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SHORT REPORT

Fibulin-2 exhibits high degree of variability, but no structural changes concordant with abdominal aortic aneurysms

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We used conformation sensitive gel electrophoresis and direct sequencing of PCR products to screen for mutations in the cDNA for fibulin-2, an extracellular matrix protein, from 11 patients with abdominal aortic aneurysms and two controls. When compared with the published reference sequence, a total of 14 single-base sequence variations were detected. Seven of the changes were neutral in that they did not result in an amino acid substitution. There were five missense changes at sites not conserved between human and mouse, and two missense changes at sites conserved between human and mouse. All but two of the sequence variants studied were also present in an additional set of 102 control alleles analyzed. One of these two changes was a missense mutation, but it did not segregate with abdominal aortic aneurysms in the family, whilst the other change was neutral. In conclusion, fibulin-2 has a large number of sequence variations in comparison with our previous analyses of type III collagen, and these variations will be useful in association studies. There was an excellent overall agreement between direct sequencing of PCR-products and conformation sensitive gel electrophoresis.

Keywords: polymorphism; mutation; missense; screening technique; cDNA; polymerase chain reaction; DNA sequencing; extracellular matrix; arterial wall; familial aneurysms

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Introduction

Fibulin-2 is a member of the extracellular matrix proteins with sequence similarity to fibulin-1, has high level of expression in the heart, and was localized to human chromosome 3p24–p25.[1] A DNA linkage study for atypical Marfan syndrome showed a significant lod score to 3p24,[2] suggesting that fibulin-2 was a candidate gene. Individuals from the pedigree had aortic dilatations and dissections or ruptures, in addition to skeletal abnormalities. A recent DNA linkage study, using two polymorphisms in the gene, however, excluded fibulin-2 as a candidate gene for this form of Marfan syndrome.[3]

A number of careful studies have demonstrated that aortic aneurysms are frequently familial, even when they are not associated with heritable disorders such as the Marfan syndrome or the type IV variant of the Ehlers-Danlos syndrome.[4–6] The efforts to identify the gene(s) involved in the development of aortic aneurysms have been directed towards analyzing candidate genes of biological significance to the vessel tissue.[4,7] Many of the known extracellular matrix genes, including the gene for type III procollagen, were candidate genes, but a number have been excluded.[4,7]

The new developments in molecular biology have provided excellent tools for screening for mutations in candidate genes for diseases.[8,9] There are, however, few if any studies in which two techniques have been compared side by side. Here we used conformation sensitive gel electrophoresis (CSGE) to screen for mutations in fibulin-2 in patients with abdominal aortic aneurysms (AAA), and compared the results with those obtained by direct sequencing of PCR products. The sequence variants identified here should be useful in association studies.[10]

Materials and Methods

Patients

The skin biopsies and blood samples were obtained after informed consent. Details on the patients (see Table 1) have been described previously.[7,11]

PCR and Sequencing

RNA isolation, cDNA synthesis, PCR, purification of PCR products and direct sequencing of them were carried out using protocols described previously.[7] Primers and PCR conditions are available from the corresponding author. Genomic DNA was extracted using an automated procedure (Genepure 340, Applied Biosystems, Foster City, CA, USA) from blood leukocytes from the buffy coat fraction purchased from the American Red Cross (Philadelphia, PA, USA), from cultured skin fibroblasts, or from whole blood.

All sequence variants detected by DNA sequencing were confirmed by restriction endonuclease digestions (see Table 1 and 2). In some cases the primers were designed to contain deliberate mismatches compared with the reference sequence so that the mismatches in conjunction with the sequence variant generated a restriction site. All but one of the variants were also studied using genomic DNA as a template in PCR. In all PCRs water controls were negative, and a cDNA clone HK-9 for fibulin-2[1] was used as a positive control.

CSGE

Samples were treated, gels run and stained as described previously.[12] CSGE analyses were performed in a blinded manner with respect to sequencing results.

