Detection of Thirty Novel FBN1 Mutations In Patients With Marfan Syndrome or a Related Fibrillinopathy

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Marfan syndrome (MFS) is a disorder of the extracellular matrix caused by mutations in the gene encoding fibrillin-1 (FBN1). Recent studies have illustrated the variability in disease severity and clinical manifestations of MFS. Useful genotype-phenotype correlations have been slow to emerge. We screened 57 unrelated patients with MFS or a Marfan-like phenotype using a combination of SSCP and/or DHPLC. We detected 49 different FBN1 mutations, 30 (62%) of which were novel. The mutations comprised 38 substitutions (78%), 10 deletions (20%), and one duplication (2%). There were 28 missense (57%), nine frameshift (18%), eight splice site (16%), and four nonsense mutations (8%). Genotype-phenotype analysis revealed that patients with an identified FBN1 mutation were more likely to have ectopia lentis and cardiovascular complications compared to those without an identifiable mutation (relative risks of 4.6 and 1.9, respectively). Ectopia lentis was also found to be more prevalent in patients whose mutations involved a cysteine substitution (relative risk 1.6) and less prevalent in those with premature termination mutations (relative risk 0.4). In our hands, we achieved 93% mutation detection for DHPLC analysis of patients who fulfilled the Ghent criteria. Further analysis of detailed clinical information and mutation data may help to anticipate the clinical consequences of specific FBN1 mutations. © 2003 Wiley-Liss, Inc.

KEY WORDS: Marfan syndrome; MFS; fibrillin; FBN1; DHPLC

INTRODUCTION

Marfan syndrome (MFS; MIM# 154700) is an autosomal dominant inherited disorder of connective tissue with an estimated incidence of 1:5,000 that primarily involves the cardiovascular, ocular and skeletal systems (Dietz and Pyeritz, 2001). The diagnosis of MFS is dependent on a set of clinical diagnostic criteria, termed the ‘Ghent nosology’ (De Paepe et al., 1996).

Mutations in the fibrillin-1 (FBN1; MIM# 134797) gene have been shown to cause MFS as well as a series of other related disorders collectively termed type-1 fibrillinopathies (Hayward and Brock, 1997). FBN1 is a large gene of 65 exons located on human chromosome 15q21. Currently identified FBN1 mutations are scattered throughout the gene and are usually unique to individual families (Dietz and Pyeritz, 2001).
*FBN1* is translated into fibrillin-1, a 350kDa glycoprotein that is one of the major structural components of the elastin-associated 10-12nm microfibrils (Sakai et al., 1986). The protein comprises several classes of repeated cysteine-rich motifs, most of which display homology to the epidermal growth factor (EGF). Forty-three of these 47 EGF motifs are thought to play a role in calcium-dependent protein stabilization due to the presence of a calcium-binding consensus sequence (Handford et al., 1991). Long stretches of these calcium-binding EGF-like motifs are interrupted by other functional domains including seven with homology to the latent transforming growth factor β1 binding protein (LTBP) (Robinson and Godfrey, 2000). A third structural motif, termed the hybrid domain, is cysteine-rich and is thought to represent a fusion of portions of the EGF and LTBP motifs (Pereira et al., 1993).

The clinical manifestations of MFS are protean and to date there have been few clinically useful genotype-phenotype correlations. The only general exceptions are the early-onset severe phenotype of neonatal Marfan syndrome (nMFS) with mutations that cluster around exons 24-32 (Putnam et al., 1996) and the associations of *FBN1* mutations with ectopia lentis (Loeys et al., 2001; Schrijver et al., 1999; Schrijver et al., 2002) or cardiovascular complications (Loeys et al., 2001).

*FBN1* spans approximately 235kb of genomic DNA and has proven to be a technical challenge with respect to mutation screening. Recent screening strategies have included sequence analysis (Matsukawa et al., 2000), single strand conformation polymorphism (SSCP) analysis (Comeglio et al., 2001), and temperature-gradient gel electrophoresis (TGGE) (Katzke et al., 2002). These conventional methods for large-scale detection of mutations are expensive, technically demanding or time consuming (Mátyás et al., 2002). Denaturing high-performance liquid chromatography (DHPLC) has been successful in overcoming many of these limitations. This approach has allowed the simple, semi-automated and cost-effective detection of single-base substitutions and small insertions/deletions (Halliday et al., 2002).

Here we present the results of mutation screening of the *FBN1* gene in 57 unrelated patients referred with a clinical diagnosis of MFS or a related phenotype. We have concurrently developed an electronic database to facilitate comparison of detailed clinical information with mutation data with a view to the identification of possible genotype-phenotype correlations.

