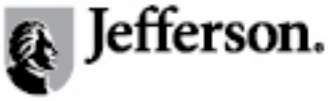

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Sequencing of cDNA from 50 Unrelated Patients Reveals That Mutations in the Triple-Helical Domain of Type III Procollagen Are an Infrequent Cause of Aortic Aneurysms

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Abstract

Detailed DNA sequencing of the triple-helical domain of type III procollagen was carried out on cDNA prepared from 54 patients with aortic aneurysms. The 43 male and 11 female patients originated from 50 different families and five different nationalities. 43 patients had at least one additional blood relative who had aneurysms. Five overlapping asymmetric PCR products, covering all the coding sequences of the triple-helical domain of type III procollagen, were sequenced with 28 specific sequencing primers. Analysis of the sequencing gels revealed only two nucleotide changes that altered the structure of the protein. One was a substitution of threonine for proline at amino acid position 501 and its functional importance was not clearly established. The other was a substitution of arginine for an obligatory glycine at amino acid position 136. In 40 of the 54 patients, detection of a polymorphism in the mRNA established that both alleles were expressed. The results indicate that mutations in type III procollagen are the cause of only about 2% of aortic aneurysms. (*J. Clin. Invest.* 1993. 91:2539–2545.) Key words: familial aneurysms • polymerase chain reaction • polymorphisms

Introduction

A number of careful studies have shown that aneurysms, even when they are not associated with well-recognized, heritable syndromes such as the Marfan syndrome (1) or Ehlers-Danlos syndrome (EDS)[1] type IV (1), are frequently familial (2–11). A recent statistical evaluation suggested that aneurysms are caused by defects in a single gene (12). The structural components of arteries and the gene products participating in the proteolysis of the structural components have been suggested as candidate genes for aneurysms (13). Mutations in the fibrillin gene located in chromosome 15 (14) have been shown to cause the Marfan syndrome (15, 16). There is a considerable variability in the phenotype among different individuals with the Marfan syndrome within the same family and among different families (17). Fibrillin 15 is, therefore, a potential candidate gene for aortic aneurysms (17). Other candidate genes for aortic aneurysms are collagens and elastin, which are important structural components of the aorta. Their contribution to the strength of an artery was elegantly demonstrated by Dobrin and his co-workers (18) by using dog and human cadaver arteries in a system where pressure could be applied onto the artery. Treatment of the artery with collagenase or elastase resulted in a considerable weakness of the structure (18). Several different types of collagens have been isolated from blood vessels, but types I and III collagen are the major components in the arterial wall (19). They are fibril-forming collagens in which three polypeptide chains called the α chains form a stable triple-helical structure (20, 21). Each α chain consists of about 340 repeating tripeptide sequences of -Gly-X-Y- in which X is frequently proline and Y is frequently hydroxyproline (20, 21). A recent study on the type III procollagen secreted by cultured skin fibroblasts from patients with aortic aneurysms demonstrated that in 2 out of 14 patients the type III procollagen was thermally unstable when assayed by brief proteinase digestion, suggesting a structural defect in the type III procollagen (22). Furthermore, a mutation in the type III procollagen gene was found in a man who expired at the age of 34 yr from a rupture of the aorta (23). The man had a history of easy bruisability and bleeding, but none of the dramatic skin changes usually associated with type IV EDS (1, 24) such as ecchymoses, abnormal scarring, and heavy pigmentation (23). He, therefore,

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1. *Abbreviations used in this paper:* EDS, Ehlers-Danlos syndrome; nt, nucleotide.

probably presented phenotypic overlap between EDS type IV and familial aneurysms. Subsequently, a mutation in the type III procollagen gene was found in a 37-yr-old woman who had a strong family history of sudden death from ruptured aortic aneurysms (25) without any evidence of EDS type IV or related disorder.

