In the present study, eight known polymorphisms were detected. Among them, two synonymous variations (V395V and K811K) were reported to have a statistically significant association with diabetes mellitus.[1] An association was made between homozygous carriers of the H611R allele and those who completed suicide.[1] Although impaired glucose tolerance in an individual (II: 3) and severe depression in another one (II: 9) were reported in the present family, we could not perform a genetic study of the individuals. Other synonymous and nonsynonymous changes (R228R, V333I, N500N, and S855S) were known as common polymorphisms.[1] T170T was a rare variant previously reported as a nonpathogenic allele in the Japanese population.[23]

The mature sodium pump (α1 and β1 subunits) is located in the plasma membrane, but the sodium pump is present transiently in the ER during its maturation.[24] Recently, Zatyka et al.[25] showed that the C-terminal domain (amino acids 652-890) of wolframin, which is known as an ER membrane protein, is important for the interaction with Na^+/K^+ ATPase β1 subunit during maturation of the sodium pump. The Na^+/K^+ ATPase deficiency is fatal to nerve tissues, and it was identified as a contributor to apoptosis and neural degenerative disease.[26] Therefore, mutations in *WFS1* may induce Na^+/K^+ ATPase deficiency in several organs, resulting in the development of Wolfram syndrome phenotypes. The disruption of the wolframin-Na^+/K^+ ATPase β1 subunit interaction may also affect potassium circulation in the inner ear, resulting in HL. Meanwhile, low-frequency SNHL is thought to be a specific audiometric configuration indicative of endolymphatic hydrops. An enlarged SP/AP ratio in ECochG reflects the specific abnormalities associated with endolymphatic hydrops.[4] Although the proband exhibited bilaterally enlarged SP/AP ratios, we could not measure ECochG in the other affected individuals. Moreover, the role of Na^+/K^+ ATPase β1 subunit in the induction of endolymphatic hydrops still remains to be elucidated. Further studies are therefore needed to clarify the mechanisms of HL induced by *WFS1* mutations.

CONCLUSION

A novel missense mutation of *WFS1* (K836T) was detected in a Japanese family with autosomal dominant, nonsyndromic hereditary HL. Affected children had mid-frequency SNHL, whereas affected adult members showed low-frequency SNHL and no vestibular dysfunction. The progression of HL in this family is thought to occur as a result of a gradual neural degeneration of the inner ear due to an ion transport deficiency.

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ORIGINAL ARTICLE

Extensive and rapid screening for major mitochondrial DNA point mutations in patients with hereditary hearing loss

Tomofumi Kato[1,2], Yutaka Nishigaki[3], Yoshihiro Noguchi[2], Hitomi Ueno[3], Hiroko Hosoya[3], Taku Ito[2], Yurika Kimura[1], Ken Kitamura[2] and Masashi Tanaka[3]

Sensorineural hearing loss (HL) is one of the most frequent clinical features in patients with mitochondrial diseases caused by mitochondrial DNA (mtDNA) mutations, and hearing is impaired in over half of all cases with mitochondrial disorders. This study analyzed 373 patients with suspected hereditary HL using an extensive and rapid suspension-array screening system for 29 major mtDNA mutations, including the m.1555A>G homoplasmic mutation in the *MT-RNR1* gene, which causes non-syndromic sensorineural HL and aminoglycoside-induced HL, and the m.3243A>G heteroplasmic mutation in the *MT-TL1* gene. This method is rapid and suitable for large-scale screening because universal 96-well plates are available for use, and because an analysis of each plate can be completed within 1 h. This system detected five different mtDNA mutations in 24 of the 373 (6.4%) patients. The m.1555A>G and m.3243A>G mutations were detected in 11 (2.9%) and 9 (2.7%) patients, respectively. In addition, three mutations, that is, m.8348A>G in the *MT-TK* gene, m.11778G>A in the *MT-ND4* gene and 15498G>A in the *MT-CYB* gene were detected in one patient for each. This screening system is useful for the genetic diagnosis and epidemiological study of both syndromic and non-syndromic HL.
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Keywords: hereditary hearing loss; MELAS; mitochondrial DNA; mutation; suspension array

INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common sensory disorder in humans. The prevalence of permanent SNHL continues to increase during childhood and it reaches a rate of about 2.7 per 1000 children before the age of 5 years and 3.5 per 1000 during adolescence.[1] Interestingly, hearing impairment is quite common in patients with mitochondrial disorders, affecting over half of all cases at some time during the course of the disease.[2] Therefore, many pathological mutations in mitochondrial DNA (mtDNA) have been reported to either cause or be associated with syndromic or non-syndromic HL.[3–6] A representative homoplasmic mutation at m.1555A>G in the *MT-RNR1* (12S ribosomal RNA) gene causes non-syndromic (isolated) hearing loss (HL) associated with a susceptibility to aminoglycoside antibiotics.[7–9] Moreover, it is not uncommon to find the m.1555A>G mutation in HL patients without defined past medication histories of aminoglycoside.[9–12] Other pathogenic mtDNA mutations have also been identified in syndromic HL with various clinical phenotypes, such as mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes, Kearns–Sayre syndrome, myoclonic epilepsy and ragged-red fibers[13] or maternally inherited diabetes and deafness.[14] Furthermore, the m.3243A>G heteroplasmic mutation in *MT-TL1* ($tRNA^{Leu(UUR)}$) gene is found in patients with non-syndromic HL.[15] Accordingly, certain mitochondrial diseases can arise from defects in the mtDNA transmitted from mothers, but there are also many cases without any apparent family history of the disease (sporadic).

It is therefore necessary to analyze the many presumable mutations in mtDNA because hearing impairment is a quite common clinical feature caused by mtDNA mutations. Over 200 point mutations in mtDNA have been listed in the mtDNA mutation database MITOMAP (http://www.mitomap.org/). However, mitochondrial single nucleotide polymorphisms (mtSNPs) are common in human mtDNA, according to the human mtSNP database (http://mtsnp.tmig.or.jp/mtsnp/index_e.shtml) and recent reports.[16–19] Consequently, it is also necessary to distinguish pathogenic mtDNA mutations from mtSNPs.

This study screened 373 unrelated Japanese patients with suspected hereditary HL by using an extensive and rapid suspension-array detection system for 29 major mtDNA mutations, including the m.1555A>G and m.3243A>G mutations, and verified the effectiveness of this system for identifying mtDNA mutations.

[1]Department of Otolaryngology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Itabashi, Tokyo, Japan; [2]Department of Otolaryngology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan and [3]Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo, Japan
Correspondence: Dr M Tanaka, Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology (TMIG), 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan.
E-mail: mtanaka@tmig.or.jp
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MATERIALS AND METHODS

Patients

The study population included 373 unrelated Japanese patients with suspected hereditary HL, who visited the outpatient clinic of the Department of Otolaryngology, University Hospital of Medicine, Tokyo Medical and Dental University. The subjects included patients with a family history of HL and those with no apparent cause of HL, even though they did not have any apparent family history of HL. The average age of the patients was 40 years, with an age range between 1 and 77 years. The patients included 144 males and 229 females, including four with the branchiootorenal syndrome, four with Pendred syndrome, one with Alport syndrome, one with Turner syndrome, one with spinocerebeller degeneration and one with renal tubular acidosis. Their mode of inheritance was autosomal dominant in 92, autosomal recessive in 52, maternal in 47, sporadic in 179 and unknown in 3. Their onset age of HL varied from newborn to 76 years of age (Table 1). The degree of HL was evaluated by pure-tone audiometry. The pure-tone average was calculated from the air-conduction audiometric thresholds at frequencies of 0.5, 1, 2 and 4 kHz.

The study protocol complied with the Declaration of Helsinki and it was also approved by the Committee on the Ethics of Human Research of the Tokyo Metropolitan Institute of Gerontology and the Institutional Review Board (IRB No. 68) of Tokyo Medical and Dental University. This study was carried out only after obtaining the written informed consent from each individual and/or the parents in the case of children.

