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# Familial Occurrence of Typical and Severe Lethal Congenital Contractural Arachnodactyly Caused by Missplicing of Exon 34 of Fibrillin-2

Mei Wang,[1] Carol L. Clericuzio,[3] and Maurice Godfrey[1,2]

[1]Munroe Center for Human Genetics and [2]Department of Pediatrics, University of Nebraska Medical Center, Omaha; and [3]Department of Pediatrics, University of New Mexico, Albuquerque

## Summary

**Genetic linkage studies have linked congenital contractural arachnodactyly (CCA), a usually mild heritable connective-tissue disorder, to FBN2, the fibrillin gene on chromosome 5. Recently, FBN2 mutations in two patients with CCA have been described. Here we report an A→T transversion at the −2 position of the consensus acceptor splice site, resulting in the missplicing of exon 34, a calcium-binding epidermal growth factor–like repeat in fibrillin-2 in a mother and daughter with CCA. Significantly, the mother exhibited a classic CCA phenotype with arachnodactyly, joint contractures, and abnormal pinnae, whereas her daughter exhibited a markedly more severe CCA phenotype, which included cardiovascular and gastrointestinal anomalies that led to death in infancy. Analysis of cloned fibroblasts showed that the mother is a somatic mosaic for the exon 34 missplicing mutation, whereas all the daughter's cells harbored the mutation.**

## Introduction

Phenotypic variability is a hallmark of most heritable connective-tissue disorders. Defects in fibrillin-1 (FBN1), Marfan syndrome (both classic and neonatal) (Dietz et al. 1991, 1992*a*, 1992*b*, 1993*a*, 1993*b*; Hayward et al. 1992, 1994; Kainulainen et al. 1992, 1994; Aoyama et al. 1993; Godfrey et al. 1993; Hewett et al. 1993, 1994*a*, 1994*b*; Milewicz and Duvic 1994; Nijbroek et al. 1995; Wang et al. 1995, and in press), familial and sporadic ectopia lentis (Kainulainen et al. 1994; Lonnqvist et al. 1994), Marfan-like skeletal deformities (Milewicz et al. 1995), and late-onset aortic aneurysms (Francke et al. 1995) are no exception.

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Address for correspondence and reprints: Dr. Maurice Godfrey, Munroe Center for Human Genetics, University of Nebraska Medical Center, 600 South 42d Street, Omaha, NE 68198-5430. E-mail: mgodfrey@unmc.edu



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Fibrillin-2 (FBN2) was a serendipitous discovery during the search for the "Marfan gene" (Lee et al. 1991). FBN2 is highly homologous, at both the nucleotide and amino acid levels, to FBN1 (Zhang et al. 1994). The temporal expression and tissue distribution of FBN2, however, are somewhat different from those of FBN1 (Mariencheck et al. 1995; Zhang et al. 1995).

Genetic linkage analyses have linked FBN2 to the relatively uncommon heritable disorder of connective tissue, congenital contractural arachnodactyly (CCA) (Lee et al. 1991; Tsipouras et al. 1992). Recently, FBN2 mutations in two unrelated individuals with classic CCA have been described (Putnam et al. 1995). CCA is an autosomal dominant disorder whose phenotypic features overlap those observed in Marfan syndrome (Epstein et al. 1968; Beals and Hecht 1971; Hecht and Beals 1972). CCA is characterized by joint contractures, abnormal pinnae, usually described as "crumpled," and kyphoscoliosis in more severe cases. The prognosis of most CCA patients is usually good, especially since they are spared the cardiovascular abnormalities associated with Marfan syndrome. Most patients live a normal life span. Importantly, some cases of severe lethal CCA have been reported (Lipson et al. 1974; Currarino and Friedman 1986; Macnab et al. 1991). In addition to the clinical characteristics that define CCA, the infants with lethal CCA often have atrial and/or ventricular septal defects, patent ductus arteriosus, interrupted aortic arch, restrictive lung disease, and duodenal atresia (Lipson et al. 1974; Currarino and Friedman 1986; Macnab et al. 1991).

Here we describe a mother and daughter with classic and severe lethal CCA, respectively. The FBN2 mutation was the identical missplicing of exon 34 in both patients. Significantly, the mother was a somatic mosaic for the FBN2 mutation, which resulted in her more classic CCA phenotype.

