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Two Novel Cases of Marfan Syndrome with *FBN1* whole Gene Deletion: Laboratory Assay and Cases Review

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Abstract

Background: Marfan syndrome is a hereditary disorder of connective tissue mainly characterized by abnormalities in the cardiovascular, skeletal and ocular systems. It is most frequently due to heterozygous point mutations in the *FBN1* gene located on chromosome 15q21.1 and coding for fibrillin-1 protein. Over 3000 *FBN1* mutations have been reported in the *FBN1* Universal Mutation Database, among these *FBN1* large rearrangements account for <2%. Several studies have shown that 15q deletions encompassing the whole *FBN1* gene are associated with Marfan syndrome and *FBN1* haploinsufficiency is involved in the pathogenesis of the disease. Since now 19 cases of *FBN1* entire deletion have been already described.

Methods: We describe two novel whole *FBN1* gene deletions in MFS cases, diagnosed according to revised Ghent criteria. Molecular and cytogenomic studies have showed a complete *FBN1* deletion, never reported so far. Moreover molecular, biochemical and immunofluorescence studies performed on dermal fibroblast clearly reported reduced levels of both *FBN1* transcript and protein, without compensatory overexpression of the normal allele.

Conclusion: We characterise two novel *FBN1* gene whole deletions in MFS diagnosed patients. Reduced levels of both *FBN1* transcript and protein have been studied to complete the molecular data on *FBN1* gene haploinsufficiency in correlation with transcript and protein levels.
We have also reviewed all the *FBN1* haploinsufficiency cases reported in literature, evaluating in each case the laboratory assay utilised and thus discussing its usefulness.

Keywords: Marfan Syndrome; *FBN1* (fibrillin-1); Gene deletion; Haploinsufficiency

Introduction

Mutations in *FBN1* (fibrillin-1) gene cause the Marfan Syndrome (MFS; OMIM #154700), a pleiotropic autosomal dominant connective tissue disorder with prominent manifestations in the skeleton, eye and cardiovascular system [1]. Fibrillin-1 is a large glycoprotein, the major component of micro fibrils. These structures constitute a main component of the extracellular matrix (ECM) in elastic connective tissue and fulfil the mechanical demands of this specialized connective tissue [3]. The physiological function of fibrillin-1 assemblies, i.e. microfibrils and elastic fibres, is to provide a scaffold that imparts specific physical properties to various tissues. Over 3000 *FBN1* mutations have been reported in the *FBN1* Universal Mutation Database (UMD, http://www.umd.be/FBN1/) with point mutations accounting for 73%; among the point mutations 80% were missense.

FBN1 gene large rearrangements account for <2% of the reported mutations, otherwise interstitial deletions involving 15q21.1 and the F*BN1* gene deletions are very rare [2].

To our knowledge there have been reported so far 19 cases of Marfan patients carrying deletions of the whole *FBN1* gene and with variable phenotypic presentations, suggesting a critical role of haploinsufficiency in the pathogenesis of the disease [4].

In this study we report two novel cases of whole *FBN1* gene deletion in patients diagnosed as MFS, based on the revised Ghent

diagnostic criteria. Laboratory assays have been performed to characterise both *FBN1* deletion and the haploinsufficiency with regard on transcript and protein level based on physiological function of fibrillin-1 assemblies.

Methods

Patients

We describe two MFS cases, a 21 year-old male (patient 1) and his mother (patient 2), both affected. Record of clinical history and physical examination were performed in the Medical Genetic Unit at Policlinico Tor Vergata Hospital. Both the proband and his mother have provided written informed consent for genetic testing.

Marfan Syndrome has diagnosed in the patient 1 according to the presence of characteristic skeletal features (dolicostenomelia, scoliosis, pes planus), aortic root aneurysm associated to ostium secundum, atrial septal defect and mitral valve prolapse (MVP). Ectopia lentis and others ocular features of MFS have been excluded during ophthalmological examination. Cardiac anomalies required surgical intervention on aortic root when the patient was 14 year-old and a mitral valvuloplasty has been performed two years later. Surgical treatment for a left inguinal hernia has also been carried out.

A CT scan has revealed multiple and bilateral renal cysts with normal renal function (normal creatinine clearance and normal BUN) and multiple apical emphysematous bullae bilaterally in the lungs. Physical examination has revealed positive wrist and thumb signs, pectus excavatum, protrusion acetabulae, flat feet and joint hypermobility; highly arched palate and crowding of teeth required orthodontic care. He measures 191 cm, weighted 70 kg with an arm span of 193.

