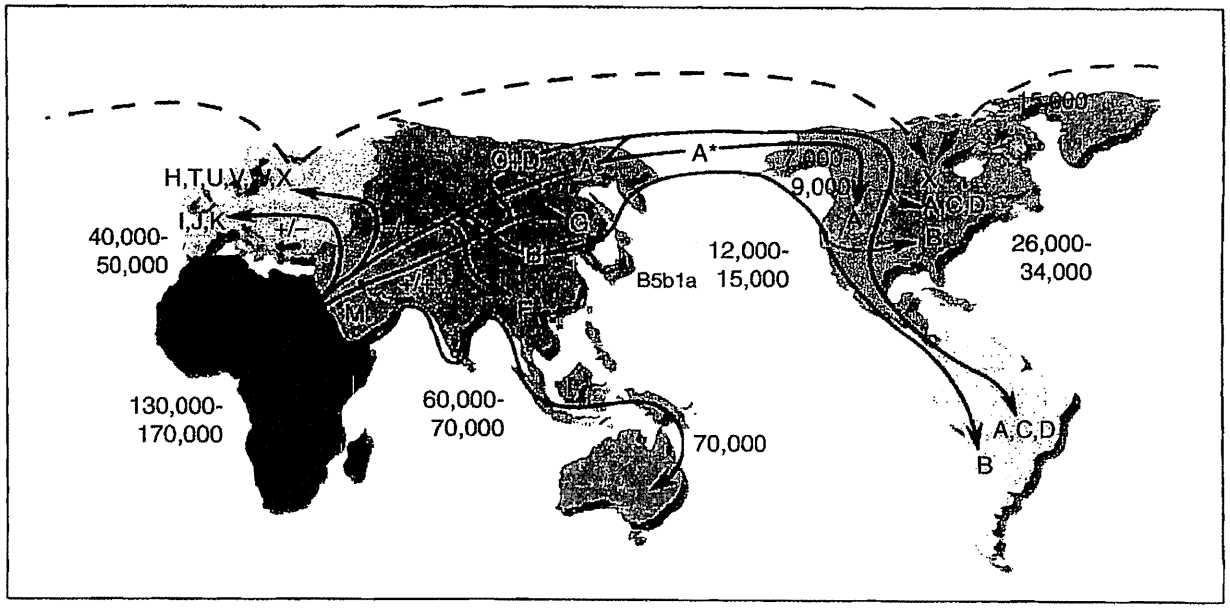

Fig. 4 Human mtDNA migrations (cited from MITOMAP: A human mitochondrial genome database. http://www.mitomap.org, 2009)

of the complex familial disease is clarified or the classical "penetrance" is explained by the multiple gene defect with the different time course (Toyo-oka et al., submitted). The SNP microarray commercially available now is still at the primitive stage to cover pathogenic mutants or variants near the responsible locus, even when a 900 k gene chip is used for the analysis (Fig. 3).

In addition, magnetic resonance spectroscopy (MRS), together with ^{1}H-magnetic resonance imaging (^{1}H-MRI), will be promising for the

elucidation of an actual effect of mutant(s) on cardiac function, especially to detect the energy metabolism with ^{31}P-MRS in the case with mtCM [31, 32], though both bore size and magnetic intensity of superconducting coils are insufficient for precise measurement of human hearts in vivo within a limited measuring time.

It is very meaningful to determine the belonging haplogroup as a risk factor. From the worldwide survey [32–37], we identified that the present mutation belonged to the B5b1a subhaplogroup (Toyo-oka et al., submitted). The same G15927A mutation in *tRNA^{Thr}* has been reported to modify the pathogenesis of other neurological diseases [38]. In addition, the current sequence was restricted to the Japanese and no similarity to Chinese or Koreans has been reported [9], which may imply that the new haplogroup has branched from East Asians after their ancestors left the Eurasian continent (Fig. 4).

Conclusions

The sequence of both nuclear and mitochondrial genomes has been identified in different cardiomyopathies. The full resequencing together with comprehensive gene analysis would clarify the controversial results obtained from PCR analysis using a partial amplification in mitochondrial gene mutation.

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ORIGINAL ARTICLE
Myocardial Disease

Prevalence and Distribution of Sarcomeric Gene Mutations in Japanese Patients With Familial Hypertrophic Cardiomyopathy

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Background: Hypertrophic cardiomyopathy (HCM), which is inherited as an autosomal dominant trait, is the most prevalent hereditary cardiac disease. Although there are several reports on the systematic screening of mutations in the disease-causing genes in European and American populations, only limited information is available for Asian populations, including Japanese.

Methods and Results: Genetic screening of disease-associated mutations in 8 genes for sarcomeric proteins, *MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1*, and *ACTC*, was performed by direct sequencing in 112 unrelated Japanese proband patients with familial HCM; 37 different mutations, including 13 novel ones in 5 genes, *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, and *TPM1*, were identified in 49 (43.8%) patients. Among them, 3 carried compound heterozygous mutations in *MYBPC3* or *TNNT2*. The frequency of patients carrying the *MYBPC3*, *MYH7*, and *TNNT2* mutations were 19.6%, 10.7%, and 8.9%, respectively, and the most frequently affected genes in the northeastern and southwestern parts of Japan were *MYBPC3* and *MYH7*, respectively. Several mutations were found in multiple unrelated proband patients, for which the geographic distribution suggested founder effects of the mutations.

Conclusions: This study demonstrated the frequency and distribution of mutations in a large cohort of familial HCM in Japan. (*Circ J* 2012; **76:** 453–461)

Key Words: Hypertrophic cardiomyopathy; Genes; Genetics

Hypertrophic cardiomyopathy (HCM) is a prevalent hereditary disease, affecting approximately 1 in 500 of the general population, and a major cause of sudden cardiac death (SCD) in the young, which is characterized by left ventricular (LV) hypertrophy accompanied by diastolic dysfunction and myofibrillar disarrays.[1–3] More than half of HCM patients have apparent family histories of the disease and/or SCD, which is consistent with the autosomal dominant genetic trait, suggesting that genetic abnormalities cause HCM.[1] The etiology of HCM, however, was unknown until

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Table 1. Distribution and Frequency of Disease-Associated Mutations in Patients With HCM

Gene	No. of mutations found in this study (novel mutations)	No. of proband patients with mutations in this study (novel mutations)	% Frequency of mutations in familial HCM patients in this study (n=112)	% Frequency of mutations in the French familial HCM cohort* (n=172)	% Frequency of mutations in the US HCM cohort** (n=389)†
MYH7	12 (2)	12 (2)	10.7	26.2	15.2
MYBPC3	13 (7)	22 (7)	19.6	26.2	18.0
MYL3	0 (0)	0 (0)	0.0	0.0	0.0
MYL2	0 (0)	0 (0)	0.0	0.6	1.8
ACTC	0 (0)	0 (0)	0.0	0.0	0.0
TNNT2	7 (1)	10 (1)	8.9	2.9	2.3
TNNI3	1 (1)	1 (1)	0.9	4.7	1.3
TPM1	4 (2)	4 (2)	3.6	0.0	0.5
Total	37 (13)	49 (13)	43.8	60.6	39.4

*_Circulation_ 2003; **107:** 2227–2232. **_J Am Coll Cardiol_ 2004; **44:** 1903–1910. †120 familial cases and 269 sporadic cases.
HCM, hypertrophic cardiomyopathy.

