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Multi-exon deletions of the *FBN1* gene in Marfan syndrome

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Abstract

Background: Mutations in the fibrillin-1 gene *(FBN1)* cause Marfan syndrome (MFS), an autosomal dominant multi-system connective tissue disorder. The 200 different mutations reported in the 235 kb, 65 exon-containing gene include only one family with a genomic multi-exon deletion.

Methods: We used long-range RT-PCR for mutation detection and long-range genomic PCR and DNA sequencing for identification of deletion breakpoints, allele-specific transcript analyses to determine stability of the mutant RNA, and pulse-chase studies to quantitate fibrillin synthesis and extracellular matrix deposition in cultured fibroblasts. Southern blots of genomic DNA were probed with three overlapping fragments covering the *FBN1* coding exons

Results: Two novel multi-exon *FBN1* deletions were discovered. Identical nucleotide pentamers were found at or near the intronic breakpoints. In a Case with classic MFS, an in-frame deletion of exons 42 and 43 removed the C-terminal 24 amino acids of the 5th LTBP (8-cysteine) domain and the adjacent 25th calcium-binding EGF-like (6-cysteine) domain. The mutant mRNA was stable, but fibrillin synthesis and matrix deposition were significantly reduced. A Case with severe childhood-onset MFS has a *de novo* deletion of exons 44–46 that removed three EGF-like domains. Fibrillin protein synthesis was normal, but matrix deposition was strikingly reduced. No genomic rearrangements were detected by Southern analysis of 18 unrelated MFS samples negative for *FBN1* mutation screening.

Conclusions: Two novel deletion cases expand knowledge of mutational mechanisms and genotype/phenotype correlations of fibrillinopathies. Deletions or mutations affecting an LTBP domain may result in unstable mutant protein cleavage products that interfere with microfibril assembly.

Background

The Marfan syndrome (MFS) is an autosomal dominant connective tissue disorder with an estimated incidence of 1 in 5,000 live births [1]. Besides the classic manifestations in the cardiovascular, ocular and musculoskeletal systems, frequent features include spontaneous pneumothorax, inguinal herniae, striae atrophicae in the skin and lumbosacral dural ectasia. There is pronounced inter- and intrafamilial variability. Age of onset and progression of the various manifestations cover a wide range [1,2].

The MFS phenotype is the result of mutations in *FBN1*, a large gene composed of 65 exons encoding the 350 kD fibrillin-1 protein that is the major constituent of microfibrils in the extracellular matrix (reviewed in [3]). To date, 201 unique *FBN1* mutations have been identified, including 70 (34.8%) cysteine substitutions, 63 (31.3%) other missense mutations, 41 (20.4%) premature termination mutations, 23 (11.4%) exon skipping mutations, and 4 other mutations, as listed in the Human Gene Mutation Database (HGMD, [http://archive.uwcm.ac.uk/uwcm/mg/search/127115.html]) [4] and reported more recently [5]. Although the *FBN1* gene, including three alternatively spliced 5' untranslated exons [6] extends over 235 kb of genomic DNA in chromosome band 15q21.1 [http://genome.cse.ucsc.edu] , no major deletions have been identified except for one report of an exon 60–62 genomic deletion [7]. Some correlations between *FBN1* mutation genotypes and phenotypes are emerging. For example, exon-skipping mutations in the central region of the gene, exons 24–32, cause severe phenotypes or neonatal presentations [8]. But there are exceptions, only four of the many other mutations in this region cause severe phenotypes, while one mutation predicted to lead to skipping of exon 24 was found in a patient with classic MFS [5]. In contrast, most cysteine substitutions cause classic MFS with a high frequency of lens dislocation, although some patients in our study did not meet the Ghent criteria for diagnosis of MFS [9]. Amino-acid substitutions not disrupting disulfide bonds may be associated with fibrillinopathies other than MFS, although mutations in the calcium-binding consensus sequence have been reported in individuals with classic MFS [5,10–12]. It is clear from the studies reported so far, that genotype-phenotype correlations are not straight-forward for *FBN1* mutations.