Extensive mtDNA pathological mutation screening by suspension array

The DNA samples were purified from the blood using a standard procedure. The mtDNA from the patients was analyzed using an extensive and rapid suspension array-based screening system that has been originally designed to identify various mtDNA point mutations that cause mitochondrial diseases.[20] In brief, multiplex PCR and a flow-metric analysis using a suspension array system (Luminex, Austin, TX, USA) was used to detect any mtDNA mutations. A total of 11 pairs of primers were used for PCR amplification and 65 sequence-specific oligonucleotide probes, which were carefully designed to detect either mutant or wild-type mtDNA even when a certain mtSNP was present in the vicinity of the mutation site. Two pairs of sequence-specific oligonucleotide probes that matched the mutant and wild-type with or without the polymorphic sites were used when mtSNPs were present in the vicinity of target mutations. Four probes were designed for the analysis of the m.1606G>A mutation in the *MT-RNR1* (12S ribosomal RNA) gene causing ataxia, myoclonus and deafness to avoid interference by the mtSNP m.1598G>A, which is found in haplogroups B5b, N9b1 and N1b in the Japanese population (frequency: 4.2%).[21] Similarly, for the detection of the m.15498G>A mutation in the *MT-CYB* (cytochrome *b*) gene,[22] two pairs of probes were designed to avoid interference by the mtSNP m.15497G>A, which is characteristic of haplogroup G1a in the Japanese population (frequency: 3.7%). The detection of the m.12706T>C mutation in the *MT-ND5* gene can be inhibited by the presence of mtSNP m.12705C>T, which is found in haplogroups A and N9a, as well as in macrohaplogroup M among the Japanese population (frequency, 81%). Nevertheless, the m.12706T>C mutation is expected to be detected even in the presence of mtSNP m.12705C>T, because four probes were designed to detect the mutant and wild-type mtDNA with or without the polymorphic sites. Therefore, this system can be used for the screening of various mtDNA point mutations, even in the presence of

Table 1 Demographic features of 373 patients with HL

Sex	
Male	144 (38.6%)
Female	229 (61.4%)
Onset age of HL (years)	
Newborn or 0	31 (8.3%)
1–3	23 (6.2%)
4–10	80 (21.4%)
11–20	43 (11.5%)
21–30	39 (10.5%)
31–40	50 (13.4%)
41–50	37 (9.9%)
51–60	31 (8.3%)
61–70	12 (3.2%)
71–80	5 (1.3%)
Unknown	22 (5.9%)
Mode of inheritance	
Autosomal dominant	92 (24.7%)
Autosomal recessive	52 (13.9%)
Maternal	47 (12.6%)
X-linked	0 (0.0%)
Sporadic	179 (48.0%)
Unknown	3 (0.8%)

Abbreviation: HL, hearing loss.

Table 2 List of 29 mtDNA mutations analyzed by the array-based extensive detection system

Nucleotide change (m.)	*Locus*	*Amino- acid change*	*Clinical phenotype*
1555A>G	*MT-RNR1*		DEAF
1606G>A	*MT-TV*		AMDF
3243A>G	*MT-TL1*		MELAS, DM, DMDF, CPEO, MM
3254C>T	*MT-TL1*		CPEO
3255G>A	*MT-TL1*		MERRF+KSS overlap
3256C>T	*MT-TL1*		MELAS
3260A>G	*MT-TL1*		MMC
3271T>C	*MT-TL1*		MELAS, DM
3280A>G	*MT-TL1*		Myopathy
3291T>C	*MT-TL1*		MELAS
3302A>G	*MT-TL1*		MM
3303C>T	*MT-TL1*		MMC
3460G>A	*MT-ND1*	Ala>Thr	LHON
8344A>G	*MT-TK*		MERRF
8348A>G	*MT-TK*		Cardiomyopathy
8356T>C	*MT-TK*		MERRF
8363G>A	*MT-TK*		MERRF, MICM+DEAF, Autism
9176T>C	*MT-ATP6*	Leu>Pro	FBSN, MILS
11777C>A	*MT-ND4*	Arg>Ser	MILS
11778G>A	*MT-ND4*	Arg>His	LHON
12315G>A	*MT-TL2*		CPEO
12706T>C	*MT-ND5*	Phe>Leu	MILS
13513G>A	*MT-ND5*	Asp>Asn	MELAS, MILS
13514A>G	*MT-ND5*	Asp>Gly	MELAS
14459G>A	*MT-ND6*	Ala>Leu	LDYT, MILS
14482C>G	*MT-ND6*	Met>Ile	LHON
14484T>C	*MT-ND6*	Met>Val	LHON
14487T>C	*MT-ND6*	Met>Val	Dystonia, MILS
15498G>A	*MT-CYB*	Gly>Asp	Histiocytoid cardiomyopathy

Abbreviations: AMDF, ataxia, myoclonus, and deafness; ATP6, ATP synthase F_0 subunit 6; CPEO, chronic progressive external ophthalmoplegia; CYB, cytochrome *b*; DEAF, maternally inherited deafness or aminoglycoside-induced deafness; DM, diabetes mellitus; DMDF, diabetes mellitus+deafness; FBSN, familial bilateral striatal necrosis; KSS, Kearns–Sayre's syndrome; LDYT, Leber hereditary optic neuropathy and dystonia; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, myopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibers; MICM, maternally inherited cardiomyopathy; MILS, maternally inherited Leigh syndrome; MM, mitochondrial myopathy; MMC, maternal myopathy and cardiomyopathy; mtDNA, mitochondrial DNA; ND, NADH dehydrogenase subunit.
Abbreviations and information about mutations are annotated in the MITOMAP database.

polymorphisms at least in an East Asian population. As shown in Table 2, this detection system simultaneously identified 29 different heteroplasmic or homoplasmic mutations in 11 genes: one in each of *MT-ND1*, *MT-ATP6*, *MT-CYB*, *MT-RNR1*, *MT-TV* ($tRNA^{Val}$) and *MT-TL2* ($tRNA^{Leu(CUN)}$) genes; two in the *MT-ND4* gene; three in the *MT-ND5* gene; four in each of *MT-TK* ($tRNA^{Lys}$) and *MT-ND6* genes; and 10 in the *MT-TL1* ($tRNA^{Leu(UUR)}$) gene. All of the 29 mtDNA mutations are listed in the MITOMAP. The median fluorescence intensity (MFI) values were calculated from an analysis of 50 microspheres of each set, which represented 50 replicate measurements, using a flowmeter equipped with a Luminex XY Platform plate reader and the Luminex 1.7 proprietary software package.[23,24] Both the MFI values for the two corresponding alleles were displayed on scatter diagrams. The mutations were considered to be heteroplasmic when both the mutant and wild-type signal intensities were detected above the cutoff values. In contrast, the mutations were homoplasmic when the mutant signal intensities were above the cutoff values, whereas the wild-type signals were below them.

Comparison of results between suspension array and direct DNA sequencing

The DNA sequences were analyzed by using an Applied Biosystems 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and Sequencher version 4.2.2 (Gene Codes, Ann Arbor, MI, USA) to compare the sequences with the revised Cambridge reference sequence,[25,26] while following the standard procedure.[27,28]

Quantification of mutation load of m.3243A>G. by restriction fragment length polymorphism analysis

The last cycle PCR-restriction fragment length polymorphism analysis on mtDNA was conducted with the m.3243A>G mutation in *MT-TL1* gene to compare MFI values, obtained by the m.3243G detection system, with the mutation loads. The 3243A>G mutant mtDNA introduces an *Apa*I restriction site detectable by restriction fragment length polymorphism analysis. The *Apa*I-digested 6-carboxyfluorescein-labeled PCR products were analyzed with the Applied Biosystems 3130*xl* Genetic Analyzer using GeneMapper software program Version 4.0 (Applied Biosystems) to quantify the proportion of the mutation as a percentage, as described previously.[20] The mutation load was also compared with the pure-tone average value.

RESULTS

Five of the 29 mutations were detected in 23 of 373 patients with SNHL by the extensive and rapid screening system (Table 3). All 373 DNA samples were analyzed by the m.1555A>G mutation detection system and the results were displayed in the scatter diagram, as shown in Figure 1a. Only the MFI value of the m.1555A>G homoplasmic mutation was detected in 11 samples (2.9%). Only 2 of the 11 patients with the m.1555A>G mutation had an apparent history of aminoglycoside exposure.

The relationship between the mutation loads and signal intensity values (MFI: 9190 ± 1590, mean ± s.d.) in the nine test DNA samples with the m.3243A>G mutation (mutational load: 23.1% ± 16.5, mean ± s.d.; range: 4–59%) was evaluated to verify the detection level of the 3243A>G detection system. The signal intensity values correlated significantly with the mutation loads (R=0.80 and P<0.001). The minimum detection limit of the m.3243A>G mutation was estimated to be approximately 2% (Supplementary Figure 1). As shown in Figure 1b, results obtained by the m.3243A>G detection system were displayed in a scatter diagram. Both mutant- (m.3243G) and wild-type-positive (m.3243A) signals were detected in nine (2.4%) samples, thus this mutation was considered heteroplasmic. The mutation loads of the m.3243A>G detected in these nine patients were between 16 and 46%, as determined by the last cycle PCR-restriction fragment length polymorphism analysis (Table 4). The clinical findings in these nine patients were evaluated with the m.3243A>G mutation, as shown in Table 4. There was no correlation between the mutation loads in the DNA extracted from the blood and the clinical severities of those patients. The onset age of HL ranged from 10 to 42 years. Although four patients suffered from diabetes and two patients showed renal dysfunction, other three patients presented isolated HL without a family history of HL.

