Clinical Investigation

Differences in Clinical Expression of Hypertrophic Cardiomyopathy Associated With Two Distinct Mutations in the β-Myosin Heavy Chain Gene

A 908Leu→Val Mutation and a 403Arg→Gln Mutation

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Background. The disease gene for hypertrophic cardiomyopathy (HCM) has been identified as the β-myosin heavy chain (β-MHC) gene in some HCM families. We describe extensive clinical evaluations in two kindreds with two distinct point mutations in the β-MHC gene.

Methods and Results. We used single-strand confirmation polymorphism (SSCP) gel analysis of polymerase chain reaction–amplified products capturing each of the four β-MHC gene exons to identify distinct missense mutations in two HCM kindreds. Clinical, ECG, and echocardiographic studies were performed in the two kindreds: kindred 2755 with amino acid 908Leu→Val mutation and kindred 2002 with amino acid 403Arg→Gln mutation. The morphological appearances of HCM were similar in these two kindreds. However, the two kindreds differed with respect to disease penetrance, age of onset of disease, and incidence of premature sudden death. Twelve of 31 adults (≥17 years) with the disease gene in kindred 2755 did not have left ventricular hypertrophy (LVH), and only 5 of these had ECG abnormalities. Thus, the disease penetrance in adults with this mutation was only 61%. None of 11 children aged <16 years had LVH. The 908 mutation was associated with a low incidence of cardiac events: Only two sudden deaths and one syncope occurred in 46 individuals with the mutant allele. In contrast, LVH was present in all 11 adults in kindred 2002 with the 403 mutation (100% disease penetrance). In addition, three of four affected children were symptomatic and had clinical evidence of HCM. The disease in this kindred was severe and resulted in six premature sudden deaths. Seven additional patients had syncope or presyncope.

Conclusions. In some kindreds, the HCM disease gene is more prevalent than indicated by echocardiography and ECG. Some point mutations may be associated with a more malignant prognosis. Preclinical identification of children with mutations associated with a high incidence of sudden death and syncope provides the opportunity to evaluate efficacy of early therapeutic interventions. (Circulation 1992;86:345–352)

KEY WORDS • cardiomyopathy, hypertrophic • β-myosin heavy chain gene mutations

Familial hypertrophic cardiomyopathy (HCM) is inherited in an autosomal dominant pattern. Linkage analysis has shown that in less than half of the kindreds, a disease gene is localized on chromosome 14q. In these studies involving a total of nine kindreds, each evaluated separately for linkage, four have shown either tight linkage or a mutation in the β-myosin heavy chain (β-MHC) gene on chromosome 14q. In the remaining five, this locus has been excluded as the site of the disease gene. Thus, nonallelic genetic heterogeneity of HCM has been established. Recently, mutations in the β-MHC gene have been described in two affected families. Despite the description of the molecular defects, the pathogenesis of the disease remains unclear. We present the contrasting clinical consequences of two mutations in the β-MHC gene.

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Methods

Patient Population

The two kindreds, 2755 and 2002, are expansions of kindreds reported previously.

Kindred 2755. We have previously shown in the unexpanded kindred evidence of linkage of the disease phenotype to β-MHC gene–linked markers (LOD score, 4.5 with no recombinations). The expanded pedigree (Figure 1) consisted of 279 family members. Of these, 194 were blood relatives and 85 were married-in spouses. Of the 279 family members, 202 individuals (165 blood relatives and 37 married-in spouses underwent 12-lead ECG, M-mode, and two-dimensional echocardiographic examinations. Blood for genotyping...
was available in 195 individuals (155 blood relatives and 40 married-in spouses).

**Kindred 2002.** There was previous evidence of linkage of the disease phenotype to the \( \beta \)-MHC gene on chromosome 14 in this kindred as well (LOD score, 2.5 with no recombinations). An expanded pedigree of 21 individuals is depicted in Figure 2. Five patients who were not examined at the National Institutes of Health (NIH) died suddenly, and the diagnosis of HCM had been made elsewhere before death or at autopsy. The remaining 15 family members, including a first-generation, married-in spouse, underwent evaluation at the NIH that included a 12-lead ECG and echocardiographic examination.

