

A Rare Branch-Point Mutation Is Associated with Missplicing of Fibrillin-2 in a Large Family with Congenital Contractural Arachnodactyly

Cheryl Maslen,^{1,2,3} Darcie Babcock,¹ Michael Raghunath,⁴ and Beat Steinmann⁵

Departments of ¹Medicine and ²Molecular and Medical Genetics and ³University Congenital Heart Research Center, Oregon Health Sciences University, Portland, OR; ⁴Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, Münster; and ⁵Division of Metabolic and Molecular Diseases, University Childrens Hospital, Zürich

Summary

Congenital contractural arachnodactyly (CCA) is an autosomal dominant disorder that is phenotypically similar to but genetically distinct from Marfan syndrome. Genetic-linkage analysis has implicated the fibrillin-2 gene (FBN2) as the CCA locus. Mutation analysis of two isolated CCA patients revealed missense mutations, indicating that defects in FBN2 may be responsible for this disorder. However, cosegregation of a mutant allele with the disease phenotype has not yet been established. We have investigated the primary cause of CCA in a large well-characterized kindred with five generations comprising 18 affected individuals. Previous studies demonstrated linkage of this family's CCA phenotype to FBN2. Mutation analysis of cDNA derived from the proband and her affected brother, using a nonisotopic RNase cleavage assay, revealed the partial skipping of exon 31. Approximately 25% mutant transcript is produced, which is apparently sufficient to cause a CCA phenotype. Sequence analysis of genomic DNA revealed an unusual base composition for intron 30 and identified the mutation, a g-26t transversion, in the vicinity of the splicing branch-point site in intron 30. Genomic DNA from 30 additional family members, both affected and unaffected, then was analyzed for the mutation. The results clearly demonstrate cosegregation of the branch-point mutation with the CCA phenotype. This is the first report of a CCA mutation in a multiplex family, unequivocally establishing that mutations in FBN2 are responsible for the CCA phenotype. In addition, branch-point mutations only very rarely have been associated with human disease, suggesting that the unusual composition of this intron influences splicing stability.

Introduction

Congenital contractural arachnodactyly (MIM 121050), also known as "Beals syndrome," is an autosomal dominant disorder that has some of the phenotypic characteristics of Marfan syndrome. The cardinal features of CCA are multiple congenital contractures, camptodactyly, arachnodactyly, kyphoscoliosis, muscle hypoplasia, and external ear malformation (Beals and Hecht 1971; Hecht and Beals 1972; Ramos-Arroyo et al. 1985; McKusick 1994). However, as in Marfan syndrome, there is significant phenotypic variability within and between families. Dolichostenomelia, micrognathia, and subluxation of the patella are among the features occasionally present (Hecht and Beals 1972; Ramos-Arroyo et al. 1985; Viljoen 1994). The most severely affected CCA patients also may manifest cardiac abnormalities including mitral valve prolapse, atrial septal defect, ventricular septal defect, and aortic hypoplasia (Anderson et al. 1984; Ramos-Arroyo et al. 1985).

There is sufficient phenotypic similarity between Marfan syndrome and CCA that, until the cloning and characterization of the fibrillin genes (FBN1 and FBN2), there was some speculation that the two disorders might be allelic. Since then, extensive studies have demonstrated that Marfan syndrome is due to a variety of mutations in FBN1 (Ramirez 1996), whereas CCA has been linked to the FBN2 locus (Lee et al. 1991; Tsiouras et al. 1992). To date, two missense mutations have been reported in fibroblast-cell strains derived from two isolated patients with CCA (Putnam et al. 1995), and a splicing defect has been characterized in an affected mother and child (Wang et al. 1996). Together these data indicate that defects in FBN2 are indeed responsible for CCA. However, significant cosegregation of the CCA phenotype with any mutation has not yet been shown. Here we report the identification of a branch-point mutation associated with the in-frame skipping of exon 31 in a large well-characterized kindred with CCA and show the segregation of this mutation with the CCA phenotype.

Subjects and Methods

Family S

We ascertained a large five-generation Swiss family with CCA and had the opportunity to examine a total

Received November 4, 1996; accepted for publication March 5, 1997.

Address for correspondence and reprints: Dr. Cheryl Maslen, Department of Medicine, L465, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201-3098. E-mail: maslenc@ohsu.edu

© 1997 by The American Society of Human Genetics. All rights reserved.
0002-9297/97/6006-0017\$02.00