Interestingly, one sample each harbored the m.8348A>G mutation in the *MT-TK* ($tRNA^{Lys}$) gene (Figure 2a), the m.11778G>A mutation in the *MT-ND4* gene (Figure 2b) and the m.15498G>A mutation in the *MT-CYB* gene (Figure 2c). None of these three mutations had been suspected until the samples were analyzed by this screening system. The sample with m.8348A>G and that with m.15498G>A were revealed as either homoplasmy or heteroplasmy with a high mutational load. The mutation load of the m.11778G>A

Table 3 The 373 DNA samples with hereditary hearing loss screened by the extensive detection system for 29 mtDNA mutations and direct DNA sequencing

mtDNA mutation	*Number*	*Frequency (%)*
m.1555A>G	11	2.9
m.3243A>G	9	2.4
m.8348A>G	1	0.3
m.11778G>A	1	0.3
m.15498G>A	1	0.3
Undetected	350	93.8

Abbreviation: mtDNA, mitochondrial DNA.

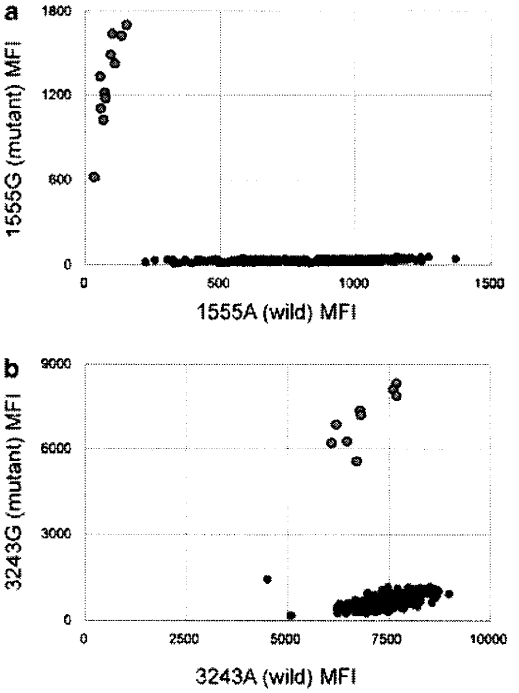

Figure 1 Scatter diagrams with the mutant median fluorescence intensity (MFI) values on the *y*-axis and the wild-type ones on the *x*-axis for the 1555A>G homoplasmic mutation (**a**) and the 3243A>G heteroplasmic mutation (**b**). All 373 DNA samples were analyzed by the m.1555A>G and m.3243A>G mutation detection systems using universal 96-well plates. Each result was merged into the two separate scatter diagrams. Red circles indicate MFI values of mutation-positive DNAs. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 4 Clinical and genetic features of the nine HL patients with m.3243A>G heteroplasmic mutation detected by the extensive mtDNA pathological mutation screening system

TMD no.	*Sex*	*PTA right (dB)*	*PTA left (dB)*	*Onset of HL (y)*	*DM*	*Renal dysfunction*	*Family history*	*Mutation load (%)*
18	M	56.3	51.3	42	+	–	–	16
22	F	30	30	25	–	–	–	26
27	M	28.8	36.3	Unknown	–	+	+	24
49	F	46.3	56.3	Childhood	+	–	+	8.6
169	F	57.5	60	10	+	–	+	25
219	F	62.5	87.5	40	–	–	–	17
268	F	31.3	38.8	27	+	–	–	23
325	M	55.0	53.8	25	–	–	–	43
583	F	43.8	45	31	–	+	+	46

Abbreviations: DM, diabetes mellitus; F, female; HL, hearing loss; M, male; mtDNA, mitochondrial DNA; PTA, pure-tone average; RFLP, restriction fragment length polymorphism.
The 3243A>G mutation loads were evaluated by RFLP analysis. Two patients, TMD 219 and 325, were not examined on pure-tone audiometry in our hospital.

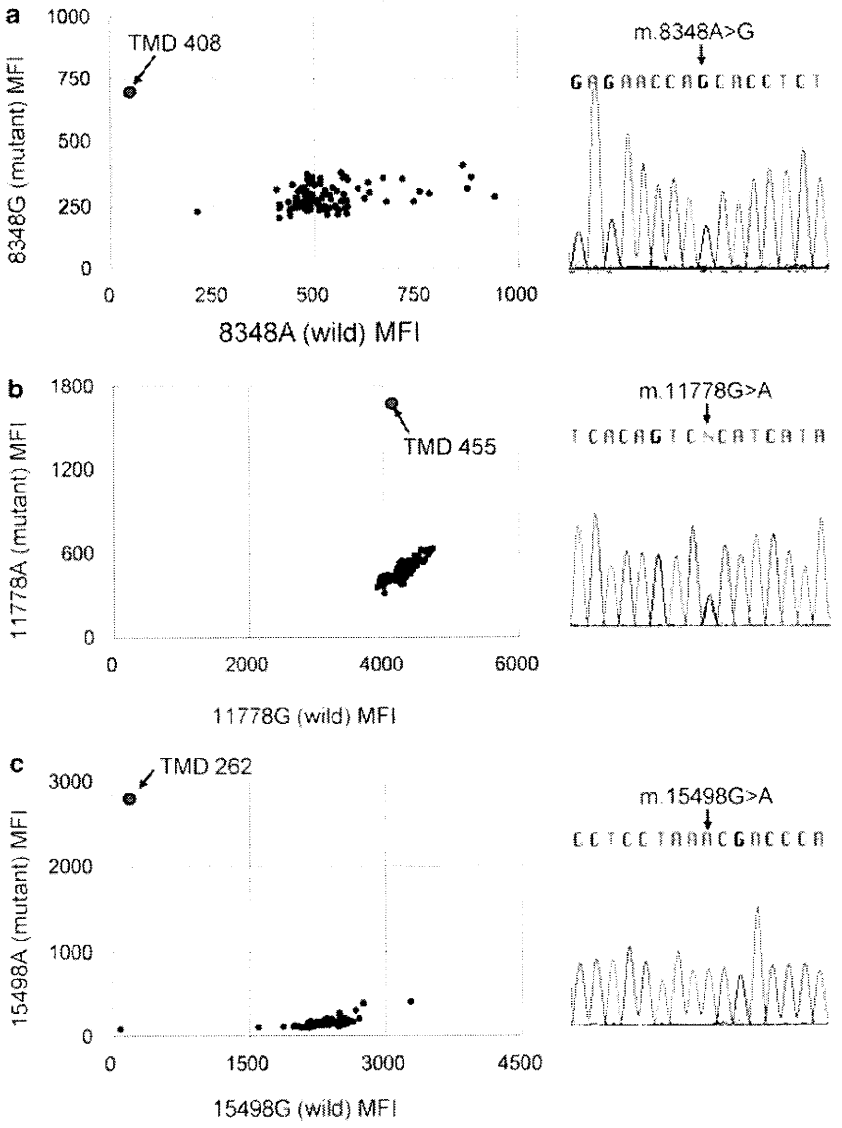

Figure 2 Scatter diagrams with the mutant median fluorescence intensity (MFI) values on the *y*-axis and wild-type ones on the *x*-axis and the electropherograms of DNA sequences for the 8348A>G homoplasmic mutation (**a**), the 11778G>A heteroplasmic mutation (**b**) and the 15498G>A homoplasmic mutation (**c**). Red circles indicate MFI values of mutation-positive DNAs. The samples were analyzed with an Applied Biosystems 3130*x*/ Genetic Analyzer (Applied Biosystems) and Sequencher version 4.2.2 (Gene Codes).

heteroplasmy was approximately 45%, as determined on the electropherogram of the DNA sequence. None of the 29 major mtDNA mutations was detected in the other 350 (93.8%) patients among the patients with suspected hereditary HL.

A total of 10,817 mutant sites in 373 DNA samples were screened using the system for detecting the 29 major mtDNA mutations. Importantly, 99.8% of the targeted mutant sites were detected by the system and only 25 (0.2%) of the mutant sites in 15 DNA samples could not be determined, because the signals of both wild-type and mutant were negative. In each instance, the DNA fragment flanking the putative mutation sites was sequenced and rare polymorphisms were found within the binding sites for both the mutant-specific and the wild-type-specific probes. Conventional DNA sequencing confirmed all the mtDNA mutations detected by this system. The evaluation of the system in this study was initially limited because DNA samples with the other mtDNA mutations were not available. The oligonucleotides complementary to the mutant-specific probes were synthesized to overcome that problem. This confirmed the specificity of all the probes for these mutations (data not shown).

DISCUSSION

This study screened 373 DNA samples from patients with suspected hereditary HL using the extensive and rapid detection system for 29 major mtDNA mutations. Accordingly, m.1555A>G and m.3243A>G mutations were detected in 11 (2.9%) and 9 (2.4%) patients, respectively. In addition, three other mutations (m.8348A>G, m.11778G>A and m.15498G>A) were detected by this screening system.

