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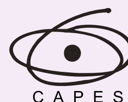

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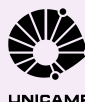

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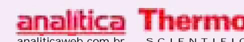

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Candidate gene linkage analysis indicates genetic heterogeneity in Marfan syndrome

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Abstract

Marfan syndrome (MFS) is an autosomal dominant disease of the connective tissue that affects the ocular, skeletal and cardiovascular systems, with a wide clinical variability. Although mutations in the *FBN1* gene have been recognized as the cause of the disease, more recently other loci have been associated with MFS, indicating the genetic heterogeneity of this disease. We addressed the issue of genetic heterogeneity in MFS by performing linkage analysis of the *FBN1* and *TGFBR2* genes in 34 families (345 subjects) who met the clinical diagnostic criteria for the disease according to Ghent. Using a total of six microsatellite markers, we found that linkage with the *FBN1* gene was observed or not excluded in 70.6% (24/34) of the families, and in 1 family the MFS phenotype segregated with the *TGFBR2* gene. Moreover, in 4 families linkage with the *FBN1* and *TGFBR2* genes was excluded, and no mutations were identified in the coding region of *TGFBR1*, indicating the existence of other genes involved in MFS. Our results suggest that the genetic heterogeneity of MFS may be greater that previously reported.

Key words: Marfan syndrome; Fibrillin-1; TGF-β; Genetic heterogeneity

Introduction

Marfan syndrome (MFS; MIM 154700) is a relatively common autosomal dominant hereditary disorder of connective tissue (1:5,000-10,000 individuals) with prominent manifestations in the skeletal, ocular, and cardiovascular systems. Many affected individuals have a characteristic habitus with tall stature, long slender limbs (dolichostenomelia), arachnodactyly, scoliosis, and pectus excavatum or carinatum. Ectopia lentis affects up to 80% of individuals with MFS and is almost always bilateral (1-3). The leading cause of premature death in untreated individuals with MFS is acute aortic dissection, which follows a period of progressive dilatation of the ascending aorta (4-6).

The disease is caused by mutations in the *FBN1* gene at 15q21.1, encoding the large cysteine-rich extracellular matrix glycoprotein fibrillin-1, the major component of microfibrils. *FBN1* spans a 230-kb genomic region with 65 exons and about 600 different reported mutations spread throughout the gene, mostly specific to each affected family (7-13).

In 2003, an unexpected link between fibrillin-1 mutations and increased transforming growth factor-β (TGF-β) signaling was established in a mouse model for MFS, revealing a novel mechanism for the pathogenesis of the condition (14,15). Subsequently, it was found that the TGF-β receptor II gene (*TGFBR2*) was mutated in patients with MFS not linked to *FBN1* (16). This provided the first genetic evidence of a direct link between abnormal TGF-β signaling and a human connective tissue disorder. Finally, it was proposed that mutations in the *TGFBR1* or *TGFBR2* gene lead to a different disease, Loeys-Dietz syndrome (LDS), with some clinical overlap with MFS (17). LDS is characterized by aortic dilatation, ocular hypertelorism, cleft lip/palate, arterial tourtoise, craniostenosis, and mental retardation. Nevertheless, there are reports of *TGFBR1*, *TGFBR2*, and *FBN1* mutations in classical MFS, non-classical MFS, thoracic aortic aneurysms and dissections (TADD), and Shprintzen-Goldberg syndrome. In neonatal MFS, ectopia lentis and Weill-Marchesani syndrome, mutations only in

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FBN1 have been reported, whereas mutations associated with LDS have been found exclusively in the *TGFBR1* and *TGFBR2* genes (18,19).

At first, identification of mutations in the *FBN1* gene appeared as the ultimate tool for confirmation of clinical diagnosis and risk assessment in MFS. As an alternative to the impractical direct identification of the disease-causing mutations in a gene as large as *FBN1*, in familial cases a successful approach to the molecular diagnosis is represented by linkage analysis using four intragenic microsatellite markers (20). Their segregation in families allows the identification of the disease haplotype.

In this study, we addressed the issue of genetic heterogeneity of MFS phenotypes by analyzing 34 families in a large cohort of Brazilian patients who met the clinical diagnostic criteria for the disease. We found 6 families in which linkage of the MFS phenotype with *FBN1* was excluded, corroborating the hypothesis of genetic heterogeneity in this disease.

Material and Methods

Subjects

Forty-seven MFS families (a total of 358 subjects) were referred to us by medical geneticists from the Medical Genetics Center (UNIFESP). All the probands met the diagnostic criteria for MFS (Ghent criteria) (6). The study was approved by the Institutional Ethics Committee of the Instituto de Biociências, Universidade de São Paulo, and all subjects gave written informed consent. Genomic DNA was extracted from leukocytes isolated from EDTA-anticoagulated whole blood (21).

