SHORT REPORT

Sensitivity of conformation sensitive gel electrophoresis in detecting mutations in Marfan syndrome and related conditions

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Objective: It has been firmly established that mutations in the gene for fibrillin 1, *FBN1*, cause Marfan syndrome (MFS). *FBN1* mutations can also cause other phenotypes, such as ectopia lentis (EL) and familial isolated thoracic aortic aneurysm and dissection (FAA). When the clinical presentation is typical, diagnosis of MFS is usually easy to make. However, there can be a marked phenotypic variation between affected subjects even in one family, and making the diagnosis can be challenging, especially in childhood. The objective of this study was to test the sensitivity of conformation sensitive gel electrophoresis (CSGE) for detecting mutations in *FBN1* in MFS and related phenotypes.
Design: Setting up CSGE analysis for the *FBN1* gene and testing the method first by screening coded samples from 17 MFS patients with previously detected *FBN1* mutations. We then used a test set consisting of 46 coded samples representing MFS, related phenotypes, and controls.
Results: Sixteen of the 17 known mutations were detected. Altogether 23 mutations were detected in a test set consisting of 46 coded samples representing MFS, related phenotypes, and controls. Nineteen of the mutations were novel. The mutation was detected in 18 of the 20 MFS patients and in one patient with familial EL, but not in a patient with sporadic MASS syndrome, any of the five sporadic annuloaortic ectasia (AAE) patients, or any of the 15 controls. A *FBN1* mutation was detected in four members of a multigeneration family with AAE, however.
Conclusions: These results indicate that CSGE is highly sensitive for the detection of mutations in *FBN1*, and that molecular diagnostics is a useful means of confirming clinical diagnoses of MFS and related disorders. Further careful investigations are needed, however, in order to correlate the interfamilial and intrafamilial clinical variabilities of fibrillinopathies and mutations in *FBN1*.

Marfan syndrome (MFS, MIM 154700) is inherited in an autosomal dominant manner and is one of the most common connective tissue disorders,[1–3] with an estimated incidence of about 1:5 000-1:10 000 in all ethnic groups. MFS is clinically highly variable and characterised mainly by the involvement of cardiovascular, skeletal, and ocular systems. Cardiovascular defects, gradual aortic dilatation leading to rupture or sudden aortic dissection, are the most severe complications, and mitral valve regurgitation and prolapse are also common. Skeletal defects typically include overgrowth of the long bones, leading to disproportionally tall stature and long fingers. MFS patients may also have kyphoscoliosis, pectus deformities, and a high arched palate. Ocular defects include ectopia lentis and myopia. Because of the clinical variability, it has proved difficult to establish a diagnosis of MFS. For this reason, there have been repeated discussions on the diagnostic criteria for MFS, the most recent agreement having been reached in 1996.[4]

About 200 mutations have already been characterised in the gene for fibrillin 1 (*FBN1*, MIM 134797) in patients with MFS (HGMD, The Human Gene Mutation Database, Cardiff; http://archive.uwcm.ac.uk/uwcm/mg/search/127115.html). In addition, a few *FBN1* mutations have been described in patients with related phenotypes such as isolated ectopia lentis (EL, MIM 129600),[5] familial isolated thoracic aortic aneurysm and dissection (FAA),[6 7] and overlap connective tissue disease or MASS syndrome (MIM 604308).[8] The Marfan-like connective tissue disorder (MFS2, MIM 154705) has the clinical characteristics of classical MFS, but displays linkage with markers at 3p25-p24.2.[9–11] Annuloaortic ectasia (AAE), also known as Erdheim cystic medial necrosis of the aorta (MIM 132900), is defined as autosomal dominant aortic dilatation/dissection without skeletal or ocular manifestations.[12] No molecular background is known for AAE.

Fibrillin 1 is the major structural component of the microfibrils that link together the different extracellular matrix components in most connective tissues, providing support for the organs and anchor cells for the matrix.[13–15] Microfibrils can also associate with elastin, forming elastic fibres that provide resilience and elasticity in tissues.[16]

The molecular diagnosis of MFS is challenging because *FBN1* is a large and complex gene estimated to be about 200 kb in size and to consist of 65 coding exons.[17 18] In addition, most MFS patients have private mutations that have been shown to be located throughout the gene. An exception is formed by neonatal MFS, a lethal form of the disease in which most mutations occur in the "neonatal region" located in the middle part of the gene and comprising exons 24 to 32.[5 19] The mutations causing MFS can be divided into three major categories. The first one, accounting for the majority, consists of missense mutations in the EGF-like domains that disrupt either an amino acid in the consensus sequence for calcium binding or one of the six highly conserved cysteine residues.[15] The other two categories consist of splicing mutations and mutations causing premature translation termination. No obvious correlation exists between the phenotype and genotype, however, despite the different nature of the mutations.

