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Inheritance of an RNA Splicing Mutation (G^{+1} ^{IVS20}) in the Type III Procollagen Gene (COL3A1) in a Family Having Aortic Aneurysms and Easy Bruisability: Phenotypic Overlap between Familial Arterial Aneurysms and Ehlers-Danlos Syndrome Type IV

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Summary

Inheritance of a single base mutation in the type III procollagen gene (COL3A1) was studied in a family with aortic aneurysms and easy bruisability. The mutation was a substitution of A for G^{+1} of intron 20 of the gene and caused aberrant splicing of RNA transcribed from the mutated allele. The phenotype in the family included aortic aneurysms that ruptured and produced death. It also included easy bruisability, but it did not include other characteristic features of Ehlers-Danlos syndrome type IV, such as ecchymoses, abnormal scarring, or prominent subcutaneous blood vessels. The data from the family, together with a review of other probands with mutations in the type III procollagen gene, indicated that there is phenotypic overlap between Ehlers-Danlos syndrome type IV and familial arterial aneurysms not associated with any of the striking changes in skin originally cited as a characteristic feature of Ehlers-Danlos syndrome type IV. In addition, the results suggested that DNA tests for mutations in the type III procollagen gene may be useful to identify individuals predisposed to developing arterial aneurysms.

Introduction

The Ehlers-Danlos syndrome (EDS) includes a heterogenous group of diseases characterized primarily by looseness of joints and variable involvement of skin and other connective tissues (Beighton 1970; McKusick 1972; Byers 1983; Uitto et al. 1986; Shamban and Uitto 1989). The type IV variant of EDS is the severest form of the disease and may produce life-threatening consequences such as rupture of arteries or hollow organs (Beighton 1970; McKusick 1972; Byers et al. 1982; Uitto et al. 1982, 1986; Byers 1983; Shamban and Uitto 1989). As originally defined by Beighton (1970), a cardinal feature of EDS type IV is that extensive ecchymoses are usually present and that bony prominences are covered with characteristically thick and darkly pigmented scars. The skin is not hyperelastic as it is in many other forms of EDS. Instead, it feels velvety and is so thin that subcutaneous vessels are visible. Also, the skin may be fragile. In addition, the joint hypermobility seen in other forms of EDS is usually limited to the digits.

Studies in cultured fibroblasts indicated that a series of probands with EDS type IV had mutations that altered either the rate of synthesis of type III procollagen or the structure of the protein (Pope et al. 1975; Byers et al. 1981; Stolle et al. 1985). Only a few mutations in the gene (COL3A1), however, have been defined. They appear similar to the mutations in the two genes for type I procollagen (COL1A1 and COL1A2) found in many probands with osteogenesis imperfecta (OI) (Prockop et al. 1989). Two mutations were single base

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substitutions that replaced codons for glycine with codons for bulkier amino acids (Tromp et al. 1989*a*, 1989*b*). In addition, Southern blot analyses of two probands with EDS type IV suggested large deletions of 3.3 and 7.5 kb in the gene (Superti-Furga et al. 1988, 1989).

Here we describe a family with a single base substitution of G^{+1} in intron 20 of the type III procollagen gene, a substitution that caused aberrant splicing of RNA transcribed from the mutated allele. The phenotype in the family included aortic aneurysms and easy bruisability but did not include other characteristic features of EDS type IV, such as ecchymoses, abnormal scarring, or prominent subcutaneous blood vessels.

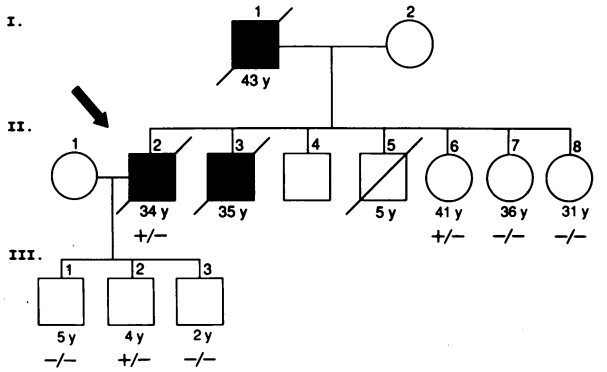

Figure 1 Proband's family. The arrow indicates the proband (II-2). A black square (■) indicates death from ruptured aortic aneurysms. One of the proband's brothers died at 5 years of age from meningitis. Plus signs (+) and minus signs (−) indicate, respectively, mutated and normal alleles for type III procollagen.