## Patients and Methods

### *Patients*

*Mother.*—The mother was a 24-year-old Caucasian woman who was born with congenital contractures of the hands, elbows, and knees. Elbow and knee con-

tractures improved spontaneously, and in childhood she underwent surgical release of finger contractures. In adolescence she was diagnosed with mitral valve prolapse. She had delivered a premature daughter (see below) who was diagnosed with CCA on day 1 of life. Subsequently, the mother was evaluated by a clinical geneticist/dysmorphologist (C.L.C.) and was diagnosed with classic CCA. Findings consistent with this diagnosis were typical facies with broad prominent forehead, broad pinnae with crumpled appearance of the superior portions, short neck, mitral valve prolapse, slender limbs with relative arachnodactyly, and mild residual camptodactyly. Skin biopsy was obtained after informed consent.

*Daughter.*—The daughter was the 1,615-g product of a 34-wk gestation who was born spontaneously to a 24-year-old gravida 1 mother (above) and unrelated 31-year-old father. Pregnancy was complicated by third-trimester polyhydramnios, "double-bubble" sign on fetal ultrasound, and preterm labor that prompted maternal transport to a tertiary-care medical facility. There were no known teratogenic exposures. After uneventful vertex vaginal delivery, the baby was taken to the newborn–intensive-care unit, where she was diagnosed with type-B interrupted aortic arch with large ventricular septal defect and moderate atrial septal defect, duodenal atresia and intestinal malrotation, single umbilical artery, vertebral anomalies, and arachnodactyly with contractures of elbows and knees. Clinical genetic/dysmorphology evaluation (by C.L.C.) when the baby was 2 h of age identified the following additional features: weight, length, and head circumference at 15th, 25th, and 50th percentiles, respectively, for 34-wk gestation; long and slender limbs and trunk with marked arachnodactyly and relatively short neck; broad square forehead with mild ocular hypertelorism and down-slanting palpebral fissures; bilateral ear findings including crumpled irregular superior helix and prominent anthelix and root of the helix; pectus excavatum; and hands showing camptodactyly with flexion of proximal interphalangeal joints. The diagnosis of severe neonatal CCA was made.

The child's postnatal course was stormy, beginning with apnea necessitating intubation on day 2 of life. The following procedures were performed: duodenal atresia repair on day 3, interrupted aortic arch repair at age 1 mo (at which time skin biopsy was obtained after informed consent), and gastrostomy tube placement for gastroesophageal reflux complicated by perforation, with subsequent jejunostomy at age 1.5 mo. She experienced multiple episodes of sepsis and remained ventilator dependent, with chronic lung disease requiring tracheostomy at age 5 mo. The patient succumbed to *Candida albicans* sepsis and renal failure at age 9 mo.

### *Fibrillin Immunofluorescence*

Skin immunostaining was performed as described elsewhere (Godfrey et al. 1990).

### *Extraction of Nucleic Acids*

*DNA.*—Extraction of DNA from dermal fibroblasts was performed by incubating $5 \times 10^6$ cells in 200 μl of lysis buffer (10 mM Tris-HCl [pH 8.0], 2 mM EDTA, 10 mM NaCl, 5% SDS, and 200 μg Proteinase K/ml) at 55°C for 16 h (Sambrook et al. 1989). In all extractions, the aqueous DNA was recovered by phenol/chloroform extractions and isopropanol precipitation. The extracted DNA was suspended in a solution of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8.0), and concentration and purity were assessed spectrophotometrically.

*RNA.*—RNA was extracted from dermal fibroblasts by established techniques (Chomczynski and Sacchi 1987). In brief, cells were lysed in situ in a guanidine thiocyanate buffer containing β-mercaptoethanol. RNA was extracted with phenol/chloroform, was precipitated with ethanol, and was stored at −80°C prior to use. RNA from cloned fibroblasts were extracted from 35-mm culture dishes by TRIzol Reagent (GIBCO-BRL), according to supplied instructions.

### *cDNA Production*

Production of cDNA from RNA was performed by a commercially available kit (Amersham), according to the manufacturer's protocol.

