The clinical presentation of Marfan syndrome is modulated by expression of wild-type FBN1 allele

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Abstract

Marfan syndrome is an autosomal dominant disorder mainly caused by mutations within FBN1 gene. The disease displays large variability in age of onset or severity and very poor phenotype/genotype correlations have been demonstrated. We investigated the hypothesis that phenotype severity could be related to the variable expression level of fibrillin-1 (FBN1) synthesized from the wild-type (WT) allele. Quantitative reverse-transcription and polymerase chain reaction was used to evaluate FBN1 levels in skin fibroblasts from 80 Marfan patients with premature termination codons and in skin fibroblasts from 80 controls. Results in controls showed a 3.9-fold variation in FBN1 mRNA synthesis level between subjects. A similar 4.4-fold variation was found in the Marfan population, but the mean level of FBN1 mRNA was a half of the control population. Differential allelic expression analysis in Marfan fibroblasts showed that over 90% of FBN1 mRNA was transcribed from the wild allele and the mutated allele was not detected. In the control population, independently of the expression level of FBN1, we observed steady-state equilibrium between the two allelic-mRNAs suggesting that FBN1 expression mainly depends on trans-acting regulators. Finally, we show that a low level of residual WT FBN1 mRNA accounts for a high risk of ectopia lentis and pectus abnormality and tends to increase the risk of aortic dilatation.

Introduction

Marfan syndrome (MFS [MIM 154700]) is a connective tissue disorder, with an autosomal dominant inheritance and a prevalence of 1 in 5000 individuals. The clinical features of MFS involve the ocular, cardiovascular and skeletal systems, skin, lung and dura. In most MFS patients, a mutation within the gene encoding fibrillin-1 (FBN1) is identified. To date, over 3000 mutations have been reported worldwide (http://www.umd.be/FBN1), widely distributed throughout the 65 coding exons of the gene with no preferred location. All molecular forms of mutations are found from missense mutations to premature termination codon (PTC) mutations arising from direct nonsense variants to whole-exon deletions. MFS phenotype variability is notable, for age of onset as well as severity of clinical manifestations. Several phenotype/genotype correlation studies have been performed (1–3), the largest through a worldwide collaboration (4) and showed no relationship, except for mutations located between exons 24 and 32 that are associated with neonatal MFS as well.
as some cases of the severe adult form of the disease (5). Moreover no environmental factor has been shown to impact MFS phenotype expression, apart from classic cardiovascular risk factors increasing cardiovascular features. Thus in the absence of genotype/phenotype correlations, the role of genetic factors beyond the initiating FBN1 mutation can be suspected.

At the molecular level, FBN1 mutations are associated with either of two mechanisms partly explaining phenotype variability: (1) a dominant negative effect, when mutant FBN1 monomers impair polymerization of monomers into a properly functioning complex, and (2) haploinsufficiency when the mutation is responsible for the absence of synthesis of the FBN1 by the mutant allele (6). PTC mutations are expected to result in FBN1 production only from the normal allele and be responsible for a decrease in the amount of FBN1 produced. We could then expect that phenotype severity may be related to the residual amount of normal protein present in the organism, as demonstrated in elliptocytosis (7) and erythropoietic protoporphyria (8). The residual expression of FBN1 in these two mechanisms (dominant negative and haploinsufficiency) has been tested with conflicting results in small studies, either in family members carrying an identical mutation or in probands (6,9,10). However, no systematic investigation has been performed.

The aim of this study was to evaluate first the extent of variability in FBN1 mRNA levels in fibroblasts from controls, as well as the relative contribution of each allele; second, to explore the existence of a relationship between the residual amount of normal FBN1 mRNA synthesis in MFS patients, all carrying a PTC mutation, and the severity of the syndrome. Our results show (i) a highly variable expression level of WT-FBN1 in both skin fibroblasts of controls and Marfan patients, (ii) that, in controls, steady-state equilibrium between the two FBN1 allelic-mRNAs suggests that FBN1 expression is mainly regulated by trans-factors and finally (iii) that a low level of WT-FBN1 mRNA is an important determinant in ectopia lentis and pectus deformity risks and tends to increase the risk of aortic dilatation.

