Mutations in the Human Muscle LIM Protein Gene in Families With Hypertrophic Cardiomyopathy

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Background—Muscle LIM protein (MLP) is an essential nuclear regulator of myogenic differentiation. Additionally, it may act as an integrator of protein assembly of the actin-based cytoskeleton. MLP-knockout mice develop a marked cardiac hypertrophy reaction and dilated cardiomyopathy (DCM). MLP is therefore a candidate gene for heritable forms of hypertrophic cardiomyopathy (HCM) and DCM in humans.

Methods and Results—We analyzed 1100 unrelated individuals (400 patients with DCM, 200 patients with HCM, and 500 controls) for mutations in the human CRP3 gene that encodes MLP. We found 3 different missense mutations in 3 unrelated patients with familial HCM but detected no mutation in the DCM group or the controls. All mutations predicted an amino acid exchange at highly conserved residues in the functionally important LIM1 domain, which is responsible for interaction with α-actinin and with certain muscle-specific transcription factors. Protein-binding studies indicate that mutations in the CRP3 gene lead to a decreased binding activity of MLP to α-actinin. All 3 index patients were characterized by typical asymmetrical septal hypertrophy. Family studies revealed cosegregation of clinically affected individuals with the respective mutations in MLP.

Conclusion—Here, we present evidence that mutations in the CRP3/MLP gene can cause HCM. (Circulation. 2003;107:1390-1395.)

Key Words: cardiomyopathy ■ genes ■ hypertrophy

Muscle LIM protein (MLP) is an LIM-only protein that shares structural and functional properties of the cysteinerich protein (CRP) subfamily. These proteins are composed of 2 LIM domains, defined by a unique double zinc finger structure. It has been suggested that the LIM domain acts as a protein-binding interface. Expression of MLP is confined to skeletal and cardiac muscle. In the myocardium, MLP is expressed at high levels in developing and adult cardiomyocytes.1

MLP acts as an essential promoter of myogenesis, and it may be a cofactor in the regulation of muscle-specific gene expression in skeletal and heart muscle.1 By linking the β-spectrin network to myofibrillar actin filaments, MLP may also stabilize the association of the contractile apparatus with the sarcolemma.2

A murine MLP gene knockout was established to gain further insight into the function of MLP.3 MLP-deficient mice develop 2 different cardiac phenotypes. Thirty-five percent to 65% of the mice develop an early phenotype characterized by 2- to 4-fold increased heart weight and a hypertrophy reaction. They die of rapid progressive congestive heart failure in the second postnatal week. The remaining fraction of animals present with an adult phenotype reminiscent of patients with dilated cardiomyopathy (DCM).

Most cases of hypertrophic cardiomyopathy (HCM) and ≈25% of cases of DCM were estimated to have a genetic cause (see recent reviews4,5). Although several disease genes have been identified, a substantial proportion of familial cases cannot presently be linked to any of the known disease genes. Human CRP3, which encodes MLP, was considered a promising candidate. Therefore, we screened 600 individuals with either DCM or HCM for mutations in CRP3. We found 3 different missense mutations in HCM families that cause amino acid substitutions in a functionally important region of MLP. Furthermore, we investigated whether mutations in

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MLP might cause alterations in its binding characteristics. These protein-binding studies demonstrate that at least 1 of the mutations significantly decreases the capacity of MLP to bind α-actinin.