We report here two multi-exon deletion mutations of *FBN1* . We located the deletion breakpoints in introns and characterized the mutant RNA and protein phenotypes. Partial deletion of an 8-cysteine (LTBP-like) domain and loss of the downstream 6-cysteine calcium-binding (cb) epidermal growth factor (EGF)-like domain caused protein instability and classic MFS. *De novo* deletion of three cbEGF-like domains encoded by exons 44–46 resulted in a severe form of MFS diagnosed in infancy. Both mutations lead to the synthesis of mutant FBN1 proteins that interfere with microfibril assembly, resulting in significantly reduced extracellular matrix microfibrils.

Patients and Methods

Clinical histories

Case 1

(FB774): Patient 1, a female, was born to a 36 yr old father and was noted to have long fingers at birth. A diagnosis of MFS was made at age 15 yr, based on bilateral lens dislocation (upward) and Marfanoid skeletal features. Diffuse red and pink cutaneous striae started to appear at age 15, when obesity developed, and progressed throughout life. As an adult, Patient 1 had a height of 186 cm with long arms and legs, a narrow highly arched palate and dental crowding, chest asymmetry and pectus deformity (upper sternum protrudes), hyperextensible large and small joints, arthritis, arachnodactyly, frequent thumb dislocations, pes planus, and thin, fragile skin. Besides ectopia lentis, ocular features included high-grade myopia and glaucoma requiring surgery at age 18 yr. A cardiac arrhythmia was treated with beta-blockers. The aortic root diameter was 4.5 cm by echocardiogram at 46 yr of age. A skin biopsy for fibroblast culture was obtained at age 40 yr. There was no family history of MFS, both parents were deceased.

Case 2

(FB 890): Born to a 27 yr old mother and 34 yr old father, Patient 2 presented as a neonate with striking arachnodactyly and pectus excavatum. At 4 months, her skeletal measurements were all above the 97th %ile (length 66 cm, arm span 67 cm, middle finger length 5 cm, foot length 10 cm) and the upper-to-lower segment ratio of 1.27 was significantly below normal for age. She had mild contractures of elbows and knees, a long face with deep-set eyes, bilateral iridodonesis and high-grade myopia. Bilateral lens dislocation was confirmed at age 3 yr. During early childhood, she developed severe progressive thoracolumbar scoliosis (>80 degrees) requiring spinal fusion procedures. Episodes of supraventricular tachycardia and cardiac arrhythmia were treated with cardioversion and beta-blockers. Starting in infancy, her aortic root dilated rapidly to a diameter of 4.5 cm at age 6 yr. She developed mitral regurgitation and mitral and tricuspid valve prolapse. Her joints became increasingly hypermobile. Before valve-replacement surgery, her severe pectus excavatum was repaired. Throughout childhood, she had underdeveloped musculature and lack of subcutaneous fat. To improve her nutritional status, she received nasogastric tube feedings at night. Photos of Pa-

tient 2 were recently published (Figure 13.2 a-d in [13]). There was no family history of MFS.

PCR and direct sequencing

Genomic DNA and total RNA were extracted from fibroblast cultures established from skin biopsies as described [14]. Long-range RT-PCR amplification of FBN1 cDNA fragments and restriction enzyme digestions were performed as previously reported [8]. Fragments migrating abnormally in agarose gel electrophoresis were excised, purified with the QIAquick Gel Extraction Kit (QIAGEN) and directly sequenced with fluorescent terminators on an ABI Prism 377 DNA sequencer (Perkin-Elmer). To define the deletion endpoints, we amplified genomic DNA with the ExpandTM PCR System (Boehringer Mannheim). The thermal profile for long-range PCR was as follows: denaturation for 2 min at 94°C, then 35 cycles of denaturation (10 s at 94°C), annealing (30 s at 60°C), and extension (8 min at 68°C), followed by a final extension step of 7 min at 68°C.

Primers used for genomic amplifications and their location in exons:

774F, 5'-AAGCCCTCTGAACAGTGTCCCATCCCAAGT-3' (exon 41);

774R, 5'-TTGCACTGTCCTGTGGAGGTGAAGCGGTAG-3' (exon 44).

890F, 5'-AGATCCCAGGGGTCTGTGAAAATGGAGTGT-3' (exon 43);

890R, 5'-TTGCACTGGCACTGGAAAGACCCCACTGTA-3' (exon 47)

We purified the genomic amplicons with exonuclease and shrimp alkaline phosphatase (United States Biochemical) and sequenced them directly with primers E41S, E43S, E44AS and E47AS [15].