### *PCR*

*Reverse Transcriptase–PCR (RT-PCR).*—Amplification of the daughter's and her mother's fibroblast cDNA, in the region of the deletion, was performed by the oligonucleotide primers from position 4258 (forward 5′-ATCAATGCTCAGTGTGTAAAT-3′) and position 4512 (reverse 5′-TTCCAGGCAGGTTATTACAT-3′), at the following conditions: denaturing at 94°C for 1.0 min, annealing at 53°C for 2.0 min, and extension at 72°C for 0.5 min, for 30 cycles. A hot start at 94°C for 5 min was used at initiation of the PCR, and a final 10-min extension at 72°C was also used.

*Genomic.*—To generate an amplicon containing intron 33 and its splice junctions, the forward primer noted above and a reverse primer, 5′-AAGGCACTGTCCGTTCTCA-3′ (position 4377), were used to amplify genomic DNA from patients and control individuals. Amplification was at the following conditions: denaturing at 94°C for 1.0 min, annealing at 53°C for 2.0 min, and extension at 72°C for 2.5 min, for 30 cycles.

### *Nucleotide Sequencing*

All PCR products were gel purified and sequenced directly. Both upper and lower bands from the patients's RT-PCR products were cut and purified separately. Direct-sequencing reactions of amplified products was performed by use of the Sequenase PCR Product Sequencing Kit (USB), according to supplied instructions with slight

modifications. In brief, amplified products were purified with Wizard PCR Preps DNA Purification System (Promega). Ten picomoles of amplification primers were annealed to 0.5 pmol of purified amplicons. Labeling reactions were set with $^{35}S$ α-dATP, according to the manufacturer's protocol.

### *Cloning of Fibroblasts*

To provide further data on the mother's mosaicism, we cloned dermal fibroblasts from both mother and daughter. Serial dilutions were used to plate one cell per two wells of flat-bottom tissue-culture-grade microtiter plates. Two microtiter plates for each individual were used. The cells were grown in F-10 media (GIBCO) supplemented with 20% fetal bovine serum. Fibroblasts from the wells that showed growth were trypsinized and expanded into single wells of 24-well tissue-culture dishes. Once confluence was reached, the cells were expanded into two 35-mm culture dishes. Thirteen clones from the daughter and eight from the mother grew sufficiently for RNA extraction.

### *Allele-Specific Oligonucleotide (ASO) Hybridization*

In order to prove that the mother was a somatic mosaic, we performed genomic PCR of serially diluted template DNA, using the amplification conditions described above, for 18 cycles. ASO hybridization was performed by loading 15 μl of each amplicon in a slot-blot apparatus. The DNA was cross-linked to the membrane by UV light and used for hybridization. Oligonucleotide probes for the normal (5′-TTT GCT TTT TTG CAC AGA TGT TGA TGA GT-3′) and mutant (5′-TTT GCT TTT TTG CAC T GA TGT TGA TGA GT-3′) alleles, differing by a single base, were synthesized and end-labeled with $^{32}P$ γ-dATP. ASO hybridization was performed by use of replicate filters with each probe. Hybridization was at 54°C for 1 h. After hybridization the filters were subject to three washes in 2 × SSPE and 0.1% SDS, for 30 s, 10 min, and 10 min, respectively. The final wash was at 56°C. The membrane was exposed on a Molecular Dynamics Exposure Cassette for 3 h at room temperature. The image plate was scanned by a Molecular Dynamics 425B PhosphorImager (fig. 6). Quantitative measurements were made by storage phosphor-signal technology and were plotted.

## Results

### *Immunohistochemistry*

Immunostaining of skin sections was performed by use of monoclonal antibodies to fibrillin-1. Analysis showed a decrease in fibrillin immunostaining at the dermal/epidermal junction and in the dermis of both daughter and mother (fig. 1*A* and *C*), compared with that in age-matched control skin sections (fig. 1*B* and *D*). Immunostaining with polyclonal antibodies directed against fibrillin-2 showed, as expected, no staining in patient or control skin (data not shown).