We report here on a systematic effort to determine the correlation between clinical and molecular diagnoses and to test the sensitivity of conformation sensitive gel electrophoresis

Abbreviations: MFS, Marfan syndrome; CSGE, conformation sensitive gel electrophoresis; EL, ectopia lentis; FAA, familial isolated thoracic aneurysm and dissection; AAE, annuloaortic ectasia

Table 1 CSGE primers

Exon	Primer name	Primer sequence	5′ end location	Product length (bp)	Annealing temperature (°C)
1	R1F	5′-GCGCACGGGTTGGGCTTGGGAATGG	–277	595	60
	R1RC	5′-ACGAACGGGGTGGGGACTAAACAAC	+154		
2	R2F	5′-GGCCATCTCTTCCTCTTCTTCTTTTTTTTAAAGTATGG	–60	227	60
	R2R	5′-CAAAAGGCCACATTCTAAGGCTC	+84		
3	R3F	5′-GATAGCAAAGTTCGTGAGGGACCTGAG	–115	347	50
	R3R	5′-GGCAGAACAGAGAAGGCAGATG	+133		
4	R4FN	5′-TGTGAGCTGTTGCCAATCTATG	–64	249	55
	R4RN	5′-CGAAGAAAATCCATCAGCACTTATCTC	+89		
5	R5F	5′-CCTTCCAGAGGACCACAAGTGTTAC	–79	269	60
	R5R	5′-CAGGAAGTAGCCATGCAGACC	+94		
6	R6F	5′-CCTCTGCATGATGGTTCCTGC	–61	309	55
	R6R	5′-CCAGAGCAAATAAGATTAATCCATTAATAATTCC	+50		
7	R7F	5′-ATATGGTAACATAATTGTGGAC	–98	275	55
	R7R	5′-TTTGCCTGCCCCCACTACACC	+51		
8	R8F	5′-CTACTGACGAATGGTTTTATATTGTGTTCTAC	–63	243	55
	R8R	5′-TTGTTATGGAACTGACTTACACAAACCATG	+54		
9	R9FB	5′-CCAGTGTGAAGTATGGAGCTG	–94	331	55
	R9R	5′-CATCTGCATCATGCACATTGCC	+79		
10	R10FB	5′-GGATGACTTCTGTGGGCCTATG	–74	467	60
	R10RB	5′-TCTCTGGAGAATAGGAAGCCTCCC	+212		
11	R11F	5′-TGCTCTGTTGTCACCAGACG	–144	378	60
	R11R	5′-GGGTAAGTTGTTACAGCAGCA	+93		
12	R12F	5′-GGAACCCAGAAAGTCTTAGAATTATGAGG	–72	267	55
	R12R	5′-CCATGGAACTCCTTTGAAGCC	+75		
13	R13FB	5′-CAAGATCATGAGTTTGCAAATGGAG	–95	374	55
	R13RB	5′-GAATATCATGGAAAATTAGGCTTC	+153		
14	R14FB	5′-TGTCCCATCTTCTCCTTCCTTAG	–169	397	55
	R14RB	5′-GTACTTTAAGTGGGGAGAATC	+105		
15	R15F	5′-GCTGCATATTATTTCCTATC	–115	376	50
	R15R	5′-CTGACCCTGTTGGTTTGTTGCTC	+138		
16	R16FB	5′-TGAGTTACACCAGGGATGATGG	–108	341	50
	R16R	5′-CTGTGAATTCCACAAATGCAAAGACCTC	+79		