Table 1 Sequence variants in fibulin-2

				*Variant**													
Patient	*Diagn.*	*Sex*	*Age*	*500*	*502*	*1115*	*1150*	*1203*	*1229*	*1278*	*1295*	*1779*	*2574*	*2629*	*2754*	*3408*	*3540*
JIMM407	EDIV	M	18	GG	AA	AA	GG	GG	AC	GG	CC	TT	AG	AG	CT	AA	TT
JIMM534	FMD	M		AA	AA	GG	AA	GG	AA	AA	CC	CC	GG	GG	CT	AG	CT
JIMM408	AAA	M	68	AG	AA	GG	AG	GG	AC	AG	CC	CC	AA	AA	TT	AG	TT
JIMM334	AAA	F	67	AA	AA	GG	AA	GG	AA	AA	CC	CC	AG	AG	CC	AA	TT
JIMM335	AAA	M	69	AA	AA	GG	AA	GG	AA	AA	CC	CC	GG	GG	CC	AG	CT
JIMM335d	AAA	M	65	AA	AA	GG	AA	GG	AA	AA	CC	CC	GG	GG	CC	GG	CC
JIMM335f	AAA	M	67	AA	AA	GG	AG	AG	AA	AA	CT	CC	GG	GG	CC	AA	TT
JIMM341	AAA	M	73	AA	AA	GG	AA	GG	AA	AA	CT	CC	AA	AA	TT	AG	CT
JIMM256	AAA	M	64	AA	AA	GG	AA	GG	AA	AA	CC	CC	AA	AA	CT	AG	TT
JIMM398	AAA	M	66	AA	AA	GG	AA	GG	AA	AA	CC	CC	GG	GG	CC	GG	CC
JIMM332	AAA	M	70	AA	AA	GG	AA	GG	AA	AA	CC	CC	AA	AA	TT	GG	CC
JIMM350	AAA	M	77	AA	AA	GG	AA	GG	AA	AA	CC	CC	AG	AG	CT	AG	CT
JIMM342	AAA	M	46	AA	AA	GG	AA	GG	AA	AA	CC	CC	AA	AA	CT	GG	CC

*Results are based on direct sequencing of RT-PCR products and confirmation of the results by restriction endonuclease digestions (see Table 2). JIMM332 has no positive family history for AAA, all the others do.

Results and Discussion

CSGE and direct sequencing of PCR products were performed independently but simultaneously to screen for mutations in the cDNA for fibulin-2 in 11 patients with AAA and two individuals with known mutations in type III collagen, one patient with the Ehlers-Danlos syndrome type IV and one patient with fibromuscular dysplasia.[7,11] Since direct sequencing of PCR-product is considered the most sensitive mutation screening technique,[8,9] it was a useful standard against which to compare CSGE. Eight overlapping PCR fragments of 453 to 670 bp to cover 96% of the coding region of fibulin-2 (nucleotides 214–3715, AA 22–1157[1]) were generated. No convenient site for a primer was available in the 5'-untranslated region which lead to the exclusion of the signal peptide of 27 AA and the first 21 AA of the mature protein. The 104 PCR products were first checked on agarose gels to verify that there was a PCR product, that the products were of the expected size, and that the positive and negative controls worked. After this, the products were run on CSGE gels, and 14 products showed multiple bands: the band of expected size and one or two other bands that migrated more slowly than the correct band, signs of heteroduplexes due to the presence of mismatches. For the direct sequencing, overlapping RT-PCR products of somewhat larger size than the products for CSGE were generated. Altogether 3502 nt (3411 nt of coding sequences) from each individual were analyzed. A minimum of 80546 nt and a maximum of 91052 nt of allelic nucleotides were analyzed from the 13 individuals, since ten of the individuals were heterozygous for at least one nucleotide (Table 1).