To sequence the junction fragments from both strands, we performed PCR and sequencing with primers 774F and reverse primer E43+355 (5'-CAA AGT CTC TGT CTG GAA GAG AAA TGA TTT TGA-3') for FB774, and with forward primer E44 -343 (5'-CCT TAG CTT CCC AAA GTG CTA GGA TTA-3') and 890R for FB890.

Quantitation of allele-specific FBN1 transcripts

Patient 1 was heterozygous for the *Rsa*I polymorphism in the 3'UTR. RT-PCR and *Rsa*I digestion were carried out as described [16]. The products of the two alleles were distinguished by SSCA and silver staining, according to the protocol provided by the manufacturer (Bio Rad, Hercules, CA), and were subsequently quantified by scanning densitometry.

Quantitative pulse-chase analysis of fibrillin-1 protein

Primary fibroblast cultures from patients and control individuals were grown to confluency and then metabolically labeled with ^{35}S-cysteine as described [17,18]. The soluble cell lysis fraction, containing newly synthesized labeled fibrillin-1 molecules, and the insoluble fraction, representing the labeled fibrillin in the extracellular matrix, were fractionated by SDS polyacrylamide gel electrophoresis and autoradiographed. Labeled fibrillin-1 was identified and quantified as described [17,18]. More recently, the results were replicated by use of a phosphorimager. The levels of fibrillin-1 production and deposition in the extracellular matrix were expressed as the percentage of values obtained for simultaneously studied normal control fibroblast cultures.

Results and Discussion

We developed a long RT-PCR approach to screen for exon-skipping and large deletion mutations in the *FBN1* gene. PCR with three sets of primer pairs amplifies overlapping fragments: exons 1 – 31 (3972 bp), exons 29 – 52 (3011 bp) and exons 47 – 65 (2971 bp) from skin fibroblast-derived total RNA. The amplicons are digested with restriction enzymes that generate individually recognizable fragments with known sequence content. A total of 60 patients were screened with this method, 55 of which met diagnostic criteria for MFS and 5 had partial manifestations. All of these skin fibroblast samples had previously been screened with a cDNA amplification/SSCA protocol [14] and a genomic DNA/exon amplification method [15] with negative results. As reported previously, the long RT-PCR method identified six samples with transcripts skipping exons 18, 31, 46, 49, 56 or 58 that were caused by nucleotide substitutions at splice sites [8]. In another patient, skipping of exon 51 was discovered to be due to a silent mutation within the exon, possibly affecting a splicing enhancer binding site [19]. Here we report abnormal fragments seen after *Taq*I digestion of the exon 29–52 amplicons (Fig. 1a). Sequence analysis of the altered cDNA fragments revealed a deletion of exons 42 and 43 in patient 1 and a deletion of exons 44–46 in patient 2 (Fig. 1b).

In both cases, we found no evidence for sequence alterations, when we amplified the deleted and flanking exons and their splice sites individually from genomic DNA (data not shown). Therefore, we suspected that these normal amplification products were derived from the normal *FBN1* alleles, and that the mutant alleles were not amplified by this approach because they carry gross genomic deletions. To test this hypothesis, primer pairs were designed from the flanking exon sequences for am-

