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Marfan syndrome (MFS) is an autosomal dominant connective-tissue disorder characterized by skeletal, ocular and cardiovascular defects of highly variable expressivity. The diagnosis relies solely on clinical criteria requiring anomalies in at least two systems. By excluding the chromosome 15 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, have localized *MFS2* to a region of 9 centiMorgans between *D3S1293* and *D3S1283*, at 3p24.2–p25. In this region, the highest lod score was found with *D3S2336*, of 4.89 (θ=0.05). By LINKMAP analyses, the most probable position for the second locus in MFS was at *D3S2335*.

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Marfan Syndrome (MFS) was the founding member of the "heritable disorders of connective tissue"[1]. The cardinal features of this autosomal dominant syndrome (estimated incidence of 1/10,000) are mostly observed in three systems: skeletal, ocular and cardiovascular[2]. These pleiotropic manifestations are associated with considerable 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[3]. Shortly after Kainulainen *et al.* mapped the classic form of MFS to chromosome 15q15–q21.3 (refs 4,5), this region was shown to contain the gene for fibrillin (*FBN1*), the major component of connective tissue microfibrills[6]. *FBN1* was definitely implicated by the detection of tight linkage[6,7] and the identification of several mutations in MFS patients[7–10]. From then on, the concept of genetic heterogeneity was set aside although it had always been suspected to explain the clinical variability of the syndrome. However, 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 fails to detect any anomaly in approximately 7% of MFS patients[11]. These observations show that the issue of genetic heterogeneity has not been addressed properly.

We have been investigating a large family, originating from the south of France, of more than 170 subjects 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 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[12,13]. These features are thus characteristic of an incomplete form of Marfan syndrome[2,3] indeed, this was the initial diagnosis[12] as several affected family members fulfilled the Berlin criteria. This family was included in the panel of families that were studied by the Marfan Syndrome Consortium to map the MFS gene[14,15]. The diagnosis of MFS thus went unchallenged until we excluded linkage between the family phenotype and the *FBN1* and *FBN2* fibrillin genes[13] that are involved in MFS[6,7] and congenital contractural arachnodactyly (CCA)[6], respectively. At that time, the clinical features of all family members were scrutinized and a controversy arose because of varying interpretations of the Berlin criteria. A compromise was reached and the terminology "Marfan-like" was provisionally adopted[13,16–18]. However, as this terminology has been disputed, because clinical findings in the majority of individuals in this family are identical to those observed in conventional Marfan syndrome[16], and since several MFS patients displaying no anomaly in fibrillin have been reported[11], the likelihood of a second locus (*MFS2*) remained a distinct possibility. In recognition of this evident genetic heterogeneity in MFS, we set out to localize *MFS2* through linkage analysis and exclusion mapping in this single exceptional family.

Exclusion map and 3p assignment

144 genetic markers from 22 autosomes were tested for linkage to the *MFS2* locus. These $(AC)_n$ microsatellite