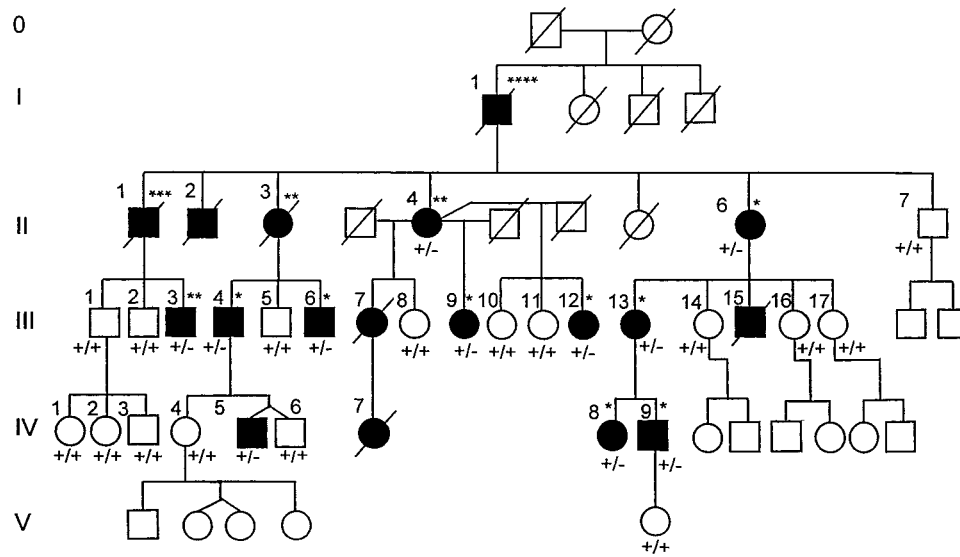


Figure 1 Pedigree of family S showing phenotypes, severity of the disorder and genotypes. $+/+$ = Homozygous wild type; $+/-$ = heterozygous, wild type, and mutant (g-26t). Blackened squares and circles denote affected males and females, respectively; a diagonal slash through a symbol indicates that the individual is deceased. Severity scores are indicated as follows: * = mild; ** = moderate; *** = severe; and **** = very severe. All 11 living affected individuals were examined (by B.S. and M.R.). Descriptions of deceased individuals and assessments of their disease severity were provided by a family member (II-4) who is best informed about the family. Her anecdotal accounts were well supported by photographic documentation and corresponded well with our clinical assessment of living family members. I-1 was the first child born to healthy, young, nonconsanguineous parents; he had severe kyphoscoliosis, a pectus excavatum, and a height of ~143 cm. He died at age 44 years. His three siblings were not affected. II-1 died at age ~70 years, from cardiac insufficiency. II-2 died at age 3 wk, from unknown cause. II-3 died at age 63 years, from carcinoma. III-7 died at age 24 years, from eclampsia. III-15 was born after 28 wk gestation and died soon thereafter; according to his mother he presented with characteristic contractures. IV-7 died soon after birth, together with her mother (III-7).

of 26 family members from generations II–V (fig. 1). Previously, this family had been included in a linkage study using markers for FBN1 and FBN2 (Lee et al. 1991). Eleven affected members were positively linked to the FBN2 locus, with a LOD score of 2.281 (for $\theta = 0$) (Velinov et al. 1992). All family members were interviewed and examined by use of a standardized protocol. Physical measurements were taken, and EDTA blood was obtained from every individual. In addition, 4-mm punch biopsies were taken from the skin of the forearm in affected individuals IV-8 and IV-9 to establish fibroblast cultures. This study was done with the informed consent of the subjects.

Nonisotopic RNase Cleavage Assay (NIRCA)

NIRCA was performed on cDNA prepared from cultured skin fibroblasts isolated from patient IV-8 and an unrelated normal control. Isolation of mRNA and subsequent preparation of total cDNA was done by use of standard techniques (Ausubel et al. 1995). First-round PCR amplification of the cDNA was done by use of the primers and conditions listed in table 1. This procedure amplifies the entire coding region of fibrillin-2, as seven fragments of ~1.5 kb each. A second round of amplification was done by use of the first-round products as template, with the primers and

conditions listed in table 2. This procedure produces 13 PCR fragments of ~750 bp each, with a T7 consensus promoter sequence added to the 5' end of the sense strand and with a SP6 consensus promoter sequence added to the 5' end of the antisense strand. Addition of the T7 and SP6 consensus promoter sequences to the second-round PCR products facilitates strand-specific in vitro transcription of cRNA from the PCR-amplified template. NIRCA then was performed with Mismatch Detect II (Ambion), according to the manufacturer's instructions.

DNA Sequence Analysis

PCR-amplified DNA fragments (cDNA and genomic) were isolated from low-melting-temperature agarose, by use of an Ultrafree-MC spin column (Millipore). The isolated cDNA was treated with Klenow polymerase to ensure full extension of the product (Ausubel et al. 1995). A second round of PCR amplification was done under the same conditions, with either a biotinylated sense or a biotinylated antisense primer. Single-stranded DNA was isolated by use of streptavidin-coated magnetic beads, according to the manufacturer's (Dyna) protocol. Solid-phase DNA sequencing was done with nested sequencing primers, by use of the Fidelity Sequencing system (Oncor).

Table 1**PCR Primers Used to Amplify Coding Region of FBN2 in Seven Overlapping Fragments**

Fragment	Position ^a	Sense Primer ^b (5'–3')	Antisense Primer (5'–3')
1	136–1495	ACAGGTTCCGGTCCGCTACAGC	GCTTACAGATATCTATTGTCTGGTTC
2	1330–2889	AGTGGCAATGGCAATGGCTATGG	GCCAGGGAACACCTCACACTCAT
3	2687–4228	CTGTTGGCTCAACATCCAGGAC	CGTCCAGATCAATACACTTGATGC
4	4088–5602	GATGTACAGATGTGGATGAGTGTG	TGATGCAGTCTGCATTCCGCTG
5	5464–6963	GTGTGCATTAACCAGATTGGCAGT	GATGCACATGAAGGTGCCGATTAG
6	6817–8302	TCCTATGAATGCACGTGCCCGAT	CGTTGATTTTGCACCTCGTAGCATG
7	8114–9645	GCAATTACGGCTGCTTAACACG	TCAGCTGCCTACAGTACCATGAG

NOTE.—The PCR reaction conditions are with standard buffer conditions (Perkin Elmer Cetus) at 94°C for 1 min and 68°C for 3 min, for 30 cycles.

^a cDNA nucleotide positions encompassed by the amplified fragment; numbering is according to Zhang et al. (1994).

^b Primers 5' to sense primer 1 have not amplified cDNA in our hands.

Allele-Specific PCR

Presence of the mutant allele was determined in a blind study of genomic DNA from family members, by use of allele-specific PCR (Bottema et al. 1993). Genomic DNA was amplified differentially by use of primers that represent the normal sequence encompassing the –26 position of intron 30 and the mutant sequence at that position (5'-ACTGTGATGAGCACATACTA-3' and 5'-ACTGTGATGAGCACATACGA-3', wild type and mutant, respectively). A second invariant PCR primer downstream from that site (in exon 32) was used as the primer pair (5'-TCGCAGTTATGAGCACCAAT-3'). Characterization of this assay demonstrated that the wild-type intron 30 primer amplified genomic DNA from both the patient and normal controls, whereas the mutant primer was capable of amplifying only genomic DNA from the affected individual in whom the mutation had been characterized. The amplifications were done by use of standard buffer conditions (Perkin Elmer Cetus) at 94°C for 1 min and at 69°C for 3 min, for 40 cycles, with a final extension at 72°C for 7 min. The products were visualized by standard ethidium bromide–agarose gel electrophoresis. In all cases the primers gave a single band of the expected size, 1.8 kb.