Subjects and Methods

The Proband and His Family (Fig. 1)

The proband (II-2) was a male who died of a massive intrathoracic and intraabdominal hemorrhage at the age of 34 years. He was admitted to an emergency ward because of acute onset of back and abdominal pain that radiated to the groin. He had thin skin but no ecchymoses or abnormal scarring. He had a well-healed 5-cm scar on his neck. He was in shock and underwent emergency surgery. He had massive left retroperitoneal hemorrhage without any obvious bleeding point. He continued to bleed postoperatively and died within 24 h. An autopsy revealed massive mediastinal, intraabdominal, and retroperitoneal hemorrhage. No distinct aneurysm or bleeding point was detected, but microscopic sections of aorta revealed an apparent decrease and disorganization of elastic fibers. All the abdominal soft tissues appeared to be unusually friable. The patient had a previous history of easy bruisability. On one occasion he developed a large hematoma from throwing a baseball. He had a history of nosebleeds and of bleeding from gums after brushing his teeth. He had no history of joint dislocations, and his wife stated he did not have loose joints or prominent cutaneous vasculature.

The proband's father (I-1) died at the age of 43 years of a ruptured aneurysm of the abdominal aorta. One of his brothers (II-3) died at the age of 35 years of a ruptured aneurysm of the proximal descending thoracic aorta. Another brother (II-5) died at the age of 5 years of meningitis. A third brother (II-4) was alive but did not want any genetic analysis or counseling. The proband's three sisters were all alive at 41 (II-6), 36 (II-7), and 31 (II-8) years of age. The eldest sister (II-6) had easy bruisability but no other signs of connective-tissue disorder. The other two sisters (II-7 and II-8) were asymptomatic. The proband also had three sons, who were 5 (III-1), 4 (III-2), and 2 (III-3) years of age. The proband's wife reported that the 4-year-old son (III-2) was similar to his father in that he had slightly thin skin and bruised easily. Otherwise the children were asymptomatic.

PCR with Templates of cDNA and Genomic DNA

To prepare a cDNA template for the polymerase chain reaction (PCR) (Saiki et al. 1985), total RNA was extracted from cultured skin fibroblasts with guanidinium isothiocyanate (Maniatis et al. 1982). The RNA was purified by centrifugation on a cesium chloride gradient and was used to synthesize double-stranded cDNA, according to a method described by Gubler and Hoffman (1983), with a commercial kit (Bethesda Research Laboratories).

In initial experiments using cDNA as template for the PCR (Saiki et al. 1985), four sets of oligonucleotides based on the cDNA sequence (Ala-Kokko et al. 1989; Tromp et al. 1989*b*) were used as primers so as to generate overlapping products that included all the coding sequences of the α chain domain (fig. 2). In subsequent experiments, an additional set of primers were used that provided a PCR product that extended from the codon for amino acid 270 of the triple-helical domain of the α1(III) chain to the codon for amino acid 892 (product 5 in fig. 2). The primers were 30-mers in which 20 nt were complementary to the cDNA and

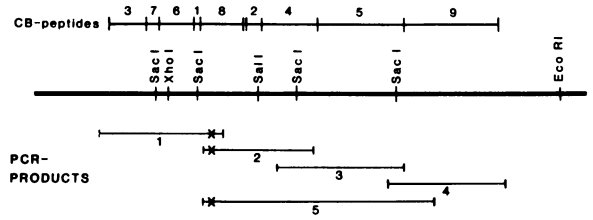

Figure 2 Schematic representation of the five PCR products prepared and analyzed here. The top line indicates the cyanogen bromide peptides (CB-peptides) of the α1(III) chain. The second line is a partial restriction map of the cDNA. X = Site of the intron insertions caused by the G^{+1} mutation in intron 20.

in which 10 nt contained sequences of restriction sites convenient for cloning (Tromp et al. 1989*b*).