17	R17F	5′-GGTGTAAGGTGACTCCCTGG	–157	353	50
	R17R	5′-GCATCCCAGATACATGGCACAGTG	+142		
18	R18F	5′-TTCCTCCTGTAGCTCCTAAGG	–112	330	50
	R18	5′-CTCTAAGCTACTCAAAGGCAG	+92		
19	R19F	5′-GCTTGAATTGATCACGTCGTTATGAC	–110	324	50
	R19R	5′-CAGGAGACTCTAATTCAGTC	+88		
20	R20FB	5′-GCAATTGGGGTCAAAGTTGAAG	–80	276	60
	R20R	5′-CTTTTGCAGGAAAAGCTGAC	+76		
21	R21FB	5′-CTTAATGTCAGCTTTTCCTGC	–158	402	60
	R21R	5′-CTTTGAAAATTCTCATGTGAGCCTAG	+106		
22	R22F	5′-CTATGTCAGAACTGCAAAGTCTGG	–76	208	55
	R22R	5′-GACAGCTTTATCCAGTCCGAG	+80		
23	F23	5′-GTTTTATGAACTTACCAGGTTC	–113	332	55
	R23	5′-ACCGAAGCTAAGTGCTCAG	+95		
24	F24	5′-CAGCAAATTATTATGTGTGCAG	–105	418	60
	R24	5′-ATCAAGTAGAGTGCTGAGATC	+85		
25	F25	5′-CAAGAACTTCCAACCTTCATG	–100	313	60
	R25R	5′-ACAGCCTTAATTCTTGCGACA	+87		
26	R26F	5′-CTTAAGGGCCAGGAGAGGG	–112	321	60
	R26R	5′-ACCTGGAACATAGGCTATGAG	+80		
27	R27FB	5′-GGAGGAGTGCTTGGTCTGG	–121	276	60
	R27RB	5′-CAAACATAAGCTTCCAACTTTGGC	+81		
28	R28F	5′-CGTGTATCGGTAAGGAGAAAGAC	–121	373	55
	R28R	5′-ACAAAACTCAGAGTACATAGAG	+128		
29	R29F	5′-GCCCTGCCTCTTAAATAGTG	–110	327	60
	R29R	5′-GACTCAAGCCTGCTTGACTCC	+93		
30	F30	5′-AATAGTCTTATGCTAGTAGGC	–113	335	55
	R30RB	5′-GGAATCTTTCTATCACTGACCC	+96		
31	R31F	5′-GTTATTATGTCTCGAGGGGAAAG	–75	276	55
	R31R	5′-CATGTATCAATCTATAATTATGATACCAATCTC	+75		
32	R32FB	5′-7 bp(CAGGACG) + CCAAAAGACATTTGTGCTGAGCC	–53 (+7 bp)*	306	55
	R32RC	5′-TAACAGAAAGGGTGGTATTTAAAACC	+124		
33	R33FB	5′-GTAGGAAAGTAACAGAGGTTGC	–146	330	55
	R33RB	5′-AATGTGGAATGCCTGGCTTCTCTGACTAGTG	+60		
34	R34F	5′-CATTGCTGCACTGGAAAGTTG	–87	281	50
	R34RB	5′-CAGGAATGTTTAAATAACCTAATCTC	+68		
35	R35F	5′-GTCTATAGGAGAAGTGCCCAG	–139	348	60
	R35R	5′-GCCCTTGTGTAGTCCCAG	+85		
36	R36F	5′-TATCTCTGAAGTGGAAGACTGC	–155	356	55
	R36R	5′-TGAGAATGGAATGTTTGGTGCTG	+78		
37	R37F	5′-CGGTAGTGTATAATGTTCATGGG	–146	436	60
	R37R	5′-CTCTACAGGGCTGAGAGGAC	+125		
38	R38F	5′-GTGGCCTTGCACTGGGGACAC	–209	402	55
	R38R	5′-CCTGGTAGCTCCTGGCACTCA	+124		
39	R39F	5′-GGAGGGGAAGGTTTCCGACC	–142	413	55