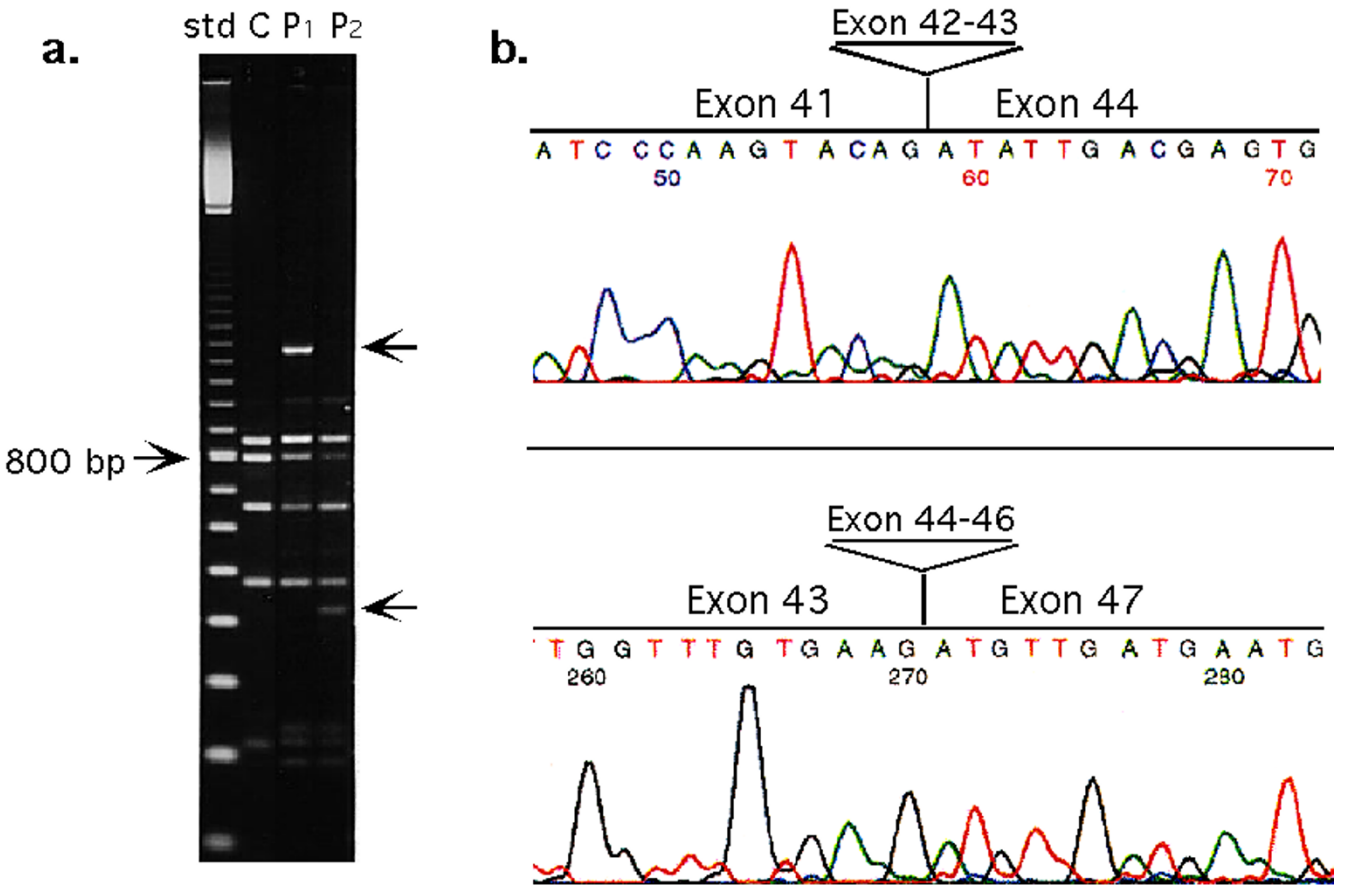

Figure 1
(a) Comparison of the *Taq*I restriction fragment patterns of long RT-PCR products containing FBN1 exons 29–52. Sample C is amplified from a normal control, PI represents patient 1 (FB774) and P2 patient 2 (and FB890). Lane 1 is a 100 bp ladder. Arrows point to the abnormal fragments in the patient lanes. (b) Direct sequencing analysis of the DNA isolated from the altered restriction fragments reveals deletion of exons 42–43 in patient 1 (top) and of exons 44–46 in patient 2 (bottom).

plification of genomic DNA. Long-range PCR analysis revealed genomic deletions, about 5 kb in size, in both patients (Fig. 2). For Case 1 (P1), a 4 kb amplicon was obtained instead of the 9 kb amplicon in the normal control sample. For Case 2 (P2), a 4.2 kb product was amplified instead of the 9.2 kb product in the normal control. In both cases, the PCR conditions used favored the amplification of the shorter (mutant) fragment, although the normal allele was also present in the genomic PCR template (Fig. 2). Direct sequencing of the deletion junction amplicons revealed the location of the intronic breakpoints. Both deletion events generated clean junctions with no missing or inserted nucleotides. In Case 1, the breakpoints are about 300 nt downstream from exons 41 and 43, while in Case 2, they are located about 300 nt upstream of exons 44 and 47. In Case 1, two sets of identical pentamers (cagta and ggaaa) were identified near the breakpoints in introns 41 and 43. In Case 2, the exchange occurred within an identical pentamer (atttt) (Fig. 2). None of these sequences are known to predispose to genomic rearrangements, although short stretches of identical sequence are often found at the sites of germline translocation and deletion breakpoints [20–22]. Genomic analysis of the parents and siblings of Case 2 indi-