Analysis of Frequency of Exon Skipping

The cDNAs derived from patients IV-8 and IV-9 were analyzed to estimate the frequency of the skipping of exon 31. Patient cDNA was PCR amplified by use of primer pair 3/2 (table 2), in separate reactions of 18, 20, 22, and 24 cycles, in the presence of 2.5 μCi ³²PαdCTP under the conditions specified in table 2, with the exception of the addition of 5% dimethylsulfoxide to the reaction to reduce any possibility of mispriming due to formation of secondary structures. Ten microliters of each reaction was electrophoresed on a 1% agarose gel. The DNA was fixed with 8% acetic acid, and the gel was dried and autoradiographed. Each reaction

consistently produced two bands, corresponding to the normal transcript and the aberrantly spliced transcript. The reactions were done three times each on three different RNA isolates, with indistinguishable results. The band intensities were determined by densitometry, and the percentage of each transcript species was calculated on the basis of the relative signal of each band in a given reaction.

Results*Clinical Presentation of Affected Members of Family S*

The details of the clinical examination are summarized in table 3 and in selected patient photographs shown in figure 2. Affected individuals could be identified immediately by their mothers at birth, on the basis of the individuals' hands, which were in clenched (fist-like) positions, and on the basis of their ears, which unequivocally were described as resembling "cabbage leaves." The auricular deformity resolved with age and was appreciable only in the youngest family member, IV-5, in whom we could confirm the palpatory impression of a cabbage leaf. This finding was, however, inconspicuous to us in all adults. Camptodactyly also improved with age, but it remained the leading phenotypic feature. Contractures of larger joints were present in varying degrees in all affected individuals. Kyphoscoliosis, thoracic deformities, and muscular hypoplasia of calves and forearms were observed in all affected individuals. In addition, all affected individuals presented with dolichostenomelia and arachnodactyly. Most of the arm spans significantly exceeded the body height but, because of contractures of elbows and fingers, are underestimated as given in table 3. The same held true for assessment of the lower-body segment, as a result of contractures of the knee. A feature present only in female affected family members was a marked truncal adipositas, which contrasted with the long and slender

Table 2
PCR Primers Used to Generate Strand-Specific Transcription Templates for NIRCA

Fragment	Position ^a	Sense/T7 Primers ^b (5'–3')	Antisense/SP6 Primers ^b (5'–3')	Annealing Temperature (°C)
1/1	157–846	GATAATACGACTCACTATAAGGGCTCTGAAGCGGGTIT	TCATTTAGGTGACACTATAAGGATAGCCTGGCAITTCATC	56.2
1/2	739–1488	GATAATACGACTCACTATAAGGGCCATGCCCTGTGAGAT	TCATTTAGGTGACACTATAAGGATATCTATTGTCIGGHTC	52.7
2/1	1357–2149	GATAATACGACTCACTATAAGGGAGGACAGGCTTCATC	TCATTTAGGTGACACTATAAGGACGACACACTCCCTT	54.4
2/2	2061–2752	GATAATACGACTCACTATAAGGGCTGTGGGATGGATGG	TCATTTAGGTGACACTATAAGGAGGGTGGCAGACATTC	56.0
3/1	2734–3541	GATAATACGACTCACTATAAGGGAGCCACTCTGAAATCT	TCATTTAGGTGACACTATAAGGATGCGCGTTTCCAATCCA	55.1
3/2	3468–4209	GATAATACGACTCACTATAAGGACATTGACGGATGTGA	TCATTTAGGTGACACTATAAGGATGTGATGGGTTAGG	54.9
4/1	4117–4935	GATAATACGACTCACTATAAGGGTGCTCATAACTGCGAC	TCATTTAGGTGACACTATAAGGATGTGATGGGTTAGG	54.7
4/2	4839–5577	GATAATACGACTCACTATAAGGGAAACGCCCTGTGAGACA	TCATTTAGGTGACACTATAAGGATGTGATGGGTTAGG	51.9
5/1	5482–6295	GATAATACGACTCACTATAAGGCAGTTTCCGCTGTGAA	TCATTTAGGTGACACTATAAGGATGTGATGGGTTAGG	51.6
5/2	6205–6945	GATAATACGACTCACTATAAGGGTTCCTGTACTAATACT	TCATTTAGGTGACACTATAAGGATGTGATGGGTTAGG	49.1
6/1	6854–7641	GATAATACGACTCACTATAAGGGAAAGATCAAAAAGATGT	TCATTTAGGTGACACTATAAGGACAGAGAACTGGCA	52.7
6/2	7528–8270	GATAATACGACTCACTATAAGGGAGTTATCAGTGTCA	TCATTTAGGTGACACTATAAGGACAGAGAACTGGCA	53.9
7/1	8170–8883	GATAATACGACTCACTATAAGGGTATTACAGAGTGGGA	TCATTTAGGTGACACTATAAGGAGGGTCAACATTCAAAAG	53.4

NOTE.—The PCR reaction conditions are with standard buffer conditions (Perkin Elmer Cetus) at 94°C for 1 min, designated annealing temperature for 0.5 min, and extension at 72°C for 1 min, for 30 cycles.

^a cDNA nucleotide positions encompassed by the amplified fragment; numbering is according to Zhang et al. (1994).

^b FBN2-specific sequences are underlined. The 3' extensions are the SP6 and T7 consensus sequences. Overlaps between the FBN2 and SP6 or T7 sequences are indicated by overbars.