Polymorphic markers

Four microsatellite markers, *mts1*, *mts2*, *mts3*, *mts4*, mapped to intron 1, 5, 28, and 43 of *FBN1*, respectively, were used for linkage analysis as previously described (20). The microsatellite markers used for *TGFBR2* were D3S3727 (an intragenic *TGFBR2* marker) and D3S3567. PCR primers and conditions were those described elsewhere (18). Fluorescently labeled PCR products were analyzed with the MegaBACE 1000 DNA Analysis system using the Genetic Profiler software, version 1.5 (Amersham Biosciences, Sweden).

Mutation analysis

The coding regions of the *TGFBR1* gene were PCR amplified from genomic DNA, and PCR products were subjected to direct sequencing (18,22).

Lod score calculations

Lod score was calculated using the computer packages MLINK of the FASTLINK version 4.2 for two-point linkage analysis (23), and MERLIN for multipoint analysis (24). The disease was coded as fully penetrant, while the disease allele frequency was set at 0.0001. Meiotic recombination frequencies were considered to be equal for males and females. Allele frequencies for the microsatellite markers were calculated by the software based on data from each family. Maximum lod score for each family was calculated by simulation, considering that all meioses were informative. Families were scored according to segregation of markers and phenotypes as: linkage (lod score ≥3.0); linkage not excluded (-2 < lod score < 3.0), or linkage excluded (lod score < -2).

Statistical analyses

Statistical analysis was performed using the BioEstat software version 5.0. The yield of mutation detection in probands with involvement in each separate organ system was confirmed by the Pearson chi-square test, or the Fisher exact test for small samples. A P value of <0.05 was considered to be significant.

Results

A total of 358 affected and unaffected individuals from 47 unrelated families were clinically assessed (Supplementary Table A). Although all probands met the Ghent diagnostic criteria for MFS, the clinical phenotype varied within and between families, as expected. The classic skeletal phenotype was observed in all 47 families, whereas in 67.6%, at least 1 individual had ectopia lentis, and in 82.3%, at least 1 individual showed dilatation of the aorta (Supplementary Table A).

Thirty-four families presented enough individuals for linkage studies to be performed. Since most of the families were not large enough to obtain a lod score above 3.0, we compared the lod score obtained for each family with the theoretical maximum lod score possible for that family (Table 1). The microsatellite marker *mts3* was not informative in many individuals, and was thus excluded from the analysis (data not shown). Initially we sought to identify those families where linkage to *FBN1* could be excluded. We could not exclude linkage of the *FBN1* haplotype with the MFS phenotype in 25 families (70.6%). A lod score greater than 3.0 was obtained for 1 of these families (family 7; Table 1). In the other families, the results of lod scores were below 3.0 due to the small number of individuals available for analysis. In 4 families (11.76%) the *FBN1* gene markers were not informative, and thus the analysis was inconclusive. Finally, in 6 families (17.64%), we excluded segregation of *FBN1* with the phenotype.

The 6 families excluded for linkage with *FBN1* were studied with polymorphic markers linked to the *TGFBR2* gene. In one family (family 43), linkage with the D3S3727 marker was not excluded, and one family was not informative for both *TGFBR2* markers. However, in 4 of the 6 families (families 4, 15, 37, and 42; Table 1) we excluded segregation of *TGFBR2* with the phenotype.

Probands of these 4 families were screened for mutations in the coding region of *TGFBR1*, but no alteration was identified.

We compared the frequency of major manifestations in each of the three affected systems in the families whose phenotype segregated with *FBN1* with those that did not segregate with *FBN1* (Table 2). We found no significant difference in the frequency of cardiac and skeletal signs, but the major manifestations in the ocular system were significantly more frequent in families segregating with *FBN1* than in those that did not segregate with this gene (Table 2).

Table 1. Linkage analysis of the Marfan syndrome phenotype with the *FBN1* and *TGFBR2* genes.