We have evaluated at the same time the mother of the 21 year-old male with MFS diagnosed, patient 2. At 56 year-old cardiac echo revealed dilatation of both the root and ascending aorta (48 and 37 mm respectively) and a mild MVP with minimal regurgitation; cardio-surgery was not required. At physical examination she presented highly arched palate, positive wrist and thumb signs, mild scoliosis, flat feet and tall stature (172 cm).

Molecular testing of *FBN1* has been performed in both cases, sequencing all the coding regions of *FBN1* gene including intron/exon boundaries but no mutations have been detected; soon after MLPA analysis has identified a novel *FBN1* gene deletion.

MLPA analysis

Genomic DNA has been extracted from peripheral whole blood leucocytes using the EZ1 Advanced XL DNA Isolation kit (Qiagen, Courtaboeuf, France). A total of 100 ng of DNA has been analysed by Multiplex Ligation dependent Probe Amplification (MLPA) using the SALSA P065 and P066 MLPA kit for Marfan Syndrome (MRC-Holland, Amsterdam; The Netherlands) containing probes for 53 of the 65 FBN1 exons and for all the 7 TGFBR2 exons. PCR products have been analyzed on a fluorescent capillary sequencer (ABI3130, Applied Biosystem, Torrence, CA, USA). Each MLPA signal has been normalized and compared to the corresponding peak area obtained in a control DNA sample. Data analysis has been performed using Coffalyser analysis software (v.140701; MRC Hollande). Deviations less than 25% have been accepted as normal.

Array-based Comparative Genomic Hybridization (aCGH)

Array –CGH has been performed using a whole genome microarray CytoChipOligo ISCA 180K (BlueGnome, Cambridge, UK). Genomic DNA has been extracted from peripheral blood lymphocytes of both patient1 and his mother (patient 2) using QIAamp DNA Midi Kit according to the manufacturer's protocol (Qiagen, Germantown, Maryland). One µg of genomic DNA has been double digested using both AluI and RsaI(New England Biolabs, Inc., UK) enzymes. Patient and pooled same-sex reference DNA (Promega, Madison, UK) has been labelled with Cy3-dCTP and Cy5-dCTP, respectively, and hybridized to the oligo-array platform as recommended by manufacturer's protocol (BlueGnome, Cambridge, UK). Data analysis has been performed using BlueFuse Multi software (BlueGnome, Cambridge, UK). Probe sequence annotation was based on NCBI Build GRCh37(hg19) of the human genome.

Preparation of Human Dermal Fibroblasts

Dermal biopsies have been obtained only from the patient 2. Skin punch biopsy specimen has been treated in sterile PBS supplemented with penicillin/streptomycin and washed 3 times, digested by 0.1% dispase (Gibco) at 4 °C overnight to remove epidermal layers. Further dermal parts have been treated by 0.1% collagenase I (Sigma) at 37 °C for 4h. The digested cells have been resuspended in DMEM (GE Healthcare) containing 10% FBS (vol/vol) (Hyclone), 2 mML-Gln (Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (Life Technologies Corporation), 0.1 mM b-mercaptoethanol (Gibco), and 1 mM (1%) nonessential amino acid (Gibco), seeded in 6-well plates at $1x10^3$ cells/cm^2, and maintained at 37 °C with 5% CO_2.

RT-qPCR analysis

Total RNA has been isolated from dermal fibroblasts using TRIzol Reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and treated by RNase-free DNase (Ambion). One µg of RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Life Technologies Corporation) and amplified. mRNA levels of FBN1 have been quantified by Sybr Green (Life Technologies Corporation) and 7500 Light Cycler Real-Time PCR System (Life Technologies). The oligonucleotide sequences for primers are as follows: 5S: Fw-TCG TCT GAT CTC GGA AGC TAA GCA, Rev-AAA GCC TAC AGC ACC CGG TAT T; FBN1:

Fw-AGC GGA GCC GAG CAG TGG, Rev-GCT GCT CCC ACT TCA GGC [5]. For normalization the 5S ribosomal gene has been used as reference gene. The comparative ΔΔCt method has been used to quantify relative gene expression levels.

Western Blot

Equal amounts of protein from cell lysates of control and MFS dermal fibroblasts have been electrophoresed into 4-10% gradient polyacrylamide/SDS gel and electro-blotted onto nitrocellulose membrane [6]. Mouse monoclonal anti-fibrillin-1 (Millipore) and mouse monoclonal anti-α-tubulin (Sigma-Aldrich St. Louis, USA) antibodies were used.