The hypothesis that aortic aneurysms are caused by increased proteolytic activity in the aorta that leads to the weakening of the arterial wall and rupture, has also been supported by several studies (26–29). There are, however, very few, if any, known examples of a gene defect producing an increase in an enzyme activity. It might, therefore, be that the increased proteolytic activity found in aneurysmal aorta is a secondary phenomenon due to some other primary defect.

In conclusion, several candidate genes for aortic aneurysms have been suggested, including (*a*) fibrillin 15 (17), (*b*) collagen (20, 21, 30), (*c*) elastin (31), (*d*) collagenase (18), (*e*) elastase (27), (*f*) tissue inhibitor of metalloproteinase (29), and (*g*) α_1-antitrypsin (28). In this article we report the detailed DNA sequencing approach to answer the question of whether most aneurysms are caused by mutations in the gene for the type III procollagen.

Methods

Patient material. Skin biopsy was obtained from all patients listed in Table I after written informed consent. In some cases, blood samples were obtained from family members of the patient after written informed consent. Skin biopsies were used to establish skin fibroblast cultures. Total RNA was isolated from cultured skin fibroblasts with a procedure involving extraction with guanidinium isothiocyanate (32). The RNA was purified by centrifugation on a cesium chloride gradient and used to synthesize first strand of cDNA with MMVL reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). Genomic DNA was isolated from cultured skin fibroblasts or blood on a Genepure 341 automated DNA extractor (Applied Biosystems, Inc., Foster City, CA).

Sequencing of cDNA. Sets of oligonucleotides (Table II) based on the cDNA sequence of type III procollagen (33–38) were used as primers so as to generate five overlapping PCR products (1, 2A, 2B, 3, and 4 in Table II) to cover all the coding sequences for the triple-helical domain of 1029 amino acids of the type III procollagen. Originally, products 2A and 2B were amplified together as one longer fragment, but since results from DNA sequencing were somewhat variable, the region was divided into two PCR fragments, after which the sequencing results improved. To produce single-stranded DNA sequencing templates, three successive PCRs, of 20 cycles each, were performed for each region.

The first PCR (39) was carried out for 20 cycles in a 30-μl reaction volume containing 0.6 pmol of each primer. 2 μl of the first PCR product was used in a second PCR that was carried out for 20 cycles in a 50 μl-reaction volume containing 10 pmol of one primer and 2.5 pmol of the other. The third PCR was carried out for 20 cycles in a 100 μl reaction volume containing 50 pmol of one primer and 1 pmol of the other primer and 2 μl of the second PCR product. The products from the third PCR were run on an agarose gel, and if they were of acceptable quality, additional third PCRs were performed to produce sufficient template DNA for sequencing.

Typically, the final results from the third PCR showed a clean amplification of the desired product on an agarose gel with two bands, one representing the double-stranded DNA and the other representing the single-stranded DNA (not shown). Routinely, cDNA from 10 different cell lines was amplified simultaneously. Water blanks were included in every experiment and they were negative.

The PCR products were purified by adsorption to powdered glass (silica) under high salt conditions using a commercial kit (Geneclean, BIO 101, Inc., Vista, CA). Since adsorption of DNA to silica powder in the presence of 6 M NaI is pH-dependent (pH of 6 M NaI should be between 6.2 and 7.2 at room temperature), the NaI solution was buffered with 50 mM Pipes and the pH was adjusted to 6.7 at room temperature with acetic acid. In addition, the pH of the asymmetric PCR sample was modified by the addition of 1 μl of 1 M Pipes, pH 6.7, per 100 μl of PCR product. Successful asymmetric PCRs were transferred to microcentrifuge tubes and three volumes of pH-buffered NaI and 5 μl of "Glassmilk" per 100 μl of PCR product were added and the mixtures rotated at room temperature. Pelleting and washing of the powdered glass were performed according to the Geneclean kit instructions except that they were performed in an Autogen 540 (an integrated microcentrifuge and pipetting station; Autogen Instruments Inc., Beverly, MA). The pellet was air-dried for 10 min at room temperature after the final wash and the adsorbed DNA eluted by resuspending the pellet in 5–30 μl of water and heating to 65°C for 10 min. The powdered glass was pelleted by 20 min centrifugation and the supernatant transferred to a new tube. Because adsorption of nucleic acids to powdered glass (under the conditions used here) is dependent on length with extremely inefficient adsorption of fragments less than 300 nt in length, the purified asymmetrical PCR products contained almost no PCR primers and were suitable to use as templates in dideoxynucleotide DNA sequencing.