For nucleotide sequencing, the PCR products were cloned into the filamentous bacteriophage M13mp18 or M13mp19, and the clones were sequenced with the dideoxynucleotide method (Sanger et al. 1977) and with recombinant T7 DNA polymerase under the conditions recommended by the manufacturer (U.S. Biochemicals). The ends of the inserts were sequenced by using the M13 universal primer. To obtain complete sequences of clones of product 5 (fig. 2), several internal primers were also employed (Tromp et al. 1989*a*).

To isolate genomic DNA templates for the PCR, DNA was extracted from 175-cm^2 flasks of cultured skin fibroblasts (Maniatis et al. 1982) or from 3–5 ml of human blood (Bell et al. 1981) anticoagulated with EDTA.

To use the genomic DNA as template for the PCR, three oligonucleotide primers were employed. The first was identical to nucleotide sequences in the middle of exon 19; the second was identical to nucleotide sequences in the 5′ end of exon 20; and the third was complementary to sequences at the 3′ end of exon 21 (Ala-Kokko et al. 1989; Tromp et al. 1989*a*). The products were cloned into M13 and were sequenced (Sanger et al. 1977).

Assays of Pepsin-resistant Type III Procollagen in the Medium of Cultured Fibroblasts

Cultures of skin fibroblasts from the proband were incubated with [^{3}H]proline for 24 h (Stolle et al. 1985). The medium proteins were precipitated with ammonium sulfate, and they were dissolved in 0.4 M NaCl and 0.01% NaN_3 in 0.1 M Tris-HCl buffer (pH 7.4). The sample was acidified by adding an equal volume of 0.5 M acetic acid, and the pH was adjusted to between 2 and 3 with dilute HCl. Pepsin (Boehringer-Mannheim) was added to a final concentration of 0.1 mg/ml, and the sample was digested at 4°C overnight. The sample was then assayed by PAGE in SDS without reduction, followed by fluorography.

Analysis of Family Members for the Mutation

To determine the presence or absence of the mutation in members of the family, the PCR was performed on genomic DNA isolated from blood. The PCR products were analyzed either by gel electrophoresis with and without prior digestion by *Bst*UI or by slot-blot hybridization (Studencki and Wallace 1984). For the slot-blot analysis, 20 μl of amplified sample and 500 μl of deionized water were mixed, and fourfold serial dilutions were made. The samples were denatured in boiling water for 10 min and rapidly cooled on ice. A sample of 125 μl was mixed with an equal volume of 20 × SSC solution (1 × SSC = 0.15 M NaCl in 0.015 M sodium citrate, pH 7.0). Duplicate filters were prepared by loading 100 μl of each sample into each of two parallel slots of a slot-blot apparatus (Schleicher and Schuell) containing a nitrocellulose filter that was equilibrated with 10 × SSC. After the liquid was slowly sucked through the filter with a weak vacuum, each well was washed twice with 200 μl of 10 × SSC. Filters were air-dried, baked for 2 h in a vacuum oven at 80°C, and prehybridized overnight at 42°C in 10 ml of 6 × SSC, 5 × Denhardt's solution, 200 μg sheared salmon sperm DNA/ml, 100 μg yeast tRNA/ml, and 0.5% Sarkosyl. Each of the duplicate filters was hybridized with γ-^{32}P-ATP-labeled 19-mer oligonucleotides corresponding to either the normal or the mutant allele. The filters were washed first with 2 × SSC for 30 min at room temperature and then with 5 × SSC for 10 min at either 57°C (for the normal oligonucleotide) or 55°C (for the mutant oligonucleotide). The calculated theoretical melting-temperature values (Meinkoth and Wahl 1984) were 52°C and 50°C, respectively.

All the PCR experiments with the DNA isolated from blood from the different family members were repeated twice using two or three different DNA isolations and three different PCR reactions. Water blanks were consistently negative.

