Mutations in the skeletal muscle α-actin gene in patients with actin myopathy and nemaline myopathy

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Muscle contraction results from the force generated between the thin filament protein actin and the thick filament protein myosin, which causes the thick and thin muscle filaments to slide past each other[1]. There are skeletal muscle, cardiac muscle, smooth muscle and non-muscle isoforms of both actin and myosin[2]. Inherited diseases in humans have been associated with defects in cardiac actin (dilated cardiomyopathy[3] and hypertrophic cardiomyopathy[4]), cardiac myosin (hypertrophic cardiomyopathy[5]) and non-muscle myosin (deafness[6]). Here we report that mutations in the human skeletal muscle α-actin gene[2] (*ACTA1*) are associated with two different muscle diseases, 'congenital myopathy with excess of thin myofilaments' (actin myopathy[7]) and nemaline myopathy[8]. Both diseases are characterized by structural abnormalities of the muscle fibres and variable degrees of muscle weakness. We have identified 15 different missense mutations resulting in 14 different amino acid changes. The missense mutations in *ACTA1* are distributed throughout all six coding exons[2], and some involve known functional domains of actin[9]. Approximately half of the patients died within their first year, but two female patients have survived into their thirties and have children. We identified dominant mutations in all but 1 of 14 families, with the missense mutations being single and heterozygous. The only family showing dominant inheritance comprised a 33-year-old affected mother and her two affected and two unaffected children. In another family, the clinically unaffected father is a somatic mosaic for the mutation seen in both of his affected children. We identified recessive mutations in one family in which the two affected siblings had heterozygous mutations in two different exons, one paternally and the other maternally inherited. We also identified *de novo* mutations in seven sporadic probands for which it was possible to analyse parental DNA.

A previous study[7] described three patients with 'congenital myopathy with excess of thin myofilaments'. The muscle biopsies from these patients showed large subsarcolemmal accumulations of thin filaments that reacted with antibodies to actin[7]. The clinical phenotype of the three patients varied; two of the patients died within the first few months of life, the third has now survived 7.5 years[7] (Table 1). On the basis of the accumulation of thin filaments, we hypothesized that mutations in the skeletal muscle gene *ACTA1* might be responsible for the disease. This would be similar to mutations of the desmin gene that cause desminopathy, a muscle disease characterized by the accumulation of desmin filaments[10]. We therefore scanned *ACTA1* (ref. 2) at chromosome 1q42 (ref. 11) for mutations in the three patients by sequencing PCR products of genomic DNA containing exons 2–7, the coding exons[2]. We identified three different single heterozygous missense mutations in the three patients (Fig. 1*a*,*b* and Table 1, patients 1–3), two of which cause the same Val163Leu amino acid change (Table 1, patients 2 and 3). These three mutations gave rise to SSCP variants (Fig. 2) that were not detected in over 100 control individuals.

As the muscle pathology of two of the patients also included intranuclear nemaline (rod) bodies and/or sarcoplasmic nemaline bodies[7], we hypothesized that an alternative diagnosis for these two patients might have been severe nemaline myopathy[12]. This form of nemaline myopathy is characterized at birth by severe hypotonia and muscle weakness, lack of spontaneous movement, feeding difficulties and respiratory insufficiency; in some patients this is combined with contractures or fractures[12]. We therefore screened *ACTA1* by sequence analysis for mutations in DNA from 25 probands with a diagnosis of severe neonatal nemaline myopathy, 3 with intermediate nemaline myopathy, 20 with milder forms and 11 nemaline myopathy patients for whom no classification was made due to incomplete clinical details. We identified single heterozygous missense mutations in ten of the nemaline myopathy probands (7 severe and 3 mild) and two heterozygous mutations in one severe nemaline myopathy proband (Table 1) by sequencing DNA. All mutations (Table 1) give rise to SSCP variants that are not found in DNA from at least 100 control individuals.

