Rapid Communication

Exclusion of Cardiac Myosin Heavy Chain and Actin Gene Involvement in Hypertrophic Cardiomyopathy of Several French Families

Ketty Schwartz, Jacques Beckmann, Cécile Dufour, Laurence Faure, Françoise Fougerousse, Lucie Carrier, Christian Hengstenberg, Daniel Cohen, Hans-Peter Vosberg, André Sacrez, Marc Ferrière, Michel Desnos, François Cambien, Olivier Dubourg, and Michel Komajda

Familial hypertrophic cardiomyopathy (FHC) is characterized by idiopathic myocardial hypertrophy, which often and predominantly involves the interventricular septum. The disease is transmitted as an autosomal dominant trait, and its major risk is sudden death. It was recently demonstrated that this disease is genetically heterogeneous and that in 13 of 18 unrelated families the morbid locus, termed FHC-1, maps to chromosome 14q11-12 in and/or very near the cardiac β-myosin heavy chain gene. We have performed linkage analysis with five chromosomal markers detecting polymorphisms in the cardiac β-myosin heavy chain gene or the cardiac actin gene (located on chromosome 15q) on eight families from different regions of France. We show that 1) it is possible to analyze medium-sized families by using highly informative microsatellite markers located in these genes and 2) the disease is not linked to the two contractile protein genes in any of these families. Moreover, 10–20% of chromosome 14 and 20–40% of chromosome 15 in the vicinity of the respective markers were excluded as possible locations for the morbid locus. These results provide new insights into the identification of the genes responsible for FHC. (Circulation Research 1992;71:3–8)

KEY WORDS • familial hypertrophic cardiomyopathy • linkage analysis • microsatellites • cardiac myosin heavy chains • cardiac actin • exclusion

From the Institut National de la Santé et de la Recherche Médicale, Unité 127 (K.S., C.D., L.F., L.C., C.H.), Hôpital Lariboisière, Paris; the Centre d’Études du Poly morphisme Humain (J.B., D.C.), Paris; the Généthon (F.F.), Evry, France; the Department of Cell Physiology (H.-P.V.), Max-Planck Institute for Medical Research, Heidelberg, FRG; the Service de Cardiologie, Hôpital Hautepierre (A.S.), Strasbourg, France; the Service de Cardiologie, Clinique Saint-Eloi (M.F.), Montpellier, France; the Service de Cardiologie, Hôpital Boucicaut (M.D.), Paris; the Institut National de la Santé et de la Recherche Médicale, Service Commun 7 (F.C.), Paris; the Service de Cardiologie, Hôpital Ambroise Paré (O.D.), Boulogne, France; and the Service de Cardiologie, Hôpital Pitité-Salpêtrière (M.K.), Paris.


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Address for correspondence: Dr. Ketty Schwartz, INSERM U127, Hôpital Lariboisière, 41 Blvd. de la Chapelle, Paris 75010, France.

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of hypertrophy may vary widely. Unexplained ventricular hypertrophy appearing on M-mode and two-dimensional echocardiography is the current basis for FHC diagnosis, which is often complicated in adolescents since hypertrophy may not develop until after growth is complete. The major risk is sudden death, which can occur even in asymptomatic persons. At the cellular level, FHC is characterized by a large extent of myocyte disarray (30% of the total tissue area versus 1.5% in normal hearts), which may contribute to the cardiac dysfunction seen with FHC.

Because of the recent and striking development of reverse genetics, substantial progress has been made in identification of the genetic defect(s) responsible for FHC. In an impressive series of works, the teams of Seidman and McKenna4–5 examined the genetic linkage between the disease and various chromosomal locations, defined by specific biallelic restriction fragment length polymorphisms (RFLPs). They found in two of four analyzed families that the disease locus, termed FHC-1, is linked to a region on chromosome 14q11-12, which contains the two cardiac myosin heavy chains (MHCs), α-MHC and β-MHC. They furthermore demonstrated that the β-MHC gene in these two families contained mutations.6,7 This locus was excluded in the other two families. FHC-1 is also linked to the disease in a group of eight unrelated US families,8 and involvement of the β-MHC gene was recently observed in three of six other pedigrees.9,10 These data provide conclusive evidence that FHC is a genetically heterogeneous dis-
ease and that certain forms are linked to or due to a defect in the \(\beta\)-MHC gene.