cont overleaf

Table 1 continued

Exon	Primer name	Primer sequence	5′ end location	Product length (bp)	Annealing temperature (°C)
	R39R	5′-ATGTGAGACATATCTACCTGGC	+145		
40	R40F	5′-GTCAACATTCATTAAGTATCAGGCC	–79	305	60
	R40R	5′-CAGTCTGATGAGTAACATCACC	+103		
41	F41	5′-GCTTGTTGAGTATCCACTTAG	–87	324	60
	R41R	5′-CGCTAAGACTGATTTCCCCAAC	+78		
42	R42F	5′-TTTTTTCAGTCCAATTATTGTTCTTTGCTGACCCCTATCC	–70	313	60
	R42R	5′-GCTGCACAGGGTGTTTGCACAG	+171		
43	R43F	5′-TTTCCATCTTGTCTTACCCTGCAC	–73	330	55
	R43R	5′-CCATGCCCTTTACTATTTCTAGAG	+131		
44	R44FB	5′-CGAAGGACATCTTGGTTGCTTCC	–179	371	60
	R44R	5′-CCAGATATCTGAAGCTTCATGAAGAC	+69		
45	R45FB	5′-CTCCTGAGAATGATAGCTAGAAG	–83	275	55
	R45R	5′-CCATATTTAGAATCAAATGAAGCTTTCAACAGC	+66		
46	F46	5′-AAGTTCTCAGCCTATGGATG	–119	389	60
	R46R	5′-GAGCACATTGTATTTGACAAGTC	+154		
47	R47F	5′-TTGGCTAAGCCAGCAAAGGC	–125	329	50
	R47R	5′-AGAACAGAGACTGCATGATTCC	+76		
48	R48F	5′-CTCCATGGTGGAATTTTATGAAC	–76	379	55
	R48RB	5′-TGCCCTTGCATTTGTTTCTG	+123		
49	R49F	5′-GGAAACTCAGTTGCCCTTTGTG	–99	314	55
	R49RC	5′-AAAAAACAGAGCTTTGCCATG	+89		
50	R50F	5′-AAGGAGTATTGCTGTGGTCCTG	–125	361	60
	R50R	5′-CTGTCTTTAAGGCCTACAGTC	+86		
51	F51	5′-CTTGTGGAGAAGCTTGTAATG	–64	199	50
	R51R	5′-AAGAATAACTAGAGAAGAAGCAG	+69		
52	R52FC	5′-CTCAGTATTCTCAATCTGCTTCTTCTC	–213	418	55
	R52RC	5′-GGTACCTATATTCATGGCTATACAGTG	+88		
53	R53FB	5′-AGCACTGCAGTCTGGATGTC	–140	330	60
	R53R	5′-GACTTGTAATCAACCAATTGTTCCC	+70		
54	R54FB	5′-ATCCATGTTTATGACAATGAAGC	–123	322	50
	R54RB	5′-AAGGGAAGCTTTGAGGGACATC	+76		
55	R55FB	5′-GAGATCATACTCAACAGAGCAG	–155	365	55
	R55R	5′-AGAACTCAGAGCCCAGGTTCC	+79		
56	R56FN	5′-GCTCCATCCTCTATAAAATGGTCAGATG	–66	242	60
	R56RS	5′-CCCAGTGTGGAGGCTGAGGTTAGG	+49		
57	R57F	5′-CTTGCTTCTTCTCACCCAGGGTAAAGTG	–128	435	55
	R57RC	5′-CCTAGGCACATATTGCAAACTCC	+100		
58	R58FB	5′-TACACTGAAGTGACCCCCTAC	–78	264	55
	R58RB	5′-20 bp + ATTTCCACTTGAGGATAAGCCATCAG	+40 (+20 bp)*		
59	R59F	5′-GTTAGACCCTGTGGAAATTGAGC	–75	282	60
	R59RB	5′-GCTTGCCTGTGGAGTTCTTAC	+84		
60	R60F	5′-CCAAATCAGACGTGGAGCTGCTTCATAG	–91	288	55
	R60RC	5′-CCCAACAGCAGAGGAAATAGAAAATAATCCC	+80		
61	R61FB	5′-GCCTTATTTGGCCTTTTCCGAGTTATCC	–105	314	60
	R61RB	5′-GATGATGAAGGTGCCAATAGCC	+80		
62	R62FB	5′-GGTAGAATAATGTGTAGGATGTG	–108	368	55
	R62R	5′-GGGTTTCAACCAGGTTAGGG	+140		
63	F63	5′-CAAGTGGCCAGATCCAATG	–76	417	60
	R63R	5′-CAAAAAGCATGGTTCTCCTCTGC	+109		
64	R64F	5′-CTCACAACTGCAAGGAACAGGC	–98	350	55
	R64RB	5′-TCCTTGGAGGAAACCACAGG	+77		
65A†	R65AF	5′-GTGTATGCAGCATAAGGCAG	–122	352	60
	R65AR	5′-TTGTGAAGTGGAGGTAGCTG	+230		
65B†	R65BF	5′-TTCCAGCTCTTACAACTCTGAC	+138	439	60
	R65BR	5′-GTCACCTGTACCTTGCTTTGG	+164		

*Random nucleotides.
†Exon 65 was analysed in two fragments, A and B.

(CSGE) for the detection of mutations in *FBN1*.[20] The results show that CSGE is sensitive and specific for this purpose. As previously established, *FBN1* mutations have been shown to be responsible for MFS as defined according to the revised clinical criteria,[4] and the present results also indicate that molecular diagnosis can be a most valuable tool for assessing carrier status in family members who do not have a typical clinical presentation of MFS, and for defining the diagnoses of Marfan-like syndromes.

METHODS

Subjects

Seventeen DNA samples from MFS patients with previously identified mutations were obtained from the Department of Human Molecular Genetics, National Public Health Institute, Helsinki, Finland, and a set of 46 DNA samples from the Clinical Genetics Unit, Helsinki University Central Hospital, which consisted of 20 samples from 13 sporadic and seven familial MFS patients, nine samples from AAE patients (five sporadic and four familial cases), one sample from a sporadic MASS patient, and one sample from a multigeneration EL family, together with 15 control samples representing five unrelated subjects, two spouses, and eight first degree non-manifesting relatives, that is, parents or sibs. The age range of the affected subjects was 7 to 61 years, and that of the controls 22 to 65 years. Patient evaluation included a clinical history and physical examination, and cardiovascular and ophthalmological consultations. Diagnosis of MFS was based on the revised diagnostic criteria.[4] A signed consent form was obtained from all the subjects.