In the process of sequencing the patient samples we identified 16 consistent non-coding differences from the previously pub-

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Table 1 • Skeletal muscle *ACTA1* mutations

Family	Patients	Diagnosis	Age at death	Exon[2]	Mutation	Amino acid substitution	Actin domain and electrostatic interactions
1	1	congenital myopathy with excess of thin myofilaments: patient 2[7]	3 months	2	GGC→CGC	Gly15Arg	hydrogen bonds with β-phosphates of ATP and ADP[9]
2	2	congenital myopathy with excess of thin myofilaments and with intranuclear and some sarcoplasmic nemaline bodies: patient 1[7]	alive at 7.5 y	4	GTG→CTG	Val163Leu	
3	3	congenital myopathy with excess of thin myofilaments and with intranuclear nemalinebodies: patient 3[7]	4 months	4	GTG→TTG	Val163Leu	
4	4	severe atypical nemaline myopathy with intranuclear nemaline bodies	2 months	2	CAC→TAC	His40Tyr	proteolytic site, myosin binding site[9], mutation inhibits filament formation[18]
5	5, 6	severe nemaline myopathy	5 and 19 days	3	CTT→CCT	Leu94Pro	mutation of flanking amino acids in yeast gives recessive phenotype[14],
				5	GAG→GTG	Glu259Val	D259A in yeast = recessive[14]
6	7, 8 and 9	milder, milder and severe forms of nemaline myopathy respectively	alive at 33, 18 and 3 y	3	AAC→AGC	Asn115Ser	
7	10	milder form of nemaline myopathy	alive at 39 y	3	ATG→GTG	Met132Val	
8	11	milder form of nemaline myopathy	alive at 3 y	4	GGC→GAC	Gly182Asp	DNase I binding site[29]
9	12, 13	severe nemaline myopathy	1 and 4 days	4	CGC→TGC	Arg183Cys	DNase I binding site[29], R183A in yeast = wild type[7] Nε...Oδ2 Asp157* Nζ2...Oδ2 Asp157*
10	14	severe nemaline myopathy	alive at 10 y	5	CGC→CAC	Arg256His	R256A in yeast = temperature sensitive mutant[14]
11	15	severe nemaline myopathy	alive at 21 months	5	CAG→CTG	Gln263Leu	
12	16	severe nemaline myopathy	9 months	6	AAC→AAG	Asn280Lys	Oδ1...O Glu276*
13	17	severe nemaline myopathy	9 months	6	GAC→GGC	Asp286Gly	forms salt bridge with Arg 39, binds other actin molecules, profilin binding site[9], D286A in yeast = lethal mutant[14] Oδ1... Oδ1 Thr203* Oδ2... Oδ1 Thr203*
14	18	severe nemaline myopathy	alive at 4 months	7	GTC→TTC	Val370Phe	myosin binding site[9]

*Protein nomenclature in Table 1 follows standard nomenclature (http://www.chem.qmw.ac.uk/iupac/AminoAcid/AA1n2.html#AA22).

lished *ACTA1* sequence[2] and determined that the CCCGCC motif in intron 3 is polymorphic, varying between 3 and 6 repeats. We have deposited the sequence differences in GenBank (Accession number AF182035).

We were able to examine DNA from the parents of seven sporadic patients (Table 1, families 1, 2, 4, 10, 11, 12 and 14). We demonstrated by SSCP analysis or restriction endonuclease digestion that the *ACTA1* mutation identified in the patient was not present in any of the parental samples. We confirmed parentage by microsatellite analysis and conclude that all seven patients carried *de novo* mutations.

We examined three families with two affected siblings. We identified two different missense mutations, Leu94Pro and Glu259Val, in the affected children in family 5 (Table 1). The Leu94Pro mutation was also present in the unaffected father and an unaffected sibling, whereas the Glu259Val mutation was present in the unaffected mother but not the unaffected sibling. This pattern of inheritance indicates that the disease associated with mutations in *ACTA1* in this family is recessive. In family 6 (Table 1), we found that the affected mother and the two affected children, but not the two unaffected children, had the same Asn115Ser *ACTA1* mutation, indicating dominant inheritance. In family 9 (Table 1) the mother did not have the Arg183Cys mutation identified in the two affected siblings, whereas the father is a somatic mosaic for this mutation (Fig. 1*c*).

