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## Chromosomal assignment of a second locus for Marfan Syndrome To Chromosome 3p24.2-p25.

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## Summary

Marfan syndrome (MFS) is an autosomal dominant connective-tissue disorder. It involves predominantly three systems (skeletal, ocular, and cardiovascular systems) and is characterized by highly variable expressivity. The diagnosis relies solely on clinical criteria requiring anomalies in at least two systems. By excluding the disease locus fibrillin 1 (FBN1) in a large French family with typical cardiovascular and skeletal anomalies, we raised the issue of genetic heterogeneity in MFS and the implication of a second locus (MFS2). Linkage analyses, performed in this family with dispersed anonymous DNA markers, have localized MFS2 to a region of less than 9 cM between *D3S1293* and *D3S1283* which map at 3p24.2-p25. In this region the highest lod score was obtained for marker *D3S2336* at 4.89 ( $\theta=0.05$ ). LINKMAP analyses indicated that the most probable position for MFS2 was at *D3S2335* (multipoint lod score in log base 10 = 5.65).

## Introduction

Marfan Syndrome (MFS) was the founding member of the "heritable disorders of connective tissue"<sup>1</sup>. The cardinal features of this autosomal dominant syndrome (estimated incidence of 1/10,000) are prominently observed in three systems: skeletal, ocular and cardiovascular<sup>2</sup>. These pleiotropic manifestations are associated with great intra- and interfamilial variability that account for complete (or classic) and incomplete (or variant) forms of the syndrome in which only two of the systems are affected. The diagnosis of MFS is entirely clinical and still difficult in some cases despite the guidelines that were set forth in the Berlin nosology<sup>3</sup>. Quickly after Kainulainen et al. mapped the classic form of MFS to 15q15-q21.3 (ref 4,5), it was shown that this chromosomal area harbored the FBN1 gene that encodes fibrillin, the major component of connective tissue microfibrils<sup>6</sup>. FBN1 was definitely implicated by the detection of tight linkage<sup>6,7</sup> followed by the identification of several mutations in MFS patients<sup>7,8,9,10</sup>. From then on, the concept of genetic heterogeneity was set

aside although it had always been suspected to explain the great clinical variability of the syndrome. However, *FBN1* mutations have been identified in only a fraction of MFS patients despite extensive screening. Furthermore, the investigation of biosynthesis and extracellular deposition of fibrillin in cultured MFS fibroblasts failed to detect any anomaly in approximately 7% of the patients<sup>11</sup>. Despite high cumulated lod scores, these observations show that the issue of genetic heterogeneity should not be disregarded in MFS.

We have been investigating a large family of more than 170 subjects, originating from the south of France, with a connective-tissue disorder. The autosomal dominant phenotype segregating in this family associates anomalies in two systems, namely the skeleton (tall stature, arm span greater than height, arachnodactyly, scoliosis, and pectus) and the heart and the aorta (mitral valve prolapse, aortic dilation, aortic dissection or rupture). However, none of the ocular features observed in classic MFS (myopia, ectopia lentis) are documented in the family<sup>12,13</sup>. These features are thus characteristic of an incomplete form of Marfan syndrome<sup>2,3</sup> and this was the initial diagnosis<sup>12</sup> since several affected subjects fulfilled the Berlin criteria. This family was included in the panel of families that were studied by the International Marfan Syndrome Consortium to map the MFS gene<sup>14,15</sup>. The diagnosis of MFS went thus unchallenged until we excluded linkage between the family phenotype and the *FBN1* and *FBN2* fibrillin genes<sup>13</sup> that are involved in MFS<sup>6,7</sup> and congenital contractural arachnodactyly (CCA)<sup>6</sup> respectively. At that time, the clinical features of all family members were scrutinized and a controversy arose because of divergent interpretation of the Berlin criteria. A compromise was reached and the terminology "Marfan-like" was provisionally adopted<sup>13,16,17,18</sup>. Since this terminology has been justifiably disputed because clinical findings in the majority of individuals in this family are identical to those observed in conventional Marfan syndrome<sup>16</sup> and since several MFS patients displaying no anomaly in fibrillin have been reported<sup>11</sup> the implication of a second locus (MFS2) could no longer be eluded. Therefore, recognition of genetic

heterogeneity in MFS prompted us to attempt localization of MFS2 through exclusion mapping in this single exceptional family.