Family*	Lod score *FBN1* (MERLIN)	Lod score *TGFBR2* (MERLIN)	Max. lod score (MERLIN)	Result *FBN1*	Result *TGFBR2*
1 (4)	-3.221	N/INF	0.3	EXCL	N/INF
2 (23)	2.104	NE	4.2	N/EXCL	NE
3 (5)	0.6	NE	0.601	N/EXCL	NE
4 (4)	-3.221	-3.221	0.3	EXCL	EXCL
5 (6)	0.600	NE	0.602	N/EXCL	NE
6 (11)	1.2	-5.754	1.204	N/EXCL	EXCL
7 (26)	3.911	NE	3.912	LINK	NE
8 (9)	0.778	NE	1.204	N/EXCL	NE
9 (18)	1.929	-9.893	1.929	N/EXCL	EXCL
13 (5)	0.6	-2.620	0.602	N/EXCL	EXCL
14 (4)	0.598	NE	0.598	N/EXCL	NE
15 (10)	-5.33	0.259	0.602	EXCL	EXCL
16 (6)	0.147	-3.272	0.147	N/EXCL	EXCL
17 (6)	0.536	NE	0.578	N/EXCL	NE
18 (19)	N/INF	-8.416	N/INF	N/INF	EXCL
19 (16)	0.283	NE	2.408	N/EXCL	NE
20 (15)	0.461	NE	0.593	N/EXCL	NE
22 (5)	0.6	NE	0.602	N/EXCL	NE
23 (11)	1.504	NE	1.504	N/EXCL	NE
24 (14)	1.2	NE	1.203	N/EXCL	NE
28 (3)	0.3	-3.221	0.3	N/EXCL	EXCL
29 (11)	1.5	NE	1.503	N/EXCL	NE
30 (3)	0.3	NE	0.3	N/EXCL	NE
31 (14)	0.903	NE	0.903	N/EXCL	NE
33 (5)	0.6	NE	0.602	N/EXCL	NE
34 (11)	0.178	NE	0.286	N/EXCL	NE
35 (16)	-1.381	NE	-0.537	N/EXCL	NE
37 (12)	-3.445	-3.133	1.158	EXCL	EXCL
38 (3)	N/INF	NE	N/INF	N/INF	NE
40 (8)	N/INF	NE	N/INF	N/INF	NE
42 (27)	-3.659	-9.03	3.421	EXCL	EXCL
43 (4)	-3.095	0.601	0.601	EXCL	N/EXCL
45 (6)	N/INF	-2.201	0.710	N/INF	EXCL
46 (5)	0.130	NE	0.139	N/EXCL	NE

*The number of family members is given within parentheses. LINK = phenotype linked with the gene (lod score ≥3.0); N/EXCL = linkage of phenotype with the gene was not excluded; EXCL = linkage of phenotype with the gene was excluded; N/INF = not informative; NE = not evaluated.

Discussion

The discovery that mutations in gene *FBN1* cause MFS has increased the chances of identifying the disease in atypical or oligosymptomatic individuals through molecular diagnosis (25). However, the more recent descriptions of mutations in other genes causing phenotypes overlapping with MFS have suggested the existence of genetic heterogeneity, or of a novel clinical entity, the LDS (18,22,25-27). The objective of the present study was to address the issue of genetic heterogeneity in MFS by characterizing a large cohort of patients with MFS phenotypes according to the gene linked to the phenotype, looking for clinical manifestations more frequent in the different groups.

We performed linkage analysis in 34 families with MFS phenotypes, which were evaluated clinically according to the criteria of Ghent. Linkage analysis performed with intragenic molecular markers in the *FBN1* gene has been used by several investigators for the diagnosis of MFS (28-31). The effectiveness of using the analysis of haplotype segregation in families with oligosymptomatic individuals, or even with individuals clinically diagnosed with MFS, has been well demonstrated (20).

In most of the families analyzed, linkage of the MFS phenotype with the *FBN1* gene was not excluded. However, we excluded linkage of the disease to this gene in 6 families whose affected individuals met the Ghent criteria for MFS, showing that either those diagnostic criteria are limited, or that the MFS phenotype can be caused by mutations in genes other than *FBN1*. We found a significant increase in the frequency of major ocular manifestations in families where linkage to *FBN1* was not excluded than in those where linkage to this gene was excluded, where none of the patients had major ocular signs. Indeed, the lower frequency of ocular manifestations in patients with MFS-like phenotypes but without *FBN1* mutations has been reported by others (12,13,19).

Among the 6 MFS families where linkage with *FBN1* was excluded, we could not exclude linkage of the phenotype with *TGFBR2* in one. However, the affected individuals did not meet the diagnostic criteria for LDS, corroborating the observations that mutations in the *TGFBR2* gene can cause MFS (12,13,19). Finally, in 4 of those 6 families we excluded linkage of the MFS phenotype with *TGFBR2*, and found no mutations in the coding region of the *TGFBR1* gene. Although one cannot rule out the existence of disease-causing mutations in promoter or intronic regions of *TGFBR1*, these results indicate the existence of additional genes that can lead to MFS.