The purified PCR products were sequenced directly with 28 sequencing primers specific for type III collagen (Table III) using Sequenase (U.S. Biochemical Corp., Cleveland, OH) or Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in the dideoxynucleotide method (40). The sequencing was carried out in sets of 12 so that DNA from 10 different cell lines, M13 control DNA and M13 clone containing type III collagen sequences that corresponded to the region in the patient sample, were sequenced simultaneously on a multiwell plate (Earley and Tromp, manuscript in preparation). The reactions were run on 6% DNA sequencing gels (Sequagel-6, National Diagnostics, Atlanta, GA). To increase the speed and ease with which the autoradiographs could be interpreted, several reactions terminated with the same dideoxynucleotide were loaded in adjacent lanes (Fig. 1). Typically, the samples from 5–10 patients were loaded so that the blocks of adjacent lanes could be interpreted like a conventional sequence ladder. A significant advantage was that it was easy to identify the appearance of a new band where the other cell lines lacked the band.

Analysis of DNA polymorphisms. Three polymorphic bases at positions nucleotides (nt) 1851, 2092, and 2244 in the coding region of type III collagen were analyzed using genomic DNA as a template in the PCR according to conditions described previously (41, 42; Wu and Kuivaniemi, manuscript in preparation). PCR products were then analyzed using allele-specific oligonucleotide hybridization technique or restriction endonuclease digestion as described previously (41, 42; Wu and Kuivaniemi, manuscript in preparation).

Results

Patient material. DNA sequencing was carried out on type III collagen cDNA from 54 patients with aneurysms. Among the 54 patients there were four pairs of siblings (JIMM408 and JIMM409, JIMM425 and JIMM425a, JIMM425b and JIMM425c, and JIMM425d and JIMM425e in Table I). Thus, the number of unrelated individuals analyzed was 50, representing 50 different families. The 50 unrelated patients were from five different nationalities: 1 Haitian, 3 Finnish, 4 Canadian, 11 Swedish, and 31 U.S. American. Most (43/50) of the patients had at least one blood relative who was also diagnosed with an aneurysm. More specifically, 15 patients had one family member with the disease, 17 patients had two family members, 6 patients had three family members, 2 patients had