The simultaneous appearance of *ACTA1* mutations with disease in the sporadic cases indicates that these mutations have a direct role in causing the actin and nemaline myopathies, analogous to the situation for other diseases such as Charcot-Marie-Tooth disease[13]. Other evidence that the missense mutations cause disease includes their absence in over 100 control individuals, the fact that all of the mutated amino acid residues are

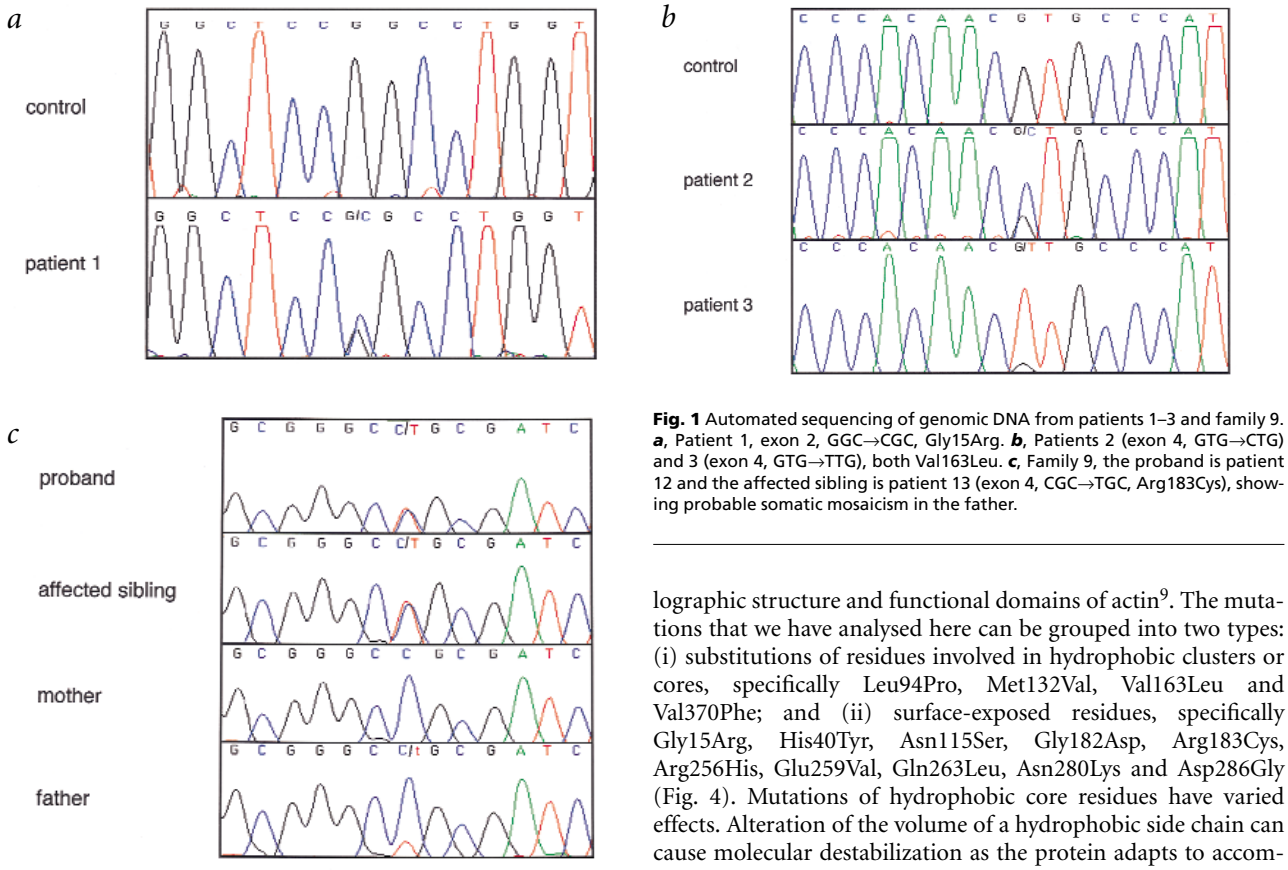

Fig. 1 Automated sequencing of genomic DNA from patients 1–3 and family 9. ***a***, Patient 1, exon 2, GGC→CGC, Gly15Arg. ***b***, Patients 2 (exon 4, GTG→CTG) and 3 (exon 4, GTG→TTG), both Val163Leu. ***c***, Family 9, the proband is patient 12 and the affected sibling is patient 13 (exon 4, CGC→TGC, Arg183Cys), showing probable somatic mosaicism in the father.

highly conserved in known actins[9] (Fig. 3), and the segregation of the mutations with the disease in families with dominant and recessive inheritance. In addition, results of studies of actin genes in yeast and *Drosophila melanogaster* support the conclusion that the missense mutations identified in *ACTA1* are pathogenic. Three mutations induced in yeast affect amino acid residues found in our study to cause dominant disease in humans (Table 1). One of the yeast mutations produced a dominant, one a recessive and one a wild-type phenotype[14]. Glu 259, mutated in the family showing recessive inheritance (Table 1, family 5), gave rise to a recessive phenotype[14] when mutated in yeast (Glu259Ala). The residues flanking the other mutated amino acid in the recessive family, Leu94, have been mutated in yeast and also gave rise to a recessive phenotype[14]. In *Drosophila*, the gene encoding the actin expressed in indirect flight muscles, *Act88F*, is mutated in a number of flightless mutants[15]. For at least one of these mutations, Gly245Asp, the muscle pathology is similar to that seen in the patients with congenital myopathy with excess of thin myofilaments[16].