Immunofluorescence assay

Immunofluorescence has been performed by staining dermal fibroblasts with a mouse monoclonal anti-fibrillin-1 antibody (Millipore, Bedford-Massachusetts, USA), followed by a rhodamine-labeled anti-mouse secondary antibody [7]. Nuclei have been counterstained using Hoechst.

Statistical analysis

Data were expressed as mean values ± standard deviation (SD). Statistical significance of differences between groups was tested by paired Student's t-test. P-values <0.05 were considered to indicate a statistically significant difference.

Results

MLPA analysis has revealed in both patients reduced relative peak areas (Figure 1A) for the 52 probes available for *FBN1* gene, demonstrating the presence of a deletion of the entire *FBN1* gene. It has been wholly inherited in proband 1 from his mother.

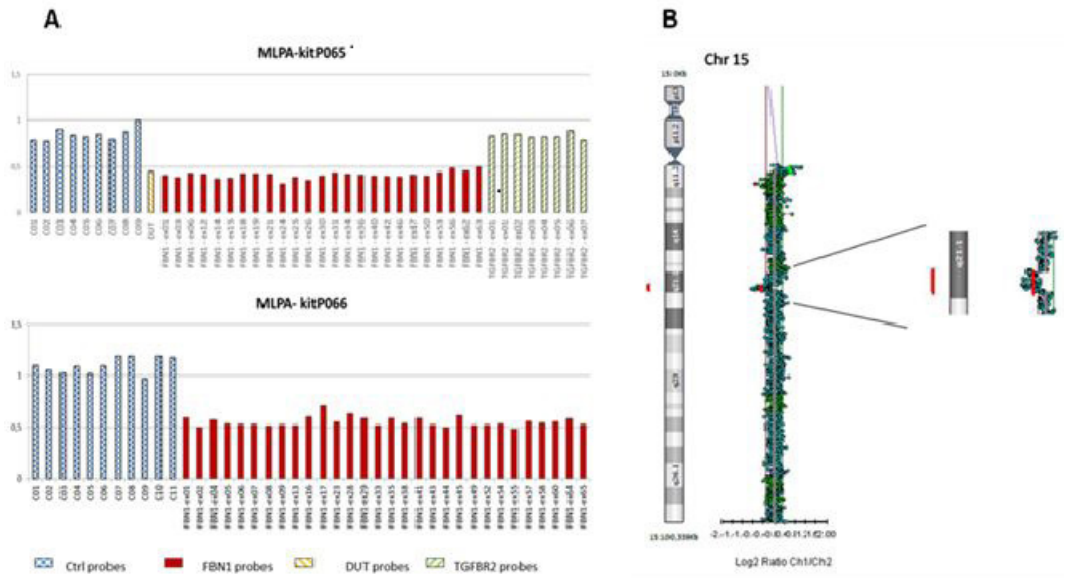

Figure 1: A) Results of MLPA analysis for family deletion (MLPA kit P065, P066, MRC-Holland). The probe signals for *FBN1*, relative to control probes and TGFBR2 probes, are reduced to about 50% and reveal the presence of single copy for *FBN1*. The same results are obtained for *DUT* gene located on chr 15q21, suggesting a larger deletion encompassing both FBN1 and *DUT*. B) Array-CGH showing the 15q21.1 deletion. On the left, the chromosome 15 profile is shown; on the right, the deleted region is highlighted (image by BlueFuse Multi software).

The other relatives, referred as not affected, have not been analysed. MLPA analysis has revealed a reduced peak area also for the probe corresponding to *DUT* gene, encoding deoxy-uridine-triphosphatase, an essential enzyme of the nucleotide metabolism. As *DUT* is located on chromosome 15q21.1 closely to FBN1 we hypothesized a larger deletion involving both genes. To better characterize the size and the gene content of the deleted region, we have performed aCGHThe analysis showed a microdeletion

Gene	OMIM	Phenotype
SLC24A5	609802	Albinism, oculocutaneous, type VI Skin/hair/eye pigmentation 4, fair/dark skin
SLC12A1	600839	Bartter syndrome, type 1
CEP152	613529	Microcephaly 9, primary, autosomal recessive Seckel syndrome 5

Table 1: Genes comprised in the reported deletion and listed in the OMIM database

on the long arm of chromosome 15 in the cytoband q21.1: arr[GRCh37] 15q21.1(48066086_49259938)x1 (Figure 1B). The microdeletion spans 1.194 Mb involving 10 known RefSeq genes including 4 OMIM genes: SLC24A5 (OMIM#609802), SLC12A1 (OMIM#600839), FBN1 (OMIM#134797) and CEP152 (OMIM#613529) (Table 1). The same analysis was carried out on the mother (patient 2) showing maternal segregation of the microdeletion 15q21.1.