Table I. Clinical Information on Patients

Code	Gender	Age*	Location of aneurysm‡	Status§	Family‖	Nationality
JIMM255	M	60	AAA	R	3 (2B,A)¶	Swedish
JIMM256	M	64	AAA, IA	O, R	1 (F)	Swedish
JIMM263	M	38	TAA	CT	0	Canadian
JIMM279	F	50	IA	O	2 (S, N)¶	Swedish
JIMM280	M	55	AAA	O	0	Swedish
JIMM281	M	60	AAA	O	0	Swedish
JIMM285	M	54	AAA, IIA	O	1 (B)	U.S.
JIMM295	M	55	AAA, IA	O, R	2 (2B)¶	Swedish
JIMM296	M	68	AAA	O	0	Swedish
JIMM332	M	70	AAA	O	0	Swedish
JIMM334	F	67	AAA	O	1 (B)	U.S.
JIMM335	M	69	AAA	O	1 (B)	U.S.
JIMM335d	M	65	AAA	O	1 (B)	U.S.
JIMM335f	M	67	AAA	O	2 (2S)	U.S.
JIMM335g	M	66	AAA	O	2 (2B)	U.S.
JIMM335h	M	64	AAA	O	1 (B)	U.S.
JIMM335i	M	73	AAA	O	2 (B, S)	U.S.
JIMM335j	M	67	AAA	O	1 (B)	U.S.
JIMM335k	M	65	AAA	O	2 (B, S)	U.S.
JIMM341	M	73	AAA	O	1 (S)	Swedish
JIMM342	M	46	AAA, IA	O, R	2 (S, F)¶	Swedish
JIMM350	M	66	AAA	O	1 (B)	Swedish
JIMM351	F	62	TAA	R	2 (F, S)	U.S.
JIMM396	M	58	AAA, IIA	O	2 (F, B)	U.S.
JIMM397	M	67	AAA	O	3 (B, 2C)	Finnish
JIMM398	M	66	AAA	U	1 (B)	Finnish
JIMM406	M	41	MAA	O	2 (D, B)	U.S.
JIMM407	M	18	AAA	R	—	U.S.
JIMM408	M	68	AAA	—	5 (F, G, A, 2B)	U.S.
JIMM409	M	63	AAA	—	5 (F, G, A, 2B)	U.S.
JIMM419	M	59	AAA	R	3 (F, 2C)	U.S.
JIMM421	F	47	AAA	O	4 (2B, M, F)	U.S.
JIMM422	F	85	AAA	O	3 (2B, F)	U.S.
JIMM425	M	70	AAA	U	1 (B)	Canadian
JIMM425a	M	71	AAA	O	1 (B)	Canadian
JIMM425b	M	65	AAA	O	2 (B)	Canadian
JIMM425c	M	62	AAA	O	2 (B)	Canadian
JIMM425d	M	69	AAA	O	1 (B)	Canadian
JIMM425e	M	70	AAA	U	1 (B)	Canadian
JIMM425g	F	65	AAA	U	1 (B)	Canadian
JIMM429	F	77	AAA	—	2 (2S)	U.S.
JIMM430	M	—	AAA	O	9 (4B, S, 2A, 2C)	U.S.
JIMM436	M	40	IA	O	4 (S, F, C, U)	U.S.
JIMM438	M	63	AAA	O	1 (B)	U.S.
JIMM443	F	34	AAA	O	3 (2B, M)¶	U.S.
JIMM445	F	68	AAA	O	2 (F, S)	U.S.
JIMM449	M	59	AAA	O	2 (B, M)	U.S.
JIMM483	F	76	AAA	U	2 (B, S)	U.S.
JIMM490	M	49	TAA	R	13 (6C, 2S, B, G, 2A, U)	U.S.
JIMM492	M	79	AAA	O	1 (B)	U.S.
JIMM497	F	73	TAA	O	3 (3B)¶	U.S.
JIMM498	M	71	AAA	O	2 (F, N)¶	U.S.
JIMM499	M	46	IA, TAA	O, R	0	Haitian
JIMM543	M	25	TAA	R	2 (F, G)	Finnish

* At the time of diagnosis.
‡ Abbreviations: AAA, abdominal aortic aneurysm; TAA, thoracic aortic aneurysm; MAA, multiple aortic aneurysms; IIA, iliac artery aneurysm; IA, intracranial artery aneurysm.
§ Status of aneurysm at the time of diagnosis. Abbreviations: U, dilation detected by ultrasonography; O, elective operation; R, rupture; CT, computerized tomography.
‖ Number of affected family members in addition to the proband. Abbreviations: S, sister; B, brother; F, father; M, mother; D, daughter; So, son; G, grandparent; C, cousin; U, uncle; A, aunt; N, niece or nephew.
¶ Some members in the family have intracranial aneurysms.

four family members, 1 patient had five family members, 1 patient had nine family members, and another patient had 13 affected family members. The age of the patients at the time of diagnosis varied between 18 and 85 yr (mean±SD = 60.3±14.1). Among the 54 patients studied there were 43 males and 11 females. The males were between 18 and 79 yr of age at the time of diagnosis (mean±SD = 59.3±13.9). The females were between 34 and 85 yr of age at the time of diagno-