Methods

Clinical Evaluation

All patients and family members gave informed consent to participate in the study in accordance with the ethical guidelines of Charité University Hospital. Blood samples were drawn for genetic investigation, medical history was taken, and an examination was performed that included a 12-lead ECG and 2D and M-mode echocardiography. All DCM patients had left heart catheterization with selective coronary angiography to exclude coronary artery disease. The diagnosis of DCM was based on World Health Organization criteria. The diagnosis of HCM was based on criteria proposed by McKenna et al.6 Additionally, tissue Doppler imaging (TDI) with determination of septal early (Ea) and late (Aa) diastolic velocities without knowledge of the genetic results. Arterial hypertension was identified in patients with HCM (unpublished data). The primer sequences were as follows:

- exon 1F 5'-CAGGGCTTTGTCACAGTCT-3'
- exon 1R 5'-AAACCCAGCCACAGAACCAC-3'
- exon 2F 5'-GAGATGGTGTCACTCTCGGG-3'
- exon 2R 5'-TTCCTCCTGTTAATGTCGTC-3'
- exon 3F 5'-CAAAGGAAGGGGAGAAGGATT-3'
- exon 3R 5'-TGGAACGGAAATGGACTGTC-3'
- exon 4F 5'-TTTGGCAAGGGGAACTTACG-3'
- exon 4R 5'-GGCTGGATTGTGTGGTTTC-3'
- exon 5F 5'-GCTGTCAAGGGACTTGAAT-3'
- exon 5R 5'-CCTCCAGGCATGAACTTAT-3'
- exon 6F 5'-TAAAGGACTGTTGCTCAGAC-3'
- exon 6R 5'-GTACGACTCACAAGGGAGG-3'

The images of the models were generated with Molscript,13 Raster3D,14 and Swiss PDB Viewer.15

Yeast 2-Hybrid Assay

The complete human α-actinin-210 and MLP cDNA sequences were amplified by PCR with a human skeletal muscle library (BD Biosciences Clontech) as a template. The cDNAs were cloned into a modified pLEXA vector or a modified pACT2 vector, respectively. The MLP Cys58Gly mutation was introduced into the full-length wild-type sequence by PCR amplification with mismatch oligonucleotides (sequences, mismatched nucleotides capitalized: 5'-ttaggagacctgcgaattctgagtgg-3' and 5'-ccagagacctgcgtagctgcgcaaa-3'). The mutated cDNA was also cloned into the modified pACT2 vector. PCR mutagenesis and cloning were done by standard procedures.17 All constructs were verified by sequencing.

The α-actinin construct was pretransformed into the Saccharomyces cerevisiae L40 reporter strain18 by a modified lithium acetate protocol.19 Subsequently, the pretransformed cells were cotransformed with the wild-type or mutant MLP prey plasmid. Selection of transformant colonies was performed on agar plates that lacked leucine and tryptophan. The transformants that appeared after 4 to 5 days' incubation at 30°C were oculated in selective medium. Cells were lysed, and β-galactosidase activity was quantified with o-nitrophenyl β-D-galacto-pyranoside (ONPG) according to the protocol on in the “Yeast Protocols Handbook” (BD Biosciences Clontech). Activities were expressed as mean arbitrary units ±SD. The β-galactosidase activity of wild-type MLP was set to 100 arbitrary units. The significance of the difference between the observed activities of wild-type and mutant MLP was tested by the Student t test.

Results

Missense Mutations in the Human MLP Gene

Three different heterozygous missense mutations in CRP3, all located in exon 3, were detected in 3 unrelated patients with familial HCM (Figure 1B). Mutations that predicted a single amino acid exchange were detected in 2 patients: a CTT→CCT mutation in codon 44 that predicted a leucine-to-proline exchange (Leu44Pro) and a TGC→GGC mutation in codon 58 that predicted a cysteine-to-glycine exchange (Cys58Gly). The third mutation affected 3 nucleotides, TCGGA→AGGGG, in the 2 adjacent codons 54 and 55, predicting an exchange of serine to arginine and glutamic acid to glycine (Ser54Arg/Glu55Gly). The mutation Leu44Pro was confirmed by an additional NlaIV restriction site generated by the mutation. The mutation Ser54Arg/Glu55Gly was confirmed with Hinfl and BgII digests, and the Cys58Gly mutation was confirmed by an additional BceI restriction site. None of the described mutations were present in 1000 control chromosomes.