Results

Well-correlated expression of FBN1 between skin and aortic adventitial fibroblasts

Comparison between FBN1 mRNA levels in skin and aortic (adventitial) fibroblasts from six subjects with aortic aneurysm showed no significant difference (Fig. 1).

Highly variable expression of FBN1 and steady-state equilibrium between the allelic-mRNAs in the control population

FBN1 mRNA levels were tested in 80 controls (age range from 0 to 68 yo, mean age 11.5 years ± 1.6 years, 44% females). Observed levels varied widely from 0.68 to 2.68 (3.9-fold), mean 1.65 ± 0.48 and presented a Gaussian distribution (Fig. 2A and Supplementary Material, Table S4). mRNA levels were related neither to sex nor age (r = 0.09, P = NS) (Supplementary Material, Fig. S1). Allele-specific expression was tested in 21 controls heterozygous for rs1042078. In each, a very stable 50/50 allelic contribution was observed (Fig. 2A).

Marfan population with PTC FBN1 mutations

Marfan population included 80 patients (age range from 18 to 68 yo, mean age 36.8 ± 1.6 years, 60% females). Nineteen patients had been operated upon for either aortic dilatation (n = 15) or dissection (n = 4), at a mean age of 37.2 ± 10.6 years. Dissection occurred in five patients, four type A who were operated upon and one type B without surgery. In the non-operated patients, aortic root diameters were 3.15 ± 1.55 standard deviation (SD) above the mean according to Roman et al. (11) with a Z score ranging from 0 (strictly normal aortic diameter) to 7.2 (i.e. aortic diameter 7.2 SD above the predicted mean). In this selected group of patients with PTC, the severity of aortic involvement varied from preventive aortic surgery at the age of 17, or aortic dissection at 33 years to normal aortic diameter (Z score = 0) at 67 years.

Forty-five patients (56%) presented ectopia lentis, requiring surgery in six patients. Major skeletal features were observed in 32 patients (40%), pectus deformity was present in 63 including 6 who had surgery for pectus; arachnodactyly was present in 66, scoliosis >20° in 37, flat feet in 32. Pneumothorax was present in 8, striae in 66 (Supplementary Material, Table S5).

No clear relationship could be observed between the severity of the features observed in one system versus the severity of the features observed in another system.

WT-FBN1 expression in Marfan patients

The mean total mRNA levels obtained in Marfan patients varied widely from 0.38 to 1.67 (4.4-fold), mean 0.87 ± 0.31 (Fig. 2B and Supplementary Material, Table S4). mRNA levels were related neither to sex nor age (r = 0.15, P = NS) (Supplementary Material, Fig. S1). The mean value was 52% of that obtained in controls. Twelve patients informative for SNP rs1042078 were studied to quantify allele-specific expression and all showed that more than 90% of FBN1 mRNA synthesized was transcribed from the wild FBN1 allele, i.e. the mutated FBN1 mRNA was not detected (Fig. 2B). This was directly confirmed by Sanger sequencing and quantification of the signal at the mutation site in 79 Marfan patients.