Table 3
Detailed Phenotype of Members of Family S Who Are Affected with CCA

	II-4	II-6	III-3	III-4	III-6	III-9	III-12	III-13	IV-5	IV-8	IV-9
Age (years)	75	69	39	49	44	45	32	46	8	28	25
Height (cm)	170	172	182	197	181	181	174	178	140	175	183
Arm span (cm)	179	175	188	202	191	185	182	183	147	186	183
Upper segment	75	88	89	95	81	87	80	89	69	81	91
Lower segment	95	84	93	102	100	94	94	89	71	94	92
Thumb sign ^a	+	?	-	-	-	+	(+)	-	++	++	(+)
Wrist sign ^a	(+)	?	-	+	+	+	+	-	++	++	+
Joint contractures:											
Fingers ^a	++	+	+	+	++	++	++	++	++	++	++
Toes ^a	++	+	+	+	+	++	++	++	++	+	-
Knees (deficit, in °)	5-10	?	10	0	?	5	0	0	0	0	0
Elbows (deficit, in °)	5-10	2.5	10	10	15-20	10-15	10	10	10-15	0	1.5
Limited pro-/supination of the hands ^a	++	++	+	++	+	++	++	++	++	++	++
Kyphoscoliosis ^a	+	+	++	++	+	+	++	+	(+)	(+)	(+)
Chest deformity ^b	exc	car	car	exc	exc	(exc)	exc	(car)	car+exc	(car)	(car)
High-arched palate ^a	-	-	+	+	+	?	+	-	+	-	-
Muscle hypoplasia ^a	+	+	+	+	+	+	+	+	+	(+)	++
Ears ^c	-	PI	-	PI	PI	CL	CL	PI	Crumpled, CL	-	-
Eyes ^d	-	cat. surg.	kerat. surg.	-16/-18 diopters	-5 diopters	-	-	-	-	-	-
Skin	Soft	Fine/striated	Soft	Fine	Fine/thin	Fine/thin	Normal	Sort/striated	Fine	Normal	Normal

^a + = Present (parentheses indicate that condition is mild); ++ = severely present; - = absent; and ? = not examined.
^b exc = pectus excavatum (parentheses indicate that condition is mild); and car = pectus carinatum (parentheses indicate that condition is mild).
^c PI = probably inconspicuous to an unprepared observer (see clinical picture); and CL = palpatory impression of a cabbage leaf.
^d cat. surg. = surgery for cataract; and kerat. surg. = surgery for keratoconus.