A RT-qPCR assay has been performed on mRNA extracted from the dermal fibroblasts obtained by skin biopsy of patient 2 to quantify *FBN1* transcript and to evaluate how the *FBN1* deletion could influence gene expression. After normalization against RNA 5S transcript, *FBN1* RNA levels have been compared to those found in dermal fibroblasts obtained from an unaffected control (CTRL). As expected, a reduction of about 50% in *FBN1* transcript has been observed in MFS patient examined (Figure 2A), excluding any compensatory effect by the expression of the normal allele, as previously described in other studies [8]. Western Blot analysis has been performed in order to confirm these results, and, indeed, we have observed a reduction of the total amount of fibrillin-1 protein from MFS fibroblasts. Densitometric and immunocytochemical analysis indicated a decrease of *FBN1* protein expression in cells derived from MFS patient compared to control cells (Figure 2B, 2C).

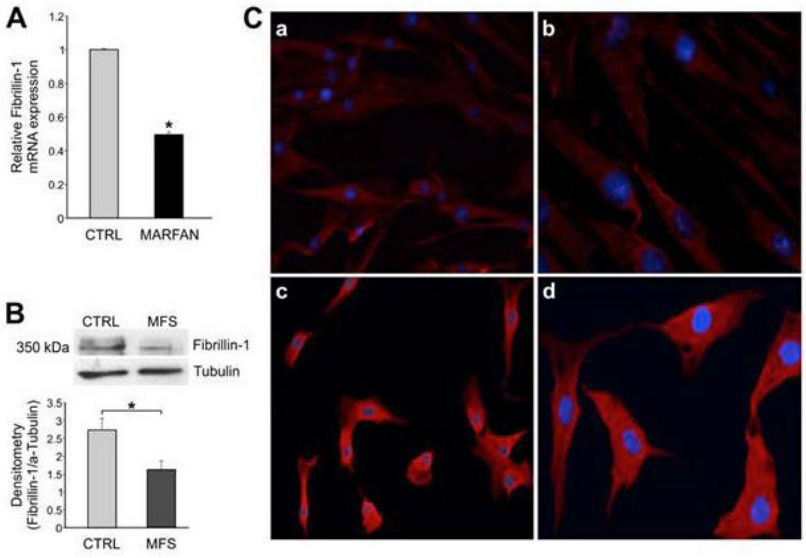

Figure 2: (A) *FBN1* transcript quantification of mutated skin fibroblast by RT-qPCR. Reverse transcription qPCR showed a 50% reduction of FBN1 mRNA respect to the wild type control (CTRL). Analysis was normalized respect to a reference gene (RNA 5S) and repeated three time by independent operators. (B) Representative blots of fibrillin-1 expression in cultured human dermal fibroblasts from control (CTRL) and Marfan Syndrome patient (MFS); semi-quantitative densitometric evaluation of fibrillin-1 protein expression in control fibroblasts compared to Marfan Syndrome fibroblasts using α-tubulin as protein leading control. *P<0.05
(C) Immunofluorescence representative images of fibrillin-1 in cultured human dermal fibroblasts from Marfan Syndrome (a, b) and control (c, d) patients at different magnification. Rhodamine-conjugated secondary antibody and Hoechst as nuclear staining. Original magnification: a, c X200; b, d X400.

Discussion

MFS is an autosomal dominant connective tissue disorder caused by mutations in the fibrillin-1 gene (*FBN1*). *FBN1* point mutations represent more than 90% of all mutations associated to MFS, while deletions and duplications involving one or more exons have been reported only in a small percentage of cases (http://www.umd.be/*FBN1*/).Whole *FBN1* gene deletion is an event rarer than a partial gene deletion; to date only 19 cases of *FBN1* haploinsufficiency have been reported with clinical findings of MFS [8-13], and the role of haploinsufficiency of fibrillin-1 in determining MFS has not been clearly defined yet. Indeed, for a long time after the discovery of the genetic basis of MFS, a traditional dominant-negative pathogenic model has been accepted where abnormal protein derived from the mutant allele could interact and interfere with protein derived from the normal.