To better understand the pathogenesis of these *ACTA1* mutations, we correlated their locations with the known X-ray crystallographic structure and functional domains of actin[9]. The mutations that we have analysed here can be grouped into two types: (i) substitutions of residues involved in hydrophobic clusters or cores, specifically Leu94Pro, Met132Val, Val163Leu and Val370Phe; and (ii) surface-exposed residues, specifically Gly15Arg, His40Tyr, Asn115Ser, Gly182Asp, Arg183Cys, Arg256His, Glu259Val, Gln263Leu, Asn280Lys and Asp286Gly (Fig. 4). Mutations of hydrophobic core residues have varied effects. Alteration of the volume of a hydrophobic side chain can cause molecular destabilization as the protein adapts to accommodate the volume change. Conformational rearrangements are also caused through such mutations; thus, mutations may have effects even at regions distal to their position through long-range communication within the actin molecule. Indeed, it has been demonstrated[17] that mutations of actin at some distance from the DNase I or profilin binding sites still affected binding of these ligands. Some of the mutations alter residues with known functions in the actin molecule. The mutated residues Gly15, His40, Gly182, Arg183 and Val370 are involved in interactions with ATP/ADP (Gly15), DNase I (Gly182, Arg183) and myosin (His40, Val370; Table 1). The surface-exposed residues Arg183 and Asn280, as well as the two residues in cardiac actin mutated in cases of dilated cardiac myopathy, Arg312 and Glu361 (ref. 3), are able to form ionic interactions with other parts of actin (Table 1). Mutation of these residues may destabilize actin.

