An Evaluation of Ribonuclease Protection Assays for the Detection of \(\beta\)-Cardiac Myosin Heavy Chain Gene Mutations

Calum A. MacRae, MRCP; Hugh C. Watkins, MRCP; John A. JARCHO, MD; Ludwig Thierfelder, MD; William J. McKenna, MD; J.G. Seidman, PhD; C.E. Seidman, MD

**Background** Ribonuclease (RNase) protection has been used to identify \(\beta\)-cardiac myosin heavy chain (MHC) gene mutations that cause familial hypertrophic cardiomyopathy (FHC). Since more than 10 different mutations within this gene have been demonstrated to cause FHC in unrelated individuals, the genetic diagnosis of this condition will involve screening the \(\beta\)-MHC gene. The accuracy with which RNase protection identifies such mutations is critical to defining the utility of this methodology in detecting mutations that cause FHC.

**Methods and Results** Twelve unrelated individuals with FHC were selected for further study because their \(\beta\)-MHC genes had been screened for mutations by use of RNase protection, and no mutation was found. We performed linkage analysis of the families of these 12 probands using polymorphic short tandem repeats within the \(\beta\)-MHC gene to determine whether FHC was genetically linked to the MHC locus on chromosome 14. FHC was not genetically linked to the MHC locus in 11 families whose \(\beta\)-cardiac MHC gene did not contain mutations detectable by RNase protection.

**Conclusions** We conclude that RNase protection is a sensitive method for screening for mutations within the \(\beta\)-cardiac MHC gene. Further, mutations in the noncoding regions of the \(\beta\)-MHC gene and mutations in the \(\alpha\)-cardiac MHC gene are not a common cause of FHC. Negative RNase protection assays of affected individuals suggest that their FHC is due to mutations at other loci. (*Circulation.* 1994;89:33-35.)

**Key Words** • hypertrophy • cardiomyopathy • genetics
• myosin

**Familial hypertrophic cardiomyopathy** (FHC) is an autosomal dominant disorder that produces unexplained ventricular hypertrophy. Affected individuals may experience substantial morbidity and premature mortality. Despite these serious sequelae, FHC has not been lost throughout human evolution, in part because affected individuals can propagate their mutation and in part because new mutations appear to contribute to this disorder. Linkage analyses have demonstrated that mutations at four different loci (located on chromosomes 1, 11, 14, and 15) can cause the same hypertrophic phenotype. 1-4 At present, only the disease-causing gene on chromosome 14 has been identified. Ten different missense mutations in the \(\beta\)-cardiac myosin heavy chain (MHC) gene have been demonstrated to cause FHC. 5-7 Further, of all individuals whose disease is caused by \(\beta\)-cardiac MHC missense mutations, only a small fraction share the same mutation (Reference 6, and H.C. Watkins, J.G. Seidman, and C.E. Seidman, unpublished data). To develop procedures for accurate genetic diagnosis of families with FHC, both locus and allelic heterogeneity must be addressed. We report here the sensitivity of ribonuclease (RNase) protection as a method for identifying missense mutations in the \(\beta\)-cardiac MHC gene that cause FHC. 6,8,9 A general problem in defining the molecular basis of a number of different autosomal dominant traits is to identify single-base mutations within a large gene. The \(\beta\)-cardiac MHC gene is organized in 40 exons that span approximately 30,000 bp of DNA. The RNase protection method described below examines only coding sequences for mutations. Hence, mutations that alter regulatory sequences or introns should not be detected. In addition, the \(\alpha\)-cardiac MHC gene located \(\approx\)4000 bp downstream was not examined. To assess the sensitivity of RNase protection for FHC mutation identification at the cardiac MHC gene locus, we have undertaken a retrospective evaluation of our results. RNase protection assays did not demonstrate any missense mutations within the \(\beta\)-cardiac MHC genes of 12 unrelated FHC probands. We obtained DNA samples from the members of the families of these 12 probands and performed genetic linkage analyses with polymorphisms within the \(\beta\)-cardiac MHC gene. In 11 families, evidence of nonlinkage between FHC and the cardiac MHC gene locus was observed. We conclude that RNase protection is a sensitive method for detecting FHC-causing mutations in the coding portions of the \(\beta\)-cardiac MHC gene. Further, mutations in noncoding regions of the \(\beta\)-cardiac MHC gene and the \(\alpha\)-cardiac MHC gene do not appear to be a significant cause of FHC.

**Methods**

**Clinical Evaluation**

These studies were performed in compliance with local institutional review board standards. A full clinical assessment
of each family member was undertaken with the individual's consent. Each subject was examined by an experienced clinician, and a 12-lead ECG, two-dimensional echocardiogram, and Doppler recordings were obtained. Standard ECG and echocardiographic criteria were used according to previously described protocols. All unaffected individuals used for linkage analyses were >21 years old. Two experienced clinicians who were unaware of the results of genetic analysis scored each individual as affected or unaffected.