**PATIENTS AND METHODS**

**Clinical Evaluation**

Fifty-seven unrelated patients were referred to our clinic or diagnostic laboratory with a possible or definite clinical diagnosis of MFS. Twenty-eight patients fulfilled the clinical criteria according to the Ghent nosology at the commencement of this study (De Paepe et al., 1996).

**PCR**

Following appropriate informed consent, DNA was isolated from peripheral blood according to standard procedures (Miller et al., 1988). All 65 exons of *FBN1* (including splice-junctions) were amplified using primers as described in Nijbroek et al. (1995), with the exception of primers to exon seven, 47, and 49 (Korkko et al., 2002), the reverse primer for exon five (5’ AGCTTTAGGTACCAGCATG 3’) and the reverse primer for exon 27 (5’ CACCCAAACATAAGCTTC 3’).

**Sequence Specific Conformation Polymorphism (SSCP)**

SSCP analysis was performed using amplified DNA fragments as previously described (Adès et al., 1996; Orita et al., 1989).

**Denaturing High Performance Liquid Chromatography (DHPLC)**

SSCP analysis was replaced by DHPLC analysis during the course of this study. DHPLC analysis was performed on an Helix™ DHPLC system (Varian Inc) according to the manufacturers’ standard operating procedures. The choice of method (small, medium or long) was determined empirically based on product size and estimated temperatures. Temperatures for successful resolution of heteroduplexes were calculated by the DHPLC Melt Program (Jones et al., 1999) available online (http://insertion.stanford.edu/melt.html) and were also experimentally determined. SSCP-negative samples were reassessed using DHPLC.
Sequencing

Any abnormally migrating band from SSCP analysis or heteroduplex from DHPLC analysis was subject to direct sequencing. This was performed on a second PCR sample following purification with Exonuclease I and shrimp alkaline phosphatase. Sequencing reactions were processed at SUPAMAC (Royal Prince Alfred Hospital) or Westmead Millenium Institute according to standard protocols. Mutation names are numbered with respect to the cDNA sequence as listed in GenBank (accession L13923.1) with the A of the ATG translation initiation start site as nucleotide +1. Intronic-exonic boundaries are as previously defined (Pereira et al., 1993).

Statistical Analysis

Clinical data and mutation results were stored in a customized database using FileMaker Pro 5.0 software (FileMaker, Inc.). This facilitated comparison of individual Ghent criteria with specific mutation categories (for example; exon/intron number, mutation type [substitution, deletion, insertion, duplication], mutation consequence [missense, nonsense, frameshift, splice site change], amino acid change, protein domain). Chi-square and logistical regression analyses were performed on exported data using Microsoft Excel 97 and SPSS for Windows v10.0.7, respectively.

RESULTS

*FBN1* mutation analysis was performed by SSCP and/or DHPLC on 57 unrelated individuals (mean age of 16 years) with typical MFS (49%), clinical features suggestive of MFS but not fulfilling the diagnostic criteria (23%) or other suspected type-1 fibrillinopathy (28%).

Screening of the *FBN1* gene uncovered 49 unique mutations (30 of which were novel) from the 57 patients examined, giving an overall mutation detection of 86%. Of the 49 identified mutations, 32 were screened and identified using SSCP and 17 by DHPLC (this included two mutations that were not detectable by SSCP). SSCP mutation detection was 90% in patients who met the Ghent criteria (26 out of 29 cases). This increased to 93% when considering only DHPLC analysis of Ghent-positive patients (14 out of 15 cases). The mutations were scattered throughout the *FBN1* sequence, with no statistically significant distribution. A summary of the genotype and clinical phenotype of the 49 patients with an identified mutation is shown in Table 1.

Of the 49 identified mutations, there were 38 substitutions (78%), 10 deletions (20%), and one duplication (2%). These mutations resulted in 28 missense coding changes (57%), nine frameshifts (18%), eight splice-site alterations (16%), and four premature termination codons (8%). The frameshifts were due to insertions and/or deletions which resulted in premature termination codons within a mean distance of 100 nucleotides.

Twenty-seven of the 49 mutations (55%) affected calcium-binding EGF modules. Twelve of these were substitutions involving the highly conserved cysteine residues, five were frameshifts (c.1469del1, c.1629_1633del5, c.2469delA, c.4179_4187del9, c.5613del), two were premature stop codons (c.4930C>T (p.Arg1644X), c.6577G>T, (p.Glu2193X)), and the remainder involved other types of mutation (c.3463G>A (p.Asp1155Asn), c.3973G>C (p.Glu1325Gln), c.4096G>A (p.Glu1366Lys), c.4270C>G (p.Pro1424Ala), c.5387G>A (p.Glu1796Glu), c.7754T>C (p.His2623Pro)) and an in-frame deletion at c.4179_4187del9 (p.Gly1394_Thr1396del).