## Results

### Exclusion map and 3p assignment

One hundred forty four genetic markers from 22 autosomes were tested for linkage to the MFS2 locus. These (AC)<sub>n</sub> microsatellite markers spanning the human genome were chosen on two criteria: their informativity and an average distance of 20 centiMorgans (cM) between adjacent markers<sup>19</sup>. Each locus was tested for linkage to MFS2 with the MLINK program<sup>20</sup>. The combined data were also analyzed with the EXCLUDE program<sup>21</sup>: a non-overlapping exclusion zone of at least 2685 cM, corresponding to 93.07% of the total genome, was established from the cumulative exclusion intervals for each marker. A strong suspicion for linkage with MFS2 was given first by marker *D3S1300* with a maximum lod score of 2.53 at  $\theta=0.17$ . This was the only locus investigated that gave a lod score higher than 1.0. For this reason, the EXCLUDE analysis indicated that the most probable position for the MFS2 locus was on chromosome 3 with a probability of 100%.

The linked marker *D3S1300* maps at 3p21 (ref 22) that also harbors a collagen gene: *COL7A1*. Mutations in this gene are associated with Epidermolysis Bullosa (EB)<sup>23,24</sup>. Although the clinical features and histologic alterations of MFS are very different from those observed in EB, we investigated a possible identity between *COL7A1* and the disease locus, since they both map to 3p21. Two intragenic RFLPs, PvuII<sup>25</sup> and AluI<sup>26</sup>, and a closely linked anonymous marker, *D3S2* (ref 27), were studied after PCR amplification. The AluI polymorphism was non-informative and the PvuII marker showed a maximum lod score of 1.58 (at  $\theta=0$ ). However, the MspI polymorphism at the *D3S2* locus showed two obligate recombinants (IV51 and IV54). Thus, the involvement of the *COL7A1* gene was excluded.

### Microsatellite markers confirm and refine localization on 3p24.2-p25

To better define the localization of MFS2, 10 polymorphic markers proximal and distal to *D3S1300*, and spanning a region of 54 cM, were studied: tel-*D3S1263*, *D3S1286*, *D3S1266*, *D3S1277*, *D3S1289*, *D3S1261*, *D3S1284*, *D3S1274*, *D3S1276*, *D3S1281*-cen. Table 2 summarizes the pairwise lod scores for these loci. Negative lod scores were obtained for the markers proximal to *D3S1300*. Among the distal markers, *D3S1286* and *D3S1266* gave the highest lod score values of 3.04 (at  $\theta=0.10$ ) and 2.47 (at  $\theta=0.07$ ) respectively. We then investigated a region of 22 cM surrounding these two markers and studied 11 other loci: tel-*D3S2338*, *D3S1293*, *D3S1599*, *D3S2336*, *D3S1583*, *D3S1567*, *D3S2335*, *D3S2337*, *D3S1283*, *D3S1609*, *D3S1619*-cen. Positive lod scores were observed for several markers (Table 2). The highest lod score was 4.89 at  $\theta=0.05$  for marker *D3S2336*. Markers *D3S1293* and *D3S1283* were mapped with somatic cell hybrids (R158 and GM11752 (3;21)) to 3p24.2-p25 (ref 22).

Thirteen-marker haplotypes were constructed (from tel-*D3S1286* to *D3S1619*-cen) (marker order is shown in figure 1). Within these, a unique 6 marker sub-haplotype (from tel-*D3S1599* to *D3S2337*-cen) was found unrecombined in all affected individuals (figure 2). This haplotype is defined by two obligate recombinants identified for loci *D3S1293* (subject IV55) and *D3S1283* (subject IV54). These data assign MFS2 to an area of less than 9 cM in the subdistal region of 3p. Surprisingly, the 6-marker haplotype is also carried by subjects IV44 and IV86 who had always been considered as "unaffected" in the linkage analyses. This observation revealed that the penetrance of the disease gene was not complete with the clinical criteria that had been used for the classification of family members. To refine the most probable position of the disease locus, LINKMAP analyses were performed. Since erroneous assumption of penetrance has a strong effect on  $\theta$  (ref. 28), these analyses were carried out with a penetrance of 0.89 estimated from the family data. These analyses (Figure 3) showed that the most probable position of the MFS2 gene was at *D3S2335* (multipoint lod score in log base 10 = 5.65) thus

confirming the regional assignment. However, since lod score variations are slight between *D3S1599* and *D3S2335*, all the positions between these two loci are almost equally likely.

