α-cardiac actin is a novel disease gene in familial hypertrophic cardiomyopathy

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We identified the α-cardiac actin gene (*ACTC*) as a novel disease gene in a pedigree suffering from familial hypertrophic cardiomyopathy (FHC). Linkage analyses excluded all the previously reported FHC loci as possible disease loci in the family studied, with lod scores varying between –2.5 and –6.0. Further linkage analyses of plausible candidate genes highly expressed in the adult human heart identified *ACTC* as the most likely disease gene, showing a maximal lod score of 3.6. Mutation analysis of *ACTC* revealed an Ala295Ser mutation in exon 5 close to 2 missense mutations recently described to cause the inherited form of idiopathic dilated cardiomyopathy (IDC). *ACTC* is the first sarcomeric gene described in which mutations are responsible for 2 different cardiomyopathies. We hypothesize that *ACTC* mutations affecting sarcomere contraction lead to FHC and that mutations affecting force transmission from the sarcomere to the surrounding syncytium lead to IDC.

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Introduction

Familial hypertrophic cardiomyopathy (FHC) is inherited as an autosomal dominant disease with a prevalence of about 1 in 500 and is characterized by unexplained hypertrophy of the heart muscle. The hypertrophy predominantly involves the interventricular septum, although any localization within the myocardium may appear. Eventually, about 10% of the patients exhibit progressive wall thinning with dilation of the left ventricle. Typical morphological changes include myocyte hypertrophy with myocytic fiber disarray surrounding areas of increased connective tissue. Clinically, the condition ranges from an asymptomatic course to incidents of dizziness and syncopes, chest pain, and symptoms of heart failure. FHC is also a major cause of sudden cardiac death among young, otherwise healthy athletes (1). Our understanding of the pathophysiological basis of the disease has increased dramatically during the last decade, not in the least because of genetic investigations. Disease-causing mutations have been described in 7 different genes, all encoding sarcomeric polypeptides. Therefore, FHC is assumed to be a disease of the heart sarcomere. The current hypothesis suggests that FHC is caused by impaired contractile performance of the sarcomere, leading to a compensatory hypertrophy of the heart (2, 3). More than 100 mutations have been reported, and missense mutations are the most prevalent type of change within the following genes encoding the polypeptides given in parentheses: *MYH7* (β-myosin heavy chain), *TNNI3* (troponin I), *TNNT2* (troponin T), *TPM1* (α-tropomyosin), *MYBPC3* (myosin-binding protein C), *MYL2* (regulatory myosin light chain), and *MYL3* (essential myosin light chain) (4). Furthermore, a disease locus on chromosome 7 has been linked to FHC, but the gene remains to be identified (5). Despite the numerous disease loci, it has not been possible to reveal mutations in the known disease genes in a substantial part of FHC families. In recent studies of French and Asian populations, it was possible to identify mutations in only 57% of the FHC cases (6, 7). These results indicate that not all FHC genes have been identified yet.

The present study reports on the investigation of a large FHC family, leading to the identification of a novel disease gene.

Methods

Clinical evaluations. Eight families were studied. Clinical investigations and blood samples for DNA analyses were obtained from family members after informed written consent, in accordance with local institutional review committee guidelines. Echocardiography was performed and analyzed by senior cardiologists. Standard 12-lead electrocardiograms were obtained with individuals in supine position during quiet respiration. Both examinations were performed and evaluated essentially as described previously (8). In brief, an individual was considered to fulfill major diagnostic criteria if the maximal wall thickness (MWT) measured by echocardiography was >13 mm or if the electrocardiogram presented either major Q-wave abnormalities, left ventricular hypertrophy, or marked repolarization alterations. Individuals were considered to fulfill minor diagnostic criteria if their electrocardiogram presented minor Q waves in at least 2 leads or minor ST-segment changes (individuals III-4 and III-8; Table 1). Minor echocardiography criteria were considered to be fulfilled by an MWT of 13 mm, mitral valve prolapse, and mitral regurgitation (individual II-8; Table 1). Before DNA analyses, all individuals were classified as having major or minor FHC phenotypes.