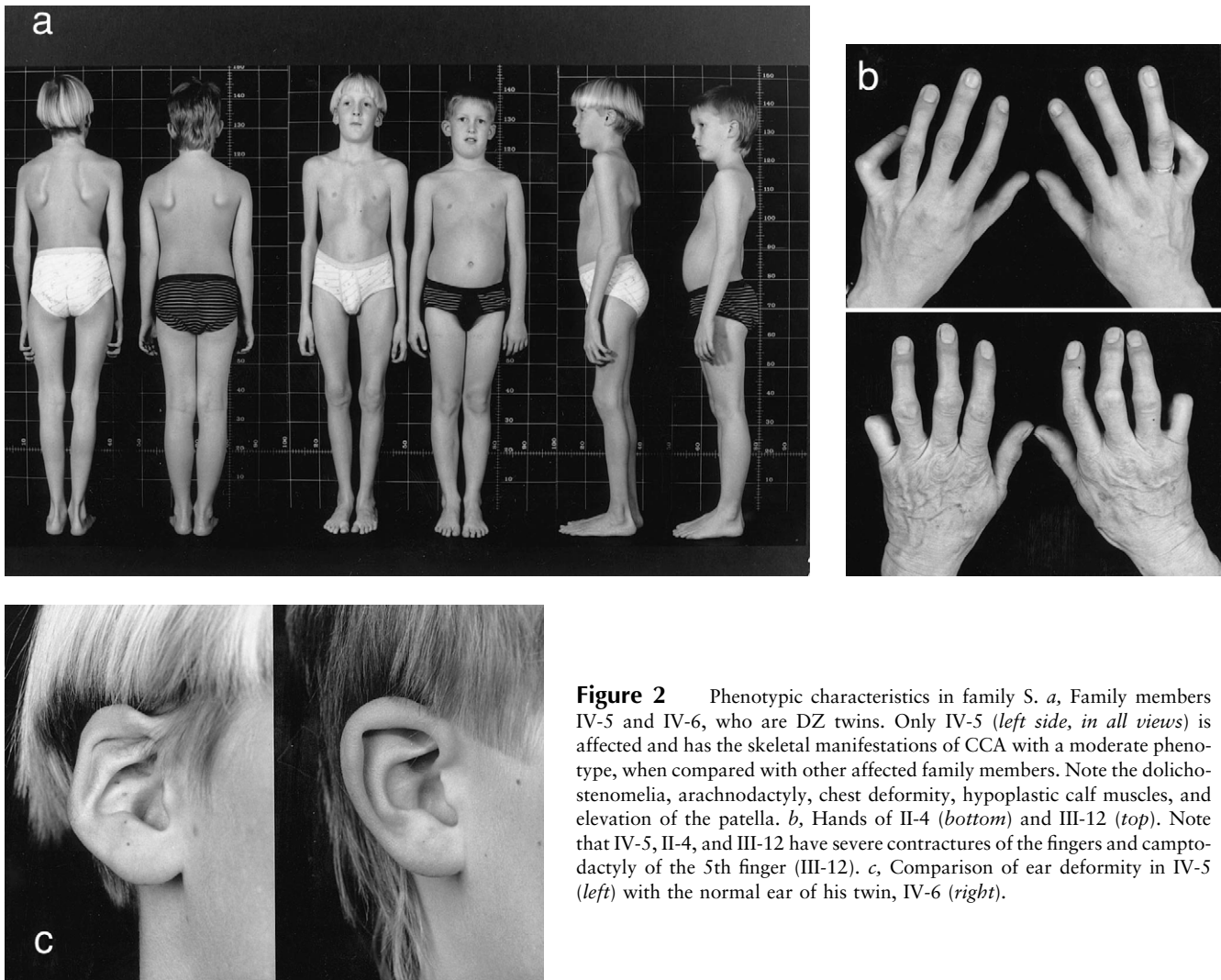


Figure 2 Phenotypic characteristics in family S. *a*, Family members IV-5 and IV-6, who are DZ twins. Only IV-5 (left side, in all views) is affected and has the skeletal manifestations of CCA with a moderate phenotype, when compared with other affected family members. Note the dolichostenomelia, arachnodactyly, chest deformity, hypoplastic calf muscles, and elevation of the patella. *b*, Hands of II-4 (bottom) and III-12 (top). Note that IV-5, II-4, and III-12 have severe contractures of the fingers and camptodactyly of the 5th finger (III-12). *c*, Comparison of ear deformity in IV-5 (left) with the normal ear of his twin, IV-6 (right).

limbs. Members III-6 and III-13 had undergone surgery for habitual patella luxation, and we also observed elevated and hypermobile patellae in IV-5.

The skin of most of the affected family members was noted to be fine and thin, and supragluteal striae were noted in individuals II-6, III-13, and IV-9. No cardiovascular abnormalities were either noted or detected in any of the affected family members. Individuals III-6 and III-9 recently had undergone echocardiography because of suspected Marfan syndrome. No abnormalities were found; in particular, there was no dilatation of the aortic root or of the sinus valsalvae. II-4 had a history of episodes of supraventricular arrhythmia, which did not require therapy; we noted a soft mesosystolic murmur. Regarding the ocular system, there was no history or clinical sign of lens dislocation or retinal detachment. Only two individuals were myopic, one of them (III-4) to a high degree. II-6 had undergone surgery for a cataract, III-3 for keratoconus. Additional features were a hearing deficit since childhood in II-6, varicosis and cox-

arthrosis in II-4, and a cleft soft palate and uvula bifida in III-9.

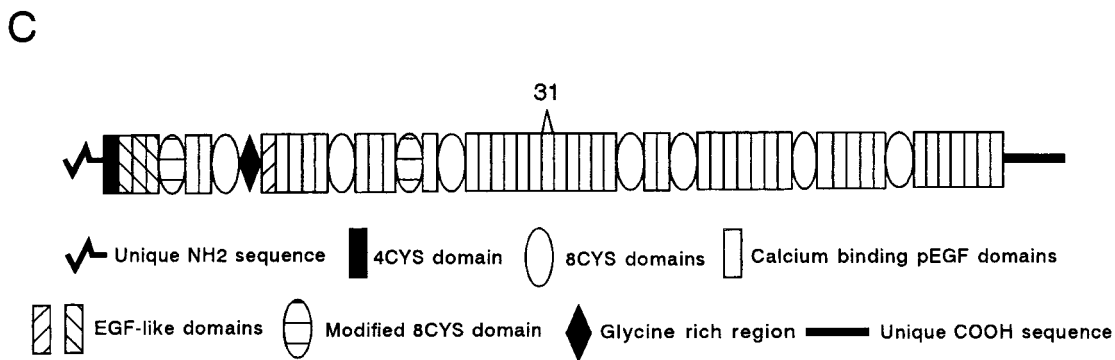
Natural History/Clinical Course of CCA in Family S

The particularly marked finger contractures obviously did not lead to major handicaps in adulthood. The grandmothers in this family worked as tailor (II-4) and waitress (II-6), lifting large and heavy trays. All others in the following generation chose professions regardless of their contractures. However, it should be noted that II-4, III-9, IV-5, and IV-9 had undergone surgery for finger and ankle contractures, without apparent success. Individuals III-3, III-6, III-9, and III-12 have chosen not to have children.

NIRCA and DNA Sequence Analysis

Analysis of cRNA derived from patient IV-8, covering the coding region of FBN2, revealed a single aberrant signal in fragment 3/2, indicating a sequence different from the normal control cRNA. All other fragments had

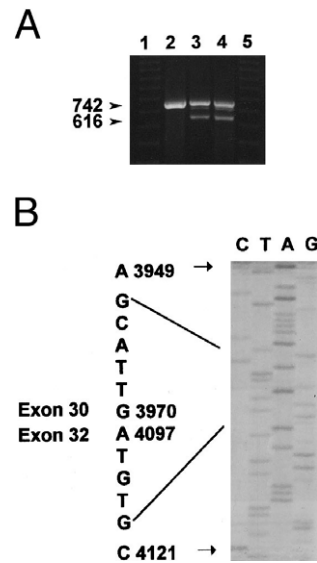
Figure 3 *a*, PCR amplification of cDNA derived from normal control and affected family members IV-8 and IV-9, by use of primer pair 3/2. Lanes 1 and 5, Size markers. Lane 2, Unrelated normal control. Lane 3, Patient IV-8. Lane 4, Patient IV-9. Amplification of the normal control (lane 2) gives the expected single band of 742 bp. Amplification of cDNA from the two patients, IV-8 and IV-9 (lanes 3 and 4, respectively), shows the normal allele of 742 bp and a second band of 616 bp. The size difference is consistent with the absence of exon 31. The difference in the intensity of the two bands suggests that the frequency of exon skipping is <100%. The numbering of the exons used here is based on homology to FBN1 and is tentative. *b*, DNA sequence analysis of the 616-bp PCR fragment representing the mutant transcript. The sequence analysis confirms that exon 31 (126 bp) is skipped in one allele of patient IV-8. Identical results were seen in a similar analysis of cDNA from her affected sibling (patient IV-9). *c*, Diagram representing the domain structure of fibrillin-2. The position of exon 31, which is partially misspliced in this family, is bracketed. The exon skipping removes one complete calcium-binding EGF domain.



normal NIRCA patterns. Closer analysis of fragment 3/2 revealed two prominent bands, at 742 bp, corresponding to the expected fragment size, and at 616 bp, consistent with a missing exon (fig. 3a). The smaller product also was detected in cDNA from patient IV-9. DNA sequence analysis of the aberrant PCR product revealed the skipping of exon 31 in both patients (fig. 3b). The relative intensities of the two bands suggested that the mutant transcript is misspliced <100% of the time. The position of the skipped exon in fibrillin-2 is shown in figure 3c. In addition, a faint third band that migrates just under the 742-bp fragment is present in the affected individuals but does not amplify out of normal cDNA. DNA sequence analysis after careful isolation of that band showed sequence only for the normal allele and for the mutant transcript described above. It is apparent from the sequencing that the third band is a heteroduplex artifact that forms at high concentrations of the PCR products.