Table 2 *FBN1* gene exon and intron sizes

Exon	Exon size	Intron size
1	164	31528*
2	83	2193*
3	99	10504*
4	96	3761*
5	96	58473*
6	198	3405*
7	126	7824*
8	126	5313*
9	159	4299*
10	180	655
11	141	1718
12	120	3382*
13	126	1341*
14	123	3436*
15	123	1092
16	153	4757*
17	54	1591
18	126	1040
19	126	485
20	120	207
21	138	868
22	51	1618
23	126	2382
24	228	1357
25	126	126
26	129	675
27	126	111
28	126	1578
29	123	1430
30	126	2037*
31	126	7006*
32	123	150
33	123	1578
34	126	1795*
35	123	2101*
36	123	309
37	165	2081
38	69	96
39	126	1547
40	123	658
41	159	2767*
42	72	3490*
43	126	3973*
44	123	3671*
45	126	1957*
46	117	1218*
47	129	720*
48	120	2696*
49	126	3806*
50	150	380
51	66	244
52	117	2255*
53	120	1607*
54	123	2067*
55	132	2202*
56	126	571
57	207	1703*
58	126	247
59	123	3300*
60	117	265
61	129	754
62	120	4919*
63	232	2801*
64	175	1193*
65	391	

*Intronic sequences were partially obtained from the public databases (GenBank accession No AC022467 and AC084757, Celera gene ID hCG38745).

Characterisation of the *FBN1* gene

About 40 to 60 bp of the sequences flanking the target sequence, an exon, and splicing consensus sequences are typically required for optimal sensitivity of mutation analysis by CSGE.[20] When the study was initiated, only partial sequences were available for most of the exon boundaries.[17] For this reason, several PCR primer pairs were designed from *FBN1* to screen BAC libraries (Genome Systems Inc). Exon boundaries were sequenced directly from the BAC clones with exon specific primers (ABI PRISM™ 377 Sequencer or ABI PRISM™ 3100 Sequencer and BigDye Terminator Cycle Sequencing Kit, Applied Biosystems), and some intronic sequences were amplified with a long range PCR kit (DyNAzyme EXT Validation Kit, Espoo, Finland).

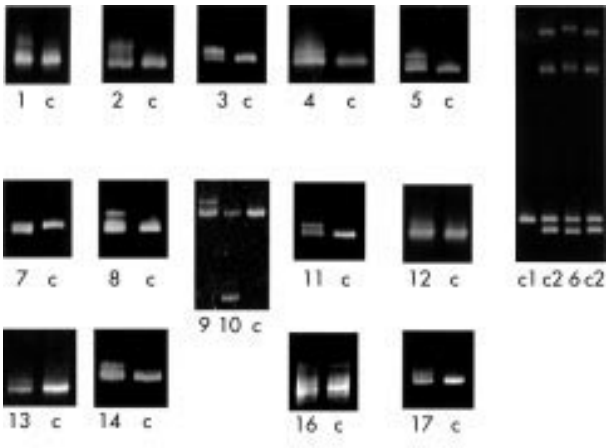

Figure 1 CSGE analysis of *FBN1* PCR products. Numbers underneath the heteroduplexes refer to the patient numbers in the data set 1 (table 3). In addition to the mutation, patient 6 is heterozygous for a polymorphism (IVS25+55-+60del TCTTTA). The same polymorphism is present in control sample 2 (c2). c, control.

Analysis of the *FBN1* gene

PCR primers were designed to amplify all 65 coding exons and the boundaries of the human *FBN1* gene. The PCR products varied in length between 199 and 595 bp, and the primers were designed so that each PCR product contained at least 40 to 60 bp of the exon flanking sequences at each end of the product (table 1). The PCR amplifications were done using 40 ng of genomic DNA, 0.25 µmol/l of forward and reverse primers, 1.5 µmol/l $MgCl_2$, 0.2 mmol/l dNTPs, and 1 U of AmpliTaq Gold polymerase (Applied Biosystems). The PCR conditions consisted of initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 25 seconds, 54-65°C for 25 seconds, and 72°C for 35 seconds, and a final extension at 72°C for 10 minutes. The PCR products were then denatured at 95°C for five minutes and annealed at 68°C for 30 minutes to generate heteroduplexes for analysis by CSGE. About 20 ng of the products was used for CSGE analysis. The conditions for CSGE analysis were essentially the same as described previously.[20] Sequence variations that were observed as heteroduplexes in CSGE analysis were identified by automated sequencing as indicated above. Before sequencing, the PCR products were treated with exonuclease I to degrade the residual PCR primers and shrimp alkaline phosphatase to dephosphorylate the residual nucleotides.[21]