Table II. Oligonucleotide Primers Used to Amplify the Coding Sequences of the α1(III) Triple-Helical Domain

PCR product	Primer name	Primer sequence	Primer location*	Size of PCR product
				bp
1	III-1	GCCGTCTAGACTGGTCCTCAGAACTATTCT	436–456	1026
	III-2	CGCAAGCTTAGCTCCTGGAAGCCCATTTGC	1423–1443	
2A	III-3	GCCGTCTAGAAGAATGGTGCCAAAGGAGAG	1305–1326	688
	III-32	CGCGAATTCTTCCCCAGGTTTTCCATTTTCT	1954–1974	
2B	III-25	CGCGGATCCAGTCAAGGATAAAGTGGTCGA	1666–1686	562
	III-4	CGCAAGCTTGACTTCCAAGACCTCCTCTTT	2189–2209	
3	III-5	GCCTCTAGACCACAAGGATTACAAGGCTTG	1909–1929	1067
	III-6	CCCGCAAGCTTAGCTCCTGGTTTCCCACTTT	2936–2955	
4	III-13	CGGAATTCTTGGGATTGCTGGGATCACT	2839–2859	933
	III-14	CGGAATTCATCAGGACTAATGAGGCTTTC	3736–3757	

* Location in cDNA sequence, nucleotide numbers according to Ala-Kokko et al. (36).

sis (mean±SD = 63.3±15.0), and all females had at least one additional blood relative who had the disease.

A majority (46 of 54) of the patients had aneurysm(s) in the abdominal aorta (see Table I). There were, however, six patients with a thoracic aortic aneurysm. In addition, three patients with intracranial aneurysms were included in the study because they had family members with aortic aneurysms. One of these cases was JIMM279 who had an intracranial aneurysm at the age of 50 yr and whose sister also had an intracranial aneurysm and the daughter of the sister had a dissecting aortic aneurysm at the age of 25 yr.

Four patients had aneurysms both in cranial arteries and in

Table III. Oligonucleotide Primers Used for Direct Sequencing of the PCR Products

PCR product	Primer name	Primer sequence
1	SP38	CTT GCC ATC TTC GCC TTT AGC T
	SP12B	CTC CTT TGG CAC CAT TCT TA
	FS8	ACC CAT TTC GCC TTT AC
	FS2	GCC TTC GAA TTG TCC AGG GGC ACC ATT TGA
	SP41	CCT CGA GCA CCG TCA TTA
	SP11	CAT TTC GTC CAT CGA AG
	SP22	CTA CCT GAT TCT CAA TCT TTT
1R	JEJ-1	CCT GGA CTG ATG GGA GCC CG
2A	SP36	TGT GTC TCC TTT GTC ACC ACC A
	SP39	GCC ACC TCG TTC TCC ATT CTT A
	SP2	CGG GCA TGC CCC TCA TT
	SP13B	CAC TTT CTC CTT GAC TT
2B	ALA2194	GGG GAC CAG CTC CAC CTC T
	SP9	CAA GCC TTG TAA TCC TT
3	SP40	ACT TTC ACC CTT GAC ACC CTG A
	SP15B	GCA CCA GGC GAT CTC TTC TCT
	SP30	CCT GGG TTA CCA TTA CTA CC
	SP4	GGG CCT CCT TCA CCT TT
	SP31	CCA GTT TCA CCT CTC TCA C
	SP19	GAC TTC CAA GAC CTC CTC TTT C
	SP14	CAC CCT TTC CTC CTT CG
4	SP42	CTA ATG AGG CTT TCT ATT TGT
	SP16	ATG GCA GCG GCT CCA AC
	SP43	CCT GGG GAG CCC TCA GAT
	SP17	CCG ATT GCA CCC TGC TG
	SP7	CTG TTT CAC CTT TGT
	SP33	ACC ACG ATC ACC CTT GCC A
	SP44	TCC AGG TTC ACC AGC TGT A

The PCR product number refers to the numbers presented in Table II. The sequencing primers used here generate antisense sequences, except primer JEJ-1 which generates sense sequences with PCR-product 1R.