Splice-Site Analysis

Splice sites were analyzed by DNA sequence analysis of genomic DNA from patients IV-8 and IV-9, to determine the cause of the missplicing of exon 31. Exhaustive



sequencing demonstrated that there were no sequence differences at the flanking splice sites for the patients compared with normal controls. However, DNA sequence analysis revealed a t→g transversion at position -26 of intron 30 (fig. 4). No other alterations were

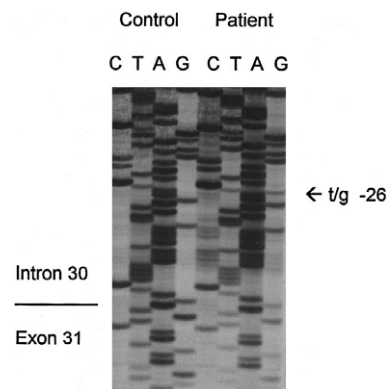


Figure 4 DNA sequence analysis of genomic DNA from an unrelated control individual (left four lanes) and patient IV-8 (right four lanes). The sequence reveals a t→g transversion at position -26 of intron 30 in the patient DNA. The intron-exon boundary between intron 30 and exon 31 is marked with a horizontal line.

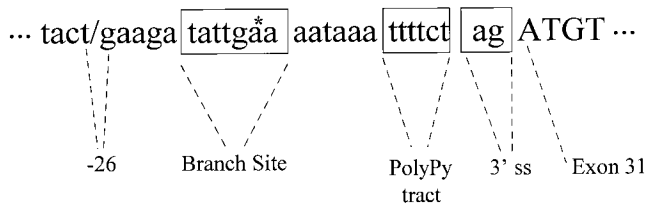


Figure 5 Diagram of the 3' end of intron 30, showing the position of the t-26g mutation, the putative branch site matching the branch-point consensus sequence YNYURAY (where Y = pyrimidine; N = any nucleotide; U = uracil; R = purine; and A = the branch-point adenosine). The proposed branch-point adenosine is marked with an asterisk. PolyPy = polypyrimidine tract; and 3' ss = the 3' splice site at the junction between intron 30 and exon 31.

found after extensive DNA sequence analysis of additional flanking intron and exon sequences extending 5' through exon 27 and 3' through exon 42, making it highly unlikely that the t-26g alteration is a polymorphism cosegregating on the mutant allele.

It was noted that intron 30 is unusual in its composition, since it has only a very short polypyrimidine tract. Analysis of the intron from -1 through -52, for a potential branch point based on the commonly used consensus sequence YNYURAY (Green 1986; Ohshima and Gotoh 1987), shows that the sequence -21 tattgaa -15 is the best match and therefore the most likely branch point, on the basis of conventional criteria (fig. 5).

Blind analysis of genomic DNA from the remaining family members by allele-specific PCR amplification revealed that the mutation was present in all affected family members but not in any of the 16 unaffected individuals (fig. 6). Cosegregation of the mutant allele with the

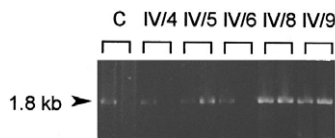


Figure 6 Example of the allele-specific PCR analysis of affected and unaffected family members. The ethidium bromide-stained gel shows the results from the analysis of affected and unaffected individuals from generation IV and of normal control genomic DNAs. For each individual there are two lanes, with PCR products for normal and mutant alleles, respectively. The two lanes are indicated by brackets, with the individual identified above. The normal control lanes are indicated by the "C." Individuals with two wild-type alleles have a band in the left lane but no product for the mutant primer in the right lane. Individuals who are heterozygous for the wild-type and mutant alleles have bands in both lanes. Only the affected individuals (IV-5, IV-8, and IV-9) are positive for the mutant allele in the analysis shown here. All of the living affected family members, along with 16 unaffected family members and 50 unrelated normal controls (not shown), were tested in a blind study using this analysis. The results are shown in the pedigree (see fig. 1). All affected individuals are positive for the mutant allele, whereas none of the unaffected family members or unrelated normal controls have the mutation. This demonstrates the cosegregation of the mutant allele with the CCA phenotype through three generations of this large family.

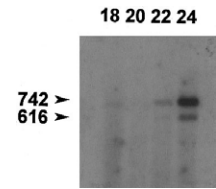


Figure 7 Radioactively labeled PCR amplification of cDNA from IV-8, by use of primer pair 3/2. Four identical samples were amplified, for 18, 20, 22, or 24 cycles; the number of cycles is indicated above the appropriate lane. The autoradiograph shows progressive accumulation of product for both the mutant (616 bp) and normal (742 bp) alleles, with no evidence of any additional bands. The film was analyzed by densitometry, to estimate the relative amounts of each transcript. Analysis of cDNA from patient IV-9 gave comparable results (data not shown).

CCA phenotype was demonstrated through three generations in this family, indicating a causal role in this disorder. In addition, the mutant allele was not detected in any of the 50 unrelated normal controls, and the normal allele was detected in all individuals analyzed (not shown).