1990 when a mutation in *MYH7* encoding cardiac β-myosin heavy chain was identified in a multiplex family with HCM by a linkage study and subsequent candidate gene analysis.[4] After the discovery of the *MYH7* mutation in HCM, extensive efforts have been made, using linkage studies and candidate gene analyses, to identify the disease-causing mutations, and hundreds of mutations in several different genes have been reported in HCM.[5]

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The sarcomere is the contractile unit of cardiac and skeletal muscles, composed of highly organized proteins represented by thick and thin filaments, which plays a crucial role in force generation.[6] Numerous HCM-associated mutations have been identified in the genes for components of thick filaments such as cardiac β-myosin heavy chain (*MYH7*), cardiac myosin binding protein-C (*MYBPC3*), ventricular essential myosin light chain (*MYL3*), and ventricular regulatory myosin light chain (*MYL2*), as well as in the genes for the components of thin filaments, including cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*), cardiac troponin C (*TNNC1*), α-tropomyosin (*TPM1*), and cardiac actin (*ACTC*).[5] Familial or hereditary HCM caused by mutations in the genes for components of the sarcomere could be defined as "sarcomere HCM", but there are only a few reports on the systematic screening of sarcomeric gene mutations in a large panel of hereditary HCM, mainly in European and American populations.[7–10] Because the frequency and distribution of disease-causing mutations could vary among the different ethnic groups, it is important for genetic counseling to elucidate the HCM-associated mutations in each ethnic group.

In this study, we performed a systematic screening of mutations in 8 genes for the components of the sarcomere in 112 unrelated proband patients with familial HCM. We report here the frequency and distribution of sarcomeric gene mutations in Japanese familial HCM.

Methods

Subjects

A total of 112 genetically unrelated Japanese patients with familial HCM were the subjects in this study. Each patient had at least one other HCM patient or case of SCD among first-degree family relatives. The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram, echocardiogram, and other special tests if necessary. The diagnostic criteria for HCM have been described previously.[11] Two hundred healthy Japanese served as control subjects. When a sarcomeric mutation was found in a proband patient, family relatives were contacted through the proband patient about the possibility of a family study. The family members willing to participate visited the relevant hospital to undergo clinical examination, including electrocardiogram and echocardiogram, and genetic testing if they consented. Informed consent was given by each subject and the research protocol conformed to the ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University, Japan.

Genetic Analyses

DNA samples extracted from the peripheral blood of subjects were used as templates to amplify each coding exon of *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1* and *ACTC*, and exons 3–25 of *MYH7* by polymerase chain reaction (PCR). Sequences of primers and the PCR conditions used in this study are available upon request. PCR products were analyzed for sequence variations by direct DNA sequencing of both strands using Big Dye Terminator chemistry (version 3.1) and ABI3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequence variations found in the patients were considered to be mutations on the basis of following criteria: (1) presence in all tested affected members of the family of each proband patient, (2) absence from 400 unrelated chromosomes of the control subjects, (3) absence from a public database of polymorphism, dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), (4) mutations at the evolutionary conserved residues, and/or (5) identification as a HCM-causing mutation in previous reports.

Statistical Analysis

Mean values for the parameter of echocardiography are expressed as mean ± SD. Differences between the stratified groups were compared with Student's t-test. A P value less than 0.05 was considered to be statistically significant.

Results

Frequency of Sarcomeric Gene Mutations in Familial HCM

To investigate the distribution of disease-causing genes for HCM in Japan, 112 consecutive proband patients of famil-

Table 2. MYBPC3 Mutations Found in This Study

Exon/Intron	Novelty*	Nucleotide change	Amino acid change	No. of patients**	Co-segregation†	Consequence	Reference††		
							USA	Europe	Asia
Int11	Novel	IVS11+1 g>t		1	NT	Splice donor site			
Ex12	Novel	GAA>TAA	Glu386ter	3	Yes	Termination codon			
Ex15		CGG>CAG	Arg502Gln	1	Yes	Missence	17	18	
Int15	Novel	IVS16–2a>g		1	NT	Splice acceptor site			
Ex16		T>del	Ser593fs/8	5	Yes	Framesift/ter			19
Int17	Novel	IVS17+1–3gtg>del		1	NT	Splice donor site			
Ex23		CGG>CAG	Arg820Gln	3	NT	Missence			20
Ex23		CGC>CTT	Arg835Leu	2	NT	Missence		10	
Ex24		TGG>TGA	Trp890ter	1	Yes	Termination codon	8		
Ex25		CG>del	Arg945fs/108	3	NT	Framesift/ter			19
Ex28	Novel	CGG>CCG	Arg1073Pro	1	NT	Missence			
Ex29	Novel	TGC>CGC	Cys1124Arg	1	NT	Missence			
Ex31	Novel	CTG>CAG	Leu1268Gln	1	Yes	Missence			

*Novel mutations; **no. of unrelated proband patients; †consistent with the co-segregation of mutation with HCM in multiplex family; ††references cited.
Int, intron; IVS, intervening sequence; NT, not tested; Ex, exon; del, deletion; ter, termination; HCM, hypertrophic cardiomyopathy.

ial HCM were searched for mutations in 8 sarcomeric genes: *MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1*, and *ACTC*. As shown in **Table 1**, we identified a total of 37 different mutations, including 13 novel mutations, in 49 patients. The mutations were most frequently identified in *MYBPC3*, which accounted for 19.6% of cases. The mutations in *MYH7* and *TNNT2* were found in 10.7% and 8.9%, respectively, of cases. On the other hand, mutations in *TNNI3* and *TPM1* were found in a few cases only, and analysis of *MYL2*, *MYL3* and *ACTC* did not reveal any mutations in this patient cohort (**Table 1**).