Asp286Gly is unique among the mutations in that it is the only one directly implicated in F-actin subunit-subunit interactions[9], and we hypothesize that mutation to glycine would alter this interaction. Another of the mutated residues, His40, is also at an interface with an adjoining actin subunit, but it is not close enough to interact with this neighbouring actin according to the current F-actin model. This presumed lack of interaction may

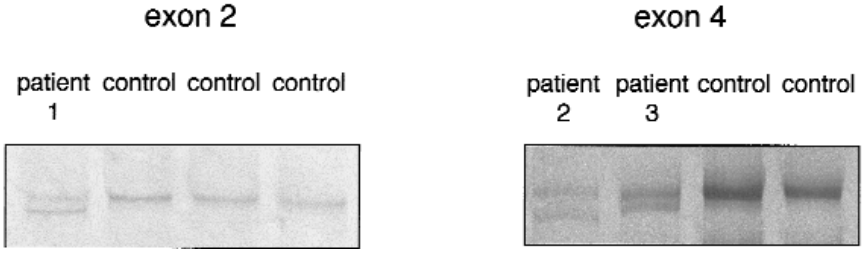

Fig. 2 SSCP analysis of the mutations in patients with congenital myopathy with excess of thin filaments.

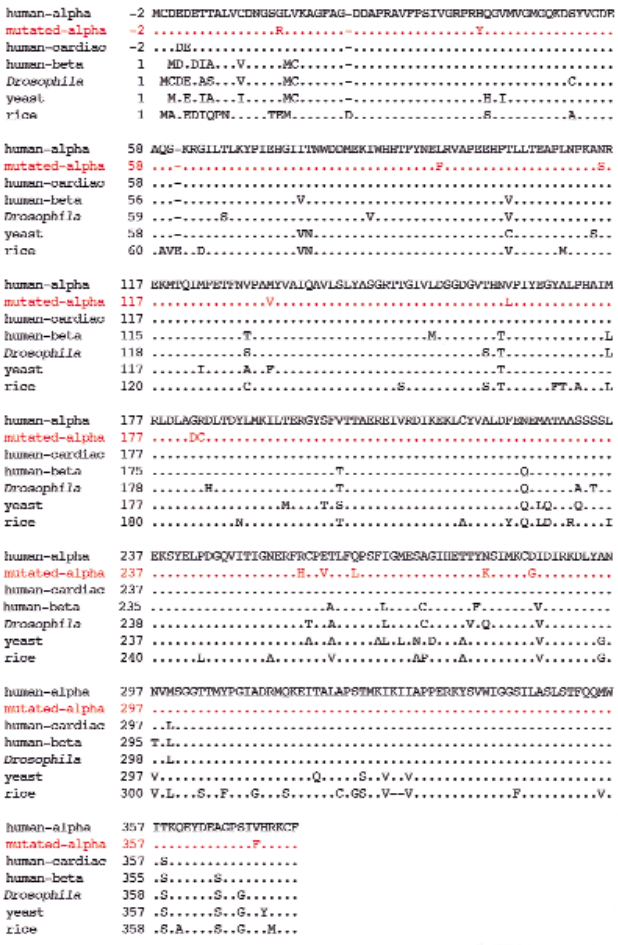

Fig. 3 Comparison of actin amino acid sequences in various species and the 15 mutated residues identified in patients with *ACTA1* mutations. All mutated residues are highly conserved. The Gly15Arg, Met132 and Asn280 residues vary only in rice, yeast and *Drosophila*, respectively.

reflect an inaccuracy in the current F-actin model, because mutation of His40 affects actin filament formation[18].

We have identified a spectrum of phenotypes associated with mutations in *ACTA1*. These are 'congenital myopathy with excess of thin myofilaments' with or without intranuclear nemaline bodies (n=3 patients), severe nemaline myopathy (n=11) and mild nemaline myopathy (n=4). Although 7 of 11 patients with *ACTA1* mutations and severe nemaline myopathy died within the first year of life, 2 are alive at 3 and 10 years. Survival with little residual disability has been described for cases with no spontaneous movement at birth, and in the typical form of nemaline myopathy it is common for patients to be severely hypotonic at birth but later to follow a non-progressive or slowly progressive course[8,19]. The two patients with the same amino acid substitution (Table 1, patients 2 and 3) showed different courses of the disease, patient 3 died from respiratory insufficiency at 4 months of age, whereas patient 2 has survived to date and is 7.5 years old. This patient has a distribution of muscle weakness different from that seen in typical nemaline myopathy[19]. In the family showing dominant inheritance, the mother, currently age 33, and daughter, 18, are mildly affected, whereas the 3-year-old son is severely affected. We conclude that the spectrum of clinical phenotypes caused by mutations in *ACTA1* result from different mutations, modifying factors affecting the severity of the disorder, variability in clinical care, or a mixture of all three factors. We identified so-called neurogenic findings at electromyographic (Table 1, patients 1, 2, 12, 14, 15 and 17) or abnormal variability in fibre size at biopsy (Table 1, patients 1–4, 12, 15, 16 and 17), even to the extent of indicating a diagnosis of spinal muscular atrophy[20]. This is not an uncommon feature in severe cases of the congenital myopathies, including X-linked myotubular myopathy[21].