Table 1 Pairwise lod scores for chromosome 3 markers and *MFS2*

Locus	Distance[a]	Lod scores at recombination fractions (θ) 0.00	0.001	0.01	0.05	0.1	0.2	0.3	0.4	Z_{max}	θ_{max}
D3S1263		−∞	−4.42	−1.47	0.40	1.02	1.28	1.08	0.6	1.28	0.18
D3S1286	0.12	−∞	−0.39	1.59	2.81	3.04	2.69	1.94	1.01	3.04	0.10
D3S2338	0.01	−∞	−4.68	−0.77	1.61	2.27	2.31	1.75	0.91	2.41	0.15
D3S1293	0.01	−∞	−1.27	0.69	1.87	2.17	2.03	1.50	0.76	2.19	0.12
D3S1599	0.01	0.71	0.71	0.69	0.63	0.54	0.37	0.21	0.08	0.71	0.00
D3S2336	0.02	−∞	3.55	4.50	4.89	4.71	3.87	2.70	1.32	4.89	0.05
D3S1567	0.03	1.50	1.49	1.47	1.37	1.23	0.94	0.62	0.31	1.50	0.00
D3S1583	0	−0.53	−0.53	−0.47	−0.30	−0.18	−0.05	0.01	0.02	–	–
D3S2335	0	−∞	3.24	4.15	4.47	4.27	3.48	2.45	1.22	4.47	0.04
D3S2337	0	−∞	3.24	4.16	4.47	4.27	3.48	2.43	1.20	4.47	0.04
D3S1283	0.02	−∞	1.07	2.97	3.94	3.99	3.38	2.40	1.19	4.02	0.07
D3S1266	0	−∞	1.07	2.01	2.46	2.42	1.97	1.32	0.60	2.47	0.06
D3S1609	0.02	2.98	2.97	2.92	2.70	2.41	1.81	1.19	0.58	2.98	0.00
D3S1619	0.07	−∞	−3.58	−0.64	1.15	1.66	1.71	1.29	0.64	1.77	0.14
D3S1277	0.01	−∞	−5.65	−2.70	−0.79	−0.15	0.22	0.17	0.003	–	–
D3S1289	0.11	−∞	−11.88	−6.76	−3.05	−1.52	−0.25	0.19	0.23	–	–
D3S1300	0.12	−∞	−6.50	−1.61	1.41	2.29	2.48	1.92	0.99	2.53	0.16
D3S1261	0.19	−∞	−11.21	−6.21	−2.74	−1.34	−0.20	0.20	0.23	–	–
D3S1284	0.08	−∞	−8.62	−4.64	−1.94	−0.88	−0.05	0.21	0.20	–	–
D3S1274	0.07	−∞	−9.44	−5.46	−2.76	−1.66	−0.68	−0.24	−0.04	–	–
D3S1276	0.01	−∞	−10.92	−5.94	−2.54	−1.18	−0.06	0.31	0.30	–	–
D3S1281	0.13	−∞	−19.81	−11.85	−6.41	−4.18	−2.12	−1.04	−0.39	–	–

[a]Distance between two adjacent markers in recombination fraction.

markers spanning the human genome were chosen on two criteria: heterozygosity and spacing (an average distance of 20 centiMorgans (cM) between adjacent markers[19]). Each locus was tested for linkage to *MFS2* using the MLINK program[20]. The combined data were also analysed with the EXCLUDE program[21]: a non-overlapping exclusion zone of at least 2,685 cM, corresponding to 93% of the genome, was established from the cumulative exclusion intervals for each marker. A strong suspicion for linkage with *MFS2* was provided first with marker *D3S1300*, with a maximum lod score of 2.53 at θ=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 *MFS2* was on chromosome 3, with a probability of 100%.

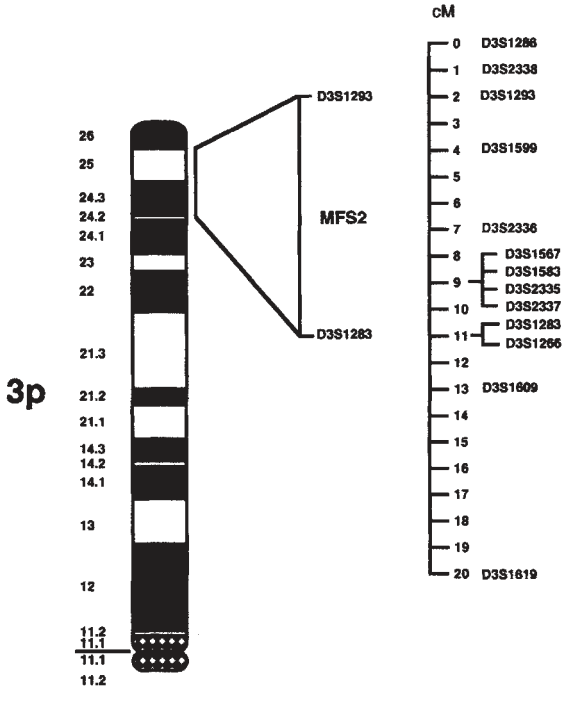

Fig. 1 The relative physical locations of the *MFS2* disease gene and 13 regional markers on the short arm of chromosome 3.