RESULTS

Characterisation of the *FBN1* gene

Screening of the genomic libraries resulted in the identification of several positive BAC clones, three of which, 21325 (clone address 47[G14]), 20802 (clone address 155[I5]), and 21283 (clone address 146[D23]), covered most of the gene and were subsequently used to identify the sequences for exon boundaries. Clone 21325 spanned from the 5′ non-coding region of the gene to intron 5, clone 20802 covered the region between intron 8 and intron 43, and clone 21283 extended from intron 35 to the 3′ non-coding region of the gene. As the clones did not cover the region between intron 5 and intron 8,

Table 3 *FBN1* mutations in data set 1

Sample		Exon	Mutation	Predicted consequence	Reference
1	F*	4	c.364C>T	$R^{122}C$	33
2	F	6	c.649T>G	$W^{217}G$	34
3	S	11	c.1468G>T	48 bp deletion	35
4	F	21	c.2668T>C	$C^{890}R$	36
5	F	24	c.2980G>T	$E^{994}X$	37
6	S	25	c.3143C>T	$I^{1048}T$	38
7	S	26	c.3220T>C	$E^{1074}R$	5
8	S	27	IVS27+1G>A	Exon skipping	37
9	F	29	c.3599A>G	$E^{1200}G$	23
10	S	29	c.3603–3662del60-bp	Del 1202–1221	38
11	S	30	c.3725G>A	$C^{1242}Y$	5
12	F	52	c.6381T>A	$D^{2127}E$	5
13	F	52	c.6453C>G	$D^{2151}W$	5
14	F	59	c.7339G>A	$E^{2447}K$	32
15	F	60–62	Del exon 60–62	Del 2486–2851†	22
16	S	60	c.7531T>C	$C^{2511}R$	5
17	F	63	c.7879G>A	$G^{2627}R$	34

*S, sporadic; F, familial.
†Mutation was not detected in the present study.

a PCR screen of the genomic libraries with two primer sets (R6F and R6R, and R8F and R8R, table 1) from this region was performed. The screening did not yield any positive clones. In addition, no PCR products were obtained from this region using long range PCR and various primer combinations. These results suggested that at least some of the introns in this region are large. Altogether about 73 000 bp of the gene was sequenced. About half of the introns were sequenced completely, and at least about 200 bp of boundaries were determined for the rest of the exons.

A complete sequence for the human *FBN1* gene has recently been released to the public databases (GenBank accession No AC022467 and AC084757, Celera gene ID hCG38745). Comparison of our sequence with these did not show any major differences. Our results and the sequence data from the databases indicated that the gene is about 240 000 bp in size (table 2).

Mutation analysis

The first stage of the analysis included designing and testing the PCR primers (table 1), followed by testing of the PCR products by CSGE. Once the optimal conditions had been found, CSGE was tested for sensitivity using the set of 17 samples from unrelated MFS patients with previously characterised *FBN1* mutations. The samples were coded and analysed anonymously. All 65 coding exons and exon boundaries in each sample were amplified and subjected to mutation analysis. A unique CSGE pattern was seen in 16 of the 17 samples (fig 1), and sequencing of the samples confirmed that these patterns were the result of mutations (table 3). No mutation was found in sample 15 by CSGE. This mutation, a three exon deletion, had been previously identified by single strand conformation polymorphism (SSCP) analysis of cDNA.[22]

The second set consisted of 46 samples from MFS patients, related phenotypes, and controls, which were again coded and analysed anonymously. In addition, the number of patients versus controls in this set was not revealed until the analysis had been completed. All 65 coding exons and exon boundaries in all the samples were amplified by PCR and analysed for heteroduplexes by CSGE. All the samples with heteroduplexes were sequenced to identify the underlying sequence variation. No false positives were detected by CSGE, and most of the sequence variations, altogether 28, were either new or previously reported neutral ones (table 4). The analysis identified 23 mutations altogether (table 5). The sample set consisted of 13 patients with sporadic and seven with familial MFS, nine patients with AAE, one with EL, one with MASS phenotype, and 15 controls. The parents of the 13 sporadic patients were found on physical examination to be unaffected, and the family histories were negative. The seven familial MFS patients were from three families: a 63 year old mother with her 32 year old daughter and 34 year old son, a 39 year old mother and her 12 year old daughter, and 11 and 12 year old sibs of healthy parents, previously reported as a case with probable gonadal mosaicism.[23] Mutations were found in 18 of the 20 MFS patients (table 5). Retrospectively, one of the MFS patients with no detectable mutation was probably a case of Ehlers-Danlos syndrome of undefined type, with mild joint hypermobility, slight aortic root dilatation, and aortic and mitral valve insufficiencies. A mutation was also detected in the patient with EL, but not in the patient with the MASS phenotype. No mutations were found in the five sporadic AAE patients.