### *Analyses of cDNA*

Heteroduplex analysis of RT-PCR products spanning the entire cDNA of FBN2 was performed by by use of 45 pairs of overlapping primers (data not shown). Amplification of a 273-bp region of the daughter's and her mother's dermal fibroblast–derived cDNA showed the appearance of two bands but showed only one in control samples (fig. 2). Both the upper and lower bands were gel purified and sequenced. Sequencing of the cDNA of both mother and daughter demonstrated a deletion of 126 bp of the FBN2-coding region in the

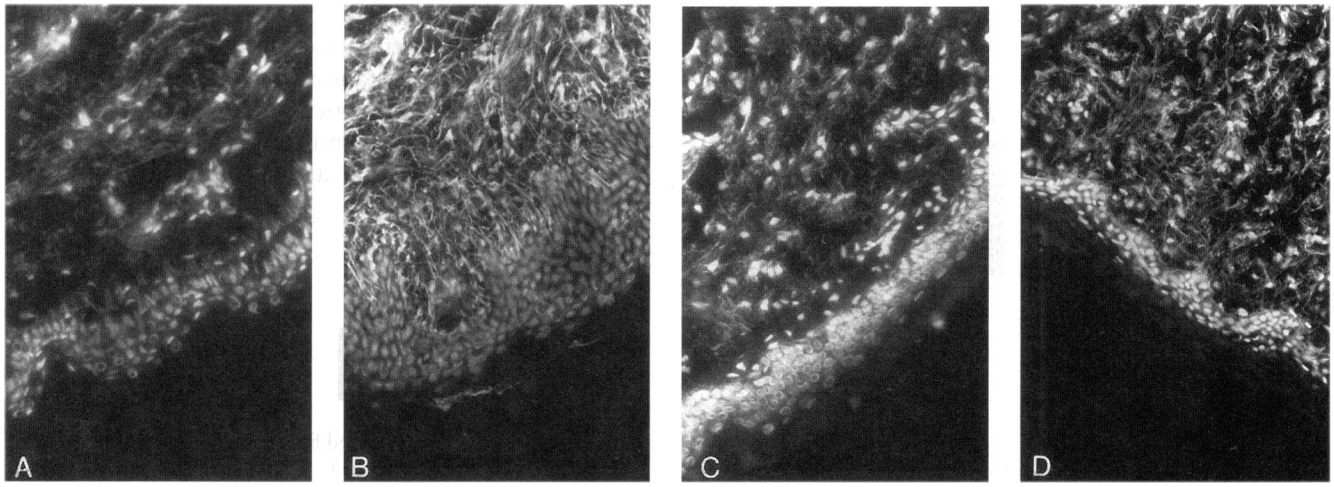


**Figure 1** Fibrillin immunostaining of skin sections of mother, daughter, and age-matched controls. The immunostaining pattern in the mother (*A*) showed an apparent decrease in immunostainable fibrils at the dermal-epidermal junction and in the dermis when compared with that in an age-matched control (*B*). Similarly, fibrillin immunostaining in the infant daughter (*C*) was less intense than that in an age-matched control (*D*). Cells were visualized by use of the nuclear dye propidium iodide. Magnification 138×.

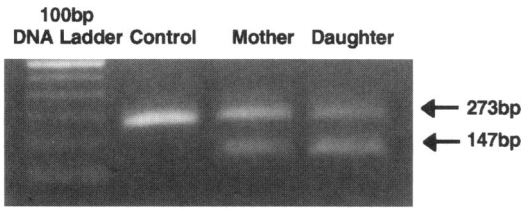


**Figure 2** RT-PCR results demonstrating the presence of one fragment in a control and two fragments in the fibroblast-derived cDNA from the mother and daughter. The marker lane is a 100-bp marker. Both upper and lower bands from the patients were direct sequenced. Note that the upper (i.e., normal) band is more intense in the mother than in the daughter.

lower band (fig. 3). The deleted region corresponds to exon 34 of FBN2 (Zhang et al. 1994) (F. Ramirez, personal communication). The deleted region begins at position 4343 and corresponds to an entire calcium-binding precursor EGF-like domain. Similar deletions were not observed in 10 additional CCA probands and 20 controls.

### *Genomic Analysis*

A set of cDNA primers was designed to amplify across intron 33 and include portions of the coding region plus the splice junctions. An amplified product of ~1.8 kb was obtained (data not shown). Direct sequencing of the amplicon that spanned intron 33 showed an A→T transversion at the −2 position of the consensus acceptor splice site (fig. 4). The same change was observed in both mother and daughter. The sequence of the donor splice site was normal.