The linked marker *D3S1300* maps at 3p21 (ref. 22) that also harbours a collagen gene, *COL7A1*. Mutations in this gene are associated with epidermolysis bullosa (EB)[23,24]. 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. Two intragenic RFLPs, *Pvu*II[25] and *Alu*I[26], and a closely linked anonymous marker, *D3S2* (ref. 27), were studied after PCR amplification. The *Alu*I polymorphism was non-informative and the *Pvu*II marker showed a maximum lod score of 1.58 (at θ=0). However, the *Msp*I polymorphism at the *D3S2* locus showed two obligate recombinants (IV51 and IV54). Thus, the involvement of *COL7A1* was excluded.

Refined localization on 3p24.2–p25

To refine the localization of *MFS2*, ten polymorphic markers proximal and distal to *D3S1300*, and spanning 54 cM, were studied: tel–*D3S1263*, *D3S1286*, *D3S1266*, *D3S1277*, *D3S1289*, *D3S1261*, *D3S1284*, *D3S1274*, *D3S1276*, *D3S1281*–cen (ref. 19). Table 1 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 θ=0.10) and 2.47 (at θ=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 (Table 2 and ref. 19). Positive lod scores

were observed for several markers (Table 1). The highest lod score was 4.89 at θ=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) (Fig. 1). Within these, a unique six marker sub-haplotype (from tel–*D3S1599* to *D3S2337*–cen) was found intact in all affected individuals (Fig. 2). This haplotype is defined by two obligate recombinants identified for *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 six-marker haplotype is also carried by subjects IV44 and IV86 who had always been considered as "unaffected" in the linkage analyses. This observation reveals that the penetrance of the disease gene is 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. As erroneous assumption of penetrance has a strong effect on θ (ref. 28), these analyses were carried out with a penetrance of 0.89 estimated from the family data. These data (Fig. 3) show that the most probable position of *MFS2* is 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 a second gene involved in MFS (*MFS2*) on chromosome 3p24.2–p25. The data from our 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[3,13]. 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, six patients and two of their parents were scored unknown[13]. Linkage analyses were thus performed under very conservative but not powerful conditions. Nevertheless, a maximum lod sore of 4.89 (at θ=0.05) was