**RNase Protection Assay**

DNA was extracted from peripheral blood samples or lymphoblastoid cell lines. RNase protection was carried out as previously described. Briefly, segments of β-MHC cDNA or individual β-MHC exons from affected probands were amplified with the polymerase chain reaction (PCR) and hybridized with complementary sense and antisense riboprobe transcribed from normal β-MHC cDNA. Digestion of the resultant RNA/DNA hybrids with RNase resulted in a characteristic pattern of labeled fragments that were resolved on polyacrylamide gel electrophoresis. All abnormal products were investigated further and sequenced to distinguish simple sequence polymorphisms from coding changes. The assay conditions were optimized to ensure the detection of all single-base mismatches. The activity of each lot of RNase A was assessed by digesting mismatch and perfect RNA/DNA hybrids with different amounts of RNase A. The optimal concentration of enzyme was one that digested the mismatch RNA/DNA hybrid to completion but digested only a small amount of the matched RNA/DNA hybrid.

**Linkage Analyses**

Linkage analyses were performed by use of two highly informative CA, short tandem repeat sequences (STRs) within the β-MHC gene, one located in a 5' untranslated sequence and the other in intron 24. With the PCR, these STRs were amplified from genomic DNA from each family member, using PCR primers that bracket the polymorphic repeat sequences as previously described. Alleles were scored by two independent observers blinded to the results of the clinical assessment. Logarithm of the odds (LOD) scores were then calculated for each family with the LINKMAP program with penetrance set at 0.98. The penetrance of 0.98 is based on our observation that in more than 100 individuals whose FHC is due to β-cardiac MHC missense mutations, clinical signs of disease were absent in only 1 genetically affected individual >21 years old. LOD scores of <−1.3 indicate odds of >20:1 against linkage.

**Results**

The β-cardiac MHC gene of FHC probands was screened for mutations by use of RNase protection. A missense mutation was identified in approximately 40% of probands studied. Twelve FHC probands in whom a β-MHC gene mutation was not identified were from families that appeared to be large enough to assess linkage (or nonlinkage) to the cardiac MHC locus. The pedigrees of these families are shown in the Figure. Genetic linkage between FHC and the β-cardiac MHC locus was assessed in each family. The likelihoods of linkage (LOD scores) for two polymorphic loci within the β-MHC gene to FHC for each family are shown in the Table.

Linkage analyses were informative in 11 of 12 families with FHC. In these 11 families, LOD scores were sufficiently negative (LOD score <1.30 equivalent to odds of <1 in 20) to exclude linkage to either the α- or β-MHC genes. The LOD score for both STRs in family 3 was noninformative and could neither confirm nor refute linkage of FHC to the MHC loci.

Subsequent analyses of families AU and OO demonstrated evidence of linkage to the CMH-2 locus on chromosome 1.

**Discussion**

The data presented here demonstrate that RNase protection is a sensitive method for identifying β-cardiac MHC missense mutations that cause FHC. Further, these studies suggest that mutations in noncoding sequences of the β-cardiac MHC gene or the α-MHC gene rarely, if ever, cause FHC.

Previous studies of RNase protection in other contexts have estimated an overall sensitivity ranging from 70% to almost 100%. The high sensitivity of the
Linkage Analyses of FHC and the β-Cardiac MHC Gene in 12 Families

<table>
<thead>
<tr>
<th>Family*</th>
<th>5' Untran.†</th>
<th>Intron 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>XX</td>
<td>-3.11</td>
<td>-3.38</td>
</tr>
<tr>
<td>WW</td>
<td>-3.20</td>
<td>-3.11</td>
</tr>
<tr>
<td>PP</td>
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<td>-0.61</td>
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<tr>
<td>ZZ</td>
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<td>O</td>
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<td>-8.41</td>
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<td>-</td>
</tr>
<tr>
<td>D</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>NN</td>
<td>-3.10</td>
<td>-2.94</td>
</tr>
</tbody>
</table>

*A families correspond to the pedigrees shown in the Figure. †Two polymorphic sites within the 5' untranslated region (5' UnTRAN.) and Intron 24 were used for linkage analyses. Logarithm of the odds score for the linkage between the β-cardiac MHC gene site and FHC in each family is indicated at 7=0. FHC indicates familial hypertrophic cardiomyopathy; MHC, myosin heavy chain.

technique in the detection of cardiac β-MHC gene mutations that we have demonstrated appears to be a function of two parameters. First, we have optimized RNase protection conditions; RNase concentration was adjusted to maximize detection of mispaired bases (see “Methods”). Second, a high proportion of mutations in FHC involve transitions at CpG dinucleotides, and these nucleotide mutations are most easily detected by RNase protection.

Although only β-MHC coding region mutations have been described in FHC, noncoding β-MHC mutations or α-MHC mutations remained a possibility on the basis of linkage analyses alone. Previous studies have not searched the noncoding regions of the β-MHC gene or the α-cardiac MHC gene for mutations that might cause FHC. The exclusion of linkage between the MHC locus and the disease in the 11 families described here implies that mutations in the noncoding regions of the β-MHC gene or in the α-cardiac MHC gene have no significant role, if any, in the genesis of FHC.

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