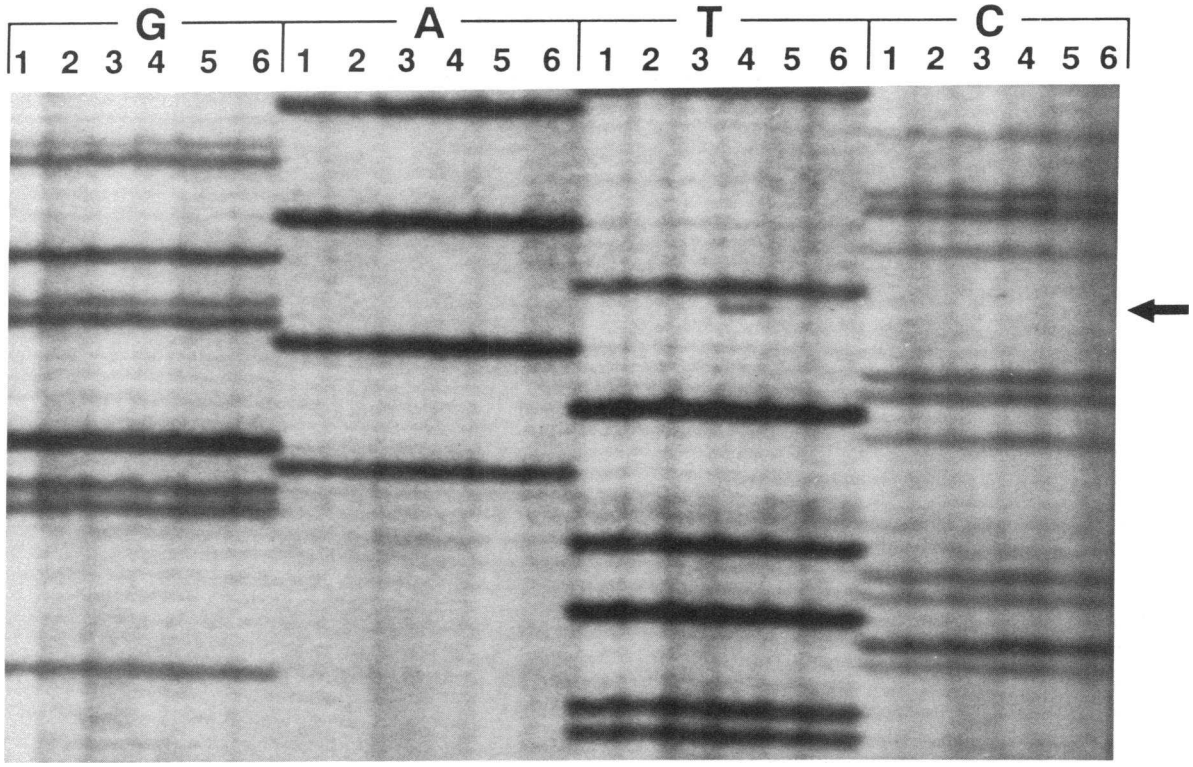

Figure 1. Autoradiogram of a DNA sequencing gel. To facilitate the interpretation of results, G reactions from six (lanes *1–6*) different individuals were loaded adjacent to each other, followed by A, T, and C reactions, in this order, from the same individuals. Lane T4 has an extra band (*arrow*), indicating the presence of G to T change in one allele of cell line JIMM498. The sequence is in antisense orientation.

the aorta. Two patients had iliac artery aneurysms. Six individuals with abdominal or thoracic aortic aneurysms had one or more family members with intracranial aneurysms. At the time of the diagnosis the aortic aneurysm had ruptured in 7/54 patients. 36 patients underwent an elective operation for their aortic aneurysms. Five patients were first diagnosed by ultrasonographic examination.