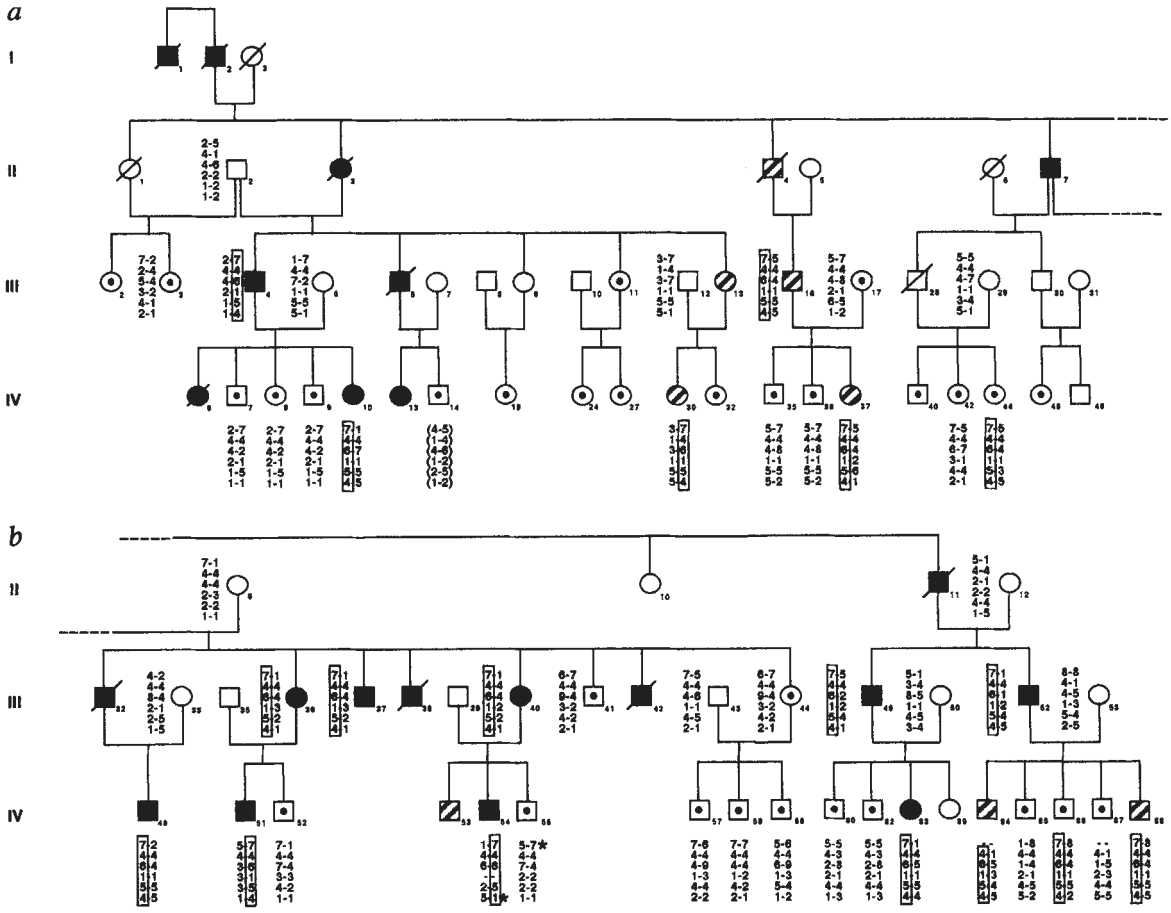

Fig. 2 Segregation of chromosome 3p24.2–p25 markers in the *MFS* kindred. (Note that (*a*) and (*b*) represent different parts of a single pedigree; (*b*) is the rightward extension of (*a*)). Haplotypes (top to bottom) at tel–*D3S1293*, *D3S1599*, *D3S2336*, *D3S2335*, *D3S2337*, *D3S1283*–cen markers are shown for each family member tested. Closed symbols, affected members; open symbols, unaffected spouses or unexamined family members; open symbols with dot, members considered unaffected; hatched symbols, members having an unknown phenotypic status; /, deceased; and *, obligate recombinants with markers *D3S1293* (subject IV55) and *D3S1283* (subject IV54).

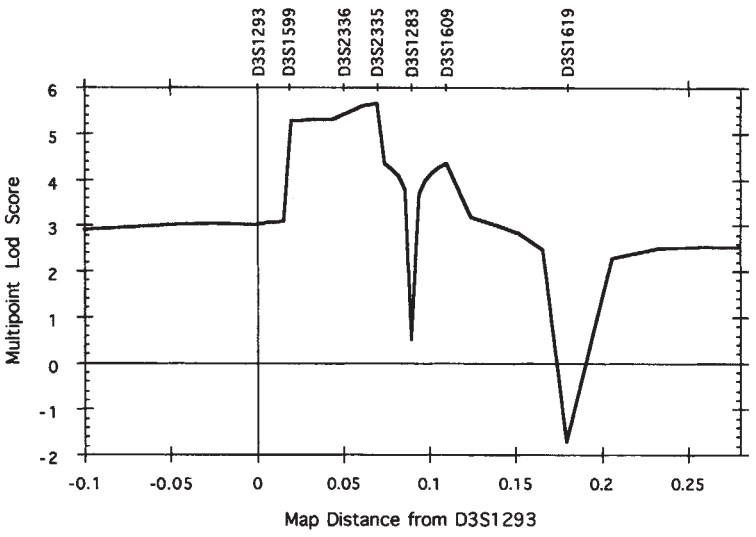

Fig. 3 Multipoint lod scores versus map distance in centiMorgans from *D3S1293*. The composite lod score curve is shown for which marker *D3S1293* was chosen arbitrarily as origin for the map. Recombination fractions were converted into cM using Haldane map function.