Sensorineural hearing loss is a quite common clinical feature in patients with mitochondrial diseases caused not only by m.1555A>G and m.3243A>G mutations, but also by the other mtDNA mutations. According to the most recent study on this topic, the prevalence of the m.1555A>G mutation is 0.21% in adults of European descent in Australia.[29] In Japan, the mutation was detected in 3% of all patients with SNHL.[30] As patients with the m.1555A>G mutation are susceptible to aminoglycoside ototoxicity,[7–9] screening for this mutation is therefore beneficial for people who are going to be administered aminoglycoside. Furthermore, this mutation has also been demonstrated in patients with non-syndromic HL without defined past medication histories of aminoglycoside, and even in patients without any apparent maternal inheritance.[9–12]

In contrast, the m.3243A>G mutation causes several major clinical phenotypes of mitochondrial disease such as myopathy, encephalopathy, lactic acidosis, stroke-like episodes, maternally inherited diabetes and deafness, chronic progressive external ophthalmoplegia and mitochondrial diabetes.[31–33] However, epidemiological evidence of the prevalence of the patients with the m.3243A>G mutation in the population still has limitations. The patients with m.3243A>G including asymptomatic cases in the general population are thought to be as common as 6.57–16.3 per 100 000.[34–36] According to the findings of recent epidemiological studies, however, Manwaring *et al.*[36] reported that the population prevalence was much higher, at 236/100 000. A population-based study of the m.3243A>G mutation revealed the mutation in 7.4% of patients with maternally inherited HL in northern Finland.[38] In this study, this mutation was demonstrated to have a prevalence of 2.4% in the cases of Japanese hereditary HL, and this frequency is very close to that previously reported in Japan.[39]

This screening system detected the m.8348A>G mutation in the *MT-TK* gene in a 6-year-old boy (patient TMD 408) with SNHL (Figure 2a). The m.8348A>G heteroplasmic mutation was reported in a patient with severe cardiomyopathy in adulthood.[40] In this study, the proband presented with SNHL at age of 3 years; but he did not display any other clinical features including cardiac symptoms. Both the 32-year-old mother and younger sister (II-2 and III-2, respectively, in Figure 3a) of the proband (TMD 408, III-1) also suffered from HL. The results of audiometry showed that the proband demonstrated a high-frequency SNHL, whereas his mother (II-2) showed moderate SNHL at the frequencies of 2 and 4 kHz. He had no apparent family history of cardiomyopathy. The grandfather (I-1) of the proband probably suffered from HL of an unknown origin, although his hearing level was not examined by audiological testing. The m.8348A>G homoplasmic transition is one of the characteristic mtSNPs of mitochondrial haplogroup H1b, and the pathogenicity of this mutation still remains controversial.[41,42] The clinical course of this patient must therefore be followed closely and further detailed investigation is required.

The m.11778G>A mutation in the *MT-ND4* gene was detected in a 28-year-old female (TMD 455, Figure 2b). At age 10, the proband developed a hearing impairment and was diagnosed to have renal dysfunction at 12 years of age. The mother (I-2 in Figure 3b), older sisters (II-2 and -4) and nieces (III-1 and -2) of the proband also suffered from HL. Her audiogram showed profound bilateral SNHL (Figure 3b). The m.11778G>A mutation in *MT-ND4* gene is the most common mutation in patients with Leber hereditary optic neuropathy.[43,44] Although impaired hearing does not generally accompany Leber hereditary optic neuropathy,[45,46] appreciable progressive auditory neuropathy in two patients with Leber hereditary optic neuropathy has been reported.[47] However, the proband (TMD 455) in this study had never complained of any visual problems as is normally observed in cases with Leber hereditary optic neuropathy, nor did any of her family members. The clinical courses of the proband and her family must therefore be closely followed because the profound HL of the proband differed from the moderate HL in the reported cases.

The screening system detected the m.15498G>A mutation in the *MT-CYB* gene in a 54-year-old female (TMD 262) with HL. The m.15498G>A (p.MT-CYB: Gly251Asp) mutation can cause histiocytoid cardiomyopathy that usually occurs in infancy or childhood.[48,49] In this study, the proband developed HL at the age of 35 years. The results of the pure tone audiogram showed high-frequency SNHL (Figure 3c). Her electrocardiogram indicated Wolff–Parkinson–White syndrome but no apparent evidence of cardiomyopathy. The proband's sister (II-4 in Figure 3c) was reported to have HL, whereas the cause of HL in their 77-year-old father (I-1) may not be the same as the proband and her sister. Assuming that the HL in the proband and her sister (II-4) is caused by the m.15498G>A mutation, the HL of the proband's father probably has a different cause. In addition, none of the children of proband was found to have HL. It is therefore necessary to follow the clinical courses of both the proband and her family.

Over the past 5 years, new technologies have been developed to screen for mtDNA mutations, including denaturing high-performance liquid chromatography;[50,51] a detection system using a mismatch-specific DNA endonuclease;[52] the Biplex Invader assay by hybridization of two overlapping oligonucleotides to the target sequence;[53] the matrix-associated laser desorption/ionization time of flight mass spectrometry assay;[54] the pyrosequncing technology to detect and estimate heteroplasmic mtDNA point mutations;[55] and an entire mtDNA resequencing chip: Mitochip.[56,57] The Mitochip uses hybridization for detecting mutations or polymorphisms, so that the probes

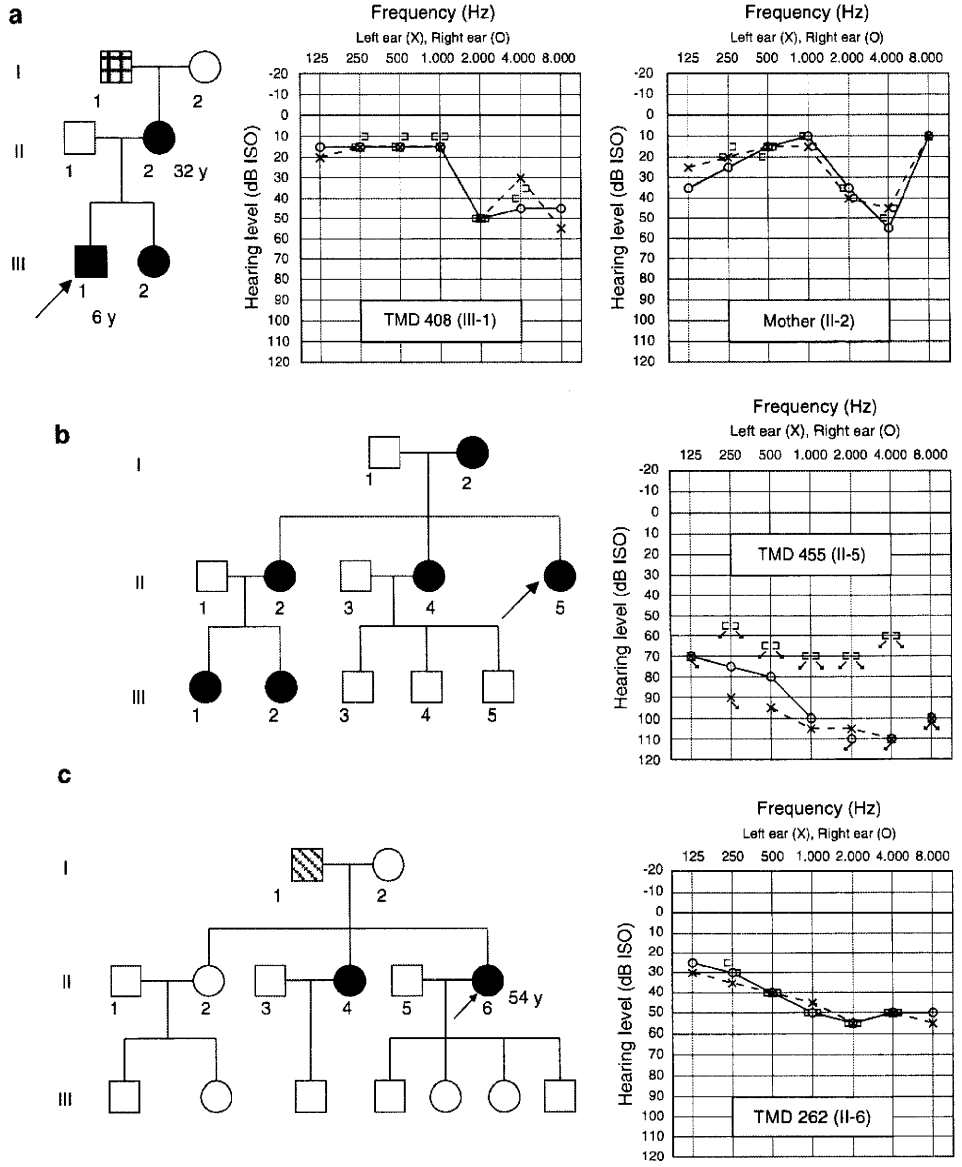

Figure 3 Pedigrees of the families and audiograms of patient TMD408 and his mother (**a**), patient TMD455 (**b**) and patient TMD262 (**c**). Clinical features are depicted: black-filled circles or squares as individuals with deafness, and pattern-filled squares as individuals with deafness of unknown cause. Arrows indicate probands. KEY on pure tone audiograms: dB, decibel hearing loss; ISO, international standards organization;], left ear bone conduction; [, right ear bone conduction; O, right ear air conduction; X, left ear air conduction.

should be designed.[56] Although version 2.0 of Mitochip has been improved for detecting mutations, the present version has not been designed for analysis of Asian mtDNA, as it is based on the revised Cambridge reference sequence.[25,26] As it is necessary to distinguish pathological mtDNA mutations from non-pathogenic mtSNPs, this study used 11 pairs of primers for multiple PCR amplifications and multiple sequence-specific oligonucleotide probes customized for the Japanese population, which were designed to carefully detect either mutant or wild-type mtDNA, even when mtSNPs were present in the vicinity of the mutation sites.