In conclusion, the analysis of a large cohort of Brazilian families with MFS indicated that *FBN1* was the gene most frequently involved in the disease, mostly when there were major ocular findings. However, this study also identified families where the MFS phenotype did not segregate with *FBN1*, revealing genetic heterogeneity in this disease. Particularly, the 4 families that did not have mutations in either the *TGFBR1* or *TGFBR2* gene, although small, represented an opportunity to search for other genes involved in the MFS phenotypes. The identification of these genes should lead to a better understanding of the molecular mechanisms involved in the normal and pathogenic physiology of the ocular, skeletal and cardiovascular systems.

Table 2. Frequency of major signs in the three affected systems in families according to linkage with *FBN1*.

	FBN1 (N/EXCL)	*FBN1* (EXCL)
Cardiac	19/24 (79%)	5/6 (83%)
Ocular	19/24 (79%)*	0/6 (0%)*
Skeletal	24/24 (100%)	6/6 (100%)

FBN1 (N/EXCL) = linkage of *FBN1* with disease was not excluded; *FBN1* (EXCL) = linkage of *FBN1* with disease was excluded. *P < 0.002 (chi-square test).

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Supplementary Table A. Clinical and genetic assessment of Marfan syndrome patients in 47 families.

Family	Patient	Ocular		Cardiac		Skeletal	Gene	
		EL	MYO	AO DIL	MR	>5 signs	*FBN1*	*TGFBR2*
1	II-1	-	+	-	-	+	EXCL	N /INF
	II-1	-	-	-	-	+		
	II-2	-	+	+	+	+		
2	II-6	-	-	+	-	-	N/EXCL	NE
	III-3	-	+	+	+	+		
	III-6	-	-	+	+	-		
	IV-4	-	+	+	+	+		
	IV-5	NE	NE	NE	NE	+		
	IV-8	-	-	-	-	+		
3	I-2	+	+	+	+	+	N/EXCL	
	II-2	+	+	+	+	+		
4	I-1	-	+	+	+	+	EXCL	EXCL
	II-1	-	+	+	+	+		
	II-2	-	+	+	+	+		
5	I-2	+	-	+	+	+	N/EXCL	NE
	II-1	+	-	+	+	+		
	II-2	+	-	+	+	+		
	II-3	-	-	-	-	-		
	II-4	NE	NE	NE	NE	NE		
6	I-2	+	+	+	+	+	N/EXCL	EXCL
	II-6	-	+	+	+	+		
	II-7	-	+	+	+	+		
	II-8	-	+	+	+	+		
	II-10	-	+	+	+	+		
7	I-2	NE	NE	+	+	+	LINK	NE
	II-1	NE	NE	NE	NE	+		
	III-1	-	-	+	+	+		
8	II-1	+	-	+	+	+	N/EXCL	NE
	III-1	-	-	-	-	+		
	III-3	+	-	+	+	+		
9	IV-5	+	-	-	+	+	N/EXCL	EXCL
	III-4	-	-	+	-	+		
	IV-1	-	-	-	-	+		
	IV-2	-	+	+	-	+		
10	I-1	NE	NE	NE	NE	+	NE	NE
11	I-1	NE	NE	NE	NE	+	NE	NE
12	I-1	NE	NE	NE	NE	+	NE	NE
13	I-1	-	+	-	+	-/+	N/EXCL	EXCL
	II-2	+	-	+	+	+		
	III-1	-	-	+	+	+		
	III-2	-	-	NE	NE	+		
14	II-1	+	+	-	+	+	N/EXCL	NE
	II-2	-	-	-	+	+		
	II-3	-	-	-	+	+		
15	II-4	-	+	+	+	+	EXCL	EXCL
	III-5	-	-	+	+	+		
	III-6	-	+	-	+	+		

Continued on next page

Supplementary Table A continued.