The continued analysis and identification of genetic abnormalities contributing to FHC may lead to new diagnostic and therapeutic tools and to an improved understanding of the mechanisms governing cardiac growth and sarcomere assembly. Therefore, the aim of the present work was to determine in a group of eight unrelated French families whether the chromosomal location of the disease locus is the same as that previously reported and, when negative, to test the hypothesis that the other major contractile protein of the cardiac sarcomere, actin, could be involved. Since it was not possible to assemble large families, we have taken advantage of the presence of two microsatellites in the \(\beta\)-MHC gene (one in the promoter region, MYO I, and one in the twenty-fourth intron, MYO II)\(^{11}\) to obtain more informative genetic markers. The same type of approach was used for the cardiac actin gene in which a polymorphic microsatellite (ACT) located in the fourth intron had recently been described.\(^{12}\)

**Materials and Methods**

**Patient Studies**

Eight unrelated FHC families were studied (Figure 1). None of the family members had a resting blood pressure \(>140/90\) mm Hg or a history of systemic hypertension. Subjects underwent detailed cardiovascular examinations including 12-lead electrocardiograms, Doppler ultrasonography, and M-mode and two-dimensional echocardiography with left and right ventricular views.\(^{13,14}\) All echocardiographic readings were reviewed independently by two cardiologists. Individuals were considered affected if echocardiography indicated an end-diastolic wall thickness of 13 mm or more in the absence of other potential causes such as hypertension or valvular stenosis. Three individuals aged 31, 37, and 67 years (No. II-5 in pedigree 706, No. III-11 in pedigree 701, and No. II-3 in pedigree 707, respectively) did not consent to complete echocardiographic assessment, but since their clinical and electrocardiographic examinations were normal, they were classified as nonaffected.

**Genetic Analysis**

Genomic DNA was extracted as previously described\(^{15}\) from blood samples obtained after informed consent in accordance with the guidelines of the Comité d’Ethique du Centre Hospitalier Universitaire de La Pitié-Salpêtrière.

RFLP analyses were performed on 4 \(\mu\)g total DNA with CRI-L436 and pSC14 probes.\(^{3}\) Digested samples were fractionated on 1% agarose gels and electrotransferred to Hybond N\(^+\) membranes (Amersham) using the automaton Mark II.\(^{16}\) Conditions for hybridization and washings were as previously described.\(^{5}\)

Microsatellite markers were analyzed using a previously reported technique,\(^{12}\) with slight modifications. The principle of this technique involves the following: 1) polymerase chain reaction amplification from genomic DNA of dinucleotide repeats: MYO I, (GT)\(_{14}\), MYO II, (TG)\(_{21}\); and ACT, (TG)\(_{25}\); 2) separation of the amplified products on a 6% sequencing gel; and 3) transfer by contact to Hybond N\(^+\) membranes. Each membrane was then hybridized with a specific labeled oligonucleotide, washed, and autoradiographed. The primers (Genset, France) used to amplify MYO I (primer 1, 5’ GTATGTGCCACAGGAGTTCC3’; primer 2, 5’ GACTATGCCGCTTGTCCAC3’) and MYO II (primer 1, 5’ ATGCCATGCTATCTGTGCC3’; primer 2, 5’ AACATCCTCAAACCTACCC3’) microsatellites were chosen from the \(\beta\)-MHC gene sequence using the program Oligo.\(^{17}\) Primer 1 was also used after 3’ end labeling with terminal transferase and (\(a^2\))dCTP to detect the amplified fragments. The primers for amplification of the ACT microsatellite were as previously described.\(^{18}\)

Each procedure, Southern blot or microsatellite analysis, was performed in duplicate, and all patterns were examined by two or three of us independently and without knowledge of disease status.

