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Differential Allelic Expression of a Fibrillin Gene (FBN1) in Patients with Marfan Syndrome

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Summary

Marfan syndrome is a connective-tissue disorder affecting cardiovascular, skeletal, and ocular systems. The major Marfan locus has been identified as the FBN1 gene on chromosome 15; this codes for the extracellular-matrix protein fibrillin, a 350-kD constituent of the 8–10-nm elastin-associated microfibrils. We identified five MFS patients who were heterozygous for an *Rsa*I restriction-site dimorphism in the 3′ UTR of the FBN1 gene. This expressed variation was used to distinguish the mRNA output from each of the two FBN1 alleles in fibroblast cultures from these five patients. Three of the patients were shown to produce <5% of the normal level of FBN1 transcripts from one of their alleles. This null-allele phenotype was not observed in 10 nonmarfanoid fibroblast cell lines.

Introduction

Marfan syndrome (MFS) is an autosomal dominant disorder of connective tissue. It has pleiotropic symptoms, including dolichostenomelia, pectus deformity, arachnodactyly, scoliosis, high and narrow palate, ectopia lentis, myopia, dilatation of the ascending aorta, aortic dissection, mitral valve abnormality, and dural ectasia (Pyeritz 1993). The fibrillin gene, FBN1, on chromosome 15 has been identified as the mutant locus in the majority of MFS patients (Kainulainen et al. 1990; Dietz et al. 1991*a*, 1991*b*; Lee et al. 1991; Magenis et al. 1991; Maslen et al. 1991; Tsipouras et al. 1992). Fibrillin is a 350-kD protein that is a major constituent of the 8–10-nm microfibrillar fibers that are nearly always closely associated with elastin deposition. At a recent meeting, some two dozen mutations were reported in the fibrillin gene, the majority of which were missense mutations or small deletions (Byers 1993; McInnes and Byers 1993; Sykes 1993). Without exception, all these mutations were first detected as changes in a cDNA copy of the fibrillin mRNA from the mutant allele. As in most laboratories, our initial screening protocol was a combination of reverse transcription (RT) linked to PCR (RT-PCR), for the detection of SSCPs (Orita et al. 1989). It led to the identification of mutations in only 2 individuals from a total of 20 (Hewett et al. 1993, 1994).

Such moderate success at recognizing mutations in MFS patients could be due in part to the assumptions inherent in such a screening program. If a significant proportion of MFS cases were caused by mutant alleles that produce unstable or no fibrillin mRNA, these would not be detected, because the cDNA would only represent mRNA from the normal allele. Heterozygosity for a polymorphism in a transcribed region of the fibrillin gene could provide a marker with which to distinguish the contribution of each allele to the fibrillin mRNA pool. In this paper we use an *Rsa*I RFLP that is in the 3′ UTR and is caused by a T/C dimorphism at nucleotide 8931 to look for differential allelic expression in the 18 patients who proved refractory to our previous screening program.

Patients and Methods

Patient Details

Patient 2615.—Patient 2615 was a 63-year-old male who was 6 feet 4 inches tall, with a low upper-segment: lower-segment ratio of 0.87. He had large ears, striae distensae, and a high palate. He demonstrated moderate kyphoscoliosis and pectus excavatum. He had a moderately dilated aortic root of 4.6 cm diameter, with mild aortic regurgitation and slight mitral valve prolapse. He had severe myopia leading to bilateral retinal detachment. He was totally blind in his right eye.

Patient 2616.—This patient was a 29-year-old male whose mother and maternal grandfather had died of MFS in their 40s. He had pneumothoraces at age 18 years, followed by a pleurectomy. He had both aortic and mitral valve regurgitation. Despite being administered regular beta-blockers, he had a slowly progressing dilatation of the aortic root that was last measured at 5.0 cm. He presented a marfanoid habitus: height of 6 feet 5 inches, marked arachnodactyly, a high-arched palate, striae distensae, and

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hyperextensible joints. He also showed bilateral subluxation of the lenses.

Patient 2618.—This patient was a 27-year-old male who was 6 feet 8 inches tall but had a normal upper-segment: lower-segment ratio. He had skeletal manifestations of MFS, including slight lower thoracic scoliosis, severe pectus excavatum, arachnodactyly, and a high palate. He had simple ears, crowding of the teeth, striae distensae, and a history of inguinal hernia. He had no ocular manifestations of MFS. His aortic root was dilated to 10 cm, with resulting severe aortic regurgitation. The proximal aortic root has now been replaced.