Linkage analysis. Pairwise and multipoint linkage analyses were carried out using the LINKAGE 5.1 package (http://linkage.rockefeller.edu/index.html) (9). The same age-dependent penetrances were used in linkage analyses of all genes except *MYBPC3*, which is known to be late onset (penetrances given in parentheses): age <30 years, 0.60 (0.45); age between 30 and 49 years, 0.80 (0.60); and age >50 years, 0.95 (0.80) (10). The frequency of phenocopies was defined as the prevalence of the disease (1 in 500). Initial linkage analyses consid-
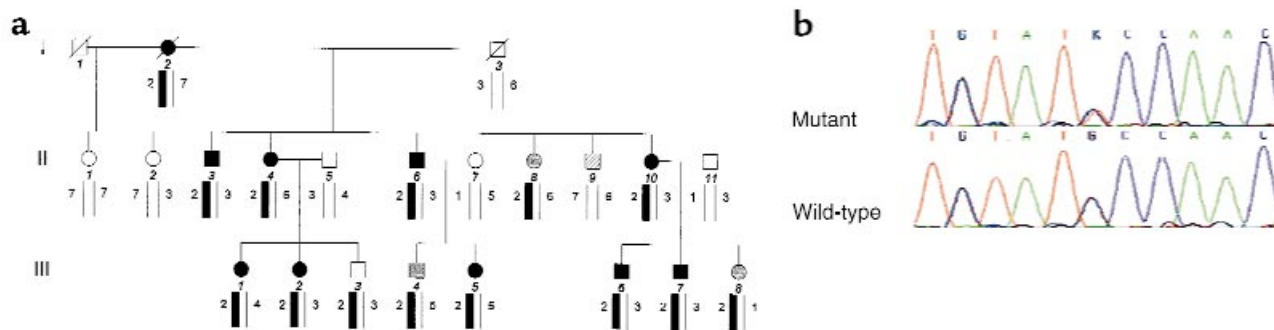
Figure 1
(**a**) Pedigree drawing of the FHC family carrying the *ACTC* Ala295Ser mutation. Individual numbers are given in italics, and symbols are defined as follows: squares, men; circles, women; diagonal slashes, deceased; open symbols, unaffected individuals; filled symbols, affected individuals fulfilling major diagnostic criteria; shaded symbols, affected individuals fulfilling minor diagnostic criteria; hatched symbols, excluded from linkage analyses because of hypertension. Alleles defined by the STR polymorphism within *ACTC* are shown in roman numerals (16). Thin bars indicate normal genotype of *ACTC*; thick bars indicate *ACTC* mutation segregating with allele 2. The genotype of individual I-3 was inferred from offspring. Individuals II-3 and II-8 have 2 children each who did not want to participate in this investigation. This may well influence the otherwise surprising observation that all individuals of the third generation carry the mutant allele. (**b**) Sequence of the *ACTC* wild-type and the G253→T mutated allele.

ered individuals fulfilling major diagnostic criteria as affected (*n* = 10 individuals), whereas individuals fulfilling minor diagnostic criteria were considered unknown and thereby excluded from these analyses (*n* = 3). In subsequent linkage analyses, individuals fulfilling minor diagnostic criteria were considered as affected and were included in the analyses. One individual was excluded from all analyses because of hypertension. Previously published polymorphic short tandem repeats (STRs) were used to perform linkage analyses of the following genes: *ACTA1* (11), *MYL3* (12), the FHC locus on chromosome 7 (5), *MYBPC3* (13), *MYH7* (14), *TPM1* (15), *ACTC* (16), *TNNI3* (17), and *MYL2* (6). *TNNT2* was localized in an interval of 6.7 cM between the markers D1S1660 and D1S504, odds 300:1, by integrating mapping information from various databases and a genetic map (12, 18, 19). Genomic DNA was isolated from either whole blood using a DNA isolation kit (Puregene D-5500; Gentra Systems, Minneapolis, Minnesota, USA) or from paraffin blocks containing lymph node tissue (individual I-2; Table 1) (20). Sense primers were labeled with the fluorescent dyes FAM, HEX, or TET. PCRs were carried out in a total volume of 6 μL with 40 ng DNA, 0.6–2.4 pmol of each primer, 50 μM dNTP, 1× standard PCR buffer (Perkin-Elmer Corp., Norwalk, Connecticut, USA), and 0.25 U AmpliTaq Gold DNA Polymerase (Perkin-Elmer Corp.). Amplifications were carried out using an initial denaturation step (10 minutes at 96°C), followed by annealing (30 seconds at 55°C), extension (1 minute at 72°C), and denaturation (30 seconds at 96°C) for 30–35 cycles, and a final extension step (8 minutes at 72°C) using a GeneAmp PCR System 9700 (Perkin-Elmer Corp.). The PCR products were analyzed on an ABI prism 377 DNA sequencer according to the manufacturer's instructions (Perkin-Elmer Corp.).

