LETTER TO JMG

Twelve novel *FBN1* mutations in Marfan syndrome and Marfan related phenotypes test the feasibility of *FBN1* mutation testing in clinical practice

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Marfan syndrome (MFS) is one of the major heritable disorders of connective tissue with a prevalence of between 1 in 5–10 000.^{1,2} It is characterised by features in the cardiovascular, ocular, and musculoskeletal systems and the Ghent criteria form a useful framework for its diagnosis.³ Mutations in *FBN1* encoding the extracellular matrix protein fibrillin-1 classically cause MFS.⁴ Fibrillin-1, comprising multiple repeating subunits, of which the most common is the calcium binding epidermal growth factor (cbEGF) domain, is a key component of 10–12 nm microfibrils.⁵

Mutation detection has not been performed routinely in MFS because of the size and complexity of *FBN1*, which contains 65 exons extending over 200 kb of genomic DNA.^{5,6} Mutations are nearly always specific to each family and to date ~300 mutations have been reported.^{7–10} *FBN1* mutations have been found in 20–80% of patients with MFS depending upon the clinical selection of patients and the mutation detection method used.^{11–17} *FBN1* mutations have been reported in a wide range of phenotypes in the Marfan spectrum¹; neonatal MFS is associated with some *FBN1* mutations in exons 24–32¹⁸; mutations of various types throughout *FBN1* have been associated with classical MFS⁸; and *FBN1* mutations have also been reported in many of the non-classical Marfan related phenotypes.¹ The Ghent criteria propose that an *FBN1* mutation is only of help diagnostically if it has been previously found in a person who independently meets the criteria for diagnosis of MFS.³

Other than mutations causing neonatal MFS, which occur in exons 24–32, correlation between genotype and phenotype is poor. The majority of patients with cysteine substitutions have classical MFS, and cysteine substitutions in exons 26–32 appear to be associated with classical disease manifesting early in life.¹⁹ Missense mutations that affect the calcium binding site of the cbEGF domain (reducing calcium binding affinity) are associated with varying phenotypes that are likely to be related to the location, calcium binding properties, and domain context of the affected domain within fibrillin-1. There is marked phenotypic variation between subjects with nonsense mutations, which is thought to be due to the degree of nonsense mediated decay of the mutant cDNA.²⁰ Intrafamilial variation of phenotype is also seen, suggesting that there are factors in addition to the *FBN1* genotype that can modify the phenotype.^{21,22} Consequently, knowledge of the underlying *FBN1* mutation in an affected proband is of relatively little help either in assigning the appropriate clinical diagnosis or in advising on the likely clinical course or severity of phenotype. Here we report our experience in the Oxford Marfan Clinic, where mutation detection has been performed on 35 families on a research basis using heteroduplex analysis of genomic amplicons with conformation sensitive gel electrophoresis (CSGE) or denaturing high performance liquid chromatography (DHPLC).

METHODS

The clinical features of the probands are given in table 1. All patients underwent a thorough clinical examination, including slit lamp examination and 2D echocardiogram. Magnetic resonance imaging (MRI) of the lumbosacral spine for evidence of dural ectasia was performed in 14 of the probands. Of the 35 probands, 22 fulfilled the Ghent criteria for MFS (table 1). Two further subjects had two major criteria but involvement of a third system was not demonstrated. The remaining 11 subjects had phenotypes related to MFS. Other family members were also examined for Marfan syndrome where practicable.