In terms of epidemiological studies, the extensive and rapid mtDNA mutation detection system using the suspension array reported in this study is suitable for large-scale screening owing to the following three advantages: first, the universal 96-well plates are available for the analysis. Second, the analysis of each plate can be completed within 1 h. Third, this system is adequate to detect heteroplasmic mutations that are a common type of mtDNA pathological mutation. The nine samples with the m.3243A>G mutation revealed positive signals with both mutant- and wild type-specific probes in this detection system. The detection limit of m.3243A>G was estimated to be approximately only 2%. The mtDNA mutation load in the impaired tissue of the patients is thought to closely correlate with the severity. In this study, however, no correlation was observed between the percentage of the m.3243A>G mutation in the DNA extracted from the blood and the clinical severities of the patients.

In conclusion, an extensive and rapid screening system using a suspension array for 29 major mtDNA mutations in patients with HL was found to be useful for the diagnosis and epidemiological study of both syndromic and non-syndromic HL. The diagnosis of mitochondrial disease is complex, but detecting mtDNA mutations in the early

stage of HL using this screening system is considered to be extremely important to select the optimal therapeutic strategies and also provide the appropriate genetic counseling for the patients.

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Review Article

Genome-Wide Association Study of Coronary Artery Disease

Naomi Ogawa,[1,2] Yasushi Imai,[1,2] Hiroyuki Morita,[1,3] and Ryozo Nagai[1,2]

[1] *Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan*
[2] *Division of Clinical Genome Informatics, Graduate School of Medicine, The University of Tokyo, Japan*
[3] *Department of Translational Research for Healthcare and Clinical Science, Graduate School of Medicine, The University of Tokyo, Japan*

Correspondence should be addressed to Ryozo Nagai, nagai-tky@umin.ac.jp

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Coronary artery disease (CAD) is a multifactorial disease with environmental and genetic determinants. The genetic determinants of CAD have previously been explored by the candidate gene approach. Recently, the data from the International HapMap Project and the development of dense genotyping chips have enabled us to perform genome-wide association studies (GWAS) on a large number of subjects without bias towards any particular candidate genes. In 2007, three chip-based GWAS simultaneously revealed the significant association between common variants on chromosome 9p21 and CAD. This association was replicated among other ethnic groups and also in a meta-analysis. Further investigations have detected several other candidate loci associated with CAD. The chip-based GWAS approach has identified novel and unbiased genetic determinants of CAD and these insights provide the important direction to better understand the pathogenesis of CAD and to develop new and improved preventive measures and treatments for CAD.

1. Introduction

Coronary artery disease (CAD) including myocardial infarction (MI) is a leading cause of death worldwide [1, 2]. The well-known conventional coronary risk factors include age, male sex, hypertension, diabetes mellitus, hypercholesterolemia, smoking and family history, which have been repeatedly demonstrated in multiple epidemiological studies [3–5]. Lifestyle and environmental factors play an important role in the pathogenesis; however, genetic predisposition is also thought to contribute to CAD/MI since these diseases cluster in families [6].

In the epidemiological studies using twins, the relative hazard of death among men from CAD when one's twin died of CAD before the age of 55 years, as compared with the hazard when one's twin did not die before 55, was 8.1 for monozygotic twins and 3.8 for dizygotic twins [7]. The recent epidemiological survey in the Framingham study showed that parental cardiovascular disease independently predicted future offspring events [8]. In this survey, participants with at least one parent with premature CAD had greater risk for events with age-adjusted odds ratios (ORs) of 2.6 for men and 2.3 for women compared with those with no parental CAD. These results support further research into genetic determinants of CAD risk. Elucidating the genetic determinants would improve risk assessment and provide better measures for prevention and treatment.

As the molecular biology and genetics had progressed, the genetic backgrounds were explored in the several genes which were thought to contribute to the pathogenesis of atherosclerosis and conventional coronary risk factors. Candidate gene studies tested the hypothesis that proteins known to be involved in the pathogenesis of atherosclerosis carry variants that affect their protein functions and the risk of developing CAD. In 1992, Cambien et al. [9] explored a possible association between CAD and a variation found in the gene encoding angiotensin-converting enzyme (ACE). The polymorphism ACE/insertion/deletion (ACE/ID) is strongly associated with the level of the circulating enzyme. They reported that the deletion homozygote (DD genotype),

which was associated with higher levels of circulating ACE, is significantly more frequent in patients with MI than in controls.

The representative variants associated with CAD/MI found by candidate gene approach are listed in Table 1. However, those candidate genes were not always reproducible in multiple studies later on. One of the reasons for poor reproducibility is that many of the study samples were not large enough with some exceptions to identify disease-associated genetic variants with odds ratio <2.0. In addition, candidate gene studies only tested a single to few variants for association with CAD and these approaches cannot discover unknown novel variants and also cannot evaluate how strong each variant contribute to the susceptibility to CAD. Therefore, the candidate gene approach resulted in only limited success in the elucidation of genetic risks for CAD.

In parallel with candidate gene studies, other strategies were carried out to interrogate the entire human genome without hypotheses on which genes may be responsible for disease risk. One of the strategies is genome-wide linkage analysis and it is based on the Mendelian cosegregation of a genetic marker within a family. However, great efforts had to be made in order to collect sufficient numbers of affected sibling pairs. Only a small number of studies were successfully performed and few genetic loci (2q21-22, Xq23-26 [10], myocyte enhancer factor-2 (*MEF2A*) [11], arachidonate 5-lipoxygenase-activating protein (*ALOX5AP*) [12], leukotriene A4 hydrolase (*LTA4H*) [13]) were detected to be associated with CAD (Table 2). These genes had never been suggested as causative genes before these family-based studies, suggesting the effectiveness of this approach in detection of novel genetic determinants. However, those associations were not always replicated. Such a family-based study has been frequently used to identify new loci in monogenic diseases, but the application of this strategy to multifactorial diseases is relatively limited.

In this situation, whole genome analysis in a case-control study design had gradually emerged around the beginning of the 21st century. Resources including the single nucleotide polymorphism (SNP) databases, major technological advances in high-throughput genotyping, and methods of data processing and statistical analysis allow researchers to confront limitations in previous approaches. Here, we introduce the representative genome-wide case-control association studies in the next two sections.

2. Establishment of J-SNP Database and Whole Genome Approach in Japan

Beginning in 2000, the Prime Minister's Millennium Project (J-SNP) was launched in Japan and about hundred thousand SNPs located in genes or in adjacent regions that might influence the coding sequence of the genes were identified in Japanese population. J-SNP established a web-based database and allowed researchers access to high quality SNP data [14]. Genome-wide association studies using this SNP database were performed in our country in many important

Table 1: The well-known genetic polymorphisms which are thought to be associated with myocardial infarction or coronary artery disease.

Location	Gene name/Polymorphisms
17q23	Angiotensin-Converting Enzyme insertion/deletion (intron 16)
1q42-q43	Angiotensinogen Met235Thr, −6G/A
3q21-q25	Angiotensin II type1 Receptor 1166A/C
8q21-q22	Aldosterone Synthase (CYP11B2) −344T/C, Lys173Arg
14q32.1-q32.2	Bradykinin B2 receptor gene −58T/C
6p24.1	Endothelin-1 Lys198Asn
7q36	eNOS Glu298Asp, −786T/C
17q21.32	Glycoprotein IIIa P1A1/A2
5q23-31	Glycoprotein Ia 807T/C
17pter-p12	Glycoprotein Ibα Thr145Met
4q28	βfibrinogen −455G/A
11p11-q12	Prothrombin 20210G/A
7q21.3-q22	PAI-1 4G/5G (promoter region)
7q21.3	Paraoxonase1 Arg192Gln, Leu54Met
8p12-p11.2	Werner Helicase Gene Cys1367Arg
1p36.3	Methylenetetrahydrofolate reductase 677C/T
16q24	NADH/NADPH oxidase p22phox 242C/T, 640A/G
5q31.1	CD14 Monocyte Receptor −260C/T
11q22.3	Stromelysin (MMP3) 5A/6A (promoter region)
20q11.2-q13.1	Gelatinase B (MMP9) −1562C/T
19q13.2	ApolipoproteinE E2/E3/E4
16q21	Cholesteryl Ester Transfer Protein (CETP) Ile405Val
9q31.1	ABCA1 gene Ile823Met
3p25	PPAR-gamma Pro12Ala, Pro115Gln
20q13.11-q13.13	Prostacyclin synthase gene
The number of 9-bp (CCGCCAGCC) repeats (promoter region)	
17q11.2-q21.1	MCP-1 −2518G/A

Table 2: Representative loci associated with CAD identified in the family-based studies.