Mutation analysis. Primers were designed according to the published genomic sequence of *ACTC* using the OLIGO 4.1 primer analysis software (MedProbe AS, Oslo, Norway) (21). Primer sequences were as follows: exon 1F: 5′-TGCAGAAACCCCCTGAAGC-3′; exon 1R: 5′-GCCATTTCCTAGATCGCTGGA-3′; exon 2F: 5′-TTCCTGACATGGTGAGAGCA-3′; exon 2R: 5′-TCGGTGACTTGGGAATGTG-3′; exon 3F: 5′-GAGCAGTGGTGTTGTCCTCAG-3′; exon 3R: 5′-AAGCAGACCCACACTGTGG-3′; exon 4F: 5′-TTCTTGCTTCAGAGCATGACTG-3′; exon 4R: 5′-AAGATTTCCAGGAAAATCGTGC-3′; exon 5F: 5′-TTGACCTGAATGCACTGTGATG-3′; exon 5R: 5′-TAGAATACCAAGACCTGCCTCG-3′ (to verify the G253→T mutation in the forward direction of exon 5, it was necessary to design the following sequencing primer downstream of the STR polymorphism [16]: 5′-GGCACTCATGAAACAACTTAC-3′); exon 6F: 5′-TTGGAACTTCAGAGTTCACTGG-3′; exon 6R: 5′-AAGAAGCATAATACCGTCATCC-3′. PCR product sizes for exons 1–6 were 223, 425, 259, 282, 312, and 242 bp, respectively. PCR was performed as already described in a final volume of 50 μL using 10 pmol of each primer and 1 U AmpliTaq Gold DNA Polymerase. The annealing temperature used to amplify exons 2 and 3 was 57°C. The PCR products were purified using a PCR purification kit (catalog no. 28104; QIAGEN Inc., Valencia, California, USA) before cycle sequencing. Forward and reverse dRhodamin terminator cycle sequencing was performed using a DNA sequencing kit (catalog no. 403042; Perkin-Elmer Corp.) with a modified protocol using 20 ng of the PCR product, 5 pmol of each primer, and 4 μL of terminator-ready reaction mixture in a final volume of 15 μL. The sequences were analyzed on an ABI prism 377 DNA sequencer as already described.

Ssp1 restriction cleavage. Two *Ssp*1 restriction sites were introduced into the PCR product of exon 5. A forward primer containing the *Ssp*1 site was designed next to the mutation (5′-TGATATCCGCAAGGACCAATAT-3′). An *Ssp*1 site was also incorporated into the reverse primer (5′-CTTAGAATACCAATATTTGCCTCG-3′) serving as a positive control. PCR amplification was performed as already described, with an annealing temperature of 55°C. *Ssp*1 cleavage was performed using 10 μL PCR product, 1× NEB2-buffer, and 15 U *Ssp*1 enzyme (New England Biolabs Inc., Beverly, Massachusetts, USA) in a final volume of 30 μL. The reaction mixture was incubated for 12 hours at 37°C, and an additional 7.5 U of *Ssp*1 enzyme was added for 2 hours. The products were analyzed by 12% PAGE and made visible by ethidium bromide staining.