Mutation detection was performed initially on 17 subjects using heteroduplex analysis of genomic amplicons and CSGE with DNA sequencing, as has been previously described.²³ In subject 12, where a mutation had not been discovered using CSGE, the cDNA was directly sequenced, as a defect had been observed in fibrillin-1 pulse chase studies.²⁴ DHPLC was used to screen for mutations in the remainder of the patients, including five cases where a mutation had not been found previously using CSGE (cases 2, 5, 7, 15, and 19). Sensitivity of the conditions used for DHPLC were tested by ensuring that DHPLC was able to identify 13 known *FBN1* mutations or polymorphisms which had been previously detected by CSGE. The 14 mutation negative subjects only analysed with DHPLC were not rescreened using CSGE, as DHPLC was the more

Key points

- Marfan syndrome is caused by mutations in *FBN1* resulting in defective fibrillin-1. It is diagnosed mainly on clinical grounds although *FBN1* genetic analysis may contribute.
- We have performed mutation detection on 35 families from the Oxford Marfan Clinic, with either classical Marfan syndrome or a Marfan related phenotype; 21 mutations were identified, of which 10 are reported here for the first time.
- In this study we found an *FBN1* mutation detection rate of 70% when this analysis was restricted to those with at least one major clinical criterion (excluding family history) and involvement of one further system.
- We suggest that this would be a suitable clinical requirement before *FBN1* testing in clinical practice.

Abbreviations: MFS, Marfan syndrome; cbEGF, calcium binding epidermal growth factor; CSGE, conformation sensitive gel electrophoresis; DHPLC, denaturing high performance liquid chromatography

Table 1 Details of the clinical features of the probands used in mutation detection along with details of the exon location, the nucleic acid change, the predicted consequence, the domain of fibrillin-1, and the method used in detection of each mutation

Patient	Age	Clinical features*							Diagnosis†	Exon	Mutation‡	Predicted consequence‡	Fibrillin-1 domain	Method of analysis
		M/S	CVS	Ocular	DE	FH	minor							
11	46	-	+	(AD)	-	+	F+	2	MFS	2	G247+1A§**	Skip exon 2	NH ₂	CSGE
3	45	-	-	+	-	+	F+	2	MFS	8	932insT§**	PTC at 348	cbEGF2	CSGE
7	65	-	-	-	+	D	F-	1	FEL	15	C1879T**	R627C††	cbEGF6	DHPLC
33	40	-	+	+	-	+	F-	2	MFS	15	G1960A	D654N	TB2	DHPLC
36	54	-	+	(AD)	+	-	S	2	MFS	18	G2168-1A	Splice defect	cbEGF7	DHPLC
40	9	-	+	+	+	D	S	0	MFS-	18	G2243A	C748Y	cbEGF7	CSGE
21	38	+	-	-	+	+	F+	0	MFS	20	G2539+1A	Splice defect	cbEGF9	DHPLC
18	30	-	+	+	-	+	F	2	MFS	35	4365delCT§	PTC at 1470	cbEGF21	CSGE
17	31	+	+	+	-	-	F	1	MFS	36	4485delC§	PTC at 1519	cbEGF22	CSGE
13	43	-	+	+	+	-	F	3	MFS	37	C4621T§**	R1541X	TB4	CSGE
14	31	-	+	+	+	-	S	2	MFS	44	G5504A§	C1835Y	cbEGF26	CSGE
15	30	-	+	+	+	D	S	0	MFS-	45	A5546-2G	Splice defect	cbEGF27	DHPLC
12	25	+	+	+	+	-	F	0	MFS	47	G5788+1A¶	Ins 33 bases	cbEGF29	Sequencing
41	10	-	+	+	+	-	S	1	MFS	48	T5929C	C1977R	cbEGF30	CSGE
1	46	-	+	+	+	+	F+	1	MFS	55	G6773A§**	C2258Y	cbEGF 35	CSGE
2	43	+	+	+	+	-	F-	0	MFS	57	A6998-2G**	Del 12 bases	TB7	DHPLC
16	43	-	+	(AD)	+	-	S	3	MFS	57	C7180T§	R2394X	TB7	CSGE
26	37	+	-	-	-	+	S	1	MFS	59	C7398A	Y2466X‡‡	cbEGF38	DHPLC
24	52	-	+	+	+	-	F	2	MFS	59	C7399T	Q2467X	cbEGF38	DHPLC
42	40	-	+	+	-	+	S	2	MFS	62	7816insT	PTC at 2607	cbEGF42	CSGE
27	17	-	-	-	+	D	F-	2	FEL	65	T8339C**	L2780P	C-term	DHPLC
29	55	+	-	-	-	+	S	2	MFS	-	-	-	-	DHPLC
30	25	+	+	+	-	+	F	0	MFS	-	-	-	-	DHPLC
5	62	-	+	+	+	-	F+	1	MFS	-	-	-	-	DHPLC
6	41	+	+	(AD)	-	+	F-	0	MFS	-	-	-	-	DHPLC
19	48	+	-	-	-	+	S	1	MFS	-	-	-	-	DHPLC
34	50	-	-	-	+	-	S	0	EL	-	-	-	-	DHPLC
22	38	-	+	+	-	D	F	2	FTAA	-	-	-	-	DHPLC
25	41	-	+	+	-	D	F	1	FTAA	-	-	-	-	DHPLC
23	48	+	-	-	-	D	F-	1	FMH	-	-	-	-	DHPLC
38	46	-	-	-	-	+	F	3	MASS	-	-	-	-	DHPLC
31	53	-	-	-	-	-	F-	3	MASS	-	-	-	-	DHPLC
37	32	-	-	-	-	-	S	1	Striae	-	-	-	-	DHPLC
35	27	-	-	-	-	-	F-	1	-	-	-	-	-	DHPLC
32	10	-	+	(AD)	-	-	S	0	SG	-	-	-	-	DHPLC