Informed consent for the studies was obtained in accordance with study protocols approved by the Institute Review Board of the National Heart, Lung, and Blood Institute.

**Echocardiography**

Echocardiographic examination was performed with a Hewlett-Packard (Sonos 500 or Sonos 1000) real-time, pulsed-array, 90° ultrasonic scanner with a 2.5-MHz...
Polymerase Chain Reaction and Southern Blot Analysis

Each family member was phlebotomized, and DNA was extracted from isolated nuclei of peripheral white cells by procedures previously described. The published sequence of the human β-MHC gene was used to design a set of intronic primers, each of which encompassed one of the 40 β-MHC gene exons and yielded a unique single fragment of the expected size in a polymerase chain reaction (PCR) amplification. Intronic rather than exonic primers were used to avoid the problem of coamplification of the highly homologous cardiac α-MHC gene. PCR was performed in a 100-μl volume using the AmpliTaq enzyme (Perkins Elmer Cetus, Norwalk, Conn.) according to the manufacturer’s recommendations with the exception that a thermocycler oven (Bios, New Haven, Conn.) was used. Each of the denaturation, annealing, and extension segments was for 40 seconds, and 35 cycles were performed. Annealing temperatures of ≥61°C were used. Radioactive labeling of the amplified fragment was accomplished through the addition of 0.1 μCi of α-32P dCTP 3000 Ci/mmol (Amersham, Chicago) to the 100-μl reaction volume.

Southern blot analysis was performed using previously described techniques together with an ammonium acetate transfer technique onto Hybond N+ nylon paper (Amersham, Chicago). The PSC14 probe was obtained from HP Vosberg HP and C. Seidman.

Single-Strand Conformation Polymorphism Detection

Polymorphisms were detected by single-strand conformation polymorphism (SSCP) analysis of PCR-amplified fragments containing each of the 41 β-MHC gene exons using a modification of the procedure described by Orita. Briefly, 1 μl of a 100-μl reaction was diluted with 9 μl of a denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), heated to 80°C, plunged into an ice bath, and resolved on a 5% polyacrylamide/10% glycerol gel run at 30 W at room temperature and also on a 5% polyacrylamide gel run at 4°C.

Sequencing

The PCR fragments showing polymorphisms by SSCP analysis were sequenced without subcloning using a Sequenase kit (USB, Cleveland, Ohio) in one of two ways. The first method was a modification of the double-stranded sequencing protocol recommended by the manufacturer. Briefly, four 100-μl PCR products starting with 100 ng of genomic DNA from the individual of interest were concentrated using a Centricon 30 (Amicon Division, W.R. Grace & Co.). The “stop volume” was further purified on a 1.0% agarose gel with ethidium bromide, and the gel band was cut out under ultraviolet light. DNA was extracted from the gel using Qiaex (Qiagen, Chatsworth, Calif.), denatured in a final concentration of 0.2N NaOH/0.2 mM EDTA, and precipitated in three times the initial volume of ethanol. After a wash in 70% ethanol, the pellet was lyophilized and resuspended in 14 μl of water. Seven microliters was then used in the sequencing reaction as recommended by the manufacturer, with the exception that 1 μl of DMSO was added to the primer/template mix before boiling for 2 minutes and cooling in ice water.