The analysis of *MYBPC3* led to the identification of 13 different mutations, including 7 mutations not described previously (**Table 2**). Among the new mutations, 3 were within the consensus sequences of splicing donor or acceptor sites, and the other 4 were missense mutations. One novel missense mutation, Leu1268Gln, was consistent with the co-segregation with HCM in a multiplex family (**Figure 1a**). Although the other mutations were not confirmed for co-segregation in each family, they were not found in 200 healthy controls and the splicing site mutations were suggested to result in the splicing abnormalities generating C-terminal truncated proteins. As for the novel missense mutations, they, except for the Arg1073Pro mutation, were found at the evolutionary conserved amino acid residues among various species, similar to the previously reported HCM-causing *MYBPC3* mutations (**Figure 2a**). The Arg1073Pro mutation was also suggested to be associated with HCM, because the 1,073rd residue is the basic amino acid, arginine (Arg) or lysine (Lys), in various species (**Figure 2a**), which was changed to proline (Pro), leading to a gross structural change.

The mutations found in *MYH7*, *TNNT2*, *TPM1* and *TNNI3* are indicated in **Table 3**. The analysis revealed a total of 25 different missense mutations, including 12 *MYH7*, 7 *TNNT2*, 4 *TPM1*, and 1 *TNNI3* mutations. Among them, 2 *MYH7*, 1 *TNNT2*, 2 *TPM1*, and 1 *TNNI3* mutations were identified for the first time in this study. These novel missense mutations were not observed in the healthy controls and occurred at the evolutionary conserved residues (**Figure 2b**). In addition, co-segregation of each novel mutation with HCM was compatible in the analyzed multiplex family of each patient (**Figures 1b–f**), although we could not test the Asp516Glu mutation in *MYH7* because the family study was denied by the proband patient.

Double Mutation Cases

Of the 49 proband patients with sarcomeric gene mutations, 46 cases carried single heterozygous mutations, while the other 3 cases carried double mutations in *MYBPC3* or *TNNT2*. Two cases were double *MYBPC3* mutations; 1 carried IVS11+1 g>t and Arg835Leu mutations, while the other had Ser593fs/8 and Cys1124Arg mutations. A female patient, who carried IVS11+1 g>t and Arg835Leu mutations, was diagnosed as HCM with ventricular tachycardia at 43 years old, and developed dilated-phase HCM at 61 years old. The other patient, who carried Ser593fs/8 and Cys1124Arg mutations, was affected with HCM of early onset with ventricular fibrillation at 17 years old. Because additional family studies for these mutations were denied by the proband patients, it was not clear whether these mutations were *cis* or *trans*. In addition, because of the lack of family studies, it was unclear whether the presence of missense mutations in these cases would worsen the clinical course, as reported previously for other double mutations.[8]

On the other hand, in another case of *TNNT2* double mutation, the Phe110Ile mutation was inherited from the patient's father and the Pro80Ser mutation from her mother (**Figure 1d**). It should be noted that her father did not manifest clinical findings of HCM, whereas her mother was diagnosed as HCM. Because the Phe110Ile mutation is reported to be a HCM-causing mutation with low penetrance,[12] her father might not develop overt HCM. The clinical manifestation of HCM was more severe in the proband patient than in her mother who possessed a single Pro80Ser mutation, because the proband patient manifested with HCM of early onset at 10 years old, whereas her mother had developed HCM in adulthood. These observations suggested that the relatively benign Phe110Ile mutation might affect the clinical expressivity of the Pro80Ser mutation in double mutation cases.

Geographic Distribution of HCM-Associated Mutations

Japan is an East Asian nation consisting of several islands. There are 4 major islands, from northeast to southwest, the Hokkaido, Honshu, Shikoku, and Kyushu Islands. Among these, Honshu Island is the largest and historically divided into 2 areas, the Honshu-Kanto area and the Honshu-Kansai area. The former includes the Tohoku area and part of the Chubu area, while the latter includes the rest of the Chubu area and