Results
Clinical investigations. The FHC family investigated (Figure 1a) displayed heterogeneous phenotypes, with the majority of affected individuals having no symptoms (Table 1). However, individual II-4 had pronounced septal hypertrophy of 30 mm, with a left ventricle outflow tract (LVOT) gradient of 64 mmHg at the age of 46 (Figure 2a). Echocardiography performed over the years showed a steady decrease in wall thickness (presently 13 mm) and the development of mild aortic regurgitation (AR). The patient had frequent incidents of dizziness and syncopes caused by nonsustained ventricular tachycardia and had progressive symptoms of heart failure due to diastolic dysfunction of the left ventricle. Heart biopsy specimens taken at ages 46 and

Figure 2

(**a**) M-mode echocardiogram of individual II-4 at age 46. The lower arrow indicates systolic anterior motion of the mitral valve typical of obstructive FHC. AO, aorta. IVS, interventricle septum varying in thickness between 20 and 30 mm. LA, left atrium. LV, left ventricle. (**b**) Endomyocardial biopsy from individual II-4's right ventricle, stained with hematoxylin and eosin. The biopsy fulfilled histological FHC criteria by the presence of marked myocyte hypertrophy, increased amounts of loose connective tissue, and disarray of the myofibrils (32).
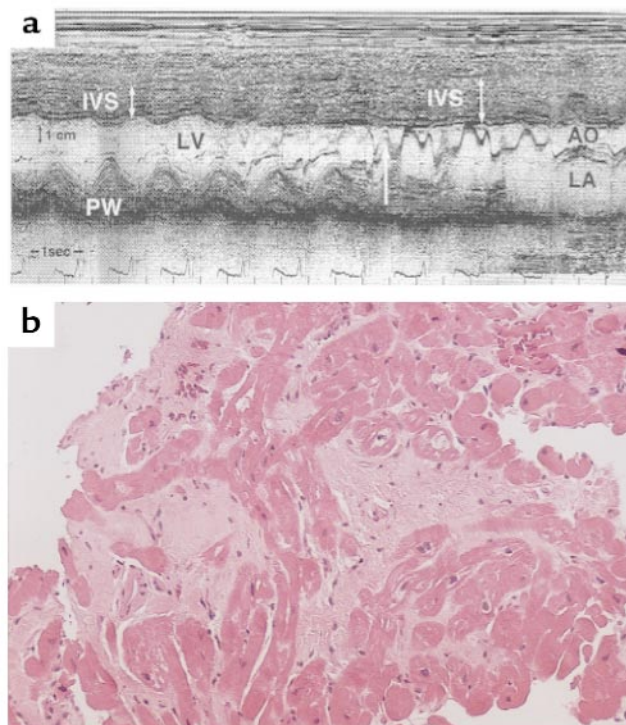
56 showed identical morphological features consistent with FHC (Figure 2b). The patient's mother, individual I-2, had progressive symptoms of heart failure beginning in her late sixties. The heart was dilated with an ejection fraction of 25%, and individual I-2 was classified as having end-stage FHC because she gave birth to 5 children fulfilling FHC diagnostic criteria. Individual III-1 had recurrent episodes of palpitations and syncopes at the age of 32 caused by an accessory atrioventricular conduction pathway known as the Wolff-Parkinson-White syndrome (WPW). Her initial echocardiogram showed borderline hypertrophy in the posterior part of the septum, which, over a 7-year period, enlarged significantly from 12 mm to 19 mm. Individual II-6 had a septal bulge in LVOT and mild AR recorded by Doppler echocardiography. It is not likely that the observed AR caused the very localized hypertrophy, as only severe AR is known to cause global hypertrophy of the left ventricle (22). In addition, individual II-6 is the father of a child (III-5) fulfilling major FHC criteria, making him an obligate carrier of the disease gene. The remaining affected family members were characterized by having an abnormal electrocardiogram, and most of them had a septal bulge in LVOT. Individual III-6 had an early onset of disease, presenting at the age of 4 with a heart murmur caused by a septal bulge in the LVOT.