Results

Analysis of cDNA Clones

Total RNA from the proband's (II-2 in fig. 1) fibroblasts was isolated and used to synthesize cDNA. The cDNA was then used as a template for PCRs. Sequenc-

ing of 156 clones of the PCR product 1 (fig. 2) indicated that three of them had an insertion of all 132 nt from intron 20, that 60 had an insertion of the first 24 nt of intron 20, that two lacked all the codons of exon 20, and that 91 had a normal sequence (fig. 3). All the clones with the 24-nt or 132-nt insertions contained a single base mutation that converted the first G of intron 20 to an A. As a result, the consensus sequence of –GT– found in most of the introns of eukaryotic genes (Shapiro and Senapathy 1987; Jacob and Gallinaro 1989) was converted to –AT–.

Parallel experiments were then carried out using genomic DNA from the proband as template for PCRs. Analysis of nine M13 clones indicated that seven had the G-to-A mutation in the first nucleotide of intron 20, whereas the remaining two clones had a normal sequence in the same region (fig. 4).

Experiments to Rule Out a Second Mutation in the Two Alleles for Type III Procollagen

Further experiments were carried out to establish that the mutation of G^{+1} to A in intron 20 was the only mutation in the two alleles for type III procollagen from the proband.

PCR products 1 and 5 in figure 2 were prepared and cloned into M13. The presence of the G^{+1}-to-A mutation was used to identify clones from the two alleles. At least two clones from each allele of PCR product 1 and at least two clones of each allele of PCR product 5 were entirely sequenced. The codons sequenced were for 15 amino acids of the N-telopeptide and for amino acids 1–892 of the α1(III) chain. They corresponded to nucleotides 468–3176 of the cDNA (Chu et al. 1985; Ala-Kokko et al. 1989). A total of over 12,000 nucleotide sequences were examined. The data revealed only 12 single base differences when compared with the previously published nucleotide sequence of the cDNA for the proα1(III) chain (Ala-Kokko et al. 1989).

Three of the 12 single base changes were found in all clones from one or both alleles. All three involved the third base of codons and did not change the amino acid encoded. One of the single base changes was at nucleotide position 912 (amino acid 137). It converted the codon of –GCT– for alanine to –GCC–, another codon for alanine. It was found in two clones from the mutant allele but not in two clones from the normal allele. The second single base change was at nucleotide position 1851 (amino acid 450), and it converted the codon of –CAA– for glutamine to –CAG, another codon for glutamine. The third single base change was at nucleotide position 2244 (amino acid 581). It converted