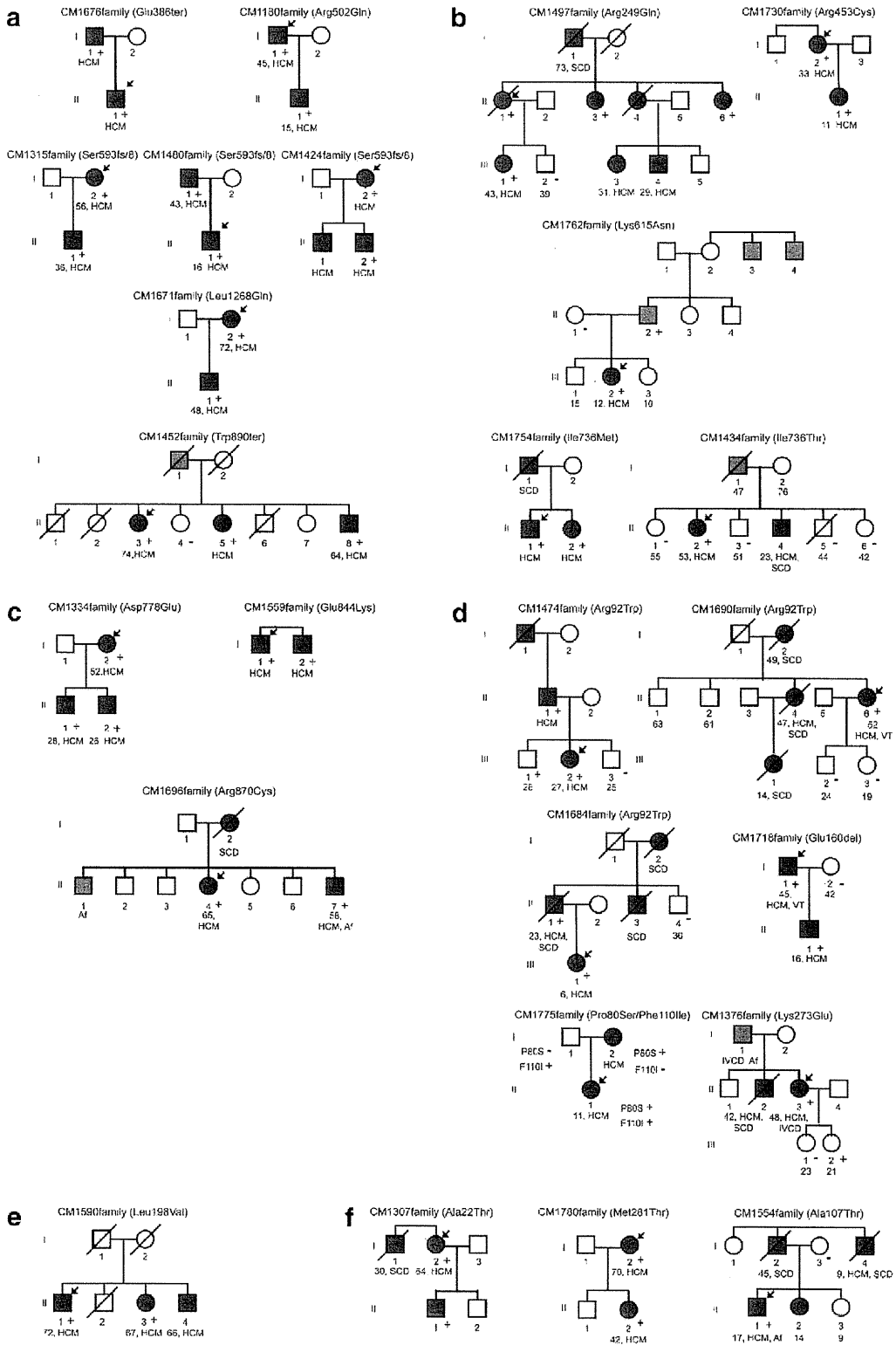

Figure 1. Pedigrees of the HCM families carrying the sarcomeric gene mutations: (**a**) *MYBPC3*, (**b**,**c**) *MYH7*, (**d**) *TNNT2*, (**e**) *TNNI3*, and (**f**) *TPM1*. Only the families of which members other than the proband patients were genotyped for the mutation, are shown. Filled red square and filled red circle indicate affected male and female, respectively. Open square and open circle represent unaffected male and female, respectively. Shadowed square represent affected male with heart disease without detailed information. Red arrows indicate the proband patients. Presence (+) or absence (–) of the mutations is noted for the analyzed individuals. HCM, hypertrophic cardiomyopathy.

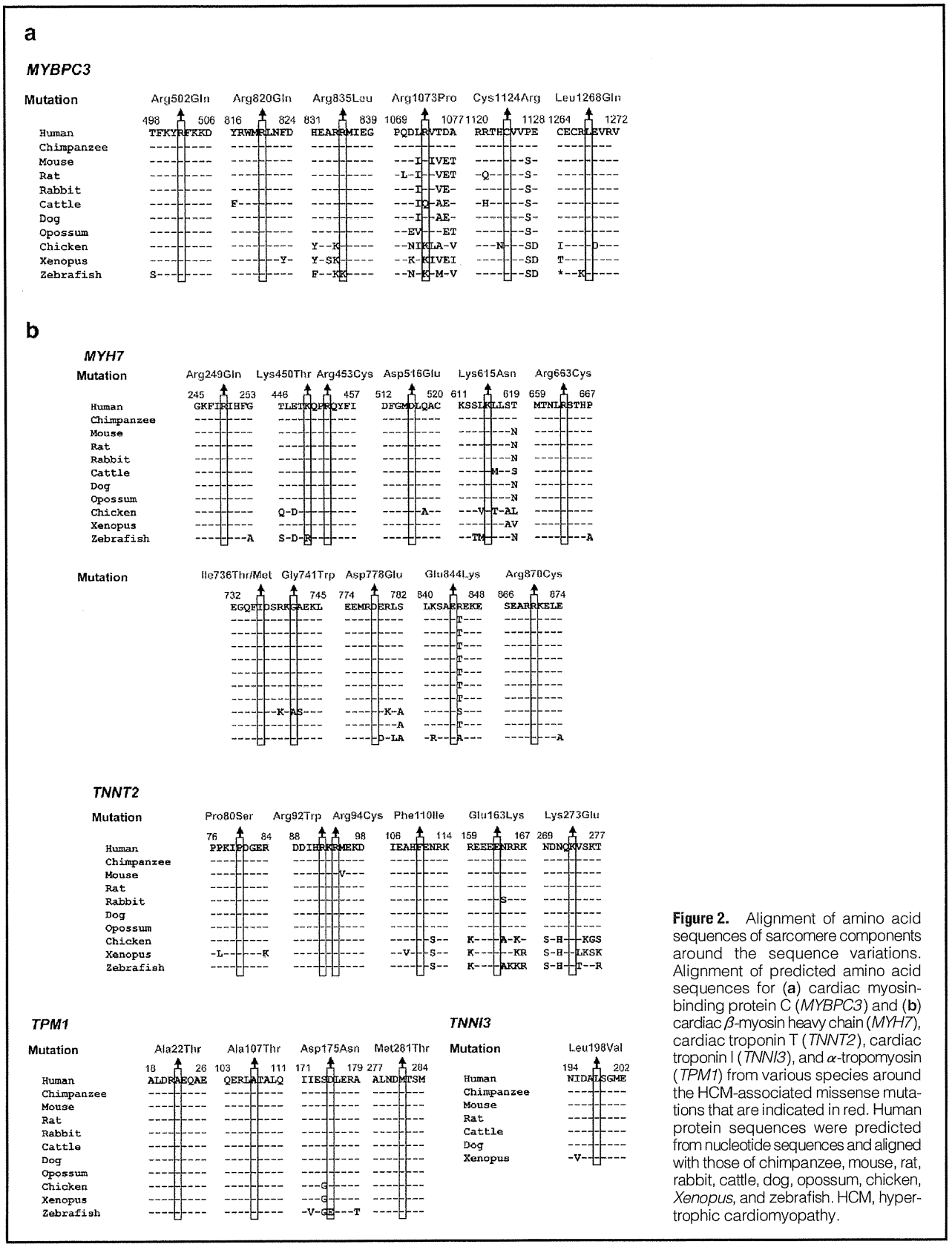

Figure 2. Alignment of amino acid sequences of sarcomere components around the sequence variations. Alignment of predicted amino acid sequences for (**a**) cardiac myosin-binding protein C (*MYBPC3*) and (**b**) cardiac β-myosin heavy chain (*MYH7*), cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*), and α-tropomyosin (*TPM1*) from various species around the HCM-associated missense mutations that are indicated in red. Human protein sequences were predicted from nucleotide sequences and aligned with those of chimpanzee, mouse, rat, rabbit, cattle, dog, opossum, chicken, *Xenopus*, and zebrafish. HCM, hypertrophic cardiomyopathy.