Genetic investigations. Linkage analyses of all the known disease loci were performed using highly polymorphic STRs. Pairwise and multipoint linkage analyses excluded linkage to all these loci, with lod scores varying between –2.5 and –6.0 using age-specific penetrances and major diagnostic criteria. Additional linkage analyses tested the candidate genes *ACTA1* (encoding α-skeletal actin) and *ACTC* (encoding α-cardiac actin), both of which are highly expressed in the adult human myocardium (23). A multipoint lod score of –5.5 excluded *ACTA1,* whereas *ACTC* was most likely to be the disease gene, showing maximal lod scores of 2.7 and 3.6 at zero recombination fraction using major and minor diagnostic criteria, respectively. Sequence analysis of *ACTC* identified a G→T mutation in position 253 of exon 5 resulting in an Ala295Ser amino acid substitution (Figure 1b). The mutation was localized on the haplotype cosegregating with the disease (Figure 1a). The presence of the mutation was confirmed by an *Ssp*1
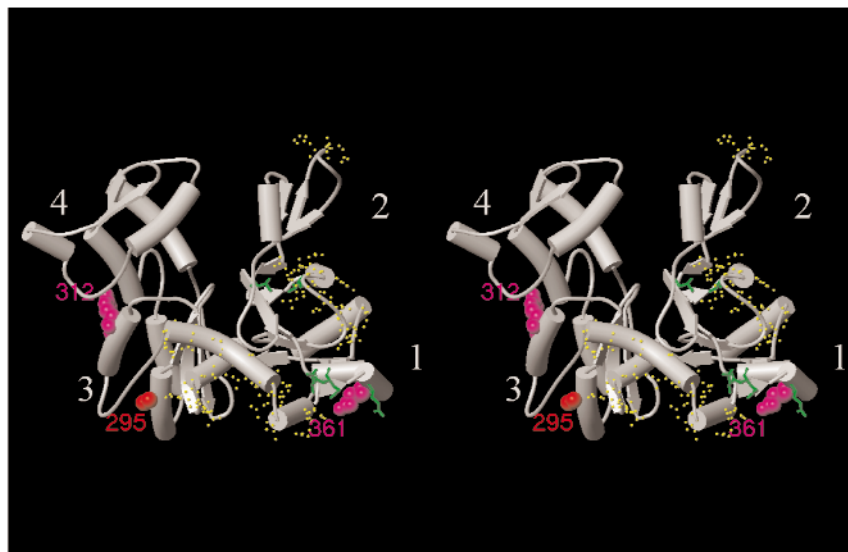

Figure 3

Schematic stereo representation of the actin monomer based on the crystal structure of actin (33). Putative binding sites to myosin are in yellow (28). The Ala295 amino acid mutated in the FHC family is shown in red and localized at the actin surface close to a possible myosin-binding site. The 2 amino acids mutated in IDC (Arg312 and Glu361) are shown in purple. The amino acid differences between α-cardiac actin and the γ-enteric actin expressed in the transgenetically modified *ACTC* knockout mouse are demonstrated in green. The figure was prepared using the protein database (34).

restriction enzyme cleavage assay. All the mutated alleles were digested as expected, leaving the wild-type allele intact (data not shown). To exclude the possiblity that the mutation identified was a common polymorphism, 300 chromosomes from healthy individuals were investigated by either sequencing and *Ssp*1 cleavage (150 chromosomes) or *Ssp*1 cleavage alone (150 chromosomes). No sequence variations were identified, confirming a previous investigation of 870 chromosomes by SSCP analysis of exon 5 (24). This is in accordance with the observation that α-cardiac actin is highly conserved throughout evolution, including the Ala295 amino acid, which is conserved in 19 different species (25). In conclusion, strong evidence was found that the Ala295Ser substitution was the disease-causing mutation and that *ACTC* is the eighth FHC gene. The expression of the actin mutation in this family gave the impression of a highly penetrant disease with diverse phenotypes and a vari-

able age of onset. Only one individual (III-3, age 28) of 13 family members carrying the mutant allele was nonpenetrant, and morbidity was low, as only 3 of 13 family members carrying the mutant allele had symptoms of the disease.

Discussion

The highly conserved actins are the major constituents of the thin filaments in the muscle sarcomere. They are involved in force generation within the sarcomere and force transmission from the sarcomere to the surrounding syncytium. In accordance with its functional role, actin is known to interact directly with the thick-filament polypeptide (β-myosin heavy chain), other thin-filament polypeptides (the light chains, troponin I, α-tropomyosin), and anchor polypeptides (actinin, dystrophin) (26–29). The Ala295Ser substitution identified is localized at the surface of actin in proximity to a putative myosin-binding site (28) (Figure 3). The side chain of alanine 295 is sit-

uated in a surface valley formed by polar and hydrophobic atoms lying between protruding charged actin residues including arginine 147, which forms the outer edge of an actin/myosin–binding site. The introduction of the polar hydroxyl group of serine may distort the surface topology, leading to an impaired myosin binding. Thereby, an adequate contraction of the sarcomere would be prevented, resulting in a compensatory hypertrophy as seen in animal models (30, 31). Recently, 2 missense mutations in *ACTC* were reported to cause idiopathic dilated cardiomyopathy (IDC) in 2 unrelated families (24). The mutations were localized in the immobilized end of actin that cross-binds to the anchor polypeptides in the Z bands. The altered actin was suggested to impair force transmission by defective attachment to the anchor polypeptides. Therefore, it seems likely that mutations in *ACTC* lead to FHC when they involve force generation within the sarcomere, and to IDC when