The non-calcium-binding EGF-like domains were involved in five of the 49 mutations (10%), two of which were substitutions involving the conserved cysteine residues (c.461G>C (p.Cys154Ser), c.496T>A (p.Cys166Ser)). There were two frameshifts (c.365_367del2 (p.Arg122fsX6), c.1378dupT (p.Cys460fsX16)) resulting in premature termination within 6 and 16 codons, respectively. One patient had a substitution resulting in a nonsense mutation (c.510C>G, (p.Tyr170X)).

The LTBP domains were involved in seven of the 49 mutations (14%). Three of these were substitutions involving cysteine residues (c.2132G>A (p.Cys711Tyr), c.4691G>A (p.Cys1564Tyr), c.4588C>T (p.Arg1530Cys)), two were frameshifts (c.4702del1 (p.Lys1568fsX13), c.7167_7169del2 (p.Leu2389fsX16)) and two were substitutions involving other amino acids (c.2113G>A (p.Ala705Thr), c.3037G>A (p.Gly1013Arg)).

Hybrid motif 1 was found to harbor a mutation in one patient. The mutation involved a c.718C>T (p.Arg240Cys) substitution. The carboxy-terminal of fibrillin-1 also contained a mutation in one patient (c.8080C>T). This resulted in a nonsense mutation (p.Arg2694X).
### Table 1: Summary of *FBN1* Mutation Analysis and Patient Phenotype

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide Change</th>
<th>Type</th>
<th>Deduced RNA or Protein</th>
<th>Domain</th>
<th>Age (years)</th>
<th>Skeletal</th>
<th>Ocular</th>
<th>CVS</th>
<th>Pulm</th>
<th>Skin</th>
<th>Dura</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>c.365_366del2A*</td>
<td>Del/Splice</td>
<td>r.spl? intronic</td>
<td>c.365_366del2A*</td>
<td>35</td>
<td>9,11,16,17</td>
<td>EL</td>
<td>diA,MVP</td>
<td>SA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>c.461G&gt;C*</td>
<td>Sub/Miss</td>
<td>p.Cys154Ser</td>
<td>EGF 3</td>
<td>14</td>
<td>7,9,16,17</td>
<td>EL</td>
<td>diA,MVP</td>
<td>SA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>c.718C&gt;T*</td>
<td>Sub/Miss</td>
<td>p.Arg204Cys</td>
<td>hybrid 1</td>
<td>33</td>
<td>EL</td>
<td>diA, MVP</td>
<td>DE</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td>c.137C&gt;G</td>
<td>Dup/Pr</td>
<td>p.Cys406X16</td>
<td>EGF 4</td>
<td>26</td>
<td>8,9,11,13,16,18</td>
<td>disA</td>
<td>SA,H</td>
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<tr>
<td>14</td>
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<td>Sub/Miss</td>
<td>p.Asp1091X26</td>
<td>cbEGF 19</td>
<td>31</td>
<td>7,9,11,15,16,18</td>
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<td>diA,MVP</td>
<td>H</td>
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<tr>
<td>26</td>
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<td>cbEGF 28</td>
<td>28</td>
<td>7,9,11,15,16,18</td>
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<tr>
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<td>cbEGF 26</td>
<td>26</td>
<td>7,9,11,15,16,18</td>
<td>EL</td>
<td>diA,MVP</td>
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<tr>
<td>33</td>
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<td>cbEGF 29</td>
<td>29</td>
<td>7,9,11,15,16,18</td>
<td>EL</td>
<td>diA</td>
<td>SA</td>
<td></td>
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<tr>
<td>46</td>
<td>c.5838C&gt;T</td>
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<td>p.Leu2389X16</td>
<td>LTBP 18</td>
<td>18</td>
<td>7,9,11,15,16,18</td>
<td>EL</td>
<td>diA,MVP</td>
<td>SA</td>
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<tr>
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<td>26</td>
<td>7,9,11,15,16,18</td>
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<td>diA,MVP</td>
<td>P DE</td>
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<td>p.Asp2442Glu</td>
<td>cbEGF 31</td>
<td>31</td>
<td>7,9,11,15,16,18</td>
<td>EL</td>
<td>diA</td>
<td>SA</td>
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<td></td>
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<tr>
<td>52</td>
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<td>p.Glu2946X</td>
<td>C-term 22</td>
<td>22</td>
<td>7,9,11,15,16,18</td>
<td>EL</td>
<td>diA,MVP</td>
<td>P SA</td>
<td></td>
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</tr>
</tbody>
</table>