The second method of sequencing involved the production of single-strand template by modification of a method described previously. Briefly, a biotin phosphoramidite (Midland Reagent Co., Midland, Tex.) was used to add a biotin molecule to the 5’ end of an oligonucleotide primer in the last step in its synthesis on the nucleic acid synthesizer (model 380B, Applied Biosystems, Foster City, Calif.). This primer, together with its mate that was not biotinolated, was used to generate the PCR-amplified fragment to be sequenced. Twenty microliters of this

Table 1. Two Mutations Identified in the β-MHC Gene and Primers Used to Generate Exon-Containing Fragments of the β-MHC Gene With Missense Mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Kindred</th>
<th>Exon</th>
<th>Residue</th>
<th>Amino acid change</th>
<th>Basepair change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2002</td>
<td>13</td>
<td>403</td>
<td>G→A</td>
<td>Arg→Gln</td>
</tr>
<tr>
<td>2</td>
<td>2755</td>
<td>23</td>
<td>908</td>
<td>C→G</td>
<td>Leu→Val</td>
</tr>
</tbody>
</table>

Size marker locus

<table>
<thead>
<tr>
<th>Fragment (basepairs)</th>
<th>Restriction enzyme site change</th>
<th>Diagnostic fragment (basepairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Exon 13 (amino acid residue 403) 5’ Primer 8,708 5’-TTACGGGAGTACACACACC-3’ 267 Dde I (site created) 129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’ Primer 5’-CTGACTTTGAACATCTCCATCCC-3’ 8,974</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Exon 23 (amino acid residue 908) 5’ Primer 13,869 5’-CCCTCTTTAGGATGTGC-3’ 404 Pvu II (site destroyed) 161</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’ Primer 5’-GGTCAGTATGCTAGAGTCC-3’ 14,272</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Identification of exon on 348 subsequent analysis ni ng an exon dredeus. Statistics the kindred.

FIGURE 3. Single-strand confirmation polymorphism gel analysis of a subset of kindred 2755. A C→G transversion in exon 23 results in a single-strand conformer with altered mobility on this gel. This band, marked with an arrow, can be seen to cosegregate with the disease in the adult members of the kindred.

The product was then incubated at room temperature, with 20 µl of magnetic beads bound to streptavidin (Dynal, Oslo, Norway) for 15 minutes. The product, now bound to beads, was then denatured with 0.2N NaOH, washed with 1X TE, and then washed with water. After the collection of the beads with a magnet, they were resuspended in 7 µl of water, and the bound template was sequenced using the complementary primer according to the manufacturer’s recommendations.

Statistics

Cumulative survival was determined by product-limit survival analysis using sudden cardiac death, syncope, and presyncope (profound impairment of consciousness not amounting to frank syncope) as time variables. Two product-limit survival functions were compared using the log rank test. A value of \( p \leq 0.05 \) was considered significant.

Results

Identification of Mutations in the β-MHC Gene

Southern blot analysis using the PSC144 marker against a Bam H 1 digest of DNA from representatives of the two kindreds showed no evidence of rearrangements in the β-MHC gene locus (data not shown). Our subsequent analysis of the β-MHC gene in these kindreds used 40 sets of intronic primer pairs, each spanning an exon and capable of producing a unique fragment in a PCR amplification. The products of all 40 primer pairs were consistently suitable for analysis. The PCR fragments amplified from DNA of the members of both kindreds were evaluated for the presence of polymorphisms on SSCP gels run at both room temperature and 4°C. Polymorphic fragments were then sequenced directly without subcloning as described in “Methods.”

These techniques detected several polymorphisms in both kindreds; however, the sequencing of all the polymorphisms showed that only two reflected changes in exon coding sequence. Each was unique to one of the two kindreds, and both involved the replacement of a highly conserved amino acid. These point mutations in exons 13 (kindred 2002) and 23 (kindred 2755), together with the primer pairs used to amplify them, are depicted in Table 1.

