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

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 lumbar 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. 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 technique.

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
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 *Phu 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
courage 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
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 splic 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, T8175C, A2168-46G, 3589+15del5bp, C3066-35T, A6496+128G, G6997+17C, and T7571-113C.\cite{11, 12, 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.\cite{4} FBN1 mutations have been reported only rarely in ectopia lentis.\cite{10, 20, 25, 28} 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, muscular-skeletal 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 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 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),\cite{13} and 90% (18/20)\cite{20} using heteroduplex analysis with CSGE, 80% (8/10)\cite{23} 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 \textit{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 \textit{FBN1} and other genes.\textsuperscript{5, 11} This is consistent with the previously reported figures of 5/32 (15%) in Marfan related phenotypes, analysed using DHPLC\textsuperscript{5} 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 \textit{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 \textit{FBN1} mutation has not been detected in the coding sequence.\textsuperscript{14, 27, 31}

Collectively this and previous studies confirm that the majority of those with a detectable \textit{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 some subjects within a family,\textsuperscript{10} 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,\textsuperscript{5} precise knowledge of the particular underlying \textit{FBN1} mutation in a proband does not help with prognosis or affect management decisions.

The principal clinical benefit of detecting an \textit{FBN1} mutation in a patient is the direct molecular test that it offers to relatives. Where an \textit{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 \textit{FBN1} mutation also allows the possibility of prenatal or preimplantation genetic diagnosis.\textsuperscript{3} 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.\textsuperscript{3} 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 \textit{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 \textit{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 \textit{FBN1}, quantitation of the proportion of transcript from each allele (where an unequal ratio of transcript could indicate an underlying \textit{FBN1} mutation), or a direct search for the mutation by sequencing the \textit{FBN1} cDNA.\textsuperscript{27, 31}

In summary, knowledge of the particular \textit{FBN1} mutation offers little direct benefit for prognosis and management to the person tested, other than in prenatal diagnosis. However, \textit{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. \textit{FBN1} testing at the service level therefore could therefore be a cost effective investigation.

\textbf{ACKNOWLEDGEMENTS}

DH was funded by a British Heart Foundation Junior Research Fellowship and SH by a BBBSRC studentship. PH acknowledges support from the British Heart Foundation and the Medical Research Council. We thank Ann Roberts for assistance with the Marfan clinic.

\textbf{REFERENCES}

Letter