DNA sequences and polymorphisms of type III collagen. The sequence analysis carried out here provided 3,232 nucleotides from each allele of the gene for type III procollagen. The region analyzed covered the coding sequences for all the 14 amino acids of the amino-terminal telopeptide, all the 1,029 amino acids of the triple-helical domain, all the 25 amino acids of the carboxyterminal telopeptide, and 8 of the 246 amino acids of the carboxy-terminal propeptide. The sequences for the amino-terminal propeptide and most of the carboxy-terminal propeptide were not analyzed. These amino acid sequences are cleaved off from the procollagen before the fibril formation can occur and they are, therefore, not present in the mature form of collagen (20).

Only two sequence variations were found in the 54 individuals (Table IV). Cell line JIMM407 had a G to A change at nt 907. The nucleotide change converted the codon at amino acid position 136 from GGG for glycine to AGG, a codon for arginine. The mutation was confirmed using genomic DNA isolated from the patient's fibroblasts (not shown). The mutation was not found in any of additional 127 individuals sequenced in the laboratory.

The patient was an 18-yr-old black male without any prior relevant medical history (43). He had suddenly developed paraparesis and bilateral loss of pulses below the waist (43). An aortogram disclosed a dissecting aneurysm of the entire aorta and obstruction of blood flow below the renal arteries (43). His autopsy findings revealed dissecting aortic aneurysm and generalized fibromuscular dysplasia (43). His father had died at the age of 36 yr in a car accident and no affected relatives were available for DNA testing. His mother did not have the mutation, but the patient's three unaffected sibs were found to have the same mutation. Ultrasound examination of the aorta on the sibs (aged 21, 20, and 16 yr) did not reveal any abnormalities.

In the cell line JIMM498 a C to A change was found at nt 2002, changing the codon CCT for proline at amino acid position 501 to ACT, a codon for threonine (Fig. 1). PCR on genomic DNA from the patient indicated that the sequence variant was real and not a PCR artifact (not shown). The change was not found in any of the other 127 individuals sequenced in the laboratory. The patient was 70 yr old, when he was operated on for an abdominal aortic aneurysm. His father also had an aortic aneurysm but was deceased and no material was available for DNA testing. The patient's nephew (a son of the patient's younger brother) was operated on for an intracranial aneurysm at the age of 28 yr. This nephew had the mutation. DNA analysis revealed that the change was also present in the patient's two unaffected brothers aged 68 and 73 yr, the younger of which had been examined by sonography and no aortic aneurysm was found. Thus, two unaffected and two affected individuals in the family had the DNA change. The change was, therefore, probably not the cause of the aneurysms.

When analyzing the sequencing results from the 54 individ-

Table IV. Sequence Variations Detected in the 54 Patients

Cell line code	Location*	Nature‡	Occurrence
JIMM407	nt 907 (E14)	Gly136 → Arg	1/128§
JIMM498	nt 2002 (E30)	Pro501 → Thr	1/128§

* E14, exon 14 of the gene for type III procollagen; E30, exon 30 of the gene for type III procollagen.
‡ Numbering starts at the beginning of the triple-helical domain of type III procollagen.
§ Only one patient was found to have the base change. A total of 127 other unrelated individuals that included the 49 other aortic aneurysm patients presented here, 55 patients with intracranial aneurysms, 5 patients with the EDS type IV, 4 patients with osteogenesis imperfecta, 5 patients with the EDS (without subclassification), 8 patients with a variety of connective tissue abnormalities without any specific diagnosis, and one apparently healthy individual were sequenced in the laboratory.

uals, special care was taken to record data on known polymorphic regions of the type III collagen. The three polymorphisms that have been found previously in the coding region of type III collagen are at positions nt 1851 (Wu and Kuivaniemi, unpublished results), 2092 (41), and 2244 (42). 40 of the 54 individuals were found to be heterozygous for at least one of the polymorphisms (Table V), indicating that both of the alleles of the gene for type III procollagen were expressed at the mRNA level. The polymorphism analysis was also carried out on genomic DNA samples isolated from the cultured skin fibroblasts of the patients. The results obtained from the analysis showed that the 40 individuals that were heterozygous in their cDNA for one or more polymorphisms were also heterozygous in their genomic DNA. The results also showed that the 14 individuals who did not show any heterozygosity in the cDNA sequences were homozygous on their genomic DNA for all three markers studied.