**Linkage analyses were done using the MLINK program.**\(^{19}\) Allele frequencies were taken from Solomon et al.\(^{9}\) for probes CRI-L436 and pSC14 and were calculated from our eight families for probes MYO I, MYO II, and ACT. In all events, results were unaffected by modification of allele frequencies at these loci. Because of the clinical variability of FHC and its age-dependent expression, results were calculated assuming two types of penetrance (full and age-related penetrance) as follows: 0.26 between 0 and 20 years of age, 0.86 between 20 and 40 years, 0.95 between 40 and 70 years, and 1.00 above 70 years. LOD (logarithm of the odds) scores were also calculated assuming two arbitrary false-positive diagnosis rates: a low rate of 0% or 1% under and above 40 years of age, respectively, and a high rate of 1% under 20 years, 2% between 20 and 40 years, and 5% above 40 years.

**Results**

**Absence of Linkage to \(\beta\)-MHC Gene**

There were three to five alleles with MYO I and three to six with MYO II, depending on the family, and indicating that, as hypothesized, these microsatellites are polymorphic. The MYO II genotype of pedigree 702 is shown in Figure 2 as an example. Each lane of the autoradiograph contains one or two major bands, representing the alleles, associated with smaller minor bands. Such a pattern is common in microsatellite analysis. Figure 1 depicts the alleles obtained for each pedigree with the myosin probes. With MYO I, some individuals in pedigrees 703, 706, 707, and 709 were obligate recombinants between \(\beta\)-MHC and the diseased phenotype, and with MYO II, recombinants were observed in all pedigrees except 708. LOD scores of \(-\infty\) at a recombination fraction (\(\theta\)) of 0 reflect these recombinational events (Table 1), and the combined use of MYO I and MYO II enabled us to exclude linkage of the disease locus to the \(\beta\)-MHC gene in seven of eight pedigrees (LOD scores of \(<-2\)). As expected, the biallelic probes were less informative, and only four families could be excluded with CRI-L436 and two with pSC14 (Table 1). Pedigree 708, a very small family, was noninformative for all markers tested (Table 1). It is possible to roughly estimate under the condition of full penetrance the size of the excluded region on chromosome 14. For MYO I and MYO II, the sum of the LOD scores is inferior to \(-2\) up to a recombination fraction of
FIGURE 1. Schematic diagrams showing pedigrees of eight French families with familial hypertrophic cardiomyopathy. Alleles are shown for probes MYO I, MYO II, and pSC14. Generation numbers are indicated to the left. ○, Unaffected female; ●, affected female; □, unaffected male; ▼, affected male. Crosses through symbols indicate unknown disease status; diagonal slashes indicate deceased individuals.
FIGURE 2. Genotype for the MYO II microsatellite in pedigree 702. Top panel: Schematic diagram of the pedigree. ○, Unaffected female; ●, affected female; □, unaffected male; ▲, affected male. Cross through symbol indicates member who could not be examined; diagonal slash indicates deceased individual. Bottom panel: Autoradiograph of amplified MYO II microsatellite hybridized with radiolabeled primer 2 (see "Materials and Methods"). Individual genotypes are listed below the gel.

10%; therefore, approximately 10 centimorgans (or $1 \times 10^7$ base pairs) on both sides of the $\beta$-MHC chain can be excluded on chromosome 14, including the gene coding for the $\alpha$-myosin heavy chain.