Relationship between WT-FBN1 mRNA levels and the clinical severity of the syndrome

Lower mRNA levels were found in patients with ectopia lentis when compared with patients without ectopia lentis (0.79 versus 1.01, P = 0.001; Fig. 3A), and a tendency for lower mRNA levels was observed in patients who had undergone surgery for ectopia...
lentis when compared with patients who had not been operated upon (0.67 ± 0.11 versus 0.82 ± 0.04 NS). In the same way, lower mean mRNA levels were found in patients with a pectus deformity (either carinatum or recurvatum) versus patients without pectus (0.83 versus 1.04; \( P = 0.01 \)), and a tendency toward lower mRNA levels in patients with either a wrist or a thumb sign (0.85 ± 0.04 versus 0.91 ± 0.07, NS). No relationship was found between mRNA expression levels and scoliosis, or protrusio acetabulae, or hyperlaxity, or any other skeletal, skin or lung feature. Finally, a non-significant trend was observed between lower mRNA \( FBN1 \) levels and greater aortic dilatation evaluated by the \( Z \) score (Fig. 3, \( r = 0.23, P = 0.06 \)), while no difference was observed between patients who had been or not been operated upon for aortic dilatation.

**Discussion**

Clinical symptoms associated with \( FBN1 \) mutations may vary greatly from moderate skeletal features to a severe Marfan phenotype associating ectopia lentis, early aortic dissection and major skeletal features. This variability has been reported across families but also within families indicating that the mutation per se is not the only determinant in the severity of the phenotype. This is in keeping with the absence of any clear-cut genotype/phenotype relationship identified despite several studies (3,12–15), notably one including over 1000 probands over the world (5). Either of two pathogenic mechanisms may result from mutations in the \( FBN1 \) gene: (1) haploinsufficiency, when the mutation gives rise to a PTC associated with nonsense-mediated decay (NMD); and (2) dominant negative effect from missense mutations, in which the mutated monomer impairs the function of its wild-type (WT) counterpart. To identify genetic modifiers of MFS, we chose to study only patients carrying a PTC mutation, thus enabling us to control for an identical initiating mechanism of the disease in MFS patients. The great clinical variability observed in this population (for example from preventive aortic surgery at 17 yo to no aortic dilatation at the age of 67) is similar to that reported in other MFS populations, reinforcing the concept that factors other than the causative mutation explain the severity of the syndrome.

The ignorance of the mechanisms underlying the variable clinical expression and penetrance defects severely limits the potential of genetic counselling. In autosomal dominant diseases such as MFS, it is tempting to speculate that the residual expression level of the WT allele may influence the outcome and severity of the disease (7). The aim of this study was thus to evaluate (1) the extent of variability in WT-\( FBN1 \) mRNA levels in fibroblasts from controls and MFS patients, as well as the relative contribution of each allele to the total amount of mRNA produced; and (2) to explore the existence of a relationship between the
severity of the syndrome and the residual amount of normal FBN1 mRNA synthesis in MFS patients all carrying a PTC mutation. The control fibroblasts originated from mostly young patients (mean age 11.5 years ± 1.6 years with a range from 0 to 68 yo). However no age effect was found in this group, nor in the MFS group it was compared with. Our results show that there is great variability of FBN1 mRNA amount synthesized by fibroblasts in the control population, with ~4-fold (0.67–2.56). To our knowledge, this is the first report of such large variability of expression in a control population because essentially comparative expression levels are reported either between cell types (16) or during different developmental stages (17). Only recently, data from the Genotype-Tissue Expression (GTEx) Project has provided some more comprehensive data that are in agreement with our results (http://www.gtexportal.org). Moreover, in our study, no differential allelic expression was observed (each allele providing 50% of total transcription level of the FBN1 locus) in this control population. Presently, the reason for this concerted expression of the two alleles is unknown. However, this observation is in favour of the effects of trans-acting regulators of FBN1 expression, i.e. regulators located outside the FBN1 locus. Only recently FBN1 expression studies have been made available through genome-wide RNA-seq analyses. Combining these with genome-wide genotyping, expression quantitative trait loci (eQTL) studies have revealed the existence of cis-eQTL at the FBN1 locus. This discrepancy with our results could be explained by the use of different biological samples (total skin instead of isolated skin fibroblasts), by the different quantification methods (genome-wide RNA sequencing instead of robust gene-specific quantitative reverse-transcription polymerase chain reaction, qRT-PCR) and, more generally, by the difficulty in identifying trans-eQTL (18).