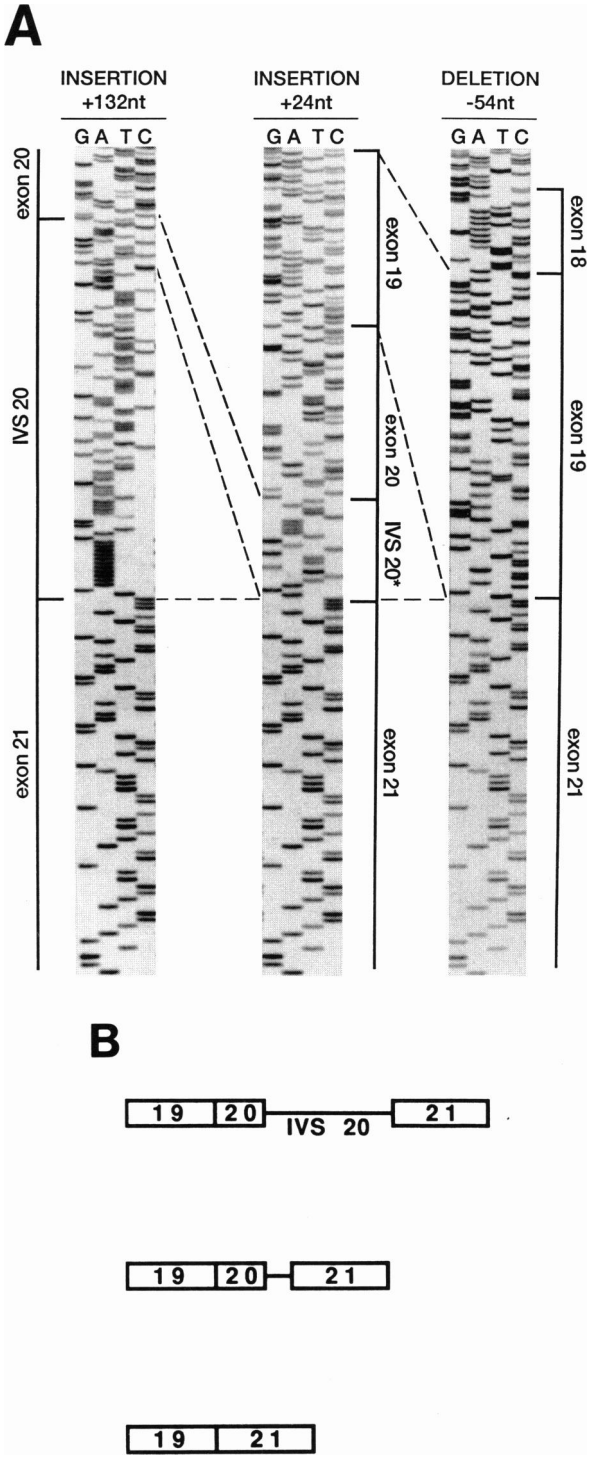

Figure 3 *A*, DNA sequences of three clones prepared from PCR products synthesized with cDNA as a template. The sequences indicate insertions of 132 nt or 24 nt from intron 20 and a deletion of the 54 nt found in exon 20. The normal sequences of exons 19–21 are shown in the middle panel. *B*, Schematic representation of the exon and intron nucleotides found in the cDNA clones.

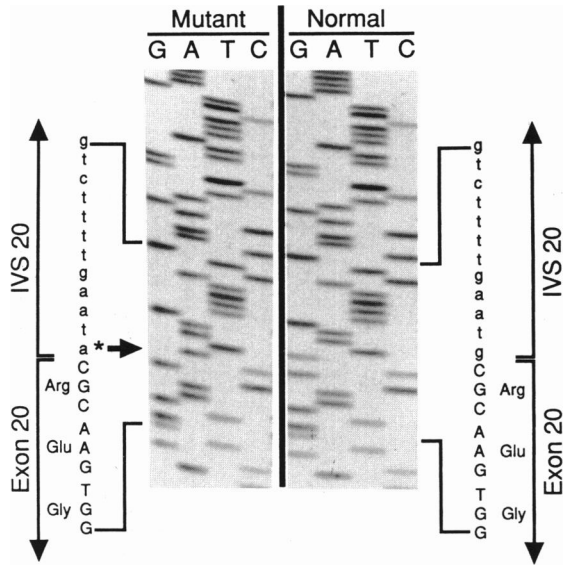

Figure 4 DNA sequence of clones prepared from PCR products synthesized with genomic DNA as template. The arrow-and-asterisk (*→) combination indicates the single G-to-A mutation of the first nucleotide in intron 20.

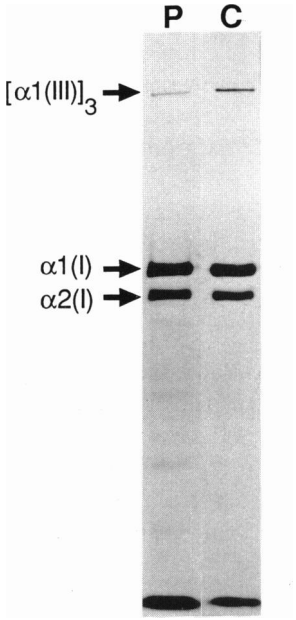

Figure 5 Pepsin-resistant type III procollagen in the medium from the proband's fibroblasts and from control fibroblasts. Fibroblasts were incubated with [^{3}H]proline for 24 h, and medium proteins were digested with pepsin as described in text. The samples were used for PAGE in SDS without reduction and then for fluorography. P = Proband; C = control; [α1(III)]$_3$ = disulfide-linked trimers of α1(III) chains of type III collagen; α1(I) and α2(I) = α chains of type I collagen.

the codon of –GGC– for glycine to a codon of –GGT, another codon for glycine. The single base changes at positions 1851 and 2244 were found in all clones from both alleles.