*Clinical features. Musculoskeletal (M/S), cardiovascular (CVS), ocular and dural ectasia (DE) are marked with a + if features were found on examination that corresponded to major involvement by the Ghent criteria.³ The aortic root was considered dilated when the maximum diameter at the sinus of Valsalva exceeded published normograms for age and body surface area (95% normal confidence limits).³⁹ AD indicates a previous aortic dissection. Dural ectasia was diagnosed based on published recommendations.⁴⁰ + = DE present, - = DE absent, blank MRI scan not performed, D MRI declined (7 and 27), delayed (22 and 25) because of aortic root surgery or prevented because of spinal deformity (23), age (40), or lost to follow up (15), but all seven of these subjects would fulfil the Ghent criteria for MFS if dural ectasia were present. FH, family history. F, familial phenotype. S, sporadic phenotype. + indicates that at least one relative was examined and fulfilled the Ghent criteria independently. - indicates that the relatives were examined and did not fulfil the Ghent criteria independently. The number of systems where minor criteria (such as M/S features, mitral valve prolapse, striae, or pneumothorax) were found (excluding those systems which also had a major criterion) is indicated. Keratometry and ultrasound of the globe to look for minor ophthalmological involvement was not performed routinely.

†Diagnosis. The diagnosis of MFS was made according to the Ghent criteria if there were at least two major criteria and minor involvement of a third system; -, third system involvement was not demonstrated in these subjects. FEL, familial ectopia lentis; EL, ectopia lentis - sporadic; FTAA, familial thoracic aortic aneurysm; FMH, familial Marfan-like habitus; SG, Shprintzen-Goldberg syndrome based on features of fatal aortic dissection, craniosynostosis, joint laxity, and arthrogryposis multiplex congenita. Two subjects had just one minor Ghent criterion but were included because of the familial nature of mild Marfan features in case 35, where four subjects had minor musculoskeletal features of MFS, and the severity of the sporadic Marfan-like striae in subject 37.

‡Nucleic acid and amino acid numbering according to Pereira *et al.*⁵ PTC, premature termination codon; Ins, insertion; Del, deletion.

§Published previously.²³

¶Published previously.²⁴

***FBN1* mutation also found in affected relatives.

††Reported previously.⁸

‡‡Reported previously.⁹

sensitive technique in our laboratory, as shown by its ability to detect three mutations (cases 2, 7, and 15) that had not been detected using CSGE.