Reporter (year)	Race	locus	LOD score	causative gene
Pajukanta et al. [10]	Finland	2q21-22	3.7	no gene identified
		Xq23-26	3.5	no gene identified
Wang et al. [11]	USA	15q26	4.19	MEF2
Helgadottir et al. [12]	Iceland	13q12-13	2.86	ALOX5AP
Helgadottir et al. [13]	Iceland	17q22	NA	LTA4H

NA = not available.

clinical fields including cardiovascular diseases, diabetes, renal dysfunction and autoimmune collagen diseases.

As the first genome-wide case-control association study in the world, Ozaki et al. [15] used 92,788 gene-based SNP markers and identified that the homozygosity in two SNPs in lymphotoxin A (*LTA*) at 6p21 was significantly associated with increased risk for MI in Japanese (odds ratio (OR) = 1.78). In vitro analyses showed that one functional SNP (Thr26Asn) caused a twofold increase in induction of several inflammation-related cell-adhesion molecules including vascular cell adhesion molecule 1 (VCAM1) in vascular smooth-muscle cells. Moreover, the SNP located in intron 1 of LTA enhanced the transcriptional level of LTA. These results indicated the variants in the LTA are risk factors for MI and implicated LTA as a novel pathogenic factor for MI. In the same year, LTA knockout mouse was shown to be resistant to atherosclerosis [16]. Double knockout mice of apolipoprotein E (ApoE) and LTA (ApoE−/−LTA−/− mice) showed less extent of atherosclerosis than ApoE−/−LTA+/+ mice, indicating LTA deteriorates atherosclerosis in vivo, consistent with the result of the genetic association study of LTA as a genetic risk for atherosclerotic disease.

Subsequently, they identified SNPs that were significantly associated with MI in lectin, galactoside-binding, soluble, 2 gene (*LGALS2*) [17], proteasome subunit alpha type 6 gene (*PSMA6*) [18], myocardial infarction associated transcript (*MIAT*) [19], inter-alpha (globulin) inhibitor 3 gene (*ITIH3*) [20] and BRCA1-associated protein gene (*BRAP*) [21] mainly by functional approaches. All the six causative genetic regions are related to inflammatory process in vasculature and are thought to contribute to the process in atherosclerotic changes through its inflammatory functions and thus increase the risk of MI.

3. Chip-Based GWAS and Novel Candidate Genetic Determinants for CAD

3.1. HapMap Project. Genome-wide association studies based on the J-SNP database (approximately 100,000 SNPs) have detected several SNPs which had significant association with myocardial infarction. However, the J-SNP database does not cover SNPs in intergene regions. In the meantime, the International HapMap Project was conducted to create a public genome-wide database of common SNPs and enable systematic studies of common SNPs for their potential role in human disease [22, 23]. The Project analyzed DNA samples from 90 people with European ancestry, 90 Yoruba people in Nigeria, 44 Japanese and 45 Han Chinese and has now genotyped over 3.1 million SNPs in each of these populations. However, testing all of these SNPs in a person's chromosomes would be extremely expensive. Adjacent SNPs across the genome are correlated each other, a phenomenon known as linkage disequilibrium and SNPs that are inherited together were compiled into "haplotypes". A haplotype block may contain many SNPs, but only a few "tag" SNPs can provide most of the information on the pattern of genetic variation in the block. The HapMap project identified these "tag" SNPs within haplotypes that uniquely identify these haplotypes. The HapMap data allowed efficient design of Chip-based genome association studies and allowed investigators to genotype far fewer SNPs while still retaining statistical power to find genetic variants related to common illness.

3.2. High throughput SNP Genotyping Platforms. The development of dense genotyping chips enables genotyping up to 1 million SNPs on a single small chip. This chip technology allowed genome-wide association studies (GWAS) to be performed on a large numbers of subjects. Chip-based GWAS typically involves genotyping approximately a few thousands cases with a disease and a few thousands of controls for about 500,000 tag SNPs. Since there are 500,000 comparisons per study, there is a high potential for false positive results. The proposed solution for that is applying the stringent P value using the Bonferroni correction for multiple tests. In that case, P value will be 0.05 divided by 500,000 and that is 0.0000001 (10^{-7}) and this stringent P value is often termed as 'genome-wide significance'. The most statistically significant variants identified in the initial case-control analysis are tested for replication in subsequent case-control studies. In GWAS method, associations between SNPs and the diseases are made free of bias of particular candidate genes. This makes the possibility of obtaining novel and unbiased information and provides the important direction to better understand the pathophysiology of the disease. For CAD, three chip-based GWAS were simultaneously reported in 2007 and all of them showed the significant association between CAD and SNPs on chromosome 9p21.

3.3. 9p21 and other Chromosomal Loci Associated with CAD/MI Detected in Chip-Based GWAS. Helgadottir et al. enrolled a total of 4587 MI cases and 12,767 controls and genotyped total 305953 SNPs using Illumina Hap300chip (Illumina) [24] (Table 3). All the participants were European descent. They identified disease association variant located in 9p21, adjacent to the tumor suppressor genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) with great statistical significance. This region had never been estimated to be associated with susceptibility to MI. They showed the allele G of the SNP rs10757278 (Figure 1) showed the strongest association with MI. The ORs for heterozygous and homozygous carriers of the risk allele G were 1.26 and 1.64, respectively. The ORs for early-onset MI (MI before the age of 50 for males and before the age of 60 for females) are 1.49 and 2.02 for heterozygous and homozygous carriers of the risk allele, respectively. They estimated the population attributable risk is 21% for MI in general and 31% for early-onset cases.

The SNPs on chromosome 9p21 associated with MI are located in the same disequilibrium block of the one which contains *CDKN2A* and *CDKN2B*. These genes encode two members of the inhibitors of CDK4 (Ink4) family of cyclin-dependent kinase inhibitors, $p16^{Ink4a}$ and $p15^{Ink4b}$, and a completely unrelated protein called ARF. The $p16^{Ink4a}$ and $p15^{Ink4b}$ which activates retinoblastoma (Rb) family members and ARF which activates p53 were shown to be upregulated in cancer cells. They play a critical role in cell proliferation and aging, senescence and apoptosis [25, 26]. However, sequencing 93 early-onset MI patients across these genes did

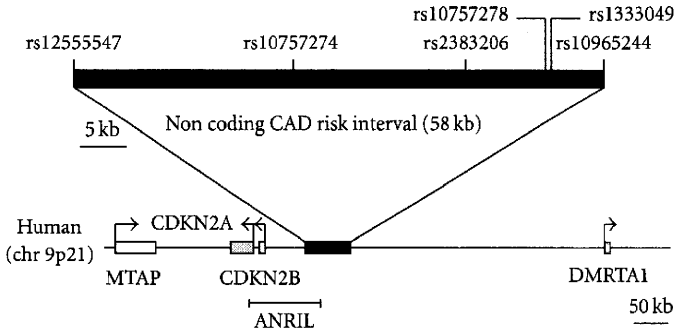

FIGURE 1

FIGURE 1: The 58 kb non-coding CAD risk interval on chromosome 9p21.

not reveal obvious causal functional variants or variants that could account for the correlation of rs10757278 to MI. The linkage disequilibrium block also contains two exons of the transcript hypothetical methylthioadenosine phosphorylase fusion protein mRNA, however the functional significance of the variants in this region remains to be elucidated.

McPherson et al. also identified a 58-kilobase region on chromosome 9p21 that was consistently associated with CAD in six independent samples consisted of 4306 cases and 20119 controls from four Caucasian populations [27]. They identified two SNPs rs10757274 and rs2383206 on 9p21 that are significantly associated with incident of CAD. The risk allele was associated with and ~15 to 20% increase risk of CAD in the 50% individuals who are heterozygous and ~30 to 40% increase in the 25% who are homozygous for the allele.

They further genotyped surrounding region of these SNPs in detail and found that eight additional SNPs at the locus spanning a 58-kb region were significantly associated with CAD. Again, the 58-kb region does not contain any annotated genes. however, the region overlaps a newly annotated noncoding RNA called noncoding RNA in the *INK* locus (*ANRIL*) [28]. *ANRIL* consists of 20 exons subjected to alternative splicing. The whole blood RNA expression levels of short variants of *ANRIL* are increased and the expression levels of the long variant are decreased in subjects homozygous for the risk alleles. There is also a positive correlation between transcript levels of the long variant of *ANRIL* and *CDKN2B* [29].

The study using genetically engineered mice showed that the deletion of orthologous 70-kb non-coding interval on mouse chromosome 4 (chr4$^{\triangle 70kb/\triangle 70kb}$mice) highly reduced cardiac expression of two neighboring genes *CDKN2A* and *CDKN2B*. Primary culture of smooth muscle cells from chr4$^{\triangle 70kb/\triangle 70kb}$mice showed increased proliferation and diminished senescence, the features relevant to atherosclerosis [30]. These findings indicated the noncoding interval is involved in the disease process via gene-regulatory effects on *CDKN2A* and *CDKN2B*.