Heterozygosity Testing

DNA was isolated from fresh blood samples by standard methods (Sambrook et al. 1989), and total RNA was extracted from dermal fibroblasts cultured in Dulbecco's minimal essential medium with 5% FCS by using guanidium thiocyanate (Chomczynski and Sacchi 1987). Ten milligrams of total RNA were reverse transcribed using a poly-T primer and a commercial cDNA synthesis kit (Amersham) in a reaction volume of 20 ml. The 3′ UTR was amplified by PCR (Saiki et al. 1988) using the primers UTR-F′ (5′-TCACCATCCAGAAACCAAA-3′) and UTR-R′ (5′-TATTGTGACATTTAT-GACA3′), for genomic DNA, and Q2S (5′-GATGAAAACGAATGCCTCAG-3′) and UTR-R′, for cDNA. The genomic UTR-F′/UTR-R′ PCR product covered residues 8618–9504 (these and all subsequent nucleotides are numbered according to Pereira et al. 1993); the cDNA Q2S/UTR-R′ PCR product spanned residues 7819–9504. Genomic PCRs were performed on 100 ng of template; 1 μl of cDNA was used for each PCR. All reactions were performed under the following conditions: 50-μl volume and 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 20 mM each dNTP, 45 pmol of each primer, and 1 unit of *Taq* polymerase (Cambio). After an initial denaturation at 95°C for 3 min, the PCR consisted of 35 cycles at 51°C for 0.5 min, 72°C for 1 min, and 94°C for 0.5 min. The reactions were carried out in an Autogene cycling water bath (Grant Instruments).

Forty microliters of the PCR products were digested with 10 units of *Rsa*I at 37°C overnight. An equal volume of dye (10% sucrose and 0.1% xylene cyanol) was added to the sample, and it was then loaded onto an 8% nondenaturing polyacrylamide gel (18 cm × 20 cm × 0.2 cm; Protean II; Biorad) before electrophoresis at 300 V for 3 h. The gel was stained with ethidium bromide, and the digestion products visualized under UV light.

PCR amplification was alternatively performed with inclusion of 1 μCi of α^{32}P-dCTP (ICN Flow) in each reaction volume. Radiolabeled PCR products were resolved on polyacrylamide as detailed above. The gel was then vacuum-dried before autoradiography at −70°C for 8–24 h.

Allele-specific Oligonucleotide Hybridization

Q2S/UTR-R′-primed PCR amplification products of patient cDNA, as well as UTR-F′/UTR-R′–primed PCR amplification products of patient genomic DNA (see above), were denatured in NaOH; separate aliquots were then immobilized onto duplicate positively charged nylon filters (Hybond N+; Amersham), according to a standard protocol (Kazazian 1989). The filters were then hybridized with one of two allele-specific oligonucleotides that had been 5′ end–labeled using T4 polynucleotide kinase and γ^{32}P-dATP (ICN Flow). The two oligonucleotides that were specific for the alleles of the T/C dimorphism at nucleotide 8931 of FBN1 were oligonucleotide RSA(−) 5′-CACATTCCCATACGTTTGC-3′ and oligonucleotide RSA(+) 5′-GCAAACGTACGGGAATGTG-3′. The differentiating bases are underlined. Hybridization, washing, and autoradiography were performed as described by Kazazian (1989). The RSA(−)- and RSA(+)-probed filters were washed at 57°C and 63°C, respectively. The hybridization signals were quantitated by excision and counting of the relevant sections of the filters on a flat-bed scintillation counter (Wallac).

Sequencing of Binding Site of Q2S Primer

PCR amplification of genomic DNA across the Q2S binding site was performed using the following two oligonucleotides: INTRON-62-S 5′-CCACACATGCCGCTTCTTAT-3′ and EXON-63-AS 5′-CGCAGAGCCACATTCATTGA-3′; the INTRON-62-S oligonucleotide was biotinylated at its 5′ end. The only exception to the PCR conditions noted above was that the annealing temperature was increased to 53°C. Streptavidin-coated magnetic beads (Dynabeads; DYNAL) were used to prepare a single-stranded template suitable for sequencing by dideoxy chain termination according to the manufacturer's instructions (Sequenase, version 2.0; USB) (Hewett et al. 1994).