The other nine single base changes were shown to be artifacts probably generated by the PCR (Tromp et al. 1989*b*). Each of these nine single base differences from the consensus sequence was found in only one clone from either the mutated or the normal allele, but it was not found in one or two other clones from the same allele.

Decrease in Pepsin-resistant Type III Procollagen in Medium from the Proband's Fibroblasts

The proband's fibroblasts were incubated with [^{3}H]proline for 24 h, and the medium proteins were examined by PAGE in SDS. The medium proteins were digested with pepsin, and the pepsin-resistant type III collagen was identified as the slowly migrating band of disulfide-linked α1(III) chains (fig. 5). The results indicated that there was a relative decrease in the amount of pepsin-resistant type III collagen in the medium. Therefore, the results were similar to those obtained with fibroblasts from several other probands having mutations that change either the rate of synthesis or the structure of type III procollagen (Byers 1983; Stolle et al. 1985; Tromp et al. 1989*b*).

Analysis of DNA from the Proband's Family for the Presence of the Mutation

The G^{+1}-to-A mutation in intron 20 destroyed a restriction site for cleavage by *Bst*UI. Therefore, members of the proband's family were assayed for the presence of the mutation by preparing PCR products with genomic DNA as template and by analyzing the products by gel electrophoresis with and without prior cleavage by *Bst*UI. As indicated in figure 6, the test confirmed the presence of the mutation in the proband's (II-2) genomic DNA. The results also indicated that the proband's 41-year-old sister (II-6) and 4-year-old son (III-2) were also heterozygous for the same mutation.

To confirm the observations, the PCR products were also used for allele-specific hybridization (Studencki and Wallace 1984) with a normal oligonucleotide and with an oligonucleotide containing the G^{+1}-to-A mutation. As indicated in figure 7, the results again confirmed the presence of the mutation in the proband's (II–2) DNA. The results also confirmed the presence of the mutation in the proband's eldest sister (II-6) and 4-year-old son (III–2).

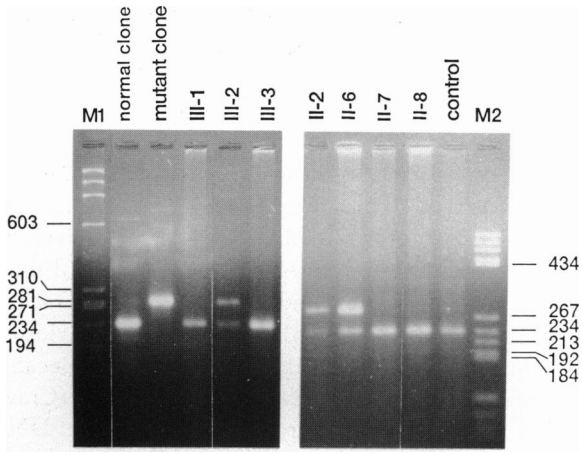

Figure 6 Analysis of genomic DNA from the proband and his family for the presence of the mutation. Genomic DNA and M13 clones were used as template for PCRs, and the PCR products were analyzed by agarose gel electrophoresis after digestion with *Bst*UI. As indicated in the text, the G-to-A mutation of the first nucleotide of intron 20 destroyed a *Bst*UI site. The mutation is present in the proband's 4-year-old son (III-2), in the proband (II-2), and in his eldest sister (II-6).