Table 4 Polymorphisms in the *FBN1* gene

Polymorphism	Reference
IVS1–39insA	
N^{625}(AAT-AAC)	24, 25, 27
IVS17–46a-g	24–27
K^{1027}(AAA-AAG)	
IVS25+55-+60delTCTTTA	
D^{1098}(GAC-GAT)	
IVS27–5g-a	24, 25, 27
IVS28+16-+20delTTTTA	25–27
IVS 34–55t-a	
IVS35–19a-g	
IVS35–74g-a	
IVS36-93c-t	
IVS37+68c-t	
IVS37–77t-g	
IVS40–35c-t	24, 25, 27
IVS40–14insT	24–27
IVS43–28^–29insCCT	
IVS48+54a-t	
I^{2110}(ATT-ATC)	
IVS51–85t-a	28
IVS51–130c-t	
IVS53–21a-t	27
D^{2285} (GAT-GAC)	24–27
IVS56+17g-c	25
Q^{2296}(CAG-CAA)	24–27
IVS56–33delG	
IVS57+63c-a	
IVS62+8a-c	

Table 5 Mutations detected in data set 2

Sample	DG*	Exon	Mutation	Predicted consequence	Reference
1095	MFS,S	2	CGT>TGT	$R^{114}C$	Novel mutation
1333	EL,F	6	CGC>TGC	$R^{240}C$	Novel mutation
1650	MFS,F	13	GAA>TAA	$E^{571}X$	Novel mutation
1651	MFS,F	13	GAA>TAA	$E^{571}X$	Same as above
1648	MFS,S	27	IVS27^{+1}G>A	Exon skipping	37
1612	MFS,F	29	GAA>GGA	$E^{1200}G$	23
1613	MFS,F	29	GAA>GGA	$E^{1200}G$	Same as above
1652	MFS,S	32	TGT>TGA	$C^{1348}X$	Novel mutation
1503	MFS,S	44	TGT>TAT	$C^{1835}Y$	39
1561	MFS,S	46	IVS47+5G>A	Exon skipping	Novel mutation
1528	MFS,S	51	TGT>CGT	$C^{2111}R$	Novel mutation
5213	MFS,S	57	c.7039^7040delAT	Frame shift	Novel mutation
1605	MFS,S	58	CGA>TGA	$R^{2414}X$	Novel mutation
1371	MFS,S	58	c.7308^7322del14 bp	Frame shift	Novel mutation
1129	AAE,F	59	TAC>TGC	$Y^{2474}C$	Novel mutation
1130	AAE,F	59	TAC>TGC	$Y^{2474}C$	Same as above
1310	AAE,F	59	TAC>TGC	$Y^{2474}C$	Same as above
1321	AAE,F	59	TAC>TGC	$Y^{2474}C$	Same as above
1599	MFS,S	62	TGC>TGG	$C^{2582}W$	Novel mutation
1306	MFS,F	63	TGT>GGT	$C^{2652}G$	Novel mutation
1418	MFS,F	63	TGT>GGT	$C^{2652}G$	Same as above
1419	MFS,F	63	TGT>GGT	$C^{2652}G$	Same as above
1588	MFS,S	65	CGA>TGA	$R^{2776}X$	40

*S, sporadic; F, familial.

The AAE family of four members with the same *FBN1* mutation Y2474C (table 5) is instructive. The proband had been referred for genetic counselling for aortic dissection at 32 years of age. He was 197 cm in height, had long extremities, normal ophthalmological findings, and no other MFS diagnostic criteria. He had three sibs. His sister, who was 5 years older, was 182 cm in height and had mild aortic dilatation (39 mm) on cardiac ultrasound examination at 40 years of age, but no other MFS criteria. She had three children, of which the oldest was an 8 year old healthy but tall daughter, +3.2 SD in height for Finnish girls. The younger sister of the proband was 180 cm tall, myopic, and had a normal aorta at 38 years of age, while the father of the proband, aged 64 years, had diabetes, was 184 cm in height, had long extremities, and minimal dilatation (45 mm) of the aortic root, but no other MFS criteria on careful evaluation. The proband had a paternal cousin one year older who had undergone aortic replacement at 35 years of age on account of dilatation. On examination, this cousin had borderline physical findings of MFS but normal ophthalmological findings. The DNA of the two latter family members was not available for analysis. Thus, based on the current criteria, none of the subjects examined had MFS, and AAE was the most likely diagnosis for the family. Since a demonstrable mutation in *FBN1* was one of the major diagnostic criteria, however, the proband, at least, definitely had MFS.

After opening the codes, all 65 coding exons and exon boundaries in the samples for the two patients, one with MFS and the other with MFS-like EDS, in whom no mutations had been detected by CSGE, were reanalysed by both CSGE and sequencing, but this reanalysis failed to detect any mutations.