Discussion

There are two general approaches using DNA techniques that can be used to identify the gene harboring the mutations causing aortic aneurysms. The first approach is to carry out linkage studies using markers that have been mapped to a particular locus on the genome and are used to test whether or not the marker is co-inherited with aortic aneurysms in families. Linkage can be established whenever the phenotype and genotype of related individuals is analyzed. Therefore, linkage can be established by analyzing large families. There are, however, several problems in the approach when studying a late-onset disease such as aortic aneurysms. It is rare to find large index families that have more than two generations of living, affected members so that pertinent samples can be obtained. In addition, diagnosis is a problem, inasmuch as the data on the incidence of aneurysms in the population indicate that few individuals develop aneurysms before the age of 50 yr and that the incidence increases with age.

Table V. Heterozygosity of the 54 Patients at Three Polymorphic Sites Located in the Coding Sequences of Type III Collagen

Location of polymorphism	Number of heterozygous individuals
nt 1851	18
nt 2092	22
nt 2244	21

Total number of patients heterozygous for at least one of the markers: 40.

The second approach, the candidate gene approach, involves the analysis of a candidate gene for absence or presence of mutations. The modern techniques of molecular biology have made the approach feasible (44). Here we were able to get definitive results from detailed DNA sequencing analysis of the gene for type III procollagen of 54 individuals from 50 unrelated families. The analysis revealed a mutation in obligatory glycine (20, 21) in one patient. The substitution of arginine for a glycine at amino acid position 136 in type III procollagen is likely to disrupt the triple-helical structure of the protein and make the protein less stable (20, 21). The finding represents a second example of a glycine to arginine mutation in the type III procollagen causing aortic aneurysms in patients without other signs of the EDS. The first report was a substitution of arginine for glycine at amino acid position 619 of the triple-helical domain of type III procollagen (25).

The other sequence variant detected in another patient substituted threonine for proline in a Y position of the Gly-X-Y repeat of collagen triple-helical domain (20, 21). Substitution of threonine for a Y-position proline is not likely to be destabilizing since six of the 14 threonines normally found in the type III collagen triple-helical domain (36) occupy the Y position. In addition, threonine and proline have been exchanged during evolution of collagens (45). Also, the mutation was present in the patient's apparently unaffected brothers suggesting that the mutation was an infrequent sequence variant. The results, therefore, indicate that mutations in the triple-helical domain of type III procollagen are the cause of ~ 2% (1/50) of aortic aneurysms. In addition, the results suggest that mutations in the promoter region or other control regions of the gene for type III procollagen are not a common cause for aneurysms, in that at least 40 of the 50 patients studied here had mRNA that was derived from both alleles of the gene.

From a theoretical perspective, DNA sequencing provides a complete record of all of the bases that comprise a gene or its coding sequence. Therefore, DNA sequencing will lead to the detection of all mutations in a target region. Furthermore, it makes it possible to exclude definitively the region from those that possibly contain mutations. Our results demonstrate that sequencing has become a feasible approach not only to establish rapidly and definitively whether or not a candidate gene harbors the mutations causing a disease phenotype, but also to determine what fraction of affected individuals have a mutation in the particular candidate gene. This approach could, therefore, be used to establish whether or not other genes that have been suggested as candidate genes, such as the genes for fibrillin (17), elastin (31), collagenase (26), elastase (27), or tissue inhibitor of metalloproteinase (29), harbor mutations causing aortic aneurysms.

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