Multipoint analysis was also performed, and the order MYO I-MYO II-pSC14 corresponding to the order of the markers on the $\beta$-MHC gene was chosen for this haplotyping. Under the assumption of full penetrance and either a high or low false-positive diagnosis rate, the same seven families showed absence of linkage (Table 2). Under the assumption of age-related penetrance, the LOD scores were less negative. They were still statistically significant in three pedigrees when a low rate of false-positive diagnosis was used, and the sum of the LOD scores over all families was strongly negative ($-17.13$). Significance decreased markedly on a family-by-family basis under the hypothesis of high false-positive diagnosis rates, and only pedigree 704 showed linkage exclusion. However, the sum was still inferior to $-2 (-4.83$ at $\theta=0$), suggesting that even under these stringent conditions, the $\beta$-MHC gene was not linked to FHC in this population. The size of the excluded region under these conditions was approximately 13.7 cM.

Absence of Linkage to the Cardiac Actin Gene

The actin microsatellite ACT is polymorphic, and we observed from two to eight alleles in the various pedigrees. Obligate recombinants were found in pedigrees 701, 702, 703, 706, 707, and 709 (not shown). Table 3 indicates that the cardiac actin gene was excluded under the assumption of full penetrance with the same high statistical significance (LOD scores of $-2$ for $\theta=0$) in the same seven families where the $\beta$-MHC gene was previously excluded. Again, pedigree 708 was noninformative. The sum allowed exclusion until the recombination fraction was 20%, equivalent to $\pm 5 \times 10^7$ base pairs or 50 centimorgans on chromosome 15. The most stringent conditions of age-related penetrance and a high rate of false-positive diagnosis still yielded a sum of less than $-2 (-6.84$ at $\theta=0$) and an excluded region of $2 \times 10^7$ base pairs.

Discussion

It is often difficult to have access to large families (over 50 members); therefore, to facilitate genetic analyses of small pedigrees we used a strategy based on the use of informative probes. Construction of the linkage map of the human genome showed that simple sequence repeats often display extensive length polymorphisms that can be rapidly detected by polymerase chain reaction amplification. In fact, it became apparent that (TG)n repeats can serve as informative and widely distributed markers.12,20 We thought that the two microsatellites contained in the $\beta$-MHC gene would also be polymorphic and more informative than standard RFLP markers. From Table 1, the advantages of these microsatellites with three to six alleles over classical biallelic RFLPs are clear: four pedigrees were informative with the classical probes, and seven were informative with the microsatellite markers. Linkage confirmation by two sets of independent polymorphic markers and increased linkage information available by haplotyping (since MYO I and MYO II are adjacent on the genome) provided additional advantages. From these results, we

<table>
<thead>
<tr>
<th>Probes</th>
<th>CRI-L436</th>
<th>pSC14</th>
<th>MYO I</th>
<th>MYO II</th>
<th>Multipoints</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
<td>0.05</td>
<td>0.00</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>701</td>
<td>$-\neq$</td>
<td>$-4.20$</td>
<td>$-\neq$</td>
<td>$-0.67$</td>
<td>$-\neq$</td>
</tr>
<tr>
<td>702</td>
<td>$-0.35$</td>
<td>$-0.20$</td>
<td>$-0.01$</td>
<td>$-0.01$</td>
<td>$-\neq$</td>
</tr>
<tr>
<td>703</td>
<td>$-\neq$</td>
<td>$-1.44$</td>
<td>0.55</td>
<td>0.42</td>
<td>$-\neq$</td>
</tr>
<tr>
<td>704</td>
<td>0.74</td>
<td>0.60</td>
<td>0.41</td>
<td>0.35</td>
<td>$-\neq$</td>
</tr>
<tr>
<td>705</td>
<td>$-\neq$</td>
<td>$-0.46$</td>
<td>0.60</td>
<td>0.54</td>
<td>$-\neq$</td>
</tr>
<tr>
<td>707</td>
<td>$-3.66$</td>
<td>$-0.58$</td>
<td>$-3.78$</td>
<td>$-0.59$</td>
<td>$-\neq$</td>
</tr>
<tr>
<td>708</td>
<td>$-0.18$</td>
<td>$-0.11$</td>
<td>$-0.10$</td>
<td>$-0.08$</td>
<td>0.00</td>
</tr>
<tr>
<td>709</td>
<td>0.42</td>
<td>0.38</td>
<td>0.18</td>
<td>0.18</td>
<td>$-3.54$</td>
</tr>
</tbody>
</table>