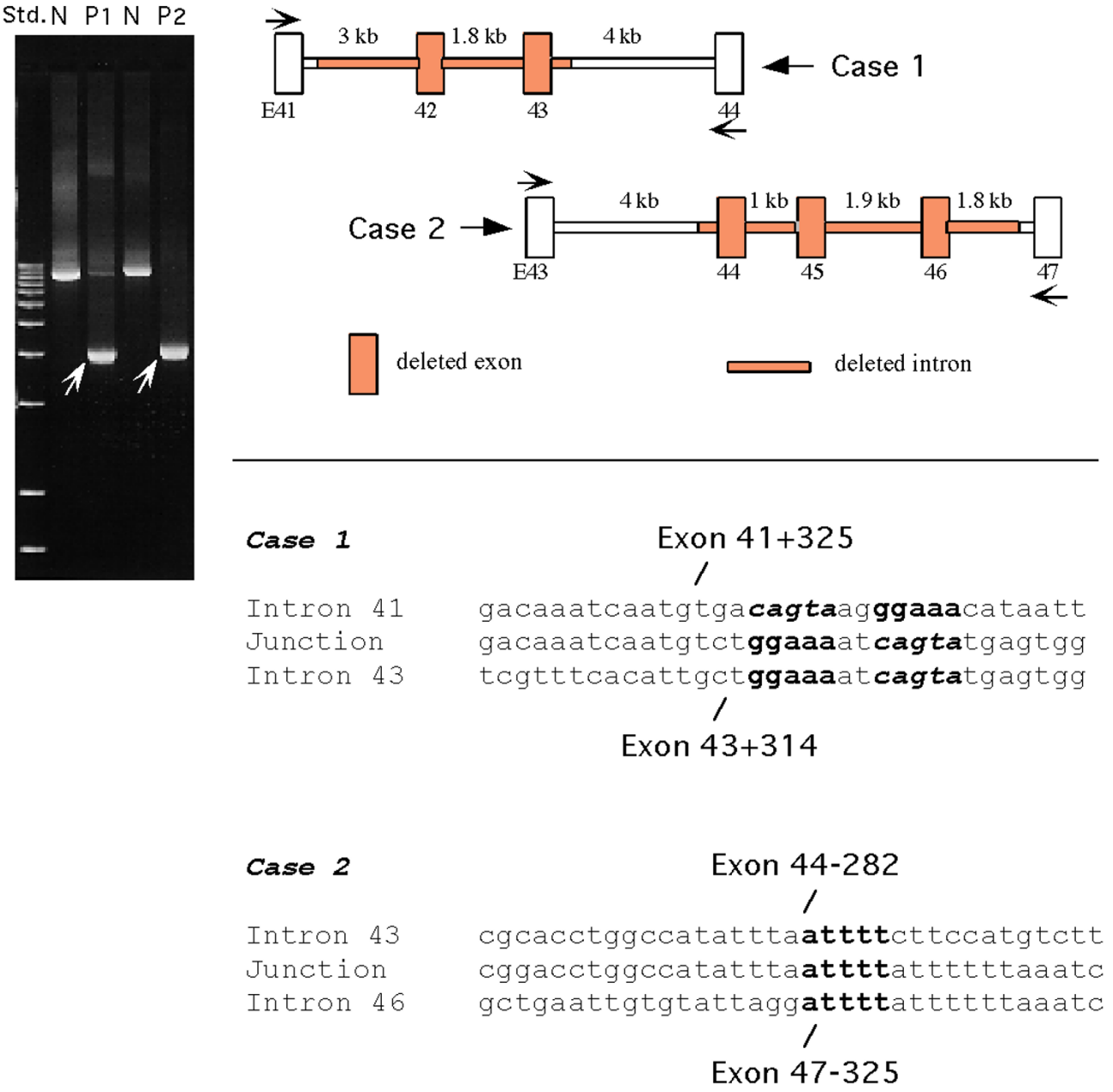

Figure 2
Left: Long PCR analysis of genomic DNA indicates about 5 kb deletions in both patients PI and P2. Lane 1 (Std) is a 1 kb ladder. Arrows point to the abnormal fragments generated by the genomic deletions. Sample N is amplified from a normal control. Right: Schematic representation of the deletion regions of the genomic DNA (shaded). Bottom: Sequence analysis of junction fragments amplified by PCR reveal identical pentamers (bold). In Case 1, the nucleotide (t) at position +325 in intron 41 is joined to the nucleotide (c) in position +314 in intron 43. In Case 2, the breakage and rejoining occurred within the pentamer (atttt) at positions -282 to -278 of intron 43 and positions -325 to -321 of intron 46.