Results

The 3′ UTR of the fibrillin gene, FBN1, was PCR amplified, using primers UTR-F′ and UTR-R′, from genomic DNA isolated from 18 individuals with MFS. The 886-bp product of this reaction spans a T/C dimorphism, at nucleotide 8931, that is detectable as an *Rsa*I RFLP (Maslen et al. 1991). A *Rsa*I restriction map of this region is shown in figure 1. *Rsa*I digestion of the amplification product gave either a 232-bp fragment from the (−) allele or a 157- and a 75-bp fragment from the (+) allele. There were five constant fragments of the following sizes: 425, 91, 63, 55, and 21 bp. Restriction of the genomic PCR products showed 5 of the 18 patients to be heterozygous for the dimorphism. The restriction patterns from four of these

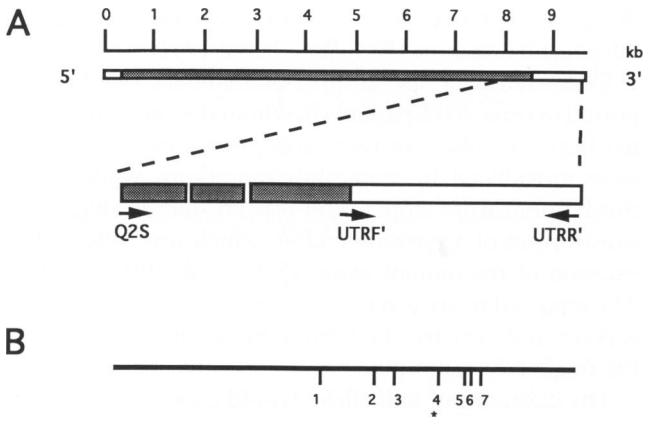

Figure 1 Location of the *Rsa*I RFLP and the primers used to amplify across the site by using PCR. *A*, 9.7 kb of the FBN1 cDNA shown in schematic form (*top*): the shaded box represents the open reading frame, and the open boxes the 5′ and 3′ UTRs. The 3′ end of the cDNA is shown in more detail (*below*). The two gaps represent the positions of the intron/exon boundaries. *B*, Positions of the seven *Rsa*I sites in this region, shown by numbered vertical bars. Site number 4 (*) is the polymorphic site.

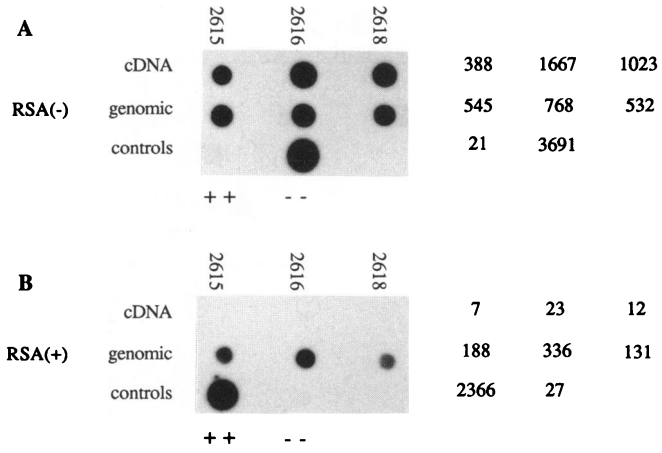

Figure 3 Hybridization of allele-specific oligonucleotides for the *Rsa*I dimorphism in the 3′ UTR of FBN1, to amplification products from genomic and cDNA templates from patients 2615, 2616, and 2618. Genomic DNA templates from both homozygous genotypes were included as controls. Filters were probed with oligonucleotide RSA1(−) (*A*) and oligonucleotide RSA1(+) (*B*). Hybridization signals (in cpm) from cut and counted spots are given in the corresponding position to the right of each photograph.

heterozygotes are shown in lanes 6–9 of figure 2 (products <157 bp are not resolved in this picture).