Table 3. *MYH7*, *TNNT2*, *TNNI3* and *TPM1* Mutations Found in This Study

Disease-causing gene [Exon/Intron]	Novelty*	Nucleotide change	Amino acid change	No. of patients**	Co-segregation†	Reference††		
						USA	Europe	Asia
MYH7								
Ex9		CGA>CAA	Arg249Gln	1	Yes	21	7	
Ex14		AAG>ACG	Lys450Thr	1	NT			22
Ex14		CGC>TGC	Arg453Cys	1	Yes	21	7	
Ex15	Novel	GAC>GAA	Asp516Glu	1	NT			
Ex16		AAG>AAC	Lys615Asn	1	Yes			22
Ex18		CGC>TGC	Arg663Cys	1	NT	8		19
Ex20		ATT>ACT	Ile736Thr	1	NT		23	
Ex20		ATT>ATG	Ile736Met	1	Yes			24
Ex20		GGG>TGG	Gly741Trp	1	NT		23	25
Ex21		GAC>GAA	Asp778Glu	1	Yes		7	
Ex22	Novel	GAA>AAA	Glu844Lys	1	Yes			
Ex22		CGC>TGC	Arg870Cys	1	Yes			26
TNNT2								
Ex6	Novel	CCC>TCC	Pro80Ser	1	Yes			
Ex8		CGG>TGG	Arg92Trp	3	Yes	27	28	29
Ex8		CGC>TGC	Arg94Cys	1	NT		30	
Ex8		TTT>ATT	Phe110Ile	3	NT	31		29
Ex11		GAG>del	Glu160del	1	Yes	31	7	
Ex11		GAG>AAG	Glu163Lys	1	NT	31		
Ex15		AAA>GAA	Lys273Glu	1	NT			29
TPM1								
Ex1	Novel	GCT>ACT	Ala22Thr	1	Yes			
Ex3	Novel	GCA>ACA	Ala107Thr	1	NT			
Ex5		GAC>AAC	Asp175Asn	1	NT	31	28	32
Ex9		ATG>ACG	Met281Thr	1	Yes	27		
TNNI3								
Ex8	Novel	CTG>GTG	Leu198Val	1	Yes			

Footnotes as in Table 2.

Table 4. Geographic Distribution of Sarcomeric Gene Mutations

Disease-causing gene	Hokkaido Island	Honshu Island		Shikoku Island	Kyushu Island
		Kanto	Kansai		
MYH7	1	1	4	1	5
MYBPC3	4	11	6	1	0
TNNT2	0	5	3	0	2
TNNI3	0	0	0	0	1
TPM1	0	3	0	1	0
NI	3	21	30	2	7
Total	8	41	43	5	15

NI, mutation was not identified in the analyzed sarcomeric genes.

the Chugoku area. Because the migration of people was less frequent and forbidden during the Edo period from the 17th to 19th centuries, especially across the middle of the Chubu area, the HCM-associated mutations might be differently distributed around Japan. We therefore investigated the geographic distribution of the HCM-associated mutations.

As shown in Table 4, the most frequent disease-causing gene in the northeastern area (Hokkaido Island and the Honshu-Kanto area including the Tohoku area) was *MYBPC3*, mutations of which were identified in 15 of 49 (30.6%) proband patients, whereas the *MYH7* mutations were identified in only 2 of 49 (4.1%) cases. In clear contrast, the most frequent disease-causing gene in the southwestern area (Shikoku and Kyushu Islands and the Honshu-Kansai area including the Chugoku area) was *MYH7*, mutations of which were detected in 10 of 63 (15.9%) cases, while the *MYBPC3* mutations were found in 7 of 63 (9.5%) cases. The other mutations in *TNNT2*, *TPM1*, and *TNNI3* were relatively infrequent and the frequencies were not largely different between the northeastern and southwestern parts.

We also investigated the geographic distributions of specific mutations, which were observed in several unrelated proband patients analyzed in this study and in our previous study.[13] As shown in **Figure 3**, *TNNT2* Glu163Lys mutation was identified in 5 patients (4 and 1 in the previous and present studies, respectively), who all were residents of Kyushu Island. The *TNNT2* Arg92Trp mutation was found in 9 patients (6 and 3 in the previous and present studies, respectively) and was preferentially identified in the southwestern region in 5 and 3 cases from Kyushu Island and the Kansai area, respectively. In addition, *MYBPC3* Ser593fs/8 mutation was observed in 12 patients (7 and 5 in the previous and present studies, respectively), and was identified in 7 and 3 cases from the Shikoku and Hokkaido Islands, respectively.

Clinical Phenotypes of HCM-Associated Mutations

Genotyping of proband patients and available family members of them allowed us to evaluate the echocardiographic param-

eters of mutation-prone patients grouped by the disease-causing genes, *MYH7*, *MYBPC3*, *TNNT2*, and *TPM1*, as compared with the patients who did not carry any sarcomeric gene mutations (Table 5). The ratios of intraventricular septum thickness (IVST) to posterior wall thickness (PWT) were significantly higher in the patients with *MYBPC3*, *MYH7*, or *TPM1* mutations than in the patients without sarcomeric gene mutations. Interestingly, a significantly higher ratio of IVST/PWT was associated with a significantly higher or lower value for IVST in patients with *MYBPC3* mutations or PWT in patients with *MYH7* or *TPM1* mutations, respectively.

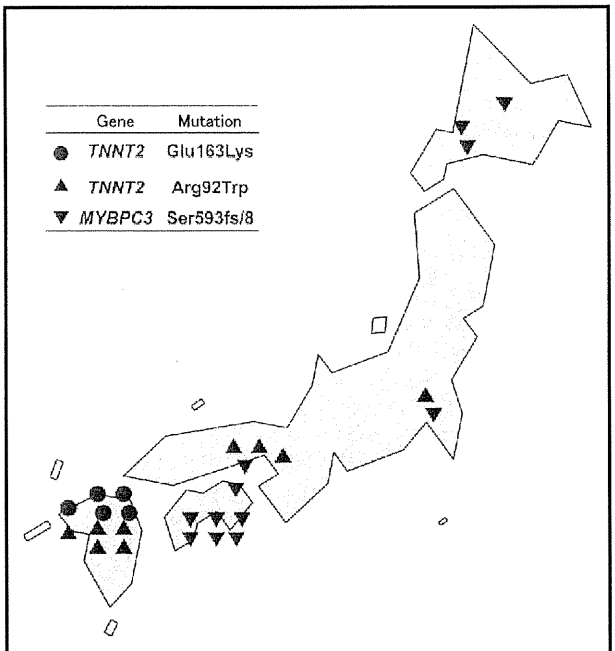

Figure 3. Distribution of same sarcomeric gene mutations found in unrelated patients. Regional distribution of unrelated patients carrying the TNNT2-Glu163Lys, TNNT2-Arg92Trp, or MYBPC3-Ser593fs/8 mutations are schematically shown on the map of Japan.

Discussion

To ascertain the frequency and distribution of HCM-associated mutations in Japan, we analyzed 8 sarcomeric genes in 112 unrelated proband patients with familial HCM. Mutations were identified in 49 cases (43.8%) as shown in Table 1. Prevalence of sarcomeric gene mutations in this Japanese cohort with familial HCM was lower than the reported prevalence in the French cohort with familial HCM[7] and that in the US cohort with familial HCM,[8] in which 60.6% and 54.2%, respectively, of the analyzed patients were found to carry sarcomeric gene mutations, while 10.8% of the US cohort with sporadic HCM had mutations.[8] We have previously reported that 76 (46.9%) and 14 (14.0%) patients, respectively, carried sarcomeric gene mutations in another set of Japanese HCM cohort composed of 162 familial cases and 100 sporadic cases.[13] Because we had screened for gene mutations using single-strand conformation polymorphism method in the previous study,[13] we might have missed several mutations and hence the prevalence of sarcomeric gene mutations might have been underestimated previously. However, we searched for mutations by direct sequencing of PCR products in this study, suggesting that the prevalence of sarcomeric gene mutations in familial HCM is relatively low in Japanese and less than half of cases, whereas in European and American populations it is over 50%. On the other hand, the prevalence of sarcomeric gene mutations in sporadic HCM appeared to be comparable between Japanese[13] and US[8] cohorts.