cated that the mutation was sporadic. Although the parents of Case 1 were not available for study, the negative family history suggests that this deletion is also *de novo*. Slightly advanced paternal age (36 yr and 34 yr, respectively) may be a factor in both cases.

The effects of the two deletions were studied at the protein level by ^{35}S-cysteine pulse-chase analysis. Both mutant mRNAs encode internally deleted proteins without a frameshift. In Case 2 with del exons 44–46, fibrillin synthesis was normal (109% of control), indicating that

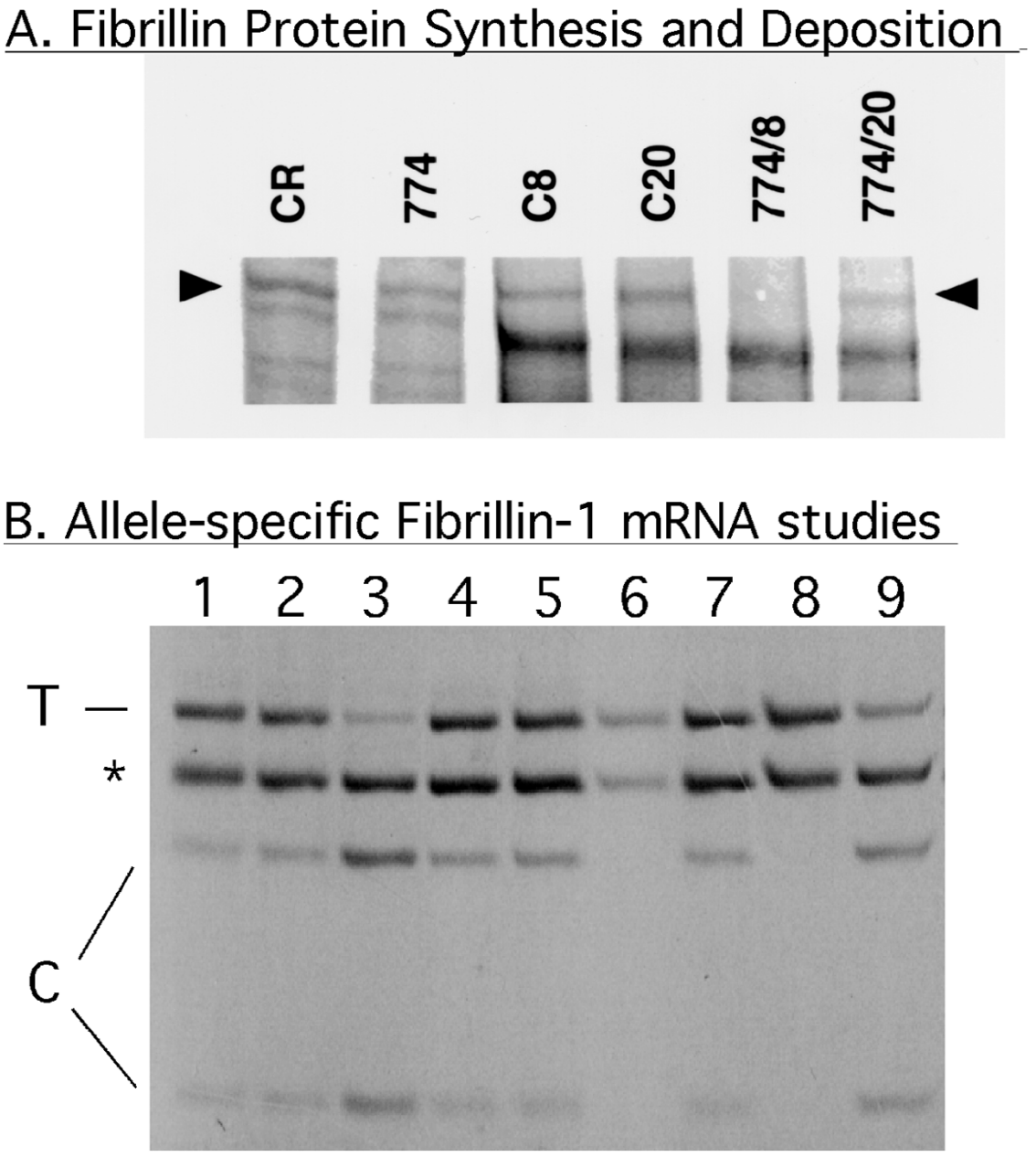

Figure 3
A. As a measure for fibrillin synthesis, the two left lanes show labeled fibrillin (arrow head) detected in the cytosol of fibroblasts from a normal control (CR) and from Case 1 (774) after a 30 min pulse with ^{35}S-cysteine. The amount in lane 774 is 55% of that in lane CR. In the right four lanes, the incorporation of pulse-labeled fibrillin (arrow head) into the extracellular matrix, after 8 hour and 20 hour chase periods, is severely reduced in Case 1 (774/8 and 774/20) compared to the normal control fibroblasts (C8 and C20). **B.** Allele-specific RNA analysis. All individuals are heterozygous for the C6236T *Rsa*I polymorphism in the *FBN1* 3'UTR. RT-PCR products from fibroblast RNA samples were digested with *Rsa*I and electrophoresed on an SSCA gel under denaturing conditions. The band labeled T represent the uncut T allele, the two bands labeled C represent the cut C allele, and the band marked by asterisk is constant. Samples 1 and 5 are normal controls, sample 2 has an exon 51 skipping mutation [19], and the sample in lane 4 is from Case 1 (774) with deletion of exons 42 and 43. Both these samples, as well as the one in lane 7 show the normal pattern of fragments indicating equal transcripts of both alleles. In contrast, sample 3 reveals predominant transcripts with the C allele, while in samples 6 and 8 only the T allele is detectable. Lane 9 shows slight skewing towards the C allele. Samples 3 and 8 have *FBN1* mutations leading to premature stop codons. For samples 6,7 and 9, no *FBN1* mutations have been identified as yet.