DISCUSSION

The aim here was to test the usefulness, sensitivity, and specificity of CSGE for the detection of *FBN1* mutations in MFS and related disorders. There have been several reports on *FBN1* mutation screening, in which the lowest detection rates, about 10%, have been mentioned when screening has taken place at the cDNA level,[22 24] while markedly higher rates have been obtained when genomic DNA has been used as a template. Four studies have been conducted using well characterised MFS patients and various mutation screening methods: mutation detection enhancement gels,[25] single strand conformation polymorphism (SSCP),[26] denaturing HPLC,[27] and a combination of SSCP with heteroduplex analysis, enzyme mediated cleavage, and direct sequencing.[28] The mutation detection rate has varied from 50%[28] to 76%[27] or 78%.[25 26]

There are a number of factors that affect the detection rate, one of the most important of which is the clinical diagnosis. It is now well established that MFS is caused by *FBN1* mutations, but these are not a common cause of MFS related phenotypes.[27]

Secondly, the type of mutation can affect the detection rate. The vast majority of *FBN1* mutations are missense or nonsense mutations, which are detectable by most screening methods, except for the mutations causing premature translation termination, which are typically associated with mRNA decay and thus cannot be detected by methods that make use of cDNA. The *FBN1* gene was found to be about 240 kb in size, and thus even larger than had previously been estimated.[18] As the introns are large, most mutation screening methods require each exon to be analysed separately. This approach will naturally not detect large, multi-exon rearrangements. The exact proportion of such mutations is not known, but entries in the database suggest that they make up only about 1-2% of all *FBN1* mutations.

The third factor is the sensitivity of the screening method. No definitive sensitivity estimates are available for most methods, but the SSCP detection rate has been reported to range between 35 and 95%.[29–31] CSGE detected mutations in 16 out of the present 17 MFS patients with known mutations (94%). The single mutation that was not detected had been previously shown to have a multi-exon deletion by SSCP analysis of cDNA,[22] a mutation type that could not be detected by most screening methods using genomic DNA. In the second set of samples, CSGE detected mutations in 18 of the 20 patients with MFS (90%). Thus, the overall detection rate of the method can be estimated to be over 90%, since it detected mutations in 34 out of 37 patients with MFS. Reanalysis of the patient with definitive MFS and the patient with MFS-like EDS by CSGE and sequencing failed to detect any causative mutation, possibly indicating a multi-exon rearrangement or genetic heterogeneity. In addition, all the heteroduplexes detected by CSGE were verified as resulting from sequence variations, indicating a high specificity for the method.

The finding of a high sensitivity and specificity for CSGE is supported by our previous results, when we tested the method for detecting sequence variations in six collagen genes, *COL1A1, COL1A2, COL2A1, COL3A1, COL9A1*, and *COL9A2*.[20] All the previously identified 75 sequence variations were detected by CSGE in that study, and the method detected 223 new sequence variations, which were all confirmed by sequencing, indicating a specificity and sensitivity of 100%.[20] It is likely, however, that this estimate is too high, because it is impossible to test sequence variations in all possible sequence contexts. Nevertheless, these results should be applicable to *FBN1* screening, because like *FBN1*, collagen genes are also large, consist of multiple exons, and contain repetitive sequences.

MFS is usually easy to diagnose in cases of typical clinical presentation, and there is no need for molecular diagnosis. It can be difficult to establish a definite MFS diagnosis in childhood, however, and occasionally in adult patients as well. It is also known that extensive phenotypic variation can occur in MFS, even within one family, and that not all family members will have MFS that meets the diagnostic criteria at the time of examination, even though they may carry a *FBN1* mutation. Molecular diagnosis could thus also benefit these subjects. This hypothesis was supported by the present findings, in that CSGE identified mutations in two members of one AAE family with some MFS findings. One of the members was a clinically unaffected 8 year old girl and the other her 43 year old mother, who had only minimal aortic dilatation.

To our knowledge, *FBN1* mutation has only been reported in one family with EL,[5 32] the members of which expressed skeletal features but no cardiovascular ones, and therefore the phenotype has not been accepted as isolated EL (MIM 154700, *FBN1* allelic variant, 0015). Only one patient with EL from a multigeneration family was included in this series as other family members were not available for the study. Based on the clinical records only, none of them had MFS. Therefore, the possibility cannot be excluded that minor skeletal features from the list of diagnostic criteria for MFS could have been detected in members of the family, but the finding that EL, as the major clinical feature, was caused by a mutation in *FBN1*, is without doubt significant.

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Accession numbers and URLs for data in this article are as follows. On-line Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim for MFS (MIM 154700), *FBN1* (MIM 134797), EL (MIM 129600), AAE (MIM 132900), MASS syndrome (MIM 604308), MFS2 (MIM 154705). The Human Gene Mutation Database Cardiff (HGMD), http://archive.uwcm.ac.uk/uwcm/mg/search/127115.html) for *FBN1* mutations.

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