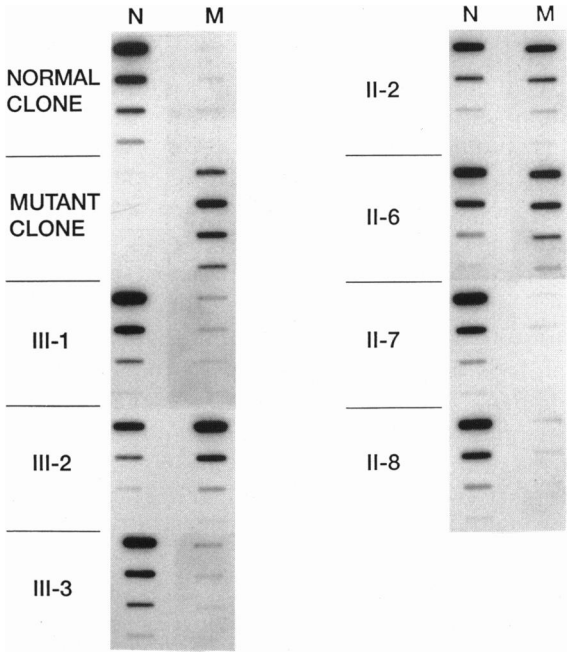

Figure 7 Allele-specific oligonucleotide hybridization of PCR products synthesized using genomic DNA or M13 clones as template. DNA from the proband (II-2), from his eldest sister (II-6), and from his 4-year-old son (III-2) are positive for the presence of the mutation. Other members of the family were negative. N = normal oligonucleotide used as a probe; M = mutant oligonucleotide used as a probe.

Discussion

The results here are the first full-length report of a mutation in the type III procollagen gene that causes aberrant RNA splicing. The proband had, in one allele for type III procollagen, a mutation that converted the first G in intron 20 to an A. The mutation caused aberrant splicing of about 50% of all the mRNA for proα1(III) chains from the proband's fibroblasts and apparently all of the RNA transcribed from the affected allele (Kuivaniemi et al., in press). The pattern of aberrant splicing was, therefore, consistent with the pattern seen with similar G^{+1}-to-A mutations in other genes (see Mitchell et al. 1986; Grandchamp et al. 1989).

The proband died of a massive thoracic and abdominal hemorrhage that was probably secondary to a ruptured artery. Both his father and one of his brothers died of ruptured aortic aneurysms. The mutation of the type III procollagen gene was dominantly inherited in the proband's family, in that the mutation was present in the proband's 41-year-old sister and in one of his three children. The 41-year-old sister and the 4-year-old son were similar to the proband in that they had easy bruisability. We have advised both these individuals that they should be followed closely for the possible development of aortic aneurysms. Surgical reports indicate that the mortality from aortic aneurysms that have ruptured is about 90%, whereas the mortality from elective surgery for aortic aneurysms is less than 10% (Crawford et al. 1981; Rutherford and Pearce 1988).

Ruptured arterial aneurysms are a prominent feature of the type IV or ecchymotic form of EDS (Beighton 1970; McKusick 1972), and mutations in the type III procollagen gene have been reported in eight probands initially classified as having EDS type IV (Pyeritz et al. 1984; Stolle et al. 1985; Superti-Furga et al. 1988; Tromp et al. 1989*a*, 1989*b*; Kontusaari et al. 1990). The proband and affected members of the family described here had slightly thin skin and easy bruisability but did not have such features as (*a*) fragile skin with prominent venous network, (*b*) extensive ecchymoses, or (*c*) thin, darkly pigmented scars over bony prominences—features that were originally cited by Beighton (1970) as being characteristic features of EDS type IV. Several authorities have continued to emphasize such striking cutaneous changes as being hallmarks of EDS type IV (McKusick 1972; Uitto et al. 1982; Shamban and Uitto 1989). In contrast, some have suggested the same diagnosis for probands having aortic

aneurysms and easy bruisability without any dramatic changes in the skin (Byers et al. 1982; Byers 1983). However, familial aortic aneurysms, predominantly of the abdominal aorta, have been reported as a distinct clinical syndrome (Collin 1985; Bergqvist and Bengtsson 1986; Darling et al. 1989). Therefore, there apparently is phenotypic overlap between EDS type IV and familial aortic aneurysms.