obtained with marker *D3S2336* (Table 1), 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 first examined, showed no clinical alteration[13]. These subjects show that the penetrance is not complete in adults, in contrast to classic Marfan syndrome, but also age-dependent. Interestingly, the six-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 genotyped and scored as "unknown" in the linkage analyses. It should be noted that the clinical findings in these five individuals are unchanged since the initial publication[13]. 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 five cloned genes[22]: *THRB* (thyroid hormone receptor beta), *RAF* (murine leukaemia viral oncogene homologue 1), *VHL* (von Hippel-Lindau disease tumour suppressor gene), *RARB* (retinoic acid receptor beta) and *ATP2B2* (ATPase, Ca^{++} 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 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[5,15] and another with the *FBN1* markers[32]. Kainulainen *et al.*[5] and Tsipouras *et al.*[32] found no evidence for genetic heterogeneity in a combined sample of 45 families. Conversely, Sarfarazi *et al.*[15] detected genetic heterogeneity in a sample of 22 families that included our French family but not among the 21 remaining pedigrees (these families are part of the sample reported by Tsipouras *et al.*[32]). 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 with two candidate loci (*FBN1* and *MFS2*) being simultaneously tested, but also in the interest of patients awaiting unequivocal molecular diagnosis for genetic conselling. 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[33], familial forms of annulo-aortic ectasia[34] and the MASS (mitral valve, aorta, skeleton and skin) phenotype[35] are still orphan syndromes. In all these diseases, the cardiovascular manifestations strongly overlap the phenotype of the French family and the implications of this locus should be investigated.

Table 2 Microsatellite tandem repeats localized to chromosome 3p24.2–25

Locus	Marker name	Primer sequence	Predominant allele size (bp)
D3S2338	AFMa037zf5	GAAGCCAGCAGTTTCTC CTGTATTGTTTTCCAGGATAAG	191
D3S2336	AFM336zb5	TCCTTTAGTGGTTTTAACACA TTTACTTGGGCATGTTTG	105
D3S2335	AFM312yf5	GCTGAATGCTTCTGAATGTAT AAGAGATGGGGTGCTTT	166
D3S2337	AFM015xd7	TACTTGGCATAGCCAGTTGA AGCCTCTGTNTTGGTTGTAT	123

Methodology

Patients and clinical evaluation. This large French family (Fig. 2 and ref. 12) 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 upper-to-lower-segment-length ratio, tall stature and increased arm span. Cardiovascular features were mitral valve prolapse associated with holosystolic mitral regurgitation, tricuspid valve prolapse and dilation of the ascending aorta. None of the affected subjects examined had ectopia lentis. 20 members who showed major cardiovascular or skeletal manifestations and were first-degree relatives of an affected member were considered as definitely affected. 17 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 six patients who showed minor skeletal anomalies and/or boderline 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 ref. 13.

DNA analysis and PCR amplification. Blood samples were collected from 59 family members and DNA was isolated according to a method described elsewhere[36]. 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 µl in a microtitre plate, using the Techne PMC3 thermocycler (Cambridge, England) or a thermocycler able to coamplify 16 microtitre plates simultaneously (IAS Products Inc.). The reaction included 5 µl of 10 × buffer (50 mM KCl, 10 mM Tris HCl pH 9, 1.5 mM $MgCl_2$, 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 µl of light mineral oil to prevent evaporation. After an initial "hot start" at 96 °C for 5 min, 1 U of *Taq* DNA polymerase (New England Biolabs) was added to each tube, then 35 cycles consisting of denaturation at 94 °C (40 s) and annealing-elongation at 55 °C (30 s) 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 µl of 0.1× TE and 12.5 µl 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 3,000 V. 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[37]. Autoradiography was carried out after the membranes were washed twice in 2× SSC, 0.1% SDS at room temperature.

Linkage analysis. Pairwise and multipoint analyses were performed using the MLINK and LINKMAP subprograms of the LINKAGE package[20] 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 allelle systems were reduced. The EXCLUDE program[21] was used to produce the exclusion map. This program estimates (i) the positional likelihood of the disease locus on each chromosome, and (ii) the percentage of probability of a locus to be on any of the 22 autosomes.

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