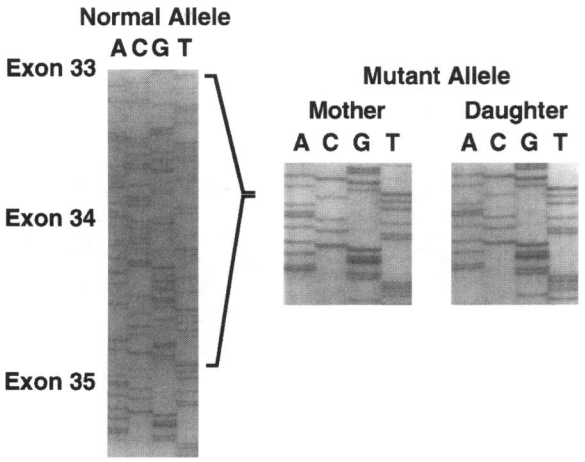


**Figure 3** Direct sequence of amplified cDNA of both the upper (normal) and lower (mutant) bands (see fig. 2) from the patients, showing a deletion of 126 bp, which corresponds to an entire precursor EGF-like exon (exon 34). The deletion begins at position 4343 and extends 126 bp. Intron/exon boundaries are shown. (The sequence is in an antisense orientation.)

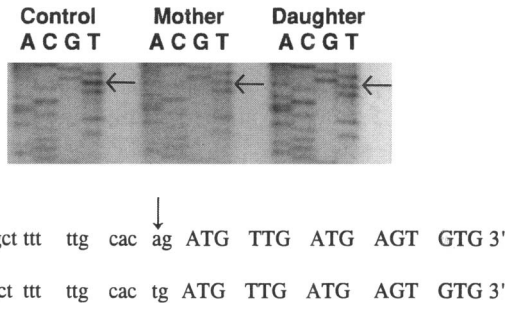


**Figure 4** Results of direct sequence analysis of amplified genomic material at the intron/exon junction of the misspliced exon, demonstrating an A→T transversion at the −2 position (*arrow*) of the consensus acceptor splice site. The bases shown in lowercase letters are the intron sequences, whereas those shown in uppercase are the exon sequences.

### *cDNA Analyses of Cloned Fibroblast Cultures*

Fibroblasts from mother and daughter were cloned by use of limiting dilutions. RT-PCR was performed on cDNAs derived from each clone. Figure 5 shows RT-PCR results from five clones from the mother and daughter. All daughter-derived fibroblast clones had both normal and mutant bands. Less than half of the clones derived from the mother's fibroblasts had the mutant band.

### *ASO Hybridization*

To prove that the mother's mosaicism was not due solely to differential splicing but that she was a true somatic mosaic at the gene level, we performed ASO hybridization of genomic PCR products, using wild-type and mutant probes that overlap the splice-site mutation. Figure 6 shows the PhosphorImager scan of the ASO. It is evident that there is no mutant allele in the control and that the mother has more normal allele than mutant allele, whereas the daughter appears to have an equal proportion of the normal and mutant alleles. The ratios of normal allele to mutant allele, between patients and control or within one patient, when phosphor signals are quantitated, are shown in table 1. The mother:control ratio for the normal allele is a little less than 1,

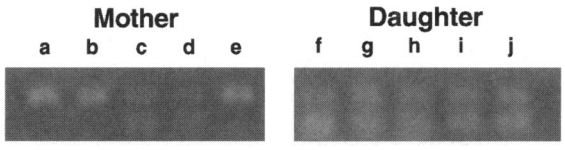


**Figure 5** RT-PCR of cloned fibroblast cultures from the mother (lanes a–e) and daughter (lanes f–j). All of the daughter's fibroblast clones showed the appearance of normal (*upper*) and mutant (*lower*) bands. The mother's fibroblast clones proved that she was a somatic mosaic for the FBN2 mutation. The mother's clones c and d had both the normal (*upper*) and mutant (*lower*) bands, whereas clones a, b, and e had only the normal (*upper*) band.