Altogether 14 different sequence variants were identified (Figure 1 and Table 1). All except one variant (nt 1779) were confirmed using genomic DNA (Table 2). Nt 1779 was located on the boundary of an intervening sequence and an exon, and we were unable to obtain necessary sequence information to design primers for this region. The 1779 variant was, however, confirmed in the RT-PCR products by restriction enzyme digestions. Since the nt 500 and 502 variants were located so close to each other, it was difficult to study them separately in the restriction enzyme assays. DNA sequencing revealed that of the 13 individuals studied, one individual was heterozygous for nt 500, whereas all the others were homozygous for both of them (Table 1).

Seven of the sequence variants were neutral polymorphisms and seven were missense variations from the reference sequence[1] (Figure 1). In the mouse sequence, 2/7 amino acids (R322 and P382) are the same as in the reference sequence,[1] and five of the sites are not conserved. R322 (nt 1115) was changed to a codon for histidine in both alleles of the individual with fibromuscular dysplasia.[7] P382 (nt 1295) was converted to a leucine in one patient with AAA. To evaluate the significance of the variants and estimate their frequencies in the general population, about 100 control alleles were analyzed for all but one of the variants (Table 2). Two of the variants (nt 1203 and 1295) were not found in the 102 control alleles tested. It is worth noting that only one aneurysm patient (JIMM 335f)

Table 2 Frequencies of sequence variants in the control group

Location of variant	*RE**	*Minor allele*	*Major allele*	*No. of alleles analyzed*	*Observed heterozygosity*
nt 500/502	Bcgl	0.040	0.960	100	0.04
nt 1115	Bst XI	0.010	0.990	102	0.02
nt 1150	Msp I	0.069	0.931	102	0.02
nt 1203	BsaI	0.000	1.000	102	0.00
nt 1229	Hph I	0.049	0.951	102	0.06
nt 1278	SmaI	0.049	0.951	102	0.06
nt 1295	Alu I	0.000	1.000	102	0.00
nt 1779	Aci I	ND	ND	ND	ND†
nt 2574	Nla III	0.284	0.716	102	0.51
nt 2629	Bsm FI	0.275	0.725	102	0.55
nt 2754	Msc I	0.314	0.686	102	0.51
nt 3408	Dde I	0.294	0.706	102	0.43
nt 3540	Fok I	0.333	0.667	96	0.50

*RE, restriction enzyme used to study variant.
†ND, not determined due to the lack of intronic sequences to design primers for genomic PCRs.

was heterozygous for the nt 1203 variant and that this change was neutral. The same patient was heterozygous for the nt 1295 variant (Table 1). The patient had two living sisters and a deceased brother, all of whom had AAA. No sample was available from the deceased brother for genotyping. One of the sisters was heterozygous for the variant, and the other was homozygous for the common allele, suggesting that the variant was not associated with AAA. Also, haplotyping the siblings using four additional sequence variants (nt 1150, 1203, 3408 and 3540) revealed that while the proband and one of his sisters had haplotypes 1 and 2, the second sister had haplotypes 3 and 4 (not shown).