The Wellcome Trust Case Control Consortium (WTCCC) study which enrolled 1926 case subjects with CAD and 2938 controls also reported the powerful association between the SNPs on chromosome 9p21 and CAD [31]. The strongest signal was seen at rs1333049, however the associations were seen for SNPs across >100 kb. Then, they further looked for replication in the German MI family study which involved 875 MI cases and 1644 controls using the GeneChip Human Mapping 500K Array Set (Affymetrix) [32]. The Same locus on chromosome 9p21 (rs1333049) had the strongest association with CAD in both studies with the risk increased by 36% per copy of the C allele. Of the nine loci which were shown to be strongly associated with CAD, two of these loci were able to replicate in the German MI study: chromosome 6q25.1 (rs6922269) and chromosome 2q36.3 (rs2943634). Further, the combined analysis of the two studies revealed four additional loci significantly associated with CAD: chromosome 1p13.3 (rs599839), 1q41 (rs17465637), 10q11.21 (rs501120) and 15q22.33 (rs17228212). In 2009, they performed another replication study with 11550 cases with CAD and 11205 controls from 9 European studies [33]. Other than the 9p21 locus, they confirmed significant association at 1p13.3 (rs599839), 1q41 (rs3008621) and 10q11.21 (rs501120). They were not able to show the significant association with 6q25.1 and 2q36.3 and there was no evidence for association with the locus at 15q22.33. The four loci (9p21, 1p13.3, 1q41 and 10q11.21) act independently and cumulatively increased the risk for CAD by 15% per additional risk allele. The genes located within or adjacent to the these four loci are listed in Table 4. The locus at chromosome 1p13.3 has been shown to be associated with increased plasma LDL cholesterol, and thus may contribute to CAD development [34–37].

The locus at 10q11.21 lies adjacent to the chemokine (C-X-C motif) ligand 12 gene (*CXCL12*) which encodes stromal cell-derived factor-1, a chemokine which plays a important role in stem-cell homing and regeneration of myocardial tissue in ischemic cardiomypopathy [38] and in promoting angiogenesis by recruiting endothelial progenitor cells from the bone marrow [39]. The SNPs at 1q41 locates within the melanoma inhibitory activity family, member 3 (*MIA3*) gene [40]. Underlying mechanism how these genetic loci affect the pathogenesis of CAD need to be further investigated.

The meta-analysis of aforementioned three studies [24, 27, 32] and 7 additional case-control studies successfully

Table 3: Representatives of chip-based GWAS of CAD/MI.

Author, year	Phenotype	No. of cases/controls	Chromosomal loci	OR
Helgadottir et al. [24]	MI	4587/12767	9p21	1.28
McPherson et al. [27]	CAD	3505/18745	9p21	1.26 (CCHS study)
			9p21	1.16 (CCHS study)
WTCCC [31]	CAD	2000/3000	9p21.3	1.47
Samani et al. [32]	CAD	2801/4582	9p21.3	1.28 (adjusted German)
			6q25.1	1.23 (adjusted German)
			2q36.3	1.08 (adjusted German)
			1p13.3	1.29
			1q41	1.2
			10q11.21	1.33
			15q22.33	1.21
CAD Consortium, [33]	CAD	11550/11205	9p21	1.2
			1p13.3	1.13
			1p41	1.1
			10q11.21	1.11
Clarke et al. [59]	CAD	7991/7946	6q26-27	1.51

Table 4: Genes located within or adjacent to the loci associated with CAD/MI.

Chromosome loci	Genes
1p33	PSRC1, CELRS2, MYBPHL, SORT1
1q41	MIA3
2q33	WDR12
2q36.3	no recognized genes
3q22.3	MRAS
6p24	PHACTR1
6q25.1	MTHFD1L
6q26-27	LPA
9p21	p16/CDKN2A, p15/CDKN2B, p14/ARF, MTAP, ANRIL
10q11.21	CXCL12
12q24	3SH2B3
21q22	MRPS6, SLC5A3, KCNE2

replicated the significant association between the risk allele (C) of the lead SNP, rs1333049 at chromosome 9p21 and risk of CAD (OR = 1.29) [41]. These study have analyzed primarily on European descent, however, since the allele frequency differs among the different ethnic groups, the risk of CAD related to the SNPs at 9p21 may differ among each ethnic group. Since then, the replicated results in other ethnics such as Chinese, Japanese and Pakistanis are published and the association of the SNPs at 9p21 with CAD seems to be consistent among various ethic groups [42–45].

It is noteworthy that the SNPs in 9p21 region are also found to be associated with variety of diseases such as ischemic stroke (OR = 1.01–1.21) [46, 47], abdominal aortic aneurysm (OR = 1.31), intracranial aneurysm (OR = 1.29) [48], peripheral artery disease (OR = 1.29) [49], incident heart failure (OR = 1.17) [50], perioperative myocardial injury after coronary artery bypass graft surgery [51], type 2 diabetes (OR = 1.20) [52, 53].

More recently, the loci other than 9p21 have been shown to be associated with CAD or MI. Erdmann et al. applied less stringent statistical thresholds on their GWAS for CAD to identify any dismissed SNPs with modest effects or low allele frequencies and they found one new locus on 3q22.3 in muscle RAS oncogene homolog (*MRAS*) (OR = 1.15), the gene thought to play an important role in inflammation [54, 55].

There is another GWAS which identified the SNPs in the gene related to inflammation to be associated with MI [56]. They found five SNPs which affect eosinophil counts in blood in Icelandic population and reported that a nonsynonymous SNP at 12q24 in SH2B adaptor protein gene (*3SH2B3*) was associated with MI significantly (OR = 1.13).

The GWAS of early-onset MI revealed 9 loci which have significant association [57]. Three of them were newly identified in the study: (i) an intergenic region between *MRPS6* (mitochondrial ribosomal protein S6), *SLC5A3* (solute carrier family 5 (sodium/myo-inositol cotransporter), member 3) and *KCNE2* (potassium voltage-gated channel, Isk-related family, member 2) on chromosome 21q22 (OR = 1.19), 6p24 in *PHACTR1* (phosphatase and actin regulator 1) (OR = 1.13) and 2q33 in *WDR12* (WD repeat domain 12) (OR = 1.17). The mechanism by which genes at these three regions increases the risk of MI needs to be elucidated. In addition to the common variant, copy number variations can be analyzed by SNP chip and there were no common or rare copy number variations associated with risk of early-onset MI in this study.

The group from the WTCCC/German MI study further conducted a genome-wide haplotype association study for

the first time and identified the *SLC22A3-LPAL2-LPA* gene cluster on 6q26-27 as a strong susceptibility locus for CAD (OR = 1.8) [58]. An increased level of Lp(a) lipoprotein is a classical hereditary risk for CAD. Clarke et al. identified three chromosomal regions (6q26-27, 9p21 and 1p13) were strongly associated with the risk of CAD using the Human CVD Bead Chip which included 48742 markers relevant to cardiovascular disease on 6500 subjects. Among them, the *LPA* locus on 6q26-27 encoding Lp(a) lipoprotein had the strongest association. They identified two *LPA* variants that were strongly associated with both an increased level of Lp(a) lipoprotein and an increased risk of CAD (OR = 1.70 and 1.92) [59]. Both variants were strongly associated with a reduced copy number in *LPA* kringle IV-type 2 repeats and an increased level of Lp(a) lipoprotein. After adjustment for the Lp(a) lipoprotein level, the association between the *LPA* genotype score and the risk of CAD was abolished. The importance of classical risk factor Lp(a) was reemphasized by the GWAS.

4. Limitation of GWAS

Initially, GWAS have been primarily assessed only on European descent and the results of these GWAS may not be applicable to other ethnics due to wide difference of distribution of SNPs and allele frequency. Further studies for various ethnicity need to be done with use of newer chips which contains 1 million SNPs to increase coverage.

The CAD associated loci have been found in regions without known gene-encoding loci. Therefore, further studies will be required to elucidate the exact functional mechanism by which these loci modulate CAD risk.

Utility of genotyping 9p21 for clinical risk assessment is controversial [60–62]. The odds ratios for CAD risk in each selected SNPs are small (around 1.2) and explain only a small proportion of the heritable, genetic component of susceptibility to the disease. Newer susceptibility loci for CAD need to be validated with replication studies and in the future, we should evaluate the genetic risks by combining multiple independent common variants susceptible for CAD.

The GWAS method is supported by the common disease-common variant hypothesis, which predicts that genetic variants causing common disease exist frequently, but each variant only have a small effect on disease susceptibility. Another hypothesis is the rare variant hypothesis. Rare variants have a minor allele frequency of less than 1%. The rare variant hypothesis postulates that common disease is caused by multiple rare variants which have a strong causative effect on disease and this hypothesis was confirmed in colorectal adenomas [63, 64]. Rare variants cannot be captured by GWAS and requires whole genome sequencing using next generation sequencing system.