The primers Q2S and UTR-R′ were used to amplify cDNA from these five individuals. The presence of two intron/exon boundaries between Q2S and UTR-R′ prevents any contaminating genomic DNA from being coamplified (fig. 1). Digestion of this cDNA amplification product gives the same dimorphic fragments as are given by genomic DNA (232 bp for the (−) allele; 157 and 75 bp for the (+) allele). The sizes of the constant fragments were 581, 425, 219, 63, 55, and 21 bp. The digestion products from four of the heterozygotes are shown in figure 2. There was no discernable product from the (+) allele in three of these patients, as indicated by the absence of the 157-bp fragment in lanes 3–5. Patients 2615, 2616, and 2618 are not producing detectable mRNA from one of their FBN1 alleles. In contrast, patient 2705 (lane 2) showed mRNA output from both alleles. Such differential allelic output was not observed in any of 10 nonmarfanoid fibroblast cell lines that were shown to be heterozygous for the 3′ UTR dimorphism (data not shown).

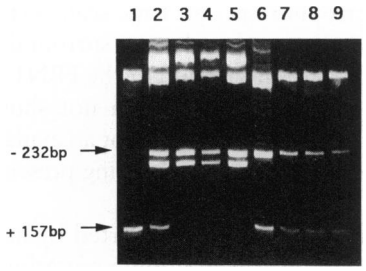

Figure 2 *Rsa*I digestion patterns of UTR PCR products. Lanes 6, 7, 8, and 9, UTRR′/UTRF′-primed genomic amplification products from patients 2705, 2615, 2616, and 2618, respectively. Lanes 2, 3, 4, and 5, Q2S/UTRR′-primed cDNA amplification products from patients 2705, 2615, 2616, and 2618, respectively. The polymorphic fragments are indicated by arrows. A genomic PCR product from a homozygous (++) individual was run in lane 1 to provide a positive control for the *Rsa*I digestion.

When hybridization of radioactively labeled allele-specific oligonucleotides was used to quantitate the reduction in the amount of the fibrillin mRNA transcribed from the *Rsa*I(+) allele (fig. 3), it produced levels of 4.1%, 2.0%, and 3.2%, relative to the level of mRNA detectable from the *Rsa*I(−) allele, in patients 2615, 2616, and 2618, respectively. This was at the limits of the sensitivity of the technique.

There remained the remote possibility that the failure to detect normal levels of *Rsa*I(+) allele transcripts in these patients was due to a polymorphism at the Q2S binding site, in linkage disequilibrium with the diagnostic *Rsa*I dimorphism, which abolished primer binding to this allele. To check this point, genomic DNA from the three individuals showing differential allelic expression was amplified using the primers INTRON 62-S and EXON 63-AS. Sequencing of these PCR products revealed no variation across the Q2S binding site.

Patient 2618 was born to unaffected parents. His mother was screened for the FBN1 3′ UTR *Rsa*I polymorphism and was found to be a (−−) homozygote. No sample was available from the father. Patients 2615 and 2616

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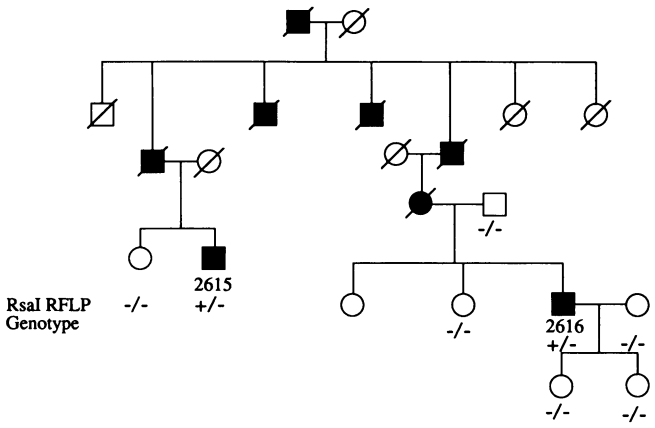

Figure 4 Pedigree of patients 2615 and 2616. The FBN1 3′ UTR *Rsa*I RFLP genotypes for each individual are shown. Individuals 2615 and 2616 are labeled.

both had one affected parent who had died from aortic complications of MFS. They were seen at clinics in London and Oxford, respectively. Originally, both were thought to be the only surviving affected members of their respective pedigrees, but additional information showed them to be first cousins once removed (fig. 4). Genotyping of the pedigree showed that the MFS phenotype cosegregated with the *Rsa*I(+) allele.