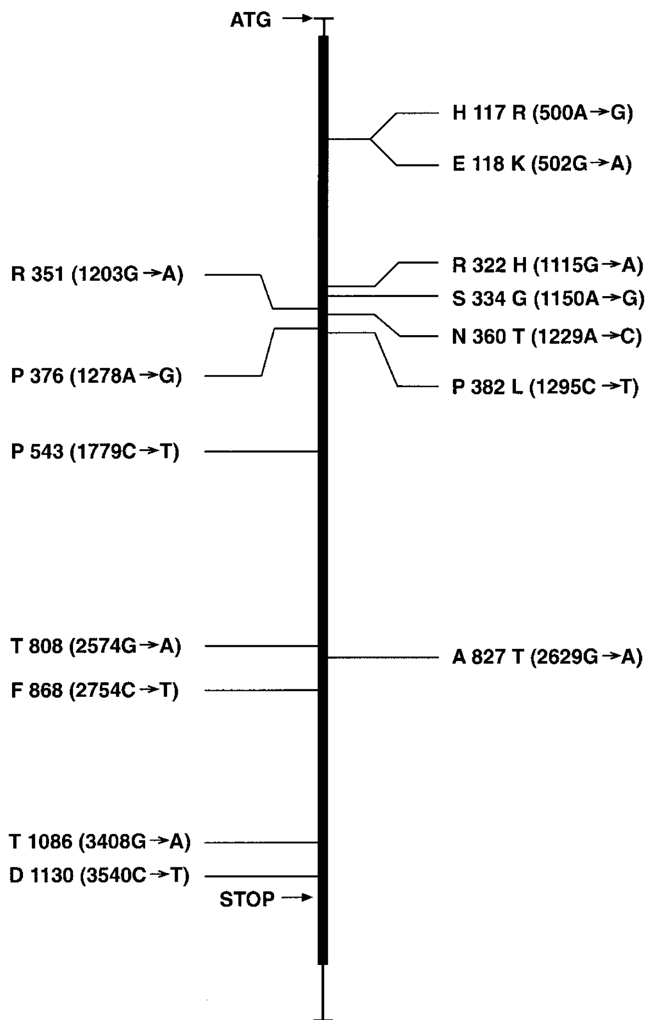

Figure 1 *Location of the 14 sequence variants in fibulin-2. The thick line in the middle represents the analyzed cDNA sequence of fibulin-2. The variants on the right are missense variants and the amino acid changes are shown using the single letter code (eg H117R, histidine codon 117 is changed to arginine). The numbers and letters in parenthesis refer to the nucleotide position and the nucleotide change on the cDNA sequence. The variants on the left are neutral variants. The numbering of the amino acids from the beginning of translation.*[1]

Five of the remaining variants were rare (Table 2). There were, however, five other variants whose minor allele frequencies were 0.28–0.33 and for which the frequency of heterozygotes was 0.43–0.55. The rare variants were present in only 0 to 2 patients, whereas the more common variants in the control population were also more frequent in the patients. There were no obvious differences in the frequencies of the sequence variants of fibulin-2 among the aneurysm patient group and the control group.

A comparison of results from the CSGE and direct sequencing of PCR products showed excellent overall agreement between the two methods. There were, however, two false positives (2/104; 1.9%) and seven false negatives (7/104; 6.7%) in the CSGE analysis. The number of false positives is well within acceptable range for a clinical screening test, and the false positives could be identified in a two-phase screening with CSGE followed by sequencing. One of the false negatives (nt 500) was missed in a PCR product in which the mismatch was only 73 bp from the end of the product, but was detected in another overlapping PCR product, in which the mismatch was more central. The nt 2754 variant was missed in all five individuals in whom it was detected by direct sequencing (and confirmed by restriction endonuclease digestions), probably due to the fact that the variant was only 43 bp away from the end of the PCR product. The overlap to the next PCR product was minimal and the variant was not present in that product. Only in the case of one variant, C to A at nt 1229, was it not possible to explain why the mismatch was not detected in CSGE. The number of false negatives could be reduced if PCR products with greater overlap were used. CSGE is, therefore, a useful, simple, fast and reliable first-step screening tool that should be complemented with DNA sequencing.

The results here made it possible to compare the frequencies of sequence variants in fibulin-2 and type III collagen. The two proteins have a coding sequence of almost the same size. In fibulin-2, altogether 14 sequence variants were found in a sequence of 3502 nt long, one variant/250 nt, which is almost five times higher a distribution of sequence variants than in type III collagen in which only three variants were found in the same individuals within 3434 nt covering the entire mature protein sequence.[7] Even analysis of over 100 unrelated individuals revealed only six variants in the

type III collagen sequences.[7,13] In summary, our findings exclude mutations in the coding sequence of fibulin-2 as the major cause for aortic aneurysms, but suggest that fibulin-2 exhibits a high degree of sequence variability. A possibility that the different variants contribute to the aneurysmal disease still exists, which could be addressed in association studies.

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