In the largest of the HCM families examined in this study (family C), all known HCM disease genes could be excluded (data not shown). Genetic analysis did not reveal any mutations that predicted amino acid exchanges in the DCM patients. All mutations were located in the LIM1 domain of MLP (Figures 2A through 2C). An alignment of the corresponding sequences revealed that all mutations affected highly conserved residues (Figure 2A). These residues were completely conserved throughout the entire family of CRPs (CRP1, CRP2, and CRP3). Additionally, the residues showed full conservation across species that included human, mouse, rat, amphibian, fish, and insects. Protein modeling suggested...
changes in the surface charge generated by the mutations found in families A and B compared with wild-type MLP (Figures 3A and 3B) and an alteration in 1 of the zinc ion binding sites generated by the mutation found in family C (Figure 3C), respectively.

A yeast 2-hybrid assay was used to investigate the potential impact of the human MLP(Cys58Gly) mutant on the interaction with the myocardial isoform of human α-actinin (α-actinin-2). Yeast cells that coexpressed α-actinin-2 and the MLP(Cys58Gly) mutant showed significantly reduced β-galactosidase activity compared with cells that coexpressed α-actinin-2 and wild-type MLP (Figure 4). This indicates reduced binding affinity of mutated MLP to α-actinin. Additionally, yeast cells that expressed mutant MLP grew significantly slower on plates that selected for activation of the H3S reporter gene (data not shown). To confirm the results of yeast 2-hybrid assays, we also performed blot overlay assays and obtained corresponding results (data not shown).
Clinical Findings

In family A, a Leu44Pro mutation was detected in 3 individuals (II:2, II:3, and II:4; Figure 1A). Individual II:2 showed marked midventricular hypertrophy at the age of 28 years. On ECG, he had T-wave inversions in leads I and aVL and deep S V2/3. Holter ECG revealed ventricular tachycardias of Lown class IVa. His 32-year-old brother (II:3) also showed midventricular hypertrophy. On ECG, he had minor repolarization changes in left ventricular leads. The oldest brother (II:4) had concentric hypertrophy and minor T-wave abnormalities. We found thus-far undetected and uncontrolled moderate arterial hypertension in him. Therefore, this individual’s clinical status was assigned “not classifiable.” All 3 brothers were asymptomatic (clinical data summarized in the Table). The father (I:1) had died at the age of 67 years of cancer. No information with respect to HCM was available on him. Cardiac hypertrophy was excluded in the mother. Individual III:1, the son of II:2, had normal results on cardiac examination.

In family B, 3 individuals were found to have the Ser54Arg/Glu55Gly mutations (II:2, II:3, and II:4). Individual II:2 reported exertional dyspnea and palpitations at the age of 44 years. He had marked concentric left ventricular hypertrophy with severely impaired diastolic compliance, first-degree AV block, and ST-segment depressions in left anterior ECG leads. Holter ECG revealed paroxysmal atrial fibrillation and ventricular tachycardias (Lown class IVb). Individual II:3 reported exertional dyspnea; echocardiography revealed pathological septal TDI indices in the absence of hypertrophy. Individual II:4 reported palpitations and angina. On ECG, she had left ventricular hypertrophy with repolarization changes and deep S V2/V3 (fulfilling 1 major plus 1 minor ECG criterion of HCM). On echocardiography, she showed regional hypertrophy in the proximal septum; normal indices were recorded on TDI. The father (I:1) had died of sudden death at the age of 77 years. He had reported palpitations and shortness of breath before his death. No echocardiographic data were available on him. Individuals I:2, II:1, and III:1 showed no pathological results on cardiac examination.