Dermal fibroblasts from MFS subjects carrying a PTC mutation in the FBN1 gene also showed great inter-individual variability, in agreement with our first observation in controls. It confirms the only results found in the literature and reported by Hutchinson et al. (10) through northern blot quantification and RNase protection assay in three affected subjects from a single family carrying a frameshift FBN1 gene mutation. Furthermore, our results show that the amount of total FBN1 mRNA synthesized in patients (mean 0.87 ± 0.31) is about half when compared with control subjects (mean 1.65 ± 0.48). Moreover, allele-specific expression showed that more than 90% of FBN1 transcript was related to

Figure 3. Relationship between mean FBN1 mRNA levels and clinical features in MFS patients. (A) Mean FBN1 mRNA levels in MFS patients with and without ectopia lentis. (B) Mean FBN1 mRNA levels in MFS patients with and without pectus deformity. (C) Mean FBN1 mRNA levels in MFS patients compared with the Z-score of aortic diameter at the level of Valsalva sinus.
the normal allele, the signal from the mutant allele being barely detected. This was expected because patient selection had been targeted for a homogenous pathogenic mechanism: haploinsufficiency through FBN1 PTC mutations leading to degradation of muta-
tant mRNA by the NMD control system. All in all, considering total and allele-specific expression levels in MFS subjects as well as in controls, it appears that FBN1 mRNA level is not allele-specific and not subject to a dose-sensitive regulating effect. Finally, ex-
pression trans-regulation appears not to be through a FBN1 sensor but through genetic and/or environmental predetermination.

In this study, a low level of WT-mRNA is associated with a greater risk of ectopia lentis and pectus in MFS patients: 75% have an ectopia lentis and all patients have a pectus in the lower quartile versus 35% with ectopia lentis and 55% with a pect-
us in the high quartile. A trend is observed for greater aortic dilat-
tion. Interestingly the only tentative genotype/phenotype correlation reported in different studies involves ectopia lentis and the presence of mutations affecting a cysteine residue in cb-EGF-like modules (calcium-binding EGF-like) (5,12). Ectopia lentis (lens displacement) seen in MFS patients is due to func-
tional alterations of the zonules which are ligaments almost ex-
clusively made of fibrillin (19). Therefore, the results of this study as well as the genotype/phenotype correlation reported previous-
ly indicate that the major pathogenic mechanism and thus the major determinant of presence or absence of ectopia lentis in MFS is the qualitative or quantitative alteration of fibrillin in the zonules. Conversely, no association was found for other clinical features suggesting that in other MFS-affected systems/ tissues, pathogenic mechanisms are more complex with qualita-
tive or quantitative alteration of fibrillin representing only one of the components. Therefore, further research is warranted to identify determinants of MFS severity in these systems.

Finally, no link was found between striae and FBN1 mRNA le-
vels despite the dermal origin of the fibroblasts. This could be ex-
plained by lack of statistical power because over 80% of patients displayed striae. Furthermore, the purpose of the study was to use patient skin fibroblasts as in vitro models because other cell types relevant for MFS-affected systems are not easily available. We were able to investigate the relevance of our model by compari-
on to aortic adventitial fibroblasts obtained at surgery for six patients. A strong correlation was obtained for FBN1 mRNA expression levels in these cells in culture. Furthermore, we found a significant association between expression levels of the WT-FBN1 and a discrete number of symptoms in different sys-

tems. These results highlight that MFS symptom severity in-
volves different mechanisms depending on the affected organ/ tissue. Therefore, the investigation of genetic variants conduct-
ing the extracellular matrix orchestra and underlying disease se-

Material and Methods
Controls and patients
Eighty control fibroblast cell lines were obtained from the ‘Centre de Ressources Biologiques-CBC Bioteck: Maladies génétiques’ from Lyon University Hospitals (Dr M.T. Zabot). This cell culture repository contains anonymized samples from patients and con-
trols in whom metabolic disorders were initially investigated and ruled out in the case of the controls. Samples were provided and used in agreement with French regulatory requirements.