**Summary of *FBN1* mutation analysis and clinical phenotype according to the Ghent nosology.** Novel mutations are indicated with an asterisk in the ‘Nucleotide Change’ column. AA=amino acid, CVS=cardiovascular system, Pulm=pulmonary system, Del=deletion, Fr=frameshift, Sub=substration, Dup=duplication, Stop=nonsense mutation, Miss=missense mutation, Splice=splice site mutation, cb=calcium-binding, EGF=epidermal growth factor-like, hybrid=hybrid motif, LTBP=latent transforming growth factor β1 binding protein, C-term=carboxy terminal. *Submitted to www.umd.necker.fr, Submitted to Am. J. Med. Genet., Adès et al., 1996, Collod-Beroud et al., 1998, Gibson et al., 1998, Adès et al., 2002, pectus carinatum, pectus excavatum requiring surgery, reduced upper:lower or increased arm-span:height ratio, positive wrist and thumb signs, scoliosis (>20°), reduced elbow extension (<170°), pes planus, proutrosia acetabulae, pectus excavatum of moderate severity, joint hypermobility, highly arched palate with dental crowding, characteristic facial appearance, EL=ectopia lentis, ILG=increased axial length of globe, MVP=mitral valve prolapse, desA=descending aorta dilation or dissection, P=spontaneous pneumothorax, SA=striae atrophica, H=recurrent or incisional inguinal/umbilical herniae, DE=dumbrosural dural ectasia.

*Mutation names are numbered with respect to the cDNA sequence as listed in GenBank (accession L13923.1) with the A of the ATG translation initiation start site as nucleotide +1. Intron-exon boundaries are as previously defined (Pereira et al., 1993).
Eight of the 49 mutations (16%) were found to be intronic and had a predicted effect on splice-site function. Seven of these were single nucleotide substitutions that resulted in a predicted loss of splice acceptor site in three cases and loss of splice donor site in the other four. The final splice site mutation was a deletion of five nucleotides in IVS40 (c.5065+3_5065+7del5) removing the predicted splice donor site.

Four of the 57 patients were diagnosed with neonatal MFS. Their identified mutations were located within exon 25 (c.3163T>G), intron 29 (c.3712+1G>C), intron 30 (c.3838+3A>T) and exon 33 (c.4179_4187del9). A total of eight patients in this study had no detectable FBN1 mutation despite clinical suggestions of a fibrillinopathy. One of these patients met the Ghent diagnostic criteria due to having a father who independently met the criteria. Another patient had Shprintzen-Goldberg syndrome (SGS) as characterized by craniosynostosis, mental retardation and a marfanoid habitus. A FBN1 mutation was not identified in this individual.

Statistical analysis of patient clinical information and mutation screening results was undertaken in an attempt to elucidate genotype-phenotype correlations. Patients harboring an identifiable FBN1 mutation were nearly twice as likely to have cardiovascular complications (aortic root dilation or mitral valve prolapse) as those in whom a mutation was not identified (p<0.05, relative risk 1.9). Patients with a detectable mutation were more than four-times as likely to have ectopia lentis as those without a detectable FBN1 mutation (p<0.05, relative risk 4.6). The prevalence of ectopia lentis was higher in patients whose mutations involved cysteine substitutions (p<0.05, relative risk 1.6) and lower in patients with premature termination mutations (p<0.05, relative risk 0.4).

DISCUSSION

Since the first report identifying a causative FBN1 mutation in MFS (Dietz et al., 1991) there have been in excess of 500 mutations described (www.umd.necker.fr). The majority of these mutations are unique to the affected families.

Of the 57 probands in this study, 28 patients (49%) fulfilled the clinical criteria according to the Ghent nosology (De Paepe et al., 1996) prior to mutation screening, 13 patients (23%) met the criteria following detection of an FBN1 mutation and 16 patients (29%) did not fulfill the Ghent criteria. Of the 16 patients who failed to meet the diagnostic criteria, nine (56%) were found to harbor an FBN1 mutation. This can be explained partially by the fact that seven of the nine patients were in the pediatric age group. These data emphasize the importance of a high index of clinical suspicion when referring for FBN1 mutation testing.