Table 1
Clinical features of the FHC family carrying the *ACTC* Ala295Ser mutation

Pedigree	Age/sex	Symptoms	Electrocardiogram	Echocardiogram	Phenotypic assignment	*ACTC* genotype
I-2[A]	*76 F	Heart failure	Atrial flutter, LBBB	End-stage FHC	FHC-major	Ala295Ser[B]
I-3[A]	*84 M	Angina, dyspnea	Previous myocardia infarctions	No data	IHD	No data
II-1	62 F	No	Normal	Normal	Normal	Normal
II-2	59 F	No	Normal	Normal	Normal	Normal
II-3	58 M	No	Minor Q waves	Septal bulge in LVOT: 14 mm, MR grade I	FHC-major	Ala295Ser
II-4	56 F	Angina, dyspnea syncopes	LBBB	Previous obstructive FHC, septum presently: 13 mm, AR grade II	FHC-major	Ala295Ser
III-1	37 F	No	Free left posteroseptal WPW	FHC, posterior septum: 19 mm	FHC-major	Ala295Ser
III-2	34 F	No	Minor Q waves	Septal bulge in LVOT: 14 mm	FHC-major	Ala295Ser
III-3	28 M	No	Normal	Normal	Normal	Ala295Ser
II-6	54 M	No	Minor Q waves	Septal bulge in LVOT: 14 mm, AR grade I	FHC-major	Ala295Ser
III-4	29 M	No	Minor Q waves and ST-segment changes	Normal	FHC-minor	Ala295Ser
III-5	25 F	No	Major Q waves	Normal	FHC-major	Ala295Ser
II-8	52 F	No	Normal	MVP, MR grade I, free anterior wall: 13 mm	FHC-minor	Ala295Ser
II-9	51 M	No	Borderline left ventricular hypertrophy	MR grade I	Excluded, known hypertension	Normal
II-10	48 F	No	Minor Q waves, borderline left ventricular hypertrophy	Septal bulge in LVOT:14 mm, MR grade I	FHC-major	Ala295Ser
III-6	26 M	No	Minor Q waves	Septal bulge in LVOT: 15 mm	FHC-major	Ala295Ser
III-7	25 M	No	Left ventricular hypertrophy	MVP	FHC-major	Ala295Ser
III-8	21 F	No	Minor Q waves	Normal	FHC-minor	Ala295Ser

[A]Clinical data on individuals I-2 and I-3 were obtained from hospital records. Echocardiogram done on individual I-2 only. [B]Mutation analysis of individual I-2 was not possible, as the amount of PCR product was only sufficient for establishing the haplotype (Figure 1a). *Deceased. AR, aortic regurgitation. IHD, ischemic heart disease. LBBB, left bundle branch block. LVOT, left ventricle outflow tract. MR, mitral regurgitation. MVP, mitral valve prolapse. SAM, systolic anterior motion of the mitral valve.

they involve force transmission from the sarcomere to the surrounding syncytium. This hypothesis is supported by analyses of an *ACTC* knockout mouse transgenetically modified to express γ-enteric actin instead of α-cardiac actin in the heart muscle (30). The 2 actins differ by 5 amino acids at positions of putative binding sites to myosin and anchor polypeptides (27, 28). The hearts of these mice shared the features of FHC: they had diastolic dysfunction and were hypertrophied, with the same histological features seen in humans. In addition, the hearts had features of IDC: they were enlarged and had impaired systolic function. Based on similarities between the findings in humans, in whom mutations in different functional domains of α-cardiac actin may lead to either FHC or IDC, and the findings in the mouse model, having features of both diseases, the suggested pathophysiological mechanism appears to be very likely.

Acknowledgments

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