Identification of the 908 C→Gln Mutation in Kindred 2755. All 40 exons of the β-MHC gene, amplified by PCR from the DNA of a subset of family members, were evaluated on SSCP gels at room temperature and 4°C. Only the fragment containing exon 23 was found to contain a polymorphism that cosegregated with the disease (Figure 3). Direct sequencing of this fragment in both directions showed a C→G transversion resulting in the replacement of a leucine by a valine at amino acid residue 908 (Figure 4). Subsequent sequencing of a cDNA clone from an affected member of the family confirmed this mutation (data not shown). This mutation destroys a Pvu II site, thus lengthening the 140 basepair fragment found in normal individuals to a 161 basepair fragment that is diagnostic of the disease in this kindred. All 19 individuals in this kindred with echocardiographic criteria for HCM had this mutation, which is shown in a subset of the kindred in Figure 5. This mutation was not seen in a survey of 100 other chromosomes.

Identification of the Arg→Gln Mutation in Kindred 2002. The same initial evaluation of the β-MHC gene exons was performed on a subset of kindred 2002. Only the two fragments containing exon 3 and exon 13 showed the presence of a polymorphism on an SSCP gel. Direct sequencing of the exon 3-containing fragment showed the mutation to lie outside of the exon and splice consensus sites. Direct sequencing in both directions of the exon 13 fragment detected a G→A transition that resulted in the replacement of an arginine by a glutamine at amino acid residue 403 (Figure 6). This mutation creates a new Dde I restriction enzyme site by changing the sequence CTCGG found in normal individuals to CTCAG, thus shortening the 161 basepair fragment found in normal individuals to a 129 basepair fragment that is found in all affected members of this

FIGURE 4. A portion of the sequence in the polymerase chain reaction–amplified fragment containing exon 23 from two affected and two unaffected individuals in kindred 2755. The C→G transversion in codon 908 results in the substitution of a valine for a leucine. The affected individuals, being heterozygotes for the mutant allele, have both the C and a G in this position. Arrows mark the level of the transversion in the sequencing gel. The G is present in the two affected individuals on the left and absent in the two normal individuals on the right.
kindred (Figure 2). The creation of a restriction site by
a mutation, as has occurred here, corroborates the
specific basepair change that was necessary to create
the enzyme recognition sequence. A survey of 100
chromosomes failed to detect another instance of the
403 mutation.

Disease Penetrance and Clinical Consequences
of the 908 Mutation

Forty-six individuals were determined to have the
disease mutation: 42 by genetic analysis, two by post-
mortem findings, and two who were obligate carriers
of the disease gene. Twelve-lead ECGs and echocardi-
grams were available in the 42 individuals with the 908
mutation confirmed by genotyping. Of these, 11 were
young (age, 1–11 years). All the 11 young patients had
normal echocardiograms except one 12-year old pa-
tient, who had an abnormal 12-lead ECG (narrow deep
Q waves in leads III, aVF, and V6). None of the blood
relatives in whom the 908 mutation was absent (n=165)
or married-in spouses (n=37) had a maximum left
ventricular wall thickness of >12 mm (Figure 7). Of the
31 older individuals with the 908 mutation (age, 17–42
years), 12 had normal echocardiograms with maximum
left ventricular wall thickness of 8–12 mm (Table 2).

Thus, in adult individuals, the disease penetrance as
estimated by echocardiography was 19 of 31, or only
61%. Of the 12 individuals with the mutation who had
normal echocardiograms, five had abnormal 12-lead
ECGs. These were mostly minor and nonspecific (Table
2). The disease penetrance in adults with this disease
mutation by ECG and echocardiographic evaluation
was therefore 24 of 31, or 77%.

The 908 mutation has been associated with a low
incidence of premature sudden death and syncope. Only
two premature (<55 years of age) sudden deaths and
one syncope occurred in the 46 individuals with the
mutation. The cumulative incidence of cardiac events
(sudden death, syncope, or presyncope) related to age
is shown in Figure 8. Thus, the cumulative sudden death-
free survival rates at 20 and 60 years of age were
97±3% and 92±6%, respectively (Figure 8A). Similarly,
the event (sudden death, syncope, or presyncope)-free rates
were 95±3% and 90±6%, respectively (Figure 8B).