the mutant fibrillin molecules are stable. The mutant protein band, expected to be 122 amino acids shorter, was not resolved on our gel system. The deposition of newly synthesized fibrillin into the matrix was greatly reduced (13 % of normal), however, suggesting that the internally deleted mutant fibrillin-1 molecules are secreted and exert a dominant-negative effect on extracellular microfibril assembly.

In Patient 1, the in-frame deletion of exons 42–43 removed the C-terminal 24 amino acids of the 5th LTBP (8-cysteine) domain and the adjacent 25th calcium-binding EGF-like domain. Fibrillin synthesis levels were 55% of control and, in addition, matrix deposition was disproportionately reduced to 16% of control (Fig. 3A). An earlier independent study of this patient's fibroblasts had revealed similar results: 34% for synthesis and 11% for deposition (T. Aoyama, unpublished data). To determine whether the low value for synthesis was due to reduced mutant transcript levels, we made use of the *Rsa*I restriction site polymorphism in the *FBN1* 3'UTR for which the patient is heterozygous. The SSCA patterns of *Rsa*I digested RT-PCR products were indistinguishable from controls, indicating that transcript levels were equal for both alleles (Fig. 3B). This leads us to conclude that the mutant mRNA is stable but that the mutant protein, containing a partially deleted LTBP-domain, if synthesized, is unstable and may be processed to smaller fragments by proteolytic cleavage. The methods we used did not allow us to detect such hypothetical proteolytic fragments.

Of more than 200 *FBN1* mutations reported, only one is a multi-exon deletion [7]. It involves deletion of exons 60–62 encoding three contiguous cbEGF-like domains [23]. This deletion co-segregated with affected status in a 3-generation family. Skeletal involvement was characterized by a moderate Marfanoid habitus with mild scoliosis and hypermobility of large and small joints. Ectopia lentis was absent. Retinal detachment was reported in one individual. Cardiovascular features included aortic dilatation in mid-life, one case of dissection at age 56 yr, and a high frequency of mitral valve prolapse associated with arrhythmia. Immunoprecipitation studies with fibrillin-specific antibodies revealed the presence of a 15 kD-smaller mutant polypeptide in the medium of metabolically labeled mutant fibroblasts [7]. The shortened molecule was incorporated into the extracellular matrix and apparently caused major disruptions of microfibrils [23].