Study Limitations

First, we did not analyze the patients for mutations in the rod region of cardiac β-myosin heavy chain. In a database of sarcomeric gene mutations in HCM (http://genepath.med.harvard.edu/seidman//cg3/), a total of 194 *MYH7* mutations are registered and among them 21 (10.8%) are found in the rod region corresponding to exons 26–40 of *MYH7*, which we did not investigate in this study. Therefore, the possibility remains that we did not detect several disease-associated *MYH7* mutations. However, such mutations might be present in a few cases, because approximately 90% of *MYH7* mutations are found in exons 3–25 in the mutation database, albeit the previous genetic analyses were mainly focused on the head and neck regions of cardiac β-myosin heavy chain as were we in this study. Second, although the mutations identified in this study were not found in 200 healthy controls, we can not exclude the possibility that they are rare polymorphisms. Further studies are required to demonstrate the effect of these mutations in causing HCM, using both in vitro and in vivo functional analyses that include mutations such as nonsense mutations, splicing mutations or frameshift mutations, which are expected to be deleterious.

Conclusions

When we focused on the geographic distribution of mutations,

Table 5. Phenotypes of HCM Patients Carrying Mutations in *MYBPC3*, *MYH7*, *TNNT2* and *TPM1*

Gene	No. of patients	Age at diagnosis (years)	% of men	LVEDD (mm)	LVESD (mm)	IVST (mm)	PWT (mm)	IVS/PW	FS (%)
MYBPC3	14	39±21	64	42.7±5.7	26.7±5.8	21.0±6.3*	11.5±2.8	1.9±0.7*	37.3±8.0
MYH7	7	38±17	57	43.5±5.4	27.5±5.9	16.5±7.0	9.3±1.8*	1.9±1.1*	39.0±9.3
TNNT2	7	34±22	57	45.0±7.9	28.7±9.6	17.7±2.1	11.9±3.4	1.6±0.4	36.8±10.7
TPM1	3	46±19	33	49.0±3.5	31.3±4.2	16.3±4.9	8.3±1.5*	2.2±0.4*	33.3±6.1
NI	30	47±18	60	46.7±5.8	29.0±7.1	14.7±4.8	11.4±2.3	1.3±0.4	38.9±8.3

*P<0.05 vs. mutation not identified group.
HCM, hypertrophic cardiomyopathy; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVST, intraventricular septal thickness; FS, fractional shortening; PWT, posterior wall thickness; NI, mutation was not identified in the analyzed sarcomeric genes.

a difference between the northeastern and southwestern regions of Japan was observed, even though we analyzed only patients who resided on the 4 main islands where the population could be considered as a relatively homogeneous ethnic group. It was also demonstrated that relatively common mutations were preferentially found in specific areas, which suggested a founder effect. On the other hand, it was unclear whether the *MYBPC3* Ser593fs/8 mutation in the Hokkaido population originated from the Shikoku population, but most of the present Hokkaido residents are descendants of immigrants from various parts of Japan, including Shikoku Island, after the Edo period. However, we could not exclude the possibility that the *MYBPC3* Ser593fs/8 arose independently in various areas, because more than half of the known mutations found in this study were previously reported in European and/or American populations (**Tables 2,3**), implying that some of the mutations were generated independently in different races or that the origin of mutations might predate the diversification of Caucasoid and Mongoloid populations. These observations suggest that the distribution of disease-causing genes might be influenced by immigration of ancestral mutation carriers. This finding in turn provides clinicians and geneticists with information about possible regional variations in the HCM phenotypes.

There were a few cases of 2 different mutations in the same disease-causing genes. Among them, a girl carrying the Pro80Ser and Phe110Ile mutations of *TNNT2* manifested both HCM and LV non-compaction of early onset. The Phe110Ile mutation, which was identified in her father who was not affected with HCM (**Figure 1d**), has been reported as a HCM mutation associated with low penetrance and favorable prognosis.[12] In contrast, the Pro80Ser mutation, which was identified in her mother who was affected with adult-onset HCM, was identified as a novel HCM-causing mutation in this study, because the Pro80 residue is highly conserved among various species from zebrafish to humans (**Figure 2b**) and this mutation was not found in the 200 healthy control subjects. Previous studies suggested that multiple mutations such as homozygous or compound heterozygous mutations in *MYBPC3* and/or *MYH7* result in more severe clinical phenotypes because of a "double dose" effect.[7,8,14] Although it was reported that homozygous *TNNT2* mutations caused more severe phenotypes than heterozygous mutations,[15,16] a compound heterozygous mutation in *TNNT2* has not been reported previously. Further continuous follow-up of the *TNNT2* double mutation patient is required to reveal the clinical phenotypes caused by the compound heterozygous mutations in *TNNT2*.

In conclusion, we report the results of a systematic screening for mutations in 8 sarcomeric genes in Japanese patients with familial HCM. We demonstrate the prevalence and geographic distribution of the sarcomeric gene mutations in Japan.

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Disclosures

Conflict of interest: none declared.

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REVIEW

Cardiovascular Therapeutics

Matricellular Proteins: New Molecular Targets To Prevent Heart Failure

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SUMMARY

Matricellular proteins are highly expressed in reparative responses to pressure and volume overload, ischemia, oxidative stress after myocardial injury, and modulate the inflammatory and fibrotic process in ventricular remodeling, which leads to cardiac dysfunction and eventually overt heart failure. Generally, matricellular proteins loosen strong adhesion of cardiomyocytes to extracellular matrix, which would help cells to move for rearrangement and allow inflammatory cells and capillary vessels to spread during tissue remodeling. Among matricellular proteins, osteopontin (OPN) and tenascin-C (TN-C) are de-adhesion proteins and upregulate the expression and activity of matrix metalloproteinases. These matricellular proteins could be key molecules to diagnose cardiac remodeling and also might be targets for the prevention of adverse ventricular remodeling. This review provides an overview of the role of matricellular proteins such as OPN and TN-C in cardiac function and remodeling, as determined by both in basic and in clinical studies.