Family	Patient	Ocular		Cardiac		Skeletal	Gene	
		EL	MYO	AO DIL	MR	>5 signs	*FBN1*	*TGFBR2*
16	II-1	-	+	NE	NE	+	N/EXCL	EXCL
	II-3	-	+	NE	NE	+		
	II-5	-	+	+	-	+		
	III-1	-	+	+	+	+		
17	II-2	+	+	+	+	+	N/EXCL	NE
	II-4	-	+	+	+	+		
	III-1	-	+	+	+	+		
	III-2	-	-	+	+	+		
18	II-1	-	+	+	-	+	N/INF	EXCL
	II-2	+	-	+	+	+		
	II-4	+	-	+	+	+		
	III-4	NE	NE	NE	NE	-/+		
	III-6	+	-	+	+	+		
	III-8	+	-	-	-	+		
	III-9	NE	NE	NE	NE	-/+		
19	II-3	NE	NE	+	+	+	N/EXCL	NE
	II-7	NE	NE	-	+	+		
	II-11	+	-	+	+	+		
	III-4	NE	NE	-	+	+		
	III-7	NE	NE	NE	NE	+		
	III-8	-	-	-	+	+		
	III-9	-	+	-	+	+		
20	II-4	+	-	-	+	+	N/EXCL	NE
	III-1	+	-	-	+	+		
	II-1	-	+	-	+	+		
	III-4	+	-	-	+	+		
	III-5	NE	NE	NE	NE	+		
	III-6	NE	NE	NE	NE	+		
21	I-1	NE	NE	NE	NE	+	NE	NE
22	III-2	+	-	-	-	+	N/EXCL	NE
	III-4	+	-	-	+	+		
23	II-6	-	+	+	-	+	N/EXCL	NE
	III-1	+	+	+	-	+		
	III-2	+	-	+	+	+		
	III-3	-	+	+	+	+		
	III-5	NE	NE	NE	NE	+		
24	II-1	NE	NE	NE	NE	+	N/EXCL	NE
	III-7	+	+	-	+	+		
25	I-1	NE	NE	NE	NE	+	NE	NE
26	I-1	NE	NE	NE	NE	+	NE	NE
27	I-1	NE	NE	NE	NE	+	NE	NE
28	I-1	-	+	+	-	+	N/EXCL	EXCL
	II-1	+	+	-	+	-/+		
	II-2	+	+	+	+	+		
29	III-2	+	-	+	-	+	N/EXCL	NE
	III-3	-	+	-	+	+		
	III-8	-	+	+	-	+		

Continued on next page

Supplementary Table A continued.

Family	Patient	Ocular		Cardiac		Skeletal	Gene	
		EL	MYO	AO DIL	MR	>5 signs	*FBN1*	*TGFBR2*
30	I-2	+	-	-	-	+	N/EXCL	NE
	II-1	+	-	+	-	+		
	II-2	+	-	-	+	+		
31	II-8	+	-	+	-	+	N/EXCL	NE
	III-4	-	-	+	+	+		
	III-5	+	+	+	+	+		
32	I-1	NE	NE	NE	NE	+	NE	NE
33	II-3	+	-	-	+	+	N/EXCL	NE
	IV-1	+	-	+	+	+		
34	III-4	-	-	+	+	+	N/EXCL	NE
	III-6	-	+	+	-	-/+		
	II-4	-	+	+	-	+		
35	III-4	+	-	+	-	+	N/EXCL	NE
	II-8	-	+	NE	NE	+		
	II-4	-	+	NE	NE	+		
	II-6	-	+	+	-	+		
	III-8	-	+	NE	NE	+		
	II-3	-	+	+	-	+		
36	I-1	NE	NE	NE	NE	+	NE	NE
37	III-3	-	-	+	-	+	EXCL	EXCL
	IV-1	NE	NE	NE	NE	+		
	III-5	NE	NE	NE	NE	+		
38	II-1	-	+	+	-	+	N/INF	NE
	I-1	-	+	+	-	+		
39	I-1	NE	NE	NE	NE	+	NE	NE
40	III-1	+	-	+	-	+	N/INF	NE
	III-2	+	+	-	-	+		
	II-2	+	-	-	+	+		
41	I-1	NE	NE	NE	NE	+	NE	NE
42	II-12	NE	NE	+	-	+	EXCL	EXCL
	II-6	NE	NE	+	-	+		
	II-8	-	+	-	-	-		
	II-10	-	-	-	+	+		
	III-7	-	-	-	-	+		
	III-5	-	+	NE	NE	+		
	III-16	-	+	NE	NE	+		
	III-17	-	+	NE	NE	+		
43	II-5	-	-	-	+	+	EXCL	N/EXCL
	I-3	-	-	-	+	-		
44	I-1	NE	NE	NE	NE	+	NE	NE
45	IV-2	+	-	+	-	+	N/INF	EXCL
46	III-1	-	+	NE	NE	+	N/EXCL	NE
	II-3	-	+	NE	NE	+		
	II-5	+	+	-	+	+		
47	I-1	NE	NE	NE	NE	+	NE	NE

EL = ectopia lentis; MYO = myopia; AO DIL = aorta dilatation; MR = mitral reflux; N/EXCL = linkage of phenotype with the gene was not excluded; LINK = phenotype linked with the gene (lod score ≥3.0); EXCL = linkage of phenotype with the gene was excluded; N/INF = not informative; NE = not evaluated.