To investigate the relevance of FBN1 expression in skin fibro-
blast with respect to expression in aortic (adventitial) fibroblasts, biopsy pairs (skin and aorta) were obtained for six patients who underwent aortic surgery (one with aortic aneurysm of undeter-

Real-time quantitative PCR
We determined mRNA levels for FBN1 by reverse transcription followed by quantitative PCR (RT-qPCR). Two sets of primers were systematically used: in exon 2 and exon 47 of FBN1. Expression of three reference genes (GAPDH, SDHA and HMBS) was also performed. Primers sequences and their Tm are shown in Supplementary Material, Table S1.

RT-qPCR analyses were carried out with the Absolute Blue qPCR SYBR Green Rox Mix (Thermo Scientific, Courtaboeuf, France), using an ABI PRISM 7300 apparatus according to the manufacturer’s instructions. Amplification was performed in a final volume of 20 µl containing 2X SYBR, 7.5 µM of each primer and 10 µl of a 1:500 dilution of the sample. Each reaction was
run in triplicate in FrameStar 96-well semi-skirted PCR plates with frosted wells and clear frames (4ti-0772 from Dutscher, Brumath, France) sealed by adhesives.

For each amplification, melting and standard curves were generated from 5-fold dilutions of cDNAs, to determine primer efficiency. Controls, lacking reverse transcriptase and carried out alongside quantitative RT-qPCR for experimental samples consistently yielded no amplification below 35 cycles. In all plates, an aliquot from a single reference cDNA pool was run to allow between plate comparisons. Mean Ct (cycle threshold) values and SD for each plate as well as melting and standard curves are provided in Supplementary Material, (Tables S2 and S3) according to minimum information for the publication of quantitative real-time PCR experiments guidelines (22).

Quantification of total FBN1 mRNA content per sample was performed by normalization using the ratio of the geometric averaging of the two FBN1 exons and the geometric averaging of the three internal control genes as described by Vandesompele et al. (23).

To determine the ratio of allele-specific FBN1 expression, rs1042078, a common single nucleotide polymorphism (SNP) located in the 3’ UTR region of the gene was studied in controls and patients. cDNAs were used and amplification of the corresponding fragments were also carried out on genomic DNA (gDNA) from the same subjects. Amplified products from gDNA and cDNA were sequenced in triplicate and the allele expression ratio was quantified using the ‘peak picker’ software as described (24). In parallel, rs1042078 alleles were genotyped in patients’ families to identify the mutation-associated allele. Finally, cDNA from all patients was Sanger sequenced at the mutation site to quantify allele-specific expression.

Statistical analysis
Statistical analysis was performed using Jumpl 7.0.1 software (SAS institute Inc.). All results are expressed as mean ± SD. Comparisons between two groups were made using one-way analysis of variance, and derived plots display means diamond (a line across each diamond represents the 95% confidence interval for each group). Simple linear regressions were used to model the relationship between two continuous variables. The significance was set at P < 0.05 for all comparisons.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
The authors thank Pr Jamel Chelly and the Cell Bank of Hôpital Cochin, Assistance Publique Hôpitaux de Paris for their cooperation throughout the VARIARFAN project.

Conflict of Interest statement. The authors declared no conflicts of interest.

Funding
This work was supported by: Assistance Publique-Hôpitaux de Paris (CRC07032—P071009), Agence Nationale de Recherche (ANR, GDPM2), Fédération Française de Cardiologie, Société Française de Cardiologie and DHU-FIRE (Assistance Publique-Hôpitaux de Paris). M.A. was supported by a grant from Fond d’Etudes et de Recherche du Corps Médical, Assistance Publique-Hôpitaux de Paris.

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