Mutant fibrillin-1 monomers included into microfibrils interfere with polymerization and lead to the formation of anomalous extracellular aggregates, culminating in tissue structural weakness and ultimately failure [14]. Thus, the role of fibrillin-1 haploinsufficiency in the pathogenesis of MFS was formerly thought to be unlikely, and this hypothesis has been further substantiated by the observation that patients harbouring a point mutation leading to premature nonsense mediated decay (NMD) and with low levels of mutant *FBN1*, also show a milder phenotype [15]. However, it is evident that some manifestations of MFS, such as bone overgrowth, mitral valve elongation and thickening, and skeletal muscle hypoplasia, are difficult to reconcile with a pathogenic model that singularly invoked compromised tissue structural integrity. Altered cellular performance, caused by dysregulation of the bioavailability of growth factors, seems to be a more likely mechanism. In particular, the degree of homology between the fibrillins and Latent Tissue Growth Factor-β Binding Proteins (LTBPs) suggested that perhaps abnormal or insufficient fibrillin-1 containing microfibrils in the tissues of MFS patients might lead to altered Tissue Growth Factor-β (TGFβ) activation [4,16].

In the last years, several studies have demonstrated that a complete *FBN1* deletion on chromosome 15q21.1 is associated to clinical findings of MFS [8-13], thus giving further support to the hypothesis that haploinsufficiency of fibrillin-1 is responsible for the phenotype.

In this study we provided an overview of all *FBN1* deletions involving the whole gene region, in order to evaluate the laboratory assay performed to analyse of *FBN1* both at protein and transcript level. To do this, we revised all the laboratory assays available in literature comparing the techniques used for the haploinsufficiency diagnosis (Table 2)[8-13, 17, 18].

Laboratory assay for FBN1 full gene deletion										
IF/Fish	**NB**	**SB**	**MLPA**	**CGH-AR-RAY**	**WB**	**RT-PCR**	**DNA Sequencing**	**MFS GHENT CRITERIA**	**N° CASE**	**Reference**
✓	✓							✓	1	Hutchinson et al. 2003
			✓					✓	1	Ades et al. 2006*
			✓	✓			✓	✓	3	Furtado et al.. 2008
✓								✓	1	Hiraki et al.. 2008
✓			✓	✓				✓	1	Faivre et al. 2010
								✓		
✓				✓				✓	1	Colovati et al. 2012
			✓	✓			✓	✓	1	Dordoni et al 2016
✓			✓	✓	✓	✓	✓	✓	2	Present cases
N° TOTAL CASES									21	

IF: immunofluorescence; NB:Northern blot analysis; MLPA: Multiplex Ligation dependent Probe Amplification ; CGH-array: Comparative Genomic Hybridization; WB: Western Blot;RT-PCR: Real time PCR; *we referred only of the haploinsufficient FBN1 case reported

Table 2: Laboratory assay for *FBN1* full gene deletion

Nowaday, few patients (n = 21 included ours cases) have been reported with FBN1 gene deletion and only for 11 of them has been performed immunoflorescence analysis for protein detection without any Western Blot or RT-qPCR analysis. To our knowledge, only Hutchinson in 2003 performed Northern Blot analysis for FBN1 mutations suggesting a potential modified role of normal FBN1 expression in clinical variability (Table 2).

In this scenario, we have fully analysed the fibrillin-1 gene deletion in our cases with several experimental assays. Our laboratory evidences demonstrated that there isn't any compensatory protein production from wild-type *FBN1* allele and that clinical variability is not depending on critical threshold in expression of *FBN1* normal allele as previously suggested [8]. Both our patients show the known clinical variability of MFS patients, supporting that there is no difference in abnormal or insufficient- fibrillin in leading to altered Tissue Growth Factor-β (TGFβ) activation.

MLPA as the useful tool to identify haploinsufficiency in *FBN1* gene deletion whilst other technical performance on *FBN1* give no further informations on molecular and clinical characterization in patients with MFS diagnosed according Ghent criteria.

Conclusion

We have reported two novel cases of a complete *FBN1* deletion associated to MFS. The deletions show a great variability in clinical manifestations in different subjects of the same family, similarly to other *FBN1* mutations. We have also demonstrated that the absence of one *FBN1* allele determines a decreased expression of the *FBN1* transcript together with reduced level of fibrillin protein and without compensatory overexpression of the normal allele in our case. Even though the 15q21.1 deletion comprises other 3 OMIM genes our patients did not show any clinical manifestation other than MFS.

In conclusion, we highlight the importance and utility of revised clinical Ghent criteria in performing additional molecular studies in those cases with MFS associated to complex phenotype particularly referring to cases with whole *FBN1* deletion.

Finally, we have provided an overview of the laboratory assays, indicating that in our experience, NGS or Sanger sequencing and MLPA analysis are the most useful molecular tools to perform a complete laboratory analysis for *FBN1* gene deletions and point mutation too.

Consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Authors' contributions

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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