In family C, a Cys58Gly mutation was found in individual III:2, in his 2 cousins (III:6 and III:8), and in the son (IV:2) of individual III:6. HCM was diagnosed in III:2 at the age of 56 years. Transaortic subvalvular myectomy (Morrow procedure) was performed because of severe outflow tract obstruction 1 year later. ECG revealed left bundle-branch block and a Sokolow-Lyon Index of 4.8 mV. Individuals III:3, III:4, III:5, and II:1 (all more than 60 years old) were clinically unaffected, but arterial hypertension was diagnosed in III:3.

A cousin (III:6) of the index patient also had HCM with marked asymmetrical septal hypertrophy and moderate outflow tract obstruction. ECG showed repolarization changes in left ventricular leads. His 42-year-old son (IV:2) reported no symptoms. On echocardiography, he showed pathological septal TDI indices in the presence of borderline septal wall thickness. Arterial hypertension was excluded. Individual III:8 reported paroxysmal palpitations. Echocardiography revealed eccentric septal hypertrophy in the presence of
borderline arterial hypertension. ECG recordings were normal. Two uncles, homozygotic twins (II:5 and II:6), and 1 cousin (III:9) of the index patient had died of sudden cardiac death in early childhood. The 44-year-old daughter (IV:1) of the index patient had normal results on cardiac examination.

**Discussion**

We identified 3 different missense mutations in human CRP3 coding for MLP in 3 families with HCM. The mutations are located in the functionally important LIM1 domain, which affects highly conserved residues. Genetically affected individuals showed mild symptoms and a great variation in the extent of hypertrophy.

For better characterization of individuals without hypertrophy, we performed TDI. This method is able to detect early pathological changes in the heart. Because the probands in the study of Nagueh et al were all less than 60 years old, and myocardial velocities decrease with age, we applied this method only to those individuals less than 60 years old. All 3 indices required to fulfill the criterion of "pathological TDI values" have been established as sensitive and specific markers of mutation-positive subjects without left ventricular hypertrophy.

The mutation-carrying individuals II:3 in family B and IV:2 in family C showed pathological TDI indices in the basal septal wall without apparent septal hypertrophy. None of the individuals without mutation presented pathological TDI indices. Individual II:4 in family B had normal TDI but ECG changes that fulfilled 1 major plus 1 minor ECG criterion for HCM. Therefore, she was classified as affected.

We consider these sequence alterations as disease-causing mutations rather than rare polymorphisms for 7 different reasons:

1. All clinically affected relatives of the 3 families had the respective mutation, whereas relatives with normal cardiac findings did not. This shows cosegregation of the mutations with the disease. Additionally, all known HCM disease genes were excluded in the largest HCM family.
2. The presence of 3 different mutations in 3 unrelated HCM families within the same exon of CRP3 makes the possible explanation of rare polymorphisms highly unlikely.
3. Neither of the MLP mutations was present in 500 controls or 400 patients with DCM. Analysis showed a significant difference of the frequency of MLP mutations in HCM patients compared with controls (P=0.0061). This argues for disease-causing mutations.
4. Further evidence for the significance of the MLP mutations comes from an alignment with the altered sequences. All the mutations predicted amino acid exchanges of highly conserved residues. We found that the altered residues were conserved throughout the family of CRPs both in humans and across species, including avian and invertebrate species.
5. Modeling suggested that the mutations detected in families A and B alter the electrostatic surface of the protein. The mutation Leu44Pro should not cause a
569. We hypothesized that the mutations influence the interaction of MLP with α-actinin, and we searched for confirming evidence. Our finding that the binding affinity of the MLP(Cys58Gly) mutant to α-actinin was significantly impaired in yeast 2-hybrid assays supports this idea. Considering the observations that MLP binds the Z-line protein α-actinin and β-spectrin at the sarcolemma, we hypothesize that the impaired interaction of mutant MLP with α-actinin results in the disturbed myocyte cytoarchitecture observed in HCM patients.

Conclusions

This is the first report describing CRP3 mutations in humans. Our data provide strong evidence that human CRP3 coding for MLP is a novel disease gene that causes HCM.

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