DHPLC analysis

All 65 exons of *FBN1* and their flanking intronic regions were amplified by PCR from genomic DNA. Primers used were as published¹³ except for 42, 47, and 49, where the primers were redesigned (table 2). PCR was performed in a 50 µl volume containing 30 ng of genomic DNA, 1 × PCR buffer II (100 mmol/l Tris-HCl, pH 8.3, 500 mmol/l KCl) (Perkin Elmer), 0.2 mmol/l dNTPs, 1.5-3.0 mmol/l MgCl₂, 50 ng each primer, and 0.05U *Pfu Turbo* DNA polymerase (Stratagene) mixed with 0.9 U *AmpliTaq gold* DNA polymerase (PE-Applied Biosystems),

and was amplified using a touchdown PCR cycle on a PTC 225 Thermal Cycler (MJ Research Inc, Massachusetts, USA). Mg²⁺ concentration and annealing temperature were optimised for each primer pair and were usually between 1.5-3.0 mmol/l MgCl₂ with an annealing temperature of 50-58°C. To encourage formation of heteroduplexes, the PCR products were denatured at 95°C for five minutes and cooled gradually to 25°C at -1°C per minute.

DHPLC analysis was carried out using the WAVE™ DNA Fragment Analysis System (Transgenomic, Cheshire, UK). The temperatures for DHPLC analysis were determined using the Transgenomic WAVEmaker™ 3.4 program (table 3) and are available on request. The PCR products were injected into a DNasep column (Transgenomic). The column mobile phase

Table 2 Details of the primer sequences that were redesigned to allow improved amplification of *FBN1* exons 42, 47, and 49

Primer sequence
42 f 5'-TATCTCCGGTCCACCT
42 r 5'-AAAGTTCTGACAATGCCGTC
47 f 5'-GGAATGTTGGGACTTTCT
47 r 5'-GGTCTTCTAAGTCCTGTAC
49 f 5'-TGATGTCTCCATCGTGTGAC
49 r 5'-AGACCACCACAAATAACATGCA

consisted of a linear acetonitrile gradient in a 0.1 mol/l triethylamine acetate buffer (TEAA), achieved by mixing buffers A (0.1 mol/l TEAA) and B (25% acetonitrile in 0.1 mol/l TEAA). The calculated gradient (at a flow rate of 0.9 ml/minute over the six minute cycle) was run for all the amplicons, at the relevant column temperatures for each fragment. Where the chromatograph peak was different from the control, indicating a potential heteroduplex, the fragment was PCR amplified using the same primers and sequenced as has been previously described.²³

RESULTS

Table 1 gives the results of *FBN1* mutation analysis. A total of 21 mutations were detected in the 35 families. Seventeen of the mutations were detected in patients who strictly fulfilled the Ghent criteria for MFS, two mutations were found in subjects with familial ectopia lentis, and two mutations were detected in subjects with only two demonstrable major criteria for MFS. Mutations were not detected in five further subjects with classical MFS (two of whom were screened using both CSGE and DHPLC) or the remaining nine subjects with phenotypes related to MFS. The *FBN1* mutations comprised seven missense mutations, four nonsense mutations, four frameshift mutations, and six splice site mutations (predicted to lead to exon skipping in four cases and shown in one case to cause a large insertion of 33 bases in the RNA transcript, and in one case to cause a deletion of the first 12 bases of exon 57). Three silent mutations (C510T, T8502C, and A3963G) were detected that did not appear to have functional significance along with eight previously identified polymorphisms: C306T, T1875C, A2168-46G, 3589+15del5bp, C5066-35T, A6496+128G, G6997+17C, and T7571-113C.^{13 16 25-27}