Disease Penetrance and Clinical Characteristics
of the 403 Mutation

Of the 20 blood relatives in kindred 2002, 15 have
been diagnosed as having HCM or the 403 mutation. Of
these, six died suddenly between the ages of 19 and 45
years and had markedly hypertrophied hearts at autopsy
examination. Of the nine living individuals with the 403
mutation, seven (age, 6–40 years) have echocardiographic
evidence of left ventricular hypertrophy and abnormal
12-lead ECGs. None of the patients had abnormal right
ventricular echocardiograms. One of the remaining
two individuals, a child aged 8 years, had an
abnormal ECG (narrow deep Q waves and left atrial
hypertrophy). The other, aged 9 months, had a normal
12-lead ECG and echocardiogram. Thus, in this
kindred, the 403 mutation was associated with early pre-
sentation of the disease and with a 100% penetrance in
adults.

The 403 mutation was also associated with a high
incidence of cardiac events: sudden death in six, syn-
cope in three, and presyncope in four patients. The
cumulative sudden death-free rates at 20, 30, and 40
years of age were 90±9%, 68±16%, and 45±17%,
respectively (Figure 8A). Similarly, the event-free rates
at 20, 30, and 40 years of age were only 70±13%,
46±14%, and 8±7%, respectively (Figure 8B).
Discussion

A detailed clinical evaluation of two of the kindreds with distinct point mutations in the β-MHC gene demonstrate that different alleles may be associated with diverse clinical presentations. Both missense mutations lay in the head or head-rod junction of the molecule, suggesting the possible occurrence of abnormal interactions between the mutated β-MHC molecule and associated proteins such as actin or myosin light chains.

A comparison of kindreds 2755 and 2002, each with distinct missense mutations (908Leu→Val and 403Arg→Gln, respectively) illustrates that the severity and penetrance of the disease may be determined by the specific mutation. Of the 195 members of kindred 2755 who were evaluated clinically, 42 individuals inherited the mutant allele. Of the 31 individuals ≥17 years of age with the mutant allele, 19 had a maximum left ventricular wall thickness of ≥13 mm. In contrast, all 123 blood relatives without the mutant allele as well as 37 unrelated spouses had a maximum left ventricular wall thickness of ≤12 mm. Thus, 12 adult members with the mutant allele failed to meet this echocardiographic criterion for HCM—a definition of HCM that was less stringent than that used traditionally to diagnose the disease (maximum left ventricular wall thickness ≥15 mm). Furthermore, seven of these 12 adults also had normal 12-lead ECGs; hence, the disease penetrance for the adults in this kindred was only 61% when the echocardiographic threshold of >12 mm was used and 77% when ECG analysis was included in the diagnostic criteria. In addition, none of 11 children with the mutant allele had left ventricular hypertrophy on echocardiogram, and only one child, aged 12 years, had an abnormal 12-lead ECG.

In contrast to kindred 2755, all 11 adults in kindred 2002 had left ventricular hypertrophy and abnormal 12-lead ECGs. Moreover, four children with the mutant allele, two had left ventricular hypertrophy and three had abnormal 12-lead ECGs. The only child with normal cardiac findings was a 9-month-old female patient. Thus, the disease penetrance in this kindred was 100% in adults by echocardiographic criteria and 93% in all ages when the ECG analysis was added to the diagnostic criteria.
TABLE 2. Clinical, ECG, and Echocardiographic Findings in Family Members of Kindred 2755 With the Disease Gene but Without Left Ventricular Hypertrophy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age (years)</th>
<th>ECG abnormality</th>
<th>Echocardiographic dimensions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LVH</td>
<td>T</td>
</tr>
<tr>
<td>1. II-6</td>
<td>F/36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. II-13</td>
<td>M/63*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. II-14</td>
<td>F/62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. III-32</td>
<td>F/31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. III-33</td>
<td>F/30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. III-34</td>
<td>M/27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. III-36</td>
<td>F/42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. III-38</td>
<td>F/34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. III-50</td>
<td>F/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. III-52</td>
<td>M/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. IV-35</td>
<td>M/17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LVH, left ventricular hypertrophy; T, T wave changes; Q, abnormal Q waves; P, abnormal P waves; Axis, abnormal QRS axis; WPW, Wolff-Parkinson-White syndrome; S, septum; PW, posterior wall; Ao, aorta; LA, left atrium; LVD, left ventricular end diastole; LVs, left ventricular end systole; RV, right ventricle; SAM, systolic anterior motion of the mitral valve; F, female; M, male; +, present; −, absent; *rheumatic mitral valve disease; **narrow, deep Q waves (<40-msec duration and >0.10 mv amplitude).