We have now identified three genes mutated in several types of nemaline myopathy: *TPM3* (encoding α-tropomyosin slow) in both dominant and recessive nemaline myopathy in which the nemaline bodies are restricted to slow, type I muscle fibres[22,23]; *NEB* (encoding nebulin) in typical non- or slowly progressive congenital nemaline myopathy[24]; and *ACTA1*. The genes mutated in other forms of nemaline myopathy, including severe nemaline myopathy causing fetal akinesia sequence[25], have yet to be identified, but may involve other sarcomeric proteins[26]. Additional phenotypes may also be caused by mutations in *ACTA1*.

Methods

PCR amplifications. We designed the primers used for PCR amplification of genomic DNA from the published human skeletal *ACTA1* sequence[2]. We situated the primers either in flanking introns or neighbouring exons. The primer sequences were: 5′ UTR/exon1 F (forward), 5′–TGGCTCAGCTTTTTG-GATTCAG–3′; 5′ UTR/exon 1 R (reverse), 5′–GGCTGACCAGGTGAACC-GACTG–3′; exon 2 F, 5′–TGAGACTTCTGCGCTGATGCA–3′; exon 2 R, 5′–GTGGCACCAGGCTGGCTTACG–3′; exon 3 F, 5′–CACCCGGAGCG-GCGTTAACG–3′; exon 3 R, 5′–GCGCGCGGGAGAGAGTGAGT–3′; exon 4 F, 5′–CGCTGAGCGCCTAGCCTCGG–3′; exon 4 R, 5′–TGCGGAGGGCA-GAAGCAGGA–3′; exon 5 F, 5′–CGGCGGCCTGAGTGAGGGCT–3′; exon 5 R, 5′–GGGGAGCGTGAGCAGAAGCT–3′; exon 6 F, 5′–CCCGGCCCGAG

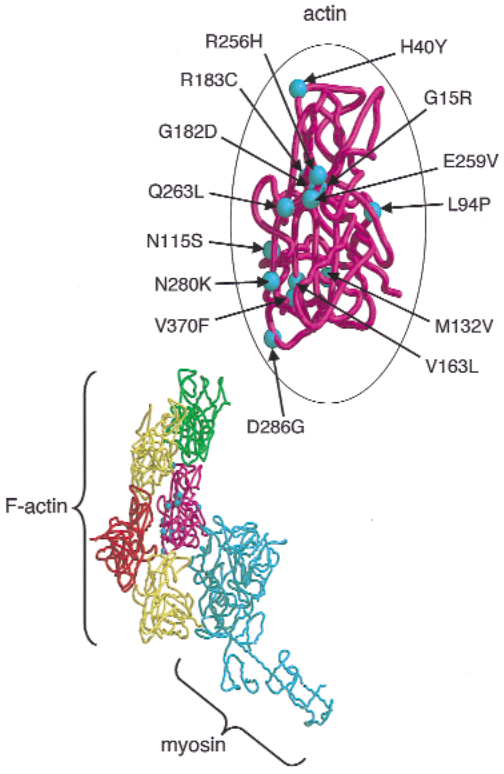

Fig. 4 Model of F-actin and myosin with actin mutations indicated. The atomic structure of F-actin is shown. Subunits are in green, yellow, magenta, red and yellow (repeat). Myosin is in cyan. The labelled insert is an enlargement of the corresponding actin subunit. The locations of *ACTA1* mutations described here are shown as cyan spheres.