DISCUSSION

Twenty-one predicted causal mutations were detected in patients from the Oxford Marfan Clinic. Causation is suggested by the type of mutation found (cysteine, nonsense, frameshift, and splice site), since these types of mutation are known to alter the structure and/or amount of fibrillin-1. Further evidence of cause and effect is suggested in cases 1, 2, 3, and 11, where in these large families the mutation was found to segregate with the disease phenotype (data not shown). The detected mutations occurred throughout the length of *FBN1* and are typical of those found in other MFS patients.⁸ *FBN1* mutations have been reported only rarely in ectopia lentis.^{9 10 28 29} In this study *FBN1* mutations were detected in two probands with familial ectopia lentis (cases 7 and 27). Case 7 (with mutation R627C) and her daughter both had ectopia lentis. For both subjects the aortic root diameter at the sinus of Valsalva was within the normal range for age and body surface area. Mitral valve prolapse was found in the daughter only. Musculoskeletal involvement included positive wrist and thumb signs in both subjects and, in addition, the mother also had a disproportionate body habitus with an

Table 3 Details of the column temperatures used in DHPLC analysis. *FBN1* mutations that had been previously identified using CSGE were used as controls to ensure that the temperatures selected for analysis were appropriate. Wherever a heteroduplex resulting from either a known mutation, new mutation, or polymorphism was identified, the temperature at which the heteroduplex was detected is indicated in bold

Exon	Column temperature (°C)	Exon	Column temperature (°C)
1	62 64	34	62
2	56 58	35	62
3	56 61	36	60
4	57 59	37	59 61
5	58 61	38	56 59
6	59 62 65	39	55 59 61
7	58 60	40	57 60
8	55 60	41	56 58 61
9	60 62 64	42	60
10	55 60 63	43	58 60
11	60 62	44	60 64
12	57 60 63	45	54 56
13	57 59	46	57 59
14	58	47	59 61
15	60 62	48	59
16	61	49	57 61
17	59 62	50	59 61 64
18	54 57	51	61
19	57	52	54 58
20	55 58	53	59
21	54 61	54	59
22	56	55	58 61
23	54 59	56	56 63
24	57 61 63	57	55 62
25	58 61	58	54 58
26	59 62	59	59
27	58 63	60	60
28	58 62	61	59 62
29	56 59 61	62	60 64
30	58	63	60 62
31	55 59	64	61
32	59	65	56 58
33	56 60		

upper segment to lower segment ratio of 0.85, and a high palate with crowding of the teeth. Lumbosacral MRI excluded the presence of dural ectasia in the daughter. Case 27 nearly meets diagnostic requirements for MFS with ectopia lentis, musculoskeletal features (a disproportionate body ratio, positive wrist and thumb signs, pes planus, joint hypermobility, and a highly arched palate with dental crowding), and striae. Protrusion acetabulae was not present, neither was there cardiovascular involvement. The patient declined lumbosacral MRI. The mother of this subject had bilateral ectopia lentis, a non-dilated aortic root, and a mild pectus excavatum. They both carried the L2780P mutation. The sister of the proband also carried L2780P but had only pes planus, a highly arched palate with crowding of the teeth, and striae. Her lenses were not dislocated. More fibrillin-1 functional studies are needed to determine how *FBN1* mutations such as these cause a less severe phenotype.

The detection rate of *FBN1* mutations in this study group overall was 21/35 (60%). In those who fulfilled the Ghent criteria for MFS, the detection rate was 17/22 (77%). This is broadly compatible with previous reports in patients fulfilling the Ghent criteria for MFS of 56% (34/61) using heteroduplex analysis with DHPLC,¹⁶ 72% (48/66),³⁰ and 90% (18/20)¹⁰ using heteroduplex analysis with CSGE, 80% (8/10)¹⁷ using single strand conformation analysis, and 66% (62/94) using CSGE or single strand conformation polymorphism (SSCP).⁹ Based on these six studies, heteroduplex analysis of genomic amplicons