Frequency of Exon Skipping

The ratio between the amount of the normal and mutant mRNA species, as determined by coamplification of the corresponding cDNAs in the presence of $^{32}\text{P}\alpha\text{dCTP}$, indicates that the mutant allele is not always misspliced (fig. 7). Estimation of the amount of mutant versus normal transcript produced by cultured skin fibroblasts derived from patients IV-8 and IV-9 indicates that the mutant transcript constitutes ~25% of the total mRNA species. This result was consistent in three separate RNA isolates. The faint third band that, in lanes 3 and 4 of the PCR amplification shown in figure 3a, is not visible in these reactions (after ≤ 24 cycles of amplification) and apparently does not form until later in amplification (i.e., after 30 cycles), when the products are more concentrated.

Discussion

This study demonstrates, for the first time, the cosegregation of a fibrillin-2 mutation with the CCA phenotype. The predominant feature in this family was congenital flexion contractures that tended to improve with age. It is noteworthy that the characteristic external ear anomalies—cabbage leaf-like auricles—vanished with increasing age and were inconspicuous in adults. Dolichostenomelia, camptodactyly, arachnodactyly, and muscle hypoplasia invariably were observed. There is no evidence of cardiovascular abnormalities or ectopia lentis. However, there is considerable intrafamilial variability. The partial skipping of exon 31, with only ~25% mutant transcript produced, is apparently sufficient to cause the CCA phenotype noted in the proband

and her brother. It is possible that the phenotypic variability within this family is due to the degree with which missplicing occurs in a given individual. However, given the two previous reports of mutations associated with CCA (Putnam et al. 1995; Wang et al. 1996), it appears that, like Marfan syndrome, there will be considerable allelic heterogeneity in CCA.

The nature of this particular mutation is especially interesting. Rather than disrupting a splice-site consensus sequence, as is commonly seen with exon skipping, the mutation is in the region of the splicing branch point, which always occurs 18–37 nucleotides upstream from the 3' splice site (Green 1986). Examples of mutations in the vicinity of the branch point resulting in exon skipping are extremely rare (Krawczak et al. 1992) but have been shown to reduce the efficiency of correct splicing when they are expressed *in vivo* as minigenes (Raben et al. 1996; Webb et al. 1996). In addition, this particular intron is peculiar in that it lacks the lengthy polypyrimidine tract that is characteristic of the introns of higher eukaryotes. The region from –3 to –20 is only 50% pyrimidine, as opposed to the >70% that is characteristic of the introns of higher eukaryotes (Ohshima and Gotoh 1987). Likewise, the region from –25 to –30 also is only 50% pyrimidine, whereas >60% would be typical. In fact, the longest uninterrupted stretch of pyrimidines anywhere in the vicinity of the branch point includes only six nucleotides. Furthermore, there is an atypical polyadenosine tract that runs from –9 to –48, with a base composition of 50% adenosine in that region. The aberrant composition of intron 30 could play a role in the exon skipping event, since it has been noted that introns with exceptionally short polypyrimidine tracts are more likely to be misspliced as the result of a mutation near the branch point (Krawczak et al. 1992). Recent evidence indicates that the length of the polypyrimidine tract is an important factor in splice-site selection (Fülöp et al. 1996). Also, although there is only a very weak sequence consensus surrounding branch points in higher eukaryotes, there is evidence that mutations directly upstream from the branch point influence branch-point selection and the efficiency of accurate splicing (Green 1986). In intron 30, the most likely branch point is –21 tattgaa –15, with the mutation immediately upstream at –26 (fig. 5). Although the influence of the immediate upstream sequence on branch-point selection is not known, splice-site choice and splicing efficiency are influenced by intramolecular base pairing of the pre-mRNA (Goguel and Rosbash 1993). Consequently, loss of a pyrimidine adjacent to the branch point could be an important factor here. Finally, cosegregation of the mutation with the CCA phenotype in this large kindred, in conjunction with the absence of any other mutation even quite distal to this region, provides substantial evidence that the mutation at the –26 position of intron 30 is the cause of exon skipping and consequent disorder

in this family. It is therefore likely that this mutation, to some degree, perturbs or destabilizes the natural secondary structure of the transcript, resulting in a partial disruption of branch formation. Missplicing occurs when the next downstream 3' splice site is utilized as an alternative. Although we cannot absolutely rule out a very-low-level use of cryptic splice sites, there is no evidence for activation of a cryptic splice site.

There has been an ongoing effort to establish phenotype-genotype correlations between the variable expression of several aspects of the Marfan syndrome phenotype and FBN1 mutations. These efforts have resulted in the identification of the so-called neonatal region of FBN1, approximately encompassing exons 23–34. Although defects in this region do not result exclusively in the severe neonatal form of Marfan syndrome, there is a clustering of mutations associated with the early, lethal Marfan phenotype. Of particular interest, a deletion in exon 31 resulted in neonatal Marfan syndrome (Nijbroek et al. 1995), and a recurrent missplicing of exon 32 has been seen in two unrelated cases of neonatal Marfan syndrome (Wang et al. 1995). The observation that mutations associated with the extreme in the Marfan phenotypic spectrum tend to cluster has driven speculation that this region of the fibrillin-1 molecule has a critical role in the structure and function of fibrillin-1 (Putnam et al. 1996). Therefore, it is interesting to note that a similar defect in the homologous region of FBN2, the skipping of exon 31, results in a relatively mild phenotype with none of the more severe abnormalities sometimes seen in CCA. In particular, this family does not have any of the cardiovascular manifestations or other unusual malformations occasionally associated with CCA (Anderson et al. 1984; Ramos-Arroyo et al. 1985). Given the large number of affected individuals characterized in this family, this finding would suggest that defects in this region of fibrillin-2 do not result in a predisposition to either cardiovascular defects or other extreme manifestations of CCA. In addition, the two previously reported FBN2 missense mutations also were detected in the exon 23–34 region, with no apparent evidence of congenital heart defect or otherwise severe phenotypic characteristics being noted in either patient (Putnam et al. 1995). However, there has been a recent report of severe lethal CCA caused by missplicing of exon 34 (Wang et al. 1996). In this case, the affected child had complex cardiovascular abnormalities as part of an extreme CCA phenotype. The mother had a mild CCA phenotype but was shown to be mosaic for the genomic mutation. This suggests that there may be some domain-specific functional similarities between fibrillin-1 and fibrillin-2 in the neonatal region—but probably no absolute correlation between the two proteins.