## Discussion

We have located in 3p24.2-p25 a second gene involved in MFS by exclusion mapping. The data from a single family place the gene between two recombinational events that define the disease haplotype in the family. Although the clinical features are identical to those observed in MFS, this diagnosis could not be made in all affected individuals using recognized criteria<sup>3,13</sup>. Therefore patient status was established independently by two of us (G. J. and O. D.) with regard to the actual clinical follow-up. Furthermore, to avoid spurious recombination in the genetic analyses, 6 patients and two of their parents were scored unknown<sup>13</sup>. Linkage analyses were thus performed under very conservative but not powerful conditions. Despite these unfavorable conditions, a maximum lod score of 4.89 (at  $\theta=0.05$ ) was obtained with marker *D3S2336* (Table 1), therefore demonstrating conclusive linkage. Careful examination of regional markers showed that two individuals (IV44 and IV86) scored as "unaffected", carried the disease-related haplotype. Since it is highly unlikely that these two subjects are both double recombinants, they reveal instances of low or non expressivity. IV44 is an adult female who only presented isolated minor skeleton anomalies. IV86, 13 years old when he was first examined, showed no clinical alteration<sup>13</sup>. These subjects show that the penetrance is not complete in adults, in contrast with classic Marfan syndrome, but also age-dependent. Interestingly, the 6-marker haplotype is also found in all the subjects for whom no definite diagnosis could be made. These patients (IV16, IV30, IV37, IV84 and IV88) were always genotyped and scored as "unknown" in the linkage analyses, in order to avoid spurious results due to misclassification. It must be noted that the clinical findings in these 5 individuals are unchanged since the initial publication<sup>13</sup>.

For the purpose of genetic counselling, all these subjects must now be considered as carriers of the disease gene.

Our data provide conclusive evidence for the assignment of a second locus for MFS to chromosome 3p24.2-p25. Positioning of the disease locus in relation to the genetic linkage map indicates that MFS2 is located within a 9 cM interval flanked by *D3S1293* and *D3S1283*. These microsatellite markers map to a region that contains 5 cloned genes<sup>22</sup>: *THRB* (Thyroid Hormone Receptor Beta), *RAF* (murine leukemia viral oncogene homolog 1), *VHL* (von Hippel-Lindau disease tumor suppressor gene), *RARB* (Retinoic Acid Receptor Beta) and *ATP2B2* (ATPase, Ca<sup>++</sup> transporting). None of these genes are likely candidates for MFS2. Furthermore, close examination of homologous loci in mouse (murine chromosomes 6 (ref 29), 9 (ref 30), and 16 (ref 31) failed to reveal any disease or developmental abnormality that could be related to the Marfan phenotype. Therefore, the assignment of MFS2 to 3p24.2-p25 opens the avenue to isolating the disease gene by the techniques of positional cloning.

For a long time, the extreme clinical variability of MFS had been attributed to an underlying genetic heterogeneity. To test this hypothesis, three heterogeneity tests were performed: two with the anonymous chromosome 15 markers initially reported as linked to the MFS locus<sup>5,15</sup> and another with the *FBN1* markers<sup>32</sup>. Kainulainen et al.<sup>5</sup> and Tsiouras et al.<sup>32</sup> found no evidence for genetic heterogeneity in a sample of 17 and 28 families respectively. Conversely, Sarfarazi et al.<sup>15</sup> detected genetic heterogeneity in a sample of 22 families that included our French family. However, no heterogeneity was detected among the 21 remaining pedigrees. Therefore, heterogeneity went undetected in a sample of 45 families (since the families reported by Sarfarazi et al. were part of the sample reported by Tsiouras et al.). Despite these findings, the question of genetic heterogeneity of MFS has not been completely settled not only because of the debate over the terminology that should be adopted for the French family's phenotype but also because of the identification of a second MFS family displaying recombination with

the *FBN1* locus (L. Peltonen, personal communication). All the above indicate the existence of a small level of heterogeneity that was previously undetected. With the mapping of MFS2, the issue should be addressed again, not only because admixture tests will be more powerful since two candidate loci (*FBN1* and MFS2) can now be simultaneously tested but also in the interest of patients awaiting unequivocal molecular diagnosis for genetic counselling. Finally, the involvement of MFS2 should also now be tested in other disorders overlapping MFS. Among these, ectopia lentis and CCA have been mapped to *FBN1* and *FBN2* respectively. However, mitral valve prolapse<sup>33</sup>, familial forms of annulo-aortic ectasia<sup>34</sup> and the MASS (mitral valve, aorta, skeleton, and skin) phenotype<sup>35</sup> are still orphan syndromes. In all these diseases, the cardiovascular manifestations strongly overlap the phenotype of the French family. Therefore the implication of this locus should be investigated.