Mutation analysis by SSCP and DHPLC resulted in the detection of 49 unique mutations from the cohort of 57 probands, giving an overall mutation detection of 86%. The detection for SSCP analysis of patients who met the Ghent criteria was 90%. This increased to 93% when one considered only DHPLC testing of Ghent-positive patients. Although this study was not designed to formally compare SSCP and DHPLC, these data indicate that DHPLC was a superior screening strategy. Of the 49 identified mutations, 30 (61%) were novel. Recent studies have reported a wide range in the prevalence of novel mutations from 14% (Rommel et al., 2002) to 60% (Katzke et al., 2002).

The 49 mutations detected were presumed causative because they fulfilled at least one of the following criteria: (1) involvement of a highly conserved cysteine residue, (2) nonsense mutation, (3) frameshift mutation, or (4) predictive involvement of splice site according to a neural network based program (www.fruitfly.org/seq_tools/splice.html). These types of mutations have all previously been shown to alter the structure or biochemical characteristics of fibrillin-1 (reviewed in Robinson et al., 2002). In addition to these criteria, pedigree analysis was performed on 17 available families and the putative mutation was found to co-segregate with the disease phenotype. The remaining five putative mutations that did not satisfy the above criteria were confirmed to be absent from 100 normal alleles (50 individuals).

A majority of the mutations identified in this study were substitutions (78%), with most of the mutations occurring within the calcium-binding EGF-like domains. These data are consistent with previously published studies (Katzke et al., 2002; Robinson et al., 2002). The mutations detected occurred throughout the entire length of FBN1 and were not statistically clustered around any mutational ‘hot-spot’. Previous studies have shown that the mutations of the early-onset severe disease phenotype of nMFS cluster around exons 24-32 (Booms et al., 1999, Putnam et al., 1996). Our study included four patients with nMFS, three of whom had mutations within this region (c.3163T>G, c.3712+1G>C, c.3838+3A>T). The fourth carried a novel deletion mutation in exon 33 (c.4179_4187del9). This contributes to the growing data in the literature of nMFS mutations outside the predicted region (Loeys et al., 2001).
Genotype-phenotype analyses revealed that patients with an identified \textit{FBN1} mutation were more likely to develop ectopia lentis or cardiovascular complications (dilatation or dissection of the ascending aorta) compared to patients without a detectable mutation. The prevalence of ectopia lentis was found to be higher in patients with cysteine substitution mutations and lower in patients with premature termination mutations. Similar correlations have been suggested previously (Loeys et al., 2001; Schrijver et al., 1999; Schrijver et al., 2002) and these data collectively highlight the importance of these correlations in anticipating the clinical consequence of specific \textit{FBN1} mutations. Further statistical analysis revealed no additional significant genotype-phenotype correlations at this stage but it is possible that analysis of larger patient cohorts will yield further useful information. It is also important to fully investigate patients as failure to perform specific tests (e.g. for dural ectasia and protrusio acetabulae) may mask potential genotype-phenotype correlations. Of the 16 patients who did not meet the Ghent criteria in this study, none were investigated for dural ectasia. Based on the clinical data obtained for these patients, at least five of them could potentially be reclassified as Ghent-positive if dural ectasia was present. This illustrates the importance of close liaison with referring clinicians in order to ensure comprehensive investigation of patients.

Knowledge about \textit{FBN1} mutations is important for early diagnosis, management and genetic counseling. The correct diagnosis of MFS has important implications with regard to appropriate cardiovascular and ophthalmologic follow-up. The use of a comprehensive database containing genotype-phenotype correlations may help to anticipate particular complications and assist clinicians in decisions relating to tailor-made patient follow-up.

Clinical diagnosis of MFS may be difficult due to the wide spectrum of disease phenotype – especially since several features of MFS can occur in the general population or in other diseases (Pyeritz, 2000). A confident diagnosis of MFS is often difficult in children due to the evolving nature of the phenotype (Lipscomb et al., 1997).

DHPLC has a proven track record for mutation detection as exemplified by its widespread use in the diagnosis of haemophilia (O’Donovan et al., 1998), cystic fibrosis (Le Marechal et al., 2001) and breast cancer (Eng et al., 2001). DHPLC is routinely used for the detection of point mutations or small insertions/deletions but despite its high sensitivity and specificity, it is not suited for the detection of large insertions/ deletions (Mátyás et al., 2002). This may be the reason for the apparent absence of an \textit{FBN1} mutation in at least one of our eight patients who screened mutation-negative by DHPLC and fulfilled the Ghent criteria. It is important to note 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. We are currently developing alternative cDNA and biochemical diagnostic strategies to further elucidate the nature of some of these elusive mutations.

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