Acknowledgments

We are grateful to the family for their participation and collaboration. In particular, the authors acknowledge the

Swiss Marfan Foundation for bringing this family to their attention. The authors also thank Dr. Robert Glanville for critical reading of the manuscript and for insightful discussion and comments and thank Cynthia Bohan for expert technical assistance. This study was supported by National Institute of Arthritis, Musculoskeletal and Skin Disease grant 5R29AR41846-03 to C.M., Swiss National Science Foundation grant 32-42198.94 to B.S., and Deutsche Forschungsgemeinschaft grants Ra 447/3-1 and Ra 447/3-2 to M.R.

References

- Anderson RA, Koch S, Camerini-Otero RD (1984) Cardiovascular findings in congenital contractural arachnodactyly: report of an affected kindred. *Am J Med Genet* 18:265–271
- Ausubel F, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1995) *Short protocols in molecular biology*, 3d ed. John Wiley & Sons, New York
- Beals RK, Hecht F (1971) Congenital contractural arachnodactyly: a heritable disorder of connective tissue. *J Bone Joint Surg* 53A:987–993
- Bottema CDK, Sarkar G, Cassady JD, Il S, Dutton CM, Sommer SS (1993) Polymerase chain reaction amplification of specific alleles: a general method of detection of mutations, polymorphisms, and haplotypes. *Methods Enzymol* 218:388–402
- Fülöp C, Cs-Szabo G, Glant TT (1996) Species-specific alternative splicing of the epidermal growth factor-like domain 1 of cartilage aggrecan. *Biochem J* 319:935–940
- Goguel V, Rosbash M (1993) Splice site choice and splicing efficiency are positively influenced by pre-mRNA intramolecular base pairing in yeast. *Cell* 72:893–901
- Green MR (1986) Pre-mRNA splicing. *Annu Rev Genet* 20:671–708
- Hecht F, Beals RK (1972) “New” syndrome of congenital contractural arachnodactyly originally described by Marfan in 1896. *Pediatrics* 49:574–579
- Krawczak M, Reiss J, Cooper DN (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet* 90:41–54
- Lee B, Godfrey M, Vitale E, Hori H, Mattei M-G, Sarfarazi M, Tsiouras P, et al (1991) Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature* 352:330–334
- McKusick VA (ed) (1994) *Mendelian inheritance in man*. Johns Hopkins University Press, Baltimore
- Nijbroek G, Sood S, McIntosh I, Francomano CA, Bull E, Pereira L, Ramirez F, et al (1995) Fifteen novel *FBN1* mutations causing Marfan syndrome detected by heteroduplex analysis of genomic amplicons. *Am J Hum Genet* 57:8–21
- Ohshima Y, Gotoh Y (1987) Signals for the selection of a splice site in pre-mRNA—computer analysis of splice junction sequences and like sequences. *J Mol Biol* 195:247–259
- Putnam EA, Cho M, Zinn AB, Towbin JA, Byers PH, Milewicz DM (1996) Delineation of the Marfan phenotype associated with mutations in exons 23–32 of the *FBN1* gene. *Am J Med Genet* 62:233–242
- Putnam EA, Zhang H, Ramirez F, Milewicz DM (1995) Fibrillin-2 (*FBN2*) mutations result in the Marfan-like disorder, congenital contractural arachnodactyly. *Nat Genet* 11:456–458
- Raben N, Nichols RC, Martiniuk F, Plotz PH (1996) A model of mRNA splicing in adult lysosomal storage disease (glycogenosis type II). *Hum Mol Genet* 5:995–1000
- Ramirez F (1996) Fibrillin mutations in Marfan syndrome and related phenotypes. *Curr Opin Genet Dev* 6:309–315
- Ramos-Arroyo MA, Weaver DD, Beals RK (1985) Congenital contractural arachnodactyly: report of four additional families and review of the literature. *Clin Genet* 27:570–581
- Tsiouras P, Del Mastro R, Sarfarazi M, Lee B, Vitale E, Child AH, Godfrey M, et al (1992) Genetic linkage of the Marfan syndrome, ectopia lentis, and congenital contractural arachnodactyly to the fibrillin genes on chromosomes 15 and 5. *N Engl J Med* 326:905–909
- Velinov MT, Raghunath M, Sarfarazi M, Kaitila I, Steinmann B, Tsiouras P (1992) Genetic linkage of congenital contractural arachnodactyly (CCA) to the fibrillin gene on chromosome 5. *Am J Med Genet* 47:159
- Viljoen D (1994) Congenital contractural arachnodactyly (Beals syndrome). *J Med Genet* 31:640–643
- Wang M, Clericuzio CL, Godfrey M (1996) Familial occurrence of typical and severe lethal congenital contractural arachnodactyly caused by missplicing of exon 34 of fibrillin-2. *Am J Hum Genet* 59:1027–1034
- Wang M, Price CE, Han J, Cisler J, Imaizumi K, Vanthienen MN, DePaepe A, et al (1995) Recurrent mis-splicing of fibrillin exon 32 in two patients with neonatal Marfan syndrome. *Hum Mol Genet* 4:607–613
- Webb JC, Patel DD, Shoulders CC, Knight BL, Soutar AK (1996) Genetic variation at a splicing branch point in intron 9 of the low density lipoprotein (LDL)-receptor gene: a rare mutation that disrupts mRNA splicing in a patient with familial hypercholesterolaemia and a common polymorphism. *Hum Mol Genet* 5:1325–1331
- Zhang H, Apfelroth SD, Hu W, Davis EC, Sanguineti C, Bonadio J, Mecham RP, et al (1994) Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *J Cell Biol* 124:855–863