Introduction

Heart failure is a major and growing public health problem in industrialized countries, mainly because of aging of the population, and the number of heart failure deaths has increased steadily despite advances in treatment. Cardiac remodeling, which is characterized by changes in the size, shape, and function of the heart, is closely associated with a worse prognosis in patients with heart failure [1]. Cellular level characteristics of cardiac remodeling include myocyte hypertrophy and loss, extracellular matrix (ECM) deposition and interstitial fibrosis, and inappropriate microvasculature and coronary flow. Moreover, the upregulation of proinflammatory cytokines can extend and trigger a second phase of elevated levels of cytokines that promote myocyte loss, interstitial fibrosis, and inappropriate coronary flow leading to left ventricular (LV) dysfunction. Cardiac remodeling might also be responsible for diastolic LV dysfunction. Recently, heart failure with preserved LV systolic function is increasingly recognized and referred to as diastolic heart failure. However, the exact cellular and molecular mechanisms of cardiac remodeling remain uncertain.

Cardiac ECM provides not only mechanical support but also cellular circumstances in which myocytes, fibroblasts, smooth muscle cells, and endothelial cells communicate and function with each other. The ECM is composed of numerous molecules including structural proteins such as fibrillar collagen, glycoproteins, proteoglycans, and so-called matricellular proteins. Matricellular proteins are a group of ECM proteins that do not contribute directly to the formation of structural elements but serve as biological mediators of cell function by interacting directly with cells or by modulating the activity of growth factors, cytokines, proteases, and other ECM proteins. Matricellular proteins are supposed to regulate cell movement, proliferation, differentiation during morphogenesis, and modulate the inflammatory and reparative response after tissue injury. They have common unique properties: (1) high levels of expression during embryonic development and in response to injury; (2) binding to many cell-surface receptors, components of ECM, growth factors, cytokines, and proteases; (3) induction of de-adhesion or counter-adhesion in contrast to the adhesivity of most matrix proteins; and (4) a grossly normal or subtle phenotype that is observed in knockout mice of most matricellular protein genes [2,3]. Matricellular proteins are a growing family which originally included thrombospondin (TSP)-1,-2, osteonectin/SPARC, tenascin-C (TN-C), TN-X, osteopontin (OPN), and CCN. Recently new members such as periostin and galectin joined the family (Table 1). It is becoming increasingly evident that matricellular proteins highly expressed in reparative responses after myocardial injury may modulate the inflammatory and

Table 1 Matricellular proteins

Osteopontin	
Thrombospondins	
TSP-1	
TSP-2	
Seceted protein acidic and rich in cysteine (SPARC)	
SPARC	BM-40, osteonectin
SPARC like 1	Hevin
Tenascin family	
Tenascin-C	
Tenascin-X	
CCN family	
CCN1	Cysteine-rich angiogenic inducer (CYP-61)
CCN2	Connective tissue growth factor (CTGF)
CCN3	Nephroblastoma overexpressed (Nov)
CCN4	Wnt-induced secreted protein-1 (WISP-1)
CCN5	WISP-2, connective tissue growth factor-like protein (CTGF-L)
CCN6	WISP-3
Galectins	
Periostin	
Plasminogen activator inhibitor type 1 (PAI-1)	
Autotaxin	

fibrotic process in ventricular remodeling, which leads to cardiac dysfunction and eventually overt heart failure [4,5]. Several other excellent reviews have discussed the biological role of TSP [6–9], osteonectin/SPARC [10,11], CCN [12–14], and periostin [15,16], thus this review will primarily focus on the role of TN-C and OPN for cardiac remodeling leading to heart failure, particularly in regard to their potential clinical application.

Osteopontin

Structure and Function

OPN is an arginine–glycine–aspartate (RGD)-containing adhesive glycoprotein generated or secreted by various cells such as macrophages, T-cells, mast cells, hematopoietic cells, VSMs, and fibroblasts. Its chief function is thought to be cellular migration and infiltration in cellular immunity as well as in angiogenesis, inhibition of cellular death, and reconstitution of ECM [17]. OPN participates in integration of intercellular networks as a cytokine or neuro-humoral factor rather than merely as an adhesion molecule. The molecular weight can range from 45 to 75 kDa, and is highly cleaved by thrombin and MMPs in various active fragments. Transcription is accelerated by growth factors such as TGFβ, EGF, TNFα, PDGF, bFGF, cytokines (IL-1α and IL-2), and endothelin [18]. OPN has 16 signal peptides in the N-terminus, Ser/Thr phosphorylation sites and a GRGDS motif [19]. A C-terminal fragment of OPN binds directly to CD44 in an RGD independent manner, inducing macrophage chemotaxis and engagement of ß3-integrin receptors [20]. Thus, OPN has a wide variety of functions mediated by the numerous receptors that match OPN and changes in bonding patterns.

Physiological Significance of OPN in Cardiac Remodeling

OPN is not expressed in healthy cardiac muscle tissue. Its expression is accelerated by mechanical stress including pressure/volume overload, hypoxia, and cardiac injury [21]. Murray et al. first examined OPN expression in human myocardial infarction (MI) [22]. Prominent OPN expression is found in cardiomyopathic hamster hearts [23] and in chronic myocarditis [24]. Singh et al. reported abundant expression of OPN mRNA expression in interstitial nonmyocytes in spontaneously hypertensive rats with heart failure [25]. In these circumstances, mechanical stress, inflammatory cytokines, and neuro-humoral factors such as angiotensin II can induce the activation of fibroblasts and participate in the repair process of myocardial damage. Activated fibroblasts or myofibroblasts are the primary mediators of tissue repair and OPN expression is required for myofibroblast differentiation [26]. *In vitro* data demonstrated that angiotensin II (AII) accelerates synthesization of OPN in rat cardiac fibroblasts [27] and that the combination of AII with IL-1β or TNF-α increases OPN mRNA more than does AII alone [28]. This upregulation of OPN involves reactive oxygen species (ROS)-mediated activation of ERK1/2 and JNK pathways. It has been demonstrated that increased early OPN expression in macrophages is a determinant of fibrosis and cardiac remodeling in myocarditis, dilated cardiomyopathy (DCM), and heart failure [29]. The primary sources of OPN are macrophages infiltrating within the necrotic and granulation tissue [30]. Meanwhile, expression of OPN by cardiomyocytes has been reported in pressure overload hypertrophied heart [31] and in streptozotocin-induced diabetic cardiomyopathy [32]. Therefore, it seems that various cells synthesize OPN in pathologic myocardium in a context-dependent manner. In OPN KO mice, LV chamber dilation after coronary ligation was accelerated associated with less fibrosis [33]. Inhibition of matrix metalloproteinases improves LV function after infarction of OPN KO mice [34]. Furthermore, OPN may facilitate angiogenesis, because capillary and arteriolar development during tissue repair after infarction is impaired [35]. Therefore, it is suggested that OPN may protect infarcted hearts from adverse remodeling by inhibiting MMPs and promoting angiogenesis and deposition of collagen in the damaged myocardium. Conversely, redundant OPN may accelerate excessive fibrosis associated with impaired cardiac function. In the angiotensin II-induced cardiac fibrosis model, perivascular and interstitial fibrosis is markedly inhibited in the OPN KO mice with retained diastolic function (Figure 1). Macrophage infiltration in the fibrotic lesion and expression of eNOS and cytokine genes were also reduced in the OPN KO mice [36]. In addition to modulation of inflammatory response, OPN regulates activity of fibroblasts. Cardiac fibroblasts isolated from OPN KO mice exhibit reduced stress fibers, focal adhesions, and lamellipodia. Adhesion to ECM proteins such as collagen I, fibronectin, laminin, and vitronectin can be reversed and AKT activity to increase cell viability can be sustained by inhibition of OPN [37]. Thus, OPN modulation might have the therapeutic potential in the remodeling processes of the injured heart.