$\theta$, Recombination fraction; multipoints, MYO I-MYO II-pSC14. LOD scores indicate the statistical likelihood that two loci are linked.
Table 2. Two-Point and Multipoint LOD Scores for Familial Hypertrophic Cardiomyopathy With β-Myosin Heavy Chain Gene Markers Under the Assumption of Age-Related Penetrance

<table>
<thead>
<tr>
<th>Probes</th>
<th>Two points</th>
<th>Multipoints</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRI-L36</td>
<td>pSC14</td>
</tr>
<tr>
<td>(θ)</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>701</td>
<td>-3.23</td>
<td>-1.75</td>
</tr>
<tr>
<td>702</td>
<td>-0.35</td>
<td>-0.21</td>
</tr>
<tr>
<td>703</td>
<td>-1.88</td>
<td>-1.12</td>
</tr>
<tr>
<td>704</td>
<td>0.65</td>
<td>0.54</td>
</tr>
<tr>
<td>706</td>
<td>-0.12</td>
<td>-0.06</td>
</tr>
<tr>
<td>707</td>
<td>-0.37</td>
<td>-0.25</td>
</tr>
<tr>
<td>708</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>709</td>
<td>0.42</td>
<td>0.38</td>
</tr>
<tr>
<td>Total</td>
<td>-3.96</td>
<td>-2.46</td>
</tr>
</tbody>
</table>

θ, Recombination fraction; High FPDR, high false-positive diagnosis rate (<20 years, 0.01; 20–40 years, 0.02; >40 years, 0.05). Values for age-related penetrance are as follows: <20 years, 0.26; 20–40 years, 0.86; 40–70 years, 0.95; >70 years, 1.00.

We were able to exclude FHC linkage from 10–20% of chromosome 14. Why none of the families showed linkage to the β-MHC gene as demonstrated previously by others is unknown. This discrepancy is possibly (but not likely) due to a particular geographic distribution of another mutated gene since the majority of previously linked families were of varied ethnic origin from across the United States and Canada.5,8,9 Diagnostic errors in our study can be discarded since extreme care was taken in the evaluation of the clinical and echocardiographic criteria. All clinical findings were typical of those classically described for FHC and identical to those used by previous authors. Atypical forms without hypertrophy have been described in two families.2 We cannot exclude the possibility that some of our completely normal individuals had some myocyte disarray, but this possibility also existed in the previous studies in which linkage was found. It should be noted that Rosenzweig et al,10 using a new diagnostic test that relies on the detection of mutations in the β-MHC gene, found in a large family that six children under 20 years of age carried the mutation without clinical symptoms. Even if a similar phenomenon existed in our pedigrees, this would not have changed the significance of our results since the age-related penetrance already allows for such a possibility.

Because myosin heavy chain mutations were previously incriminated in some forms of FHC, another obvious candidate gene was actin. We detected fewer alleles for the cardiac actin microsatellite than did Litt and Luty12 (eight instead of 12 alleles), probably because of the smaller population size. Nonetheless, the results were highly informative (Table 3), and we were able to exclude linkage of FHC to the cardiac actin gene and to 20–40% of chromosome 15 (total length, 10×107 base pairs). However, this does not rule out the possibility that another sarcomeric actin could be involved, since we recently demonstrated that the skeletal isoform, whose gene is located on chromosome 1, is the major constituent of the adult human heart.21

In conclusion, the results presented here based on eight French families rule out genetic linkage of an FHC disease allele to α- and β-MHC and to cardiac actin genes and confirm that the expression of FHC is compounded by both clinical and genetic heterogeneity.

Acknowledgments

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