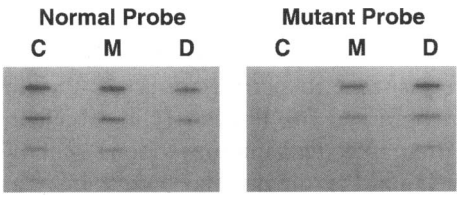


**Figure 6** ASO hybridization of normal and mutant alleles in genomic DNA from dermal fibroblasts of the mother (lanes M), daughter (lanes D), and control (lanes C), in a PhosphorImager scan of the ASO analysis. The probe to the wild-type allele is in the left panel (Normal Probe), and that to the mutant allele is on the right panel (Mutant Probe). Note that the control lane show no hybridization to the mutant allele.

whereas the daughter:mother or daughter:control ratio is ~0.5. Furthermore, when the daughter:mother ratio for the mutant allele is quantitated, it is almost 2.0. When the normal allele:mutant allele ratios for the mother and daughter are quantitated, they are 3.0 and 0.9, respectively. Thus, these data show that the mother has more cells that are homozygous for the normal allele than cells that are normal/mutant allele heterozygotes. The daughter has approximately equal populations of both alleles, indicating that all her cells are normal/mutant allele heterozygotes.

## Discussion

Coordinate regulation of matrix components during elastogenesis has been the subject of a great deal of speculation (Cleary and Gibson 1983). The recent identification of the specific genes that encode some elastic-fiber elements (Bashir et al. 1989; Gibson et al. 1991; Pereira et al. 1993; Zhang et al. 1994) has enabled the study of structural, as well as temporal, associations between some of these components (Brown Augsburger et al. 1994; Mariencheck et al. 1995; Yin et al. 1995; Zhang et al. 1995). The study of disease phenotypes frequently yields some understanding of normal pathophysiology. The fibrillin microfibrilopathies are no exception. For example, Zhang et al. (1994) elegantly showed differential expression of fibrillin-1 and fibrillin-2 in human ear cartilage. Abnormally shaped—that is, crumpled—auricular helices are a hallmark of CCA, now known to be caused by defects in FBN2. On the other hand, most people with Marfan syndrome (FBN1 mutations) do not have abnormally shaped ears, although some individuals with neonatal Marfan syndrome do have crumpled ears (Godfrey et al. 1995).

There are additional similarities between neonatal Marfan syndrome and severe lethal CCA. For example, arachnodactyly, joint contractures, and some facial characteristics are present in both groups. Importantly, although both have severe cardiovascular abnormalities that lead to very early death, the specific cardiac changes are quite different (table 2). That observation led Lipson et al. (1974, p. 6) to speculate >20 years ago that "the cardiovascular defects in CCA reflect incomplete organ development," when compared with Marfan syndrome. Perhaps overly simplistic, this idea may be borne out, given expression of FBN1 and FBN2 in development (Mariencheck et al. 1995; Zhang et al. 1995). Further, Zhang et al. (1995) suggest that expression of fibrillin-2 directs the assembly of elastic fibers during early embryogenesis whereas fibrillin-1 provides the major struc-

## Table 1

**Ratios of ASO Hybridization of Genomic PCR from Scan Shown in Figure 6**

| | Probe to Normal Allele Ratio | Probe to Mutant Allele Ratio |
|---|---|---|
| Mother:control | .88 | ... |
| Daughter:control | .51 | ... |
| Daughter:mother | .57 | 1.91 |
| | Normal Allele:Mutant Allele Ratio | |
| Mother | | 3.0 |
| Daughter | | .90 |

NOTE.—Data were derived by phosphor-image quantitation.

## Table 2

**Comparison of Clinical Manifestations, between Neonatal MFS and Severe Lethal CCA**

| Manifestation | Neonatal Marfan Syndrome[a] | Severe Lethal CCA[b] |
|---|---|---|
| Cardiovascular: | | |
| Mitral valve prolapse | 9/9 | 0/6 |
| Tricuspid valve prolapse | 9/9 | 0/6 |
| Mitral valve insufficiency | 9/9 | 0/6 |
| Aortic root dilatation | 9/9 | 1/6 |
| Atrial septal defect | 0/9 | 4/6 |
| Ventricular septal defect | 0/9 | 4/6 |
| Interrupted aortic arch | 0/9 | 4/6 |
| Single umbilical artery | 0/9 | 3/6 |
| Skeletal: | | |
| Arachnodactyly | 9/9 | 6/6 |
| Flexion contractures | 8/9 | 6/6 |
| Scoliosis | 0/6 | 3/6 |
| Vertebral anomalies | 0/6 | 2/5[c] |
| Gastrointestinal: | | |
| Duodenal atresia | 0/9 | 3/5[c] |
| Esophageal atresia | 0/9 | 1/5[c] |
| Intestinal malrotation | 0/9 | 2/5[c] |