Patient 2 described here also has a deletion of three contiguous cbEGF-like domains, those encoded by exons 44–46. This deletion resulted in a more severe phenotype with onset in infancy and a rapidly progressing clinical course. Thus, it appears that deletion of three contiguous EGF-like domains can have different effects depending on their location within the fibrillin-1 molecule. Deletion of the EGF-like domains encoded by exons 60–62 in the C-terminal domain of fibrillin-1 results in a much less severe phenotype than deletion of three EGF-like domains in the second longest stretch of EGF-like domains (between exons 43–49). It has been reported that 40% of mutations identified in the region between exons 59–65, but only 7% of mutations in other regions, were associated with mild Marfan-like phenotypes [12]. Our results are in line with the notion of this general genotype-phenotype correlation.

Deletion of one EGF-like domain due to exon skipping, especially in the region between exon 24 and 32, usually causes severe neonatal-onset MFS [8,24,25]. Similar deletions in other regions of fibrillin-1 may also result in early onset of cardiovascular symptoms [8]. Patient 1, described here, had a genomic deletion of exons 42 and 43 that results in an in-frame deletion of part of the 5th LTBP-like domain and the adjacent downstream cbEGF-like domain. Diagnosed as a teenager, patient 1 had the skeletal and skin findings of MFS, bilateral dislocated lenses and, at the age of 46 yr, a mildly dilated aortic root. In contrast to the cases with deleted EGF-like domains, her mutant protein was unstable. We obtained a similar result in a case of exon 51 skipping due to a silent C>T transition at a CpG dinucleotide [19]. In-frame deletion of exon 51 removes 22 amino acids of the 6th LTBP-like domain. In this patient's fibroblasts, fibrillin synthesis was 41% of control and deposition was reduced to 5%, while the mutant mRNA level was normal as shown here in Fig. 3. In addition, we found that certain cysteine substitutions in EGF-like domains and located immediately N-terminal of LTBP-like domains, cause protein instability and greatly reduced matrix deposition [9]. Taken together, our data support a model in which misfolding or partial deletion of LTBP-like domains results in proteolytic cleavage of the mutant fibrillin molecules. We hypothesize that truncated intermediates interfere with microfibril assembly and, thus, produce the low levels of matrix deposition observed. This hypothesis is not only based on analysis of Case 1 reported here but on a similar analysis of five previously reported cases, one with exon 51 skipping [19] and four with cysteine substitutions immediately N-terminal of the LTBP like domains [9]. It is also possible, however, that proteolytic fragments of the mutant protein associate with the normal length fibrillin molecules and inhibit their secretion. Future focused mechanistic studies are needed to test our hypothesis.

The FBN1 mutation detection rate for individuals with clinically diagnosed MFS is currently about 75% when the gene is completely screened with a DNA-based PCR amplification protocol of individual exons [15,26]. Ge-

nomic deletions such as the ones reported here are not a frequent cause of human disease and would be missed by this approach. To search for additional large genomic rearrangements, we carried out Southern analyses of *Bam-HI*, *Sac*I and *Bgl*II digested genomic DNA from 18 fibroblasts strains that were negative for *FBN1* mutation screening by other methods, but had shown abnormal fibrillin biosynthetic profiles by pulse-chase analysis. The blots were hybridized successively with the three overlapping long-range RT-PCR fragments covering the FBN1 coding region, that were generated from a normal cDNA template as described above. While we identified several restriction fragment length polymorphisms, no evidence for major deletions or rearrangements was found (data not shown). Thus, we have only detected two genomic deletions out of 105 unique disease-causing FBN1 mutations identified in our laboratory. The Human Gene Mutation Database (as of 08/14/01) contains 23,345 unique mutations in 1069 genes. Of those, 1166 (5%) are gross deletions [http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html] [27] suggesting that more FBN1 genomic deletions await recognition. Multi-exon deletions are easily detected with the long RT-PCR method we used here, that requires RNA from fibroblast cultures, while Southern blot analyses of genomic DNA are much more laborious and more difficult to interpret. Whereas most published genotype-phenotype correlation studies compare the nucleotide changes at the DNA level with the list of phenotypic features, we feel strongly that studies of the mutant alleles at the RNA and protein level are necessary to take some of the guesswork out of these correlations.

Competing interests

None declared

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