Clinical data were reported on seven of the eight probands having mutations in the type III procollagen gene who were initially classified as EDS type IV (Pyeritz et al. 1984; Stolle et al. 1985; Superti-Furga et al. 1988; Tromp et al. 1989*a*, 1989*b*; Kontusaari et al., in press). With one exception, the probands or one of their first-degree relatives had arterial aneurysms (table 1). Six of the seven probands, however, did not have the characteristic skin changes of EDS type IV that have been cited by Beighton (1970) and others (McKusick 1972; Uitto et al. 1982; Shamban and Uitto 1989). Most of the probands developed hematomas from minor trauma. The aneurysms found in the seven individuals or members of the families (table 1) were widely distributed among the thoracic aorta, abdominal aorta, and other arteries, including the subclavian, cervical, hepatic, and celiac arteries. Therefore, there are no ready criteria with which to distinguish these individuals, who were classified as having EDS type IV, from individuals having familial aneurysms (Collin 1985; Bergqvist and Bengtsson 1986; Darling et al. 1989).

These considerations raise the possibility that mutations in the type III procollagen gene may well account for a considerable fraction of the 1%–2% of adult males who die of ruptured aneurysms of the abdominal aorta (Collin 1985; Bergqvist and Bengtsson 1986; Darling et al. 1989)—and even of the 8% or so of individuals who die of intracranial aneurysms (Weir 1987). Because early surgical intervention is frequently effective (Crawford et al. 1981; Rutherford and Pearce 1988), DNA tests for mutations in the type III procollagen gene may be of considerable value in identifying people at risk for developing arterial aneurysms. Many different mutations in the gene may be present in different families, and the initial definition of a single base mutation such as the one defined here requires considerable effort with current technologies. However, procedures for defining single base mutations are rapidly improving and may soon be practical for screening of families (see Cotton 1989; Gibbs et al. 1989; Higuchi et al. 1990; Trayst-

Table 1

Clinical Manifestations in Probands and Relatives with Mutations in the Gene for Type III Procollagen

Type III Mutation[a]	Arterial Aneurysms[b]	Easy Bruisability[c]	Ruptured Bowel	Skin					Joint Laxity			Spontaneous Pneumothorax
				Prominent Vessels	Fragile	Hyperextensible	Soft	Ecchymoses and Scarring	Fingers	Other Joints	Dislocations	
Probable deletion	+	+	0	+	+	NA	+	+	NA	NA	+	+
Glycine substitution:												
Gly^{619}-Arg	+	+	0	0	0	0	0	0	±	0	0	0
Gly^{790}-Ser[d]	+	±	+	0	0	0	+	0	0	0	0	0
Gly^{883}-Asp[d]	0	+	0	±	0	0	+	0	+	+	0	+
G^{+1} substitution:												
$G^{+1\ IVS16}$	+	+	0	+	0	0	+	0	±	±	+	0
$G^{+1\ IVS20}$	+	+	0	0	0	0	+	0	0	0	0	+
$G^{+1\ IVS42}$	+	NA	0	+	0	0	+	0	±	+	+	NA

NOTE.—NA = no information available.

[a] The probable deletion was reported by Superti-Furga et al. (1988); the three glycine substitutions were reported by Pyeritz et al. (1984), Kontusaari et al. (1990), and Tromp et al. (1989*a*, 1989*b*); the $G^{+1\ IVS20}$ mutation is the mutation described here. (Preliminary data on this mutation and the two other G^{+1} mutations have been presented previously in abstract form [Zhao et al. 1990]).

[b] Locations of the aneurysms are as follows: the epigastric artery has the probable deletion; the abdominal aorta, thoracic aorta, hepatic artery, and celiac artery have the Gly^{619}-Arg mutation; the subclavian artery has the Gly^{790}-Ser mutation; the cervical artery has the $G^{+1\ IVS16}$ mutation; the thoracic aorta and abdominal aorta have the $G^{+1\ IVS20}$ mutation described here; and the aorta has the $G^{+1\ IVS42}$ mutation.

[c] Hematomas from minor trauma.

[d] Probands initially classified by us as having EDS type IV (Tromp et al. 1989*a*, 1989*b*).

man et al. 1990; Ganguly and Prockop, in press). Once a mutation is defined, it is relatively simple, with the PCR, to analyze members of the family for the presence or absence of the mutation (figs. 6 and 7).

Acknowledgments

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