## Methodology

### Patients and clinical evaluation

This large French family (see figure 2)<sup>12</sup> was ascertained following the death of a 39 year old male subject from aortic dissection. A family investigation was undertaken. Subjects at risk underwent careful physical examination, echocardiography, and slit-lamp examination. Skeletal findings included arachnodactyly, narrow arched palate, pectus excavatum, scoliosis, increased lower-upper body segment ratio, tall stature, and increased arm span. Cardiovascular features were mitral valve prolapse associated with holosystolic mitral regurgitation, tricuspid valve prolapse, and dilatation of the ascending aorta. None of the affected subjects examined had ectopia lentis. Twenty members who showed major cardiovascular or skeletal manifestations and were first-degree relatives of an affected member were considered as definitely affected. Seventeen family members with no abnormality in any of the systems and 12 subjects who presented with isolated minor skeletal or cardiovascular findings, were considered unaffected.

Finally no diagnosis could be made for 6 patients who showed minor skeletal anomalies and/or borderline aortic dilation or mitral valve prolapse and regurgitation. These subjects were scored as "unknown" in the genetic analyses. Complete individual clinical features are listed in <sup>13</sup>.

#### DNA analysis and PCR amplification

Blood samples were collected from 59 family members and DNA was isolated according to a method described elsewhere<sup>36</sup>.

All PCR amplifications were performed under the following standard conditions. Forty ng DNA from each patient were used as template. The PCR was carried out in a final volume of 50  $\mu$ l in a microtiter plate, using the Techne PMC3 thermocycler (Cambridge, England) or a thermocycler able to coamplify 16 microtiter plates simultaneously (IAS Products Inc.). The reaction included 5  $\mu$ l of 10 X buffer (50 mM KCl, 10 mM Tris HCl pH 9, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton and 0.01% gelatine), 50 pmol of each primer and 31 mM each of dATP, dTTP, dGTP and dCTP. Samples were overlaid with 20  $\mu$ l of light mineral oil to prevent evaporation. After an initial "hot start" at 96°C for 5 min, 1 unit of Taq DNA polymerase (NBL) was added to each tube, then 35 cycles consisting of denaturation at 94°C (40s) and annealing-elongation at 55°C (30s) were carried out followed by an extension step at 72°C for 2 min. Aliquots from 16 PCR reactions from given DNA samples were pooled, precipitated, and resuspended in 5  $\mu$ l of 0.1X TE and 12.5  $\mu$ l of sequencing dye. Finally, they were loaded onto a 6% denaturing polyacrylamide DNA sequencing gel. Electrophoresis was performed for 3 to 5 h at 40 to 50 mA at 3000V. After transfer on Hybond N+ membranes, each forward primer was labelled using terminal transferase (Boehringer). Hybridization was performed at 42°C overnight in the AMASINO medium<sup>37</sup>. Autoradiography was carried out after the membranes were washed twice in 2XSSC, 0.1% SDS at room temperature.

#### Linkage analysis

Pairwise and multipoint analyses were performed using the MLINK and LINKMAP subprograms of the LINKAGE package<sup>20</sup> assuming an autosomal dominant disease gene with a frequency of 0.00002 and equal female to male recombination rates. In the MLINK analyses, the penetrance value used was 1.0 since there was no evidence of skipped generations in the pedigree and since very conservative diagnostic criteria were used. Allele frequencies were calculated from the study of unrelated members of the family. In the LINKMAP analyses, the penetrance value was inferred from family data and was set at 0.89. Furthermore, the allele systems were reduced.

The EXCLUDE program<sup>21</sup> was used to produce the exclusion map. This program estimates 1) the positional likelihood of the disease locus on each chromosome, 2) the percentage of probability of a locus to be on any of the 22 autosomes.

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