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

D J Halliday, S Hutchinson, L Lonie, J A Hurst, H Firth, P A Handford, P Wordsworth

Marfan syndrome (MFS) is one of the major heritable disorders of connective tissue with a prevalence of between 1 in 5-10 000. It is characterised by features in the cardiovascular, ocular, and musculoskeletal systems and the Ghent criteria form a useful framework for its diagnosis. Mutations in FBN1 encoding the extracellular matrix protein fibrillin-1 classically cause MFS. 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.

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. Mutations are nearly always specific to each family and to date ~300 mutations have been reported. 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. FBN1 mutations have been reported in a wide range of phenotypes in the Marfan spectrum; neonatal MFS is associated with some FBN1 mutations in exons 24-32; mutations of various types throughout FBN1 have been associated with classical MFS; and FBN1 mutations have also been reported in many of the non-classical Marfan related phenotypes. 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.

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. 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. Intramolecular variation of phenotype is also seen, suggesting that there are factors in addition to the FBN1 genotype that can modify the phenotype. 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. Mutation detection was performed initially on 17 subjects using heteroduplex analysis of genomic amplicons and CSGE with DNA sequencing, as has been previously described. 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. 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 sensitive method used.

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
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 published 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 mM Tris-HCl, pH 8.3, 500 mM MgCl2) (Perkin Elmer), 0.2 mM each dNTPs, 1.5-3.0 mM 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 mM 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 WAVEMaker3 3.4 program (table 3) and are available on request. The PCR products were injected into a DNAsep column (Transgenomic). The column mobile phase

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*Clinical features: Musculoskeletal (M/S), cardiovascular (CVS), ocular and dural ectasia (DE) are marked with ± if features were found on examination that corresponded to major involvement according to the Ghent criteria. The aortic root was considered dilated when the maximum diameter of the sinus of Valsalva exceeded published normograms for age and body surface area (95% normal confidence limits). AD indicates a previous aortic dissection. Dural ectasia was diagnosed based on published recommendations. + = 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 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 27.

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

§Published previously.

¶Reported previously.

††Reported previously.

‡‡Reported previously.

§§Published previously.

**FBN1 mutation also found in affected relatives.

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

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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, C5066-7G, 7571-113C, 35T, A6496+128G, G6997+17C, and T7571-113C. 

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. Four FBN1 mutations have been reported only rarely in ectopia lentis. 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 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. Protrusio acetabulæ 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 dental crowding, 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, 72% (48/66), and 90% (18/20) using heteroduplex analysis with CSGE. 80% (8/10) using single strand conformation analysis, and 66% (62/94) using CSGE or single strand conformation polymorphism (SSCP). 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 sub-
jects fulfilling the Ghent criteria. In our population, the detect-
tion 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
genoms. 31 32 33 This is consistent with the previously reported fig-
ures of 5/32 (15%) in Marfan related phenotypes, analysed
using DHPLC or 9/77 (12%) using CSGE or SSCP. It should
be noted that analysis of genomic amplicons will not detect
mutations caused by changes in the regulatory or promoter
regions of FBN1, but in several subjects within a family, 9 14
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, 5 15 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 preimplan-
tation genetic diagnosis. 34 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. 35 However, this method typically requires
large numbers of family samples and is of no use in the ∼30%
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 (exclud-
ing 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 under-
lving FBN1 mutation), or a direct search for the mutation by
sequencing the FBN1 cDNA. 27 28

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,
creating a precise diagnosis in relatives with equivocal
features. Precise early molecular diagnosis could obviate the
need for repeated clinical screening including echocardi-
ography 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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Mutations in the fibrillin gene: SSCP screening of exons 15–21 in Marfan syndrome.


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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