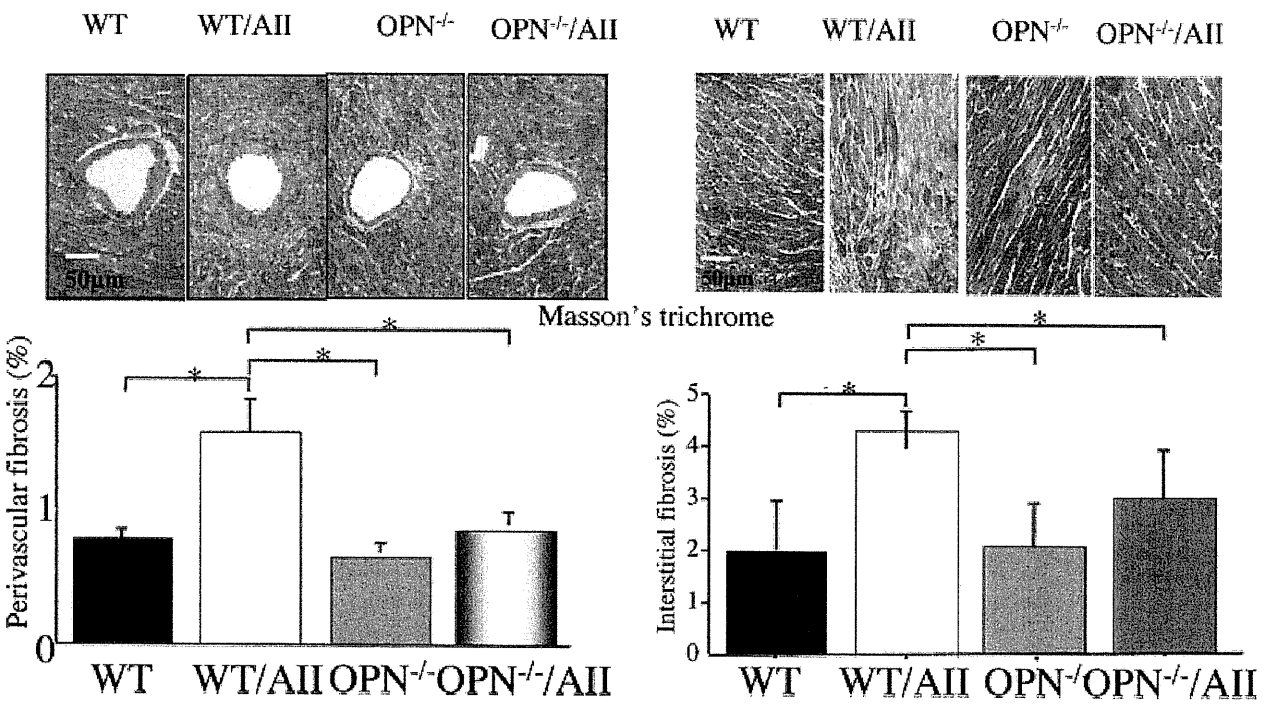

Figure 1 Left ventricular interstitial and perivascular fibrosis. OPN-deficiency significantly reduced the AII-induced elevation of BP. AII treatment could increase the LVW/BW both in WT and OPN-/- mice. These panels show representative short-axis images of myocardium stained with Masson's trichrome. Both perivascular and interstitial fibrosis were significantly increased after AII treatment and OPN deficiency nearly abolished AII-induced fibrosis.

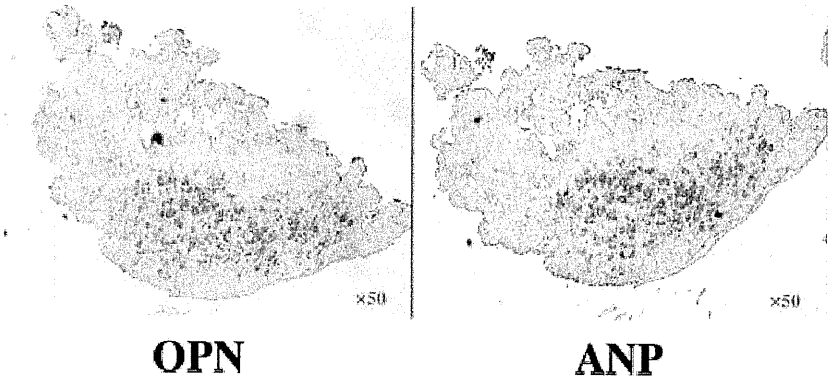

Figure 2 OPN and cardiac fibrosis. The relationship between OPN and cardiac fibrosis was examined using endomyocardial biopsies from patients with heart failure due to DCM. In patients with or without OPN expression, OPN-positive patients had a higher fibrosis ratio determined by Masson–Goldner staining.

Clinical Applications

Analysis of myocardial biopsies obtained from patients with DCM showed that OPN was highly expressed in fibroblasts and mononuclear cells on immunostaining, and was related to myocardial hypertrophy, an increase in LV diastolic dimension (LVDd), a decrease in ejection fraction, and an increase in type-I collagen [38]. We also found OPN expression in myocardial biopsies from a group of patients with DCM, hypertrophic cardiomyopathy (HCM), and ventricular tachycardia [39]. While neither OPN nor atrial natriuretic peptide (ANP) was expressed in the group with normal hearts, OPN protein expression was detected in cardiac fibroblasts of all ANP-positive patients with DCM, HCM, or idiopathic ventricular tachycardia, and this expression was associated with the area of myocardial fibrosis (Figure 2). It has also been reported that OPN expression of circulating CD4+ T cells and plasma OPN levels reflect the severity of heart failure and cardiac remodeling [40]. On the other hand, Graf et al. have demonstrated that *in situ* hybridization of OP was predominantly in cardiomyocytes not in infiltrated cells from 12 patients undergoing heart transplantation due to ischemic or idiopathic cardiomyopathy [31]. It is suggested that OPN released from cardiomyocytes induces cardiac hypertrophy resulting from impaired relaxation through activation of fibroblasts. Recently, it has been demonstrated that the plasma OPN level can be a new biomarker for