Although the disease penetrance by echocardiographic evaluation was significantly less in kindred 2755 than in 2002, there were many members of kindred 2755 who were symptomatic and had severe left ventricular hypertrophy. Indeed, the morphological appearances in affected members of the two kindreds did not differ significantly. Despite these findings, the incidence of premature sudden death in kindred 2755 was significantly lower than in kindred 2002 (Figure 8). A high incidence of premature sudden death associated with the 403 mutation was also present in a previously reported French-Canadian kindred with the identical mutation.1,5 In this French-Canadian kindred, seven premature sudden deaths occurred in 44 affected members.

Kindred 2002 is the second family with the 403 mutation described in the literature. The fact that both kindreds with this mutation exhibit a high incidence of premature sudden death lends credence to the concept of mutation-associated disease characteristics. The occurrence of the same point mutation in two kindreds that we believe are unrelated is intriguing although not novel in genetics. It is possible that a common ancestor is responsible for a “common founder effect.”15 Comparison of the locus polymorphism haplotypes in these two kindreds would address that question. “Hot spots” for mutations have been proposed as an explanation for the independent occurrence of the same mutation. Previously described in the gene encoding factor VIII,16 these hot spots involve methylation of a cytosine in a CpG dinucleotide and its subsequent spontaneous deamination to thymine. This event however, did not apply to the 403 mutation. A further consideration is that myosin is such a critical protein that only a limited number of mutations is compatible with life, and identical mutations would be expected to occur in unrelated kindreds.

Only one of the two mutations involved a change in the charge of the amino acid residue, i.e., the 403 mutation in exon 13. Thus, although a change in charge may be disruptive and may lead to a defect in the
function of the myosin molecule, a substitution without a charge change in a critical region may have a similar effect. The question of how these point mutations may cause HCM is still undetermined. Changes that result from these base substitutions may affect the functional integrity of the molecule itself, its interaction with other myosin molecules (mutated or wild type), or other molecules that interact with the myosin head. The latter possibility is particularly interesting, as exon 13 encodes a region that may bind actin. The 908 mutation in exon 23 is located in the S′ end of the rod. Traditionally, the invariant proline at residue 838 in the human β-MHC gene has been considered to mark the border between the head and rod portion of the molecule. The use of specific monoclonal antibodies and physical mapping by electronmicroscopic studies on a Acathamoeba myosin II have suggested that the true border is closer to the 900 amino acid residue. Thus, the mutation at residue 908, although involving the relatively conservative substitution of a valine for a leucine, occurred at what may be an important transition portion of the myosin molecule.

Careful clinical evaluation of HCM pedigrees with distinct myosin mutations should help to delineate mutation-specific patterns of disease, as has been described in this study. These, in turn, may prove useful in prognosis and management of patients. At the molecular level, evaluation of the set of mutant myosin molecules with respect to motility, ATP binding and hydrolysis, actin binding and myosin light chain binding may further elucidate the etiology of the disease and suggest strategies for intervention in the disease process.

Acknowledgments

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References

Differences in clinical expression of hypertrophic cardiomyopathy associated with two distinct mutations in the beta-myosin heavy chain gene. A 908Leu----Val mutation and a 403Arg----Gln mutation.

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