CTTCTGCTC–3′; exon 6 R, 5′–CCGACAGCCCGCGCAGGCCACC–3′; exon 7 F, 5′–CTCCAGGGTGAGGTCTCCCC–3′; and exon 7 R, 5′–TATGTACACGTTATAAACACTG–3′. We used PCR volumes (25 µl) consisting of genomic DNA (50 ng), primers (50 ng each), $MgCl_2$ (2 mM), deoxynucleotides (200 µM each), *Taq* polymerase (1.1 U) and 4% dimethylsulphoxide (DMSO; 3% DMSO, 5′ UTR PCR). All amplification conditions included an initial 4 .5 min at 94 ºC during which the enzyme was added. We performed a 40-cycle, 3-step PCR programme for amplification of 5′ UTR/exon 1 and exons 2, 3 and 7. We employed 30 s for the denaturation (94 ºC) and annealing steps (5′ UTR/exon 1, 58 ºC; exon 2, 62 ºC; exon 3, 64 ºC; exon 7, 60 ºC) and either 30 s (exons 2, 3 and 7) or 1 min (5′ UTR/exon 1) for each extension step (72 ºC). We used a two-step PCR programme for amplification of exons 4, 5 and 6 with 40 cycles of 30 s at 94 ºC and 45 s at 70 ºC.

Sequencing. We performed sequencing reactions with the BigDye Terminator mix (ABI) and an amplification temperature of 55 ºC. We sequenced each purified PCR product (Qiaquick spin columns, Qiagen) using the original primers used for amplification. We electrophoresed the purified products of the sequencing reactions on either an upgraded ABI 373 or an ABI 377 Automated Sequencer.

SSCP analyses. We used a variety of conditions for SSCP analysis of the mutations. These included nondenaturing acrylamide gels with Bis/acrylamide ratios of 19:1, 29:1, 75:1 and 99:1, 0.5×MDE gels and running temperatures between 4 ºC and ambient RT for 2–16 h. We either silver stained the polyacrylamide gels for DNA visualization[27] or radiolabelled the PCR products by inclusion of 1 µCi ^{32}P-dCTP per 10 µl reaction.

Enzyme digestions. The mutations identified in three patients introduced restriction sites into *ACTA1* (patients 12 and 13, *Bbv*I; patient 18, *Mbo*II). We designed mismatch primers for patients 1, 2, 3, 5, 6 and 15 to introduce *Bst*NI (patients 2, 3, 5 and 6), *Ban*I (patient 1) and *Fnu*4HI (patient 15) sites only if the putative mutation was present. We used mismatched primers (ex 2 F mismatch, 5′–CCGTGTGCGACAATGG**G**T**G**C–3′, patient 1; ex 3 R mismatch, 5′–GTGCTCCTCGGGAGCCACGC**C**A–3′, patients 5 and 6; ex 4 F mismatch, 5′–GTAGCCCTCATAAATGG**C**CA–3′, patients 2 and 3; and ex 5 R mismatch, 5′–GGGCTCACCGATGAAGG**T**G**C**GC–3′, patient 15) with the opposite strand primer from the original exonic primer pair. We incubated PCR products with the appropriate restriction endonuclease according to the manufacturer's instructions (New England Biolabs). We electrophoresed aliquots (10 µl) of these digestion reactions through 8%, 19:1 polyacrylamide gels and visualized bands by silver staining.

Protein modelling. We extracted the three-dimensional coordinates for this analysis from the Protein Database at Brookhaven (PDB). We constructed a 'complete' atomic model of F-actin using the Cα electron microscopy model[28] (PDB identification code, 1ALM), superimposing the complete coordinates of G-actin[29] (PDB identification code, 1ATN) on each subunit of the F-actin. We also included the proposed position of myosin in the model. We performed mutational analysis on a Silicon Graphics O2 using the program O (ref. 30).

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