In addition, other types of variants, such as insertion-deletion variant, block substitution and inversion variant, so called structural variants may account for important contributors to the diseases and are also hard to detect by the chip-based method. The next generation sequencing method is also helpful to find these structural variants.

5. Conclusion

(1) The SNPs Data from the HapMap project and development of new chip technology enabled genotyping large amount of common variants simultaneously and contributed to efficiently identify gene loci affecting susceptibility to common diseases including CAD.

(2) The region at 9p21 was shown to be significantly associated with CAD in 2007 and comprehensive replication across multiple studies provides unequivocal evidence that this locus is associated with CAD in European descent. This region is also associated with abdominal aneurysm, intracranial aneurysm and type 2 diabetes, and seems to be a very important region for various diseases.

(3) Since the odds ratios of the risk allele at 9p21 for CAD are small, screening for this risk allele probably affects little, if any, to the each individual's risk prediction. Using genomic tests to improve existing risk models would likely require combining the effects of multiple common genetic variants.

(4) Rare variants and structural variants which cannot be captured by GWAS need to be searched by whole genome sequencing.

(5) GWAS approach has identified novel and unbiased genetic contributors to CAD and these insights provide the important direction to better understand the pathogenesis of CAD and to develop new and improved preventive measures and treatments for CAD.

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Sarcomere Gene Mutations in Hypertrophy and Heart Failure

Hiroyuki Morita · Ryozo Nagai · J. G. Seidman · Christine E. Seidman

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Abstract Despite considerable progress in identifying and modifying risk factors that cause cardiovascular disease, heart failure has emerged as an important medical and socioeconomic problem. Hypertrophic remodeling, a common response to many cardiovascular disorders, increases the risk of heart failure. Discovery of the genetic basis of hypertrophic cardiomyopathy has allowed consideration of whether these genes also contribute to pathologic remodeling that occurs in the context of common acquired cardiovascular disorders. Evidence supporting a shared etiology has emerged from the recent identification of sarcomere protein mutations and sequence variants in community-based populations with hypertrophy and heart failure. These findings imply that harnessing genetic testing for hypertrophic mutations may help define patients at risk for heart failure. In the future, mechanistic insights into hypertrophic remodeling, combined with strategies to prevent this pathology, are expected to reduce the burden of heart failure.

H. Morita
Department of Translational Research for Healthcare and Clinical Science, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

R. Nagai
Department of Cardiovascular Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

J. G. Seidman · C. E. Seidman (✉)
Department of Genetics, Harvard Medical School, NRB Room 256, 77 Avenue Louis Pasteur, Boston, MA 02115, USA
e-mail: CSeidman@genetics.med.harvard.edu

C. E. Seidman
Cardiovascular Division, Brigham and Women's Hospital and Howard Hughes Medical Institute, Boston, MA, USA

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Introduction

Heart failure, the pathophysiologic state in which blood delivery is inadequate for tissue requirement, arises in the context of a wide range of antecedent cardiovascular diseases. Despite many technological advances in the diagnosis of and aggressive therapeutic interventions for underlying cardiovascular diseases, heart failure has reached epidemic proportions. In the USA, there are an estimated 5.7 million affected individuals; each year, more than 670,000 new cases are diagnosed [1]. Heart failure statistics around the world are similarly dismal; in Japan, there are more than 1 million patients with heart failure [43]. The economic costs associated with this condition are enormous; in the USA, there were 1.1 million hospitalizations for heart failure in 2006, consuming an estimated US$37.2 billion [27]. Despite this considerable investment, more than 300,000 deaths due to heart failure occurred.

Many cardiovascular disorders underlie heart failure, including coronary atherosclerosis, hypertension, diabetes, valvular disease, congenital malformations, rhythm disturbances, and cardiomyopathies. While these conditions have unrelated pathophysiologic mechanisms, cardiac hypertrophy is often an important morphologic feature that emerges with each condition. Cardiac hypertrophy is also an established risk factor for heart failure [11, 16, 25]. Epidemiologic data indicate that elderly patients with eccentric or concentric ventricular hypertrophy have adjusted hazard ratios for incident congestive heart failure (e.g., the ratio of the number of new congestive heart failure events in patients with hypertrophy to the number of new congestive heart failure events in patients

without hypertrophy) of 2.95 and 3.32, respectively [14]. The association of hypertrophy and heart failure raises the hypothesis that a better understanding of the genes and molecules that remodel the heart along a hypertrophic process may provide insights that define at-risk patients. The risk for heart failure development, when combined with preventive interventions, may be diminished.

Over the past decade, single-gene mutations have been discovered to be causes of cardiac hypertrophy. In this review, we focus on hypertrophic cardiomyopathy (HCM), a monogenic disorder caused by more than 900 unique single-gene mutations, and we also consider how variation in HCM genes contributes to hypertrophy accompanying other cardiovascular conditions. HCM, while often highlighted as the most common cause of sudden death in young athletes [29], is also an important cause of heart failure in patients of any age [28, 37]. Recent studies emphasize this important relationship: one HCM mutation found in millions of individuals of Southeast Indian ancestry was demonstrated to increase the risk of heart failure by sevenfold [10].

HCM is defined by an unexplained increase in left ventricular wall thickness (reviewed by [30]), a morphologic abnormality that is estimated to occur in 1 of 500 adults in the general population [31]. Hemodynamic manifestations of HCM include vigorous systolic function, which can exceed normal parameters, and impaired relaxation, which can elevate ventricular diastolic pressure. Only 10–15% of HCM patients develop an end-stage ('burnt-out') phenotype in which the ventricle dilates and systolic function is diminished. Yet despite a preserved systolic function, most HCM patients experience heart failure symptoms, including exertional dyspnea, orthopnea, paroxysmal nocturnal dyspnea, and fatigue—clinical manifestations of an intrinsic deficit that limits the relaxation of thickened heart muscle. With superimposition of arrhythmias, particularly atrial fibrillation, which occurs in 25% of HCM patients [56], symptoms worsen. Atrial contraction promotes filling of the noncompliant hypertrophied ventricle during end diastole, and loss of atrial kick can acutely precipitate reduction of cardiac output and further elevation of left atrial pressure. Cardiac collapse and/or acute pulmonary edema may result, especially when hypertrophy is marked and ventricular contraction is very rapid.

HCM as a Genetic Disorder

Mutations in 11 genes that encode proteins of the cardiac sarcomere, including components of thick and thin filaments and proteins with regulatory functions, cause HCM. Comprehensive genetic analyses on individuals with inherited familial disease [36, 49, 52] indicate that approximately 60% of cases are due to a mutation in a sarcomere protein gene [5]. Most of these pathogenic mutations are unique to the affected individuals within one family or within a few families. More than 900 distinct HCM mutations have been identified (http://cardiogenomics.med.harvard.edu/). Mutations occur predominantly in genes that encode cardiac β-myosin heavy chain (*MYH7*), cardiac myosin-binding protein C (*MYBPC3*), cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*), cardiac troponin C (*TNNC1*), α-tropomyosin (*TPM1*), cardiac actin (*ACTC*), essential myosin light chain (*MYL3*), and regulatory myosin light chain (*MYL2*). Defects in *MYH7* and *MYBPC3* predominate in frequency and account for half of all pathogenic HCM mutations. Mutations in other genes, including α-cardiac myosin heavy chain (*MYH6*), titin (*TTN*), MLP (*CSRP3*), telethonin (*TCAP*), metavinculin (*VCL*), myozenin 2 (*MYOZ2*), junctophilin 2 (*JPH2*), and CARP (*ANKRD1*), have been implicated in HCM, but occur substantially less commonly [3, 6, 15, 18, 24, 45, 53].

The considerable genetic heterogeneity (hundreds of different mutations in scores of genes) of HCM predicts that most mutations arose recently in human evolution. HCM has a deleterious impact on life expectancy and, while modest, this provides sufficient negative selection on most HCM mutations for these to be lost over several generations. However, because the emergence of a new mutation in one of the two copies of any sarcomere protein gene is sufficient to cause HCM (i.e., mutations are dominant), new mutational events account for both the high prevalence of HCM in the general population and the large number of family-specific mutations. Recent studies also indicate that some cases of HCM are due to ancient founding mutations that have been maintained throughout human evolution [10, 22]. Selective evolutionary pressure on these founding mutations is presumed to be neutral, as the clinical expression of HCM and adverse outcomes associated with these mutations are delayed until late in life, well after the reproductive years. However, with increases in human longevity, the impact of founding HCM mutations has become apparent, and older mutation carriers are now recognized to have late-onset hypertrophic remodeling and to be at high risk for heart failure.

Pathophysiology of HCM Mutations

HCM missense mutations result in the insertion of an erroneous amino acid residue into the contractile peptide. Most of these mutant sarcomere peptides are stable and become incorporated into myofilaments, where they impact sarcomere function [50]. Studies on the biophysical properties of myosin molecules with HCM mutations indicate that these increase actin-activated ATPase activity,