[a] Data are from Godfrey et al. (1995).
[b] Data are from Lipson et al. (1974), Currarino and Friedman (1986), Macnab et al. (1991), Godfrey et al. (1995), and present study.
[c] Data from one patient are not known.

tural—that is, "load bearing"—function of the microfibrils. The phenotypic differences between the cardiovascular abnormalities in severe lethal CCA and Marfan syndrome would support that distinction. In addition, given the much lower expression of FBN2 versus FBN1 in adult tissues (Zhang et al. 1995), the abnormal immunostaining in skin sections, when antibodies to fibrillin-1 are used, in both mother and daughter (fig. 1) are suggestive of the role that fibrillin-2 must play in the formation of the microfibrils. Antibody probes provide a useful window onto the components and organization of various tissues. Thus, it appears that this particular FBN2 mutation distorts the microfibrillar array as measured by immunofluorescence using anti–fibrillin-1 antibodies. Therefore, in a model that requires coordinate regulation of various components, an abnormality in any one constituent may adversely affect the entire structure.

In the study described here, we have identified the identical missplicing of a single EGF-like domain of FBN2 in a mother and her daughter. The clinical phenotypes and courses of the two affected individuals were very different indeed. The mother had classic CCA with predominantly self-limiting contractures, mild mitral valve prolapse, and abnormally shaped ears. The daughter, on the other hand, had severe lethal CCA, including multiple cardiac and hollow viscera abnormalities that led to her death in infancy. This is the first report of mosaicism in the fibrillin microfibrilopathies. These results may be of benefit to this mother should she choose prenatal diagnosis of CCA in future pregnancies.

The occurrence of mild disease in a parent and of severe disease in a child because of parental somatic mosaicism has been observed for both type I and type II collagen (Constantinou et al. 1990; Wallis et al. 1990; Edwards et al. 1992; Winterpacht et al. 1993). Since, as noted by Edwards et al. (1992) and Winterpacht et al. (1993), the abnormal allele was present in both the germ cells and fibroblasts, the mutation must have occurred early in embryonic development. Cloning of the mother's and the daughter's fibroblasts (fig. 5) strongly suggested that the mother was a somatic mosaic. ASO hybridization of amplified genomic DNA showed a 3:1 normal allele:mutant allele ratio in the mother's skin fibroblasts, proving that the mother was indeed a somatic mosaic, at the gene level (fig. 6 and table 1). The degree of mosaicism in any other organ or tissue is not known, since no additional sampling could be performed.

It may be interesting to speculate that FBN2 mutations in later stages of ontogeny may be responsible for some of the cardiac septal defects in children. The absence of the severe cardiovascular abnormalities in the mother (she does have mitral valve prolapse) may be due to the relatively low abundance of abnormal FBN2 mRNA greatly diluted by the normal FBN2 message. It is possible that cartilage (ear and joints) are more sensitive and that the cardiovascular system (aorta and septa) are more resistant to perturbations of fibrillin-2. This has been observed for fibrillin-1, in which mutations causing premature-termination codons generally result in relatively mild cardiovascular disease but classic skeletal phenotypes (Dietz et al. 1993*a*, 1993*b*). Moreover, substitutions for cysteine in the "neonatal region" of FBN1 (Kainulainen et al. 1994; Dietz and Pyeritz 1995) and in the homologous region of FBN2 (Putnam et al. 1995) also result in disease that is less severe than that caused by either substitutions of other amino acids or exon-skipping mutations. In light of these findings and the clustering of mutations in the homologous region of FBN1 in patients with neonatal Marfan syndrome (Kainulainen et al. 1994; Milewicz and Duvic 1994; Nijbroek et al. 1995; Wang et al. 1995, and in press), it appears that similar regions of both fibrillin-1 and fibrillin-2 are critical to providing optimal structural or functional integrity to the microfibrils and/or elastic fibers in which they are found.

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