Discussion

We have used an *Rsa*I dimorphism in the 3′ UTR of the FBN1 gene to differentiate between the fibroblast mRNA output of each allele in heterozygotes. In three of five MFS heterozygotes tested, the output from one allele was depressed below our detection threshold of 5%. Cells from 10 heterozygous individuals without MFS showed a balanced output from both alleles. Subsequent pedigree investigation showed that two MFS patients showing this abnormal cellular phenotype are first cousins once removed and therefore are not independent occurrences. However, this shows that the cellular phenotype is segregating with and affecting the output of the mutant fibrillin allele in this pedigree, which, in turn, is evidence in favor of a causal connection between the phenomenon and the disease phenotype.

Reduction in the abundance of mutant-allele mRNA and, ultimately, of its protein product, has been observed in many inherited disorders. In osteogenesis imperfecta (OI), reduced output from the mutant COL1A1 allele, detected by an expressed *Mnl*1 dimorphism, is a frequent cause of the mild, Sillence type 1, form of the disease (Willing et al. 1992), although the general mutational mechanism is not yet understood. Among the globin disorders, reductions in stable β-globin mRNA has been shown to be due both to premature termination and to splicing defects (Orkin and Kazazian 1984; Weatherall 1991).

Depressed allele-specific mRNA abundance has been reported in three MFS patients in whom the causal mutation has been identified. In two cases premature stop codons were introduced by frameshift mutations, while in the third a premature stop codon was created by single-base substitution of a tyrosine codon, which was followed by excision of the mutant exon (Dietz et al. 1993*a*, 1993*b*). The reported relative abundance of the mutant transcripts was 6% and 16% for the frameshift mutants and 25% for the single-base mutation.

The existence of null alleles would explain some earlier results on the biochemistry of fibrillin production in cultured dermal fibroblasts taken from patients with MFS. One-third of these samples synthesized half as much fibrillin as did samples from control individuals, but there was no difference either in the rate its secretion from the cell or in its rate of incorporation into the extracellular matrix (Milewicz et al. 1992). The patients exhibiting this biochemical profile had a classical features of MFS.

One issue raised is whether the null-allele phenotype described in this paper is due to a reduction in the output of normal gene products, as is the case in the thalassemias, or whether a structural mutation has resulted in an abnormal gene product whose mRNA is unstable, or indeed whether both mechanisms could give rise to the null phenotype. It is well known that even 10% of abnormal collagen chains, when introduced into otherwise normal molecules and fibrils, can sufficiently disrupt their organization, to cause a severe clinical phenotype in experimental mice (Stacey et al. 1988), and coculture experiments with normal and MFS fibroblasts have shown that 10% mutant/normal mixtures disrupt microfibril structure (Raghunath et al. 1993). The patients in the present study had classical symptoms and were not particularly mildly affected, which might argue in favor of a low-level output of very disruptive mutant fibrillin molecules. There was no evidence, from pulsed-field gel electrophoresis, of large-scale gene rearrangements or mutations immediately surrounding the proposed start site of transcription of the FBN1 gene in these patients (Pereira et al. 1993) (data not shown). Clearly, complete deletion of the locus is not an explanation, since the test depends on both alleles being present in genomic DNA.

At a recent meeting it was reported by many independent research groups that a fibrillin mutation was discovered in only 1 in every 10 MFS patients screened (Byers 1993). The frequent occurrence of the null phenotype may explain the relatively low success rate of current mutation-detection strategies that test cDNA copies of fibrillin mRNA from cultured skin fibroblasts. Clearly, in patients with the null phenotype such techniques would be testing a cDNA population in which all or almost all copies of the

mRNA came from the normal allele. Our finding that two of four unrelated, testable MFS patients demonstrate the null phenotype suggests that the phenomenon may be widespread; but its true extent will only become clear with further work. Only 5 of 18 MFS patients were testable heterozygotes for the expressed *Rsa*I dimorphism; and the discovery and use of other expressed variants to distinguish allelic outputs would clearly be useful.

The existence of the null phenotype is of particular importance to laboratories wishing to set up their own service for prenatal or presymptomatic MFS diagnosis based on detection of abnormalities in the FBN1 gene. First, as explained above, if it is not first determined that both alleles are being expressed in a patient, it would be unwise to base a mutation-screening program exclusively on cDNA. Second, detection of the null-allele phenotype could itself sufficiently define the mutant allele, without recourse to further analysis. Once defined using the fibroblasts from one heterozygous patient in a pedigree, the mutant allele could be easily detected in other family members, even *Rsa*I homozygotes, by using genomic DNA alone. This would naturally depend on informative genotypes; and it may be necessary to establish phase with other more informative markers.

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