using a technique such as DHPLC might be expected to give an *FBN1* mutation detection rate of up to 68% (187/273) in subjects fulfilling the Ghent criteria. In our population, the detection rate for those who had a Marfan related phenotype was only 2/11 (18%), despite the fact that they were all analysed using DHPLC, which has been shown to be a highly sensitive technique for mutation detection in *FBN1* and other genes.^{27 31 32} This is consistent with the previously reported figures of 5/32 (15%) in Marfan related phenotypes, analysed using DHPLC¹⁶ or 9/77 (12%) using CSGE or SSCP.⁹ It should be noted that analysis of genomic amplicons will not detect mutations caused by changes in the regulatory or promoter regions of *FBN1* or deletion of a whole gene. Further analysis of the fibrillin-1 protein or of the fibrillin-1 cDNA may show mutations in those patients where an *FBN1* mutation has not been detected in the coding sequence.^{14 24 27 33}

Collectively this and previous studies confirm that the majority of those with a detectable *FBN1* mutation have clear clinical features of MFS. Mutation detection can therefore often confirm an underlying defect in fibrillin-1 in those with a clinical diagnosis of classical MFS, but uncommonly in those with a Marfan related phenotype. It is clear from the observation that clinical features, such as aortic dissection, often occur in several subjects within a family,³⁴ that individual mutations do correlate with the phenotype. However, knowledge of the mutation alone is usually insufficient to predict the phenotype in isolation from clinical observations in other family members. With the lack of correlation between genotype and phenotype,^{9 35} precise knowledge of the particular underlying *FBN1* mutation in a proband does not help with prognosis or affect management decisions.

The principal clinical benefit of detecting an *FBN1* mutation in a patient is the direct molecular test that it offers to relatives. Where an *FBN1* mutation is detected, the first line investigation for relatives can become mutation detection rather than echocardiography and slit lamp examination. This can be particularly helpful in the screening of children at an early stage and potentially may save them and their families several years of repeated screening and anxiety. It may also help in the investigation of families dispersed geographically and managed by different centres. Knowledge of the *FBN1* mutation also allows the possibility of prenatal or preimplantation genetic diagnosis.³⁶ In large families, linked markers can be used to show that the phenotype segregates with a particular haplotype, and this approach has been advocated for service use.^{37 38} However, this method typically requires large numbers of family samples and is of no use in the ~30% of cases that are sporadic.

Our data, taken with the results of previous studies, indicate that for routine service there would be a high detection rate of *FBN1* mutations using heteroduplex analysis of genomic amplicons by DHPLC, provided that those selected for analysis had clear features of Marfan syndrome. We suggest that a minimum requirement of one major Ghent criterion (excluding family history) and involvement of a second system in a patient would be appropriate. This would allow the detection of the majority of *FBN1* mutations identifiable by this method, yet still be compatible with a reasonable detection rate. In this study it would have given a detection rate of 21/30 (70%). Where a mutation was not detected by heteroduplex analysis of genomic amplicons by DHPLC, further analysis at the cDNA level could be performed if there was a particular need to know the causative mutation, such as for use in prenatal diagnosis. This could involve restriction enzyme analysis to identify the presence of a large insertion or deletion in *FBN1*, quantitation of the proportion of transcript from each allele (where an unequal ratio of transcript could indicate an underlying *FBN1* mutation), or a direct search for the mutation by sequencing the *FBN1* cDNA.^{14 27 33}

In summary, knowledge of the particular *FBN1* mutation offers little direct benefit for prognosis and management to

the person tested, other than in prenatal diagnosis. However, *FBN1* mutation testing allows confirmation of the underlying genetic defect and allows its use for diagnosis in relatives, enabling a precise diagnosis in relatives with equivocal features. Precise early molecular diagnosis could obviate the need for repeated clinical screening including echocardiography and slit lamp examination, thereby offsetting the cost of mutation analysis. *FBN1* testing at the service level could therefore be a cost effective investigation.

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