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An *FBN1* Deep Intronic Variant is Associated with Pseudoexon Formation and a Variable Marfan Phenotype in a Five Generation Family

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Abstract

Exome sequencing of genes associated with heritable thoracic aortic disease (HTAD) failed to identify a pathogenic variant in a large family with Marfan syndrome (MFS). A genome-wide linkage analysis for thoracic aortic disease identified a peak at 15q21.1, and genome sequencing identified a novel deep intronic *FBN1* variant that segregated with thoracic aortic disease in the family (LOD score 2.7) and was predicted to alter splicing. RT-PCR and bulk RNA sequencing of RNA harvested from fibroblasts explanted from the affected proband revealed an insertion of a pseudoexon between exons 13 and 14 of the *FBN1* transcript, predicted to lead to nonsense mediated decay (NMD). Treating the fibroblasts with an NMD inhibitor, cycloheximide, greatly improved the detection of the pseudoexon-containing transcript. Family members with the *FBN1* variant had later onset aortic events and fewer MFS systemic features than typical for individuals

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AUTHOR CONTRIBUTIONS

Dianna M. Milewicz and Dong-chuan Guo designed the study and write manuscript; Yang Yu and Xue Zhu provide support on exome and genome data analysis; Xueyan Duan and Kathleen Mimnagh designed and performed RT-PCR and sequencing assays. Alana C. Cecchi and David R. Murdock analyzed RNA-seq data; Isabella C. Marin and Walter V. Torres acquired clinical information from family and analyzed; Kwanghyuk Lee and Suzanne M. Leal performed genetic statistical analyses; Marsha M. Wheeler, Josh Smith, and Michael J. Bamshad provided supports on whole exome and whole genome sequencing assay and data analyses.

CONFLICT OF INTEREST

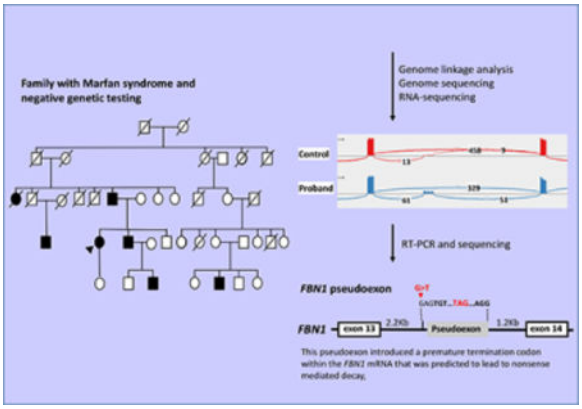
Dr. David R. Murdock was previously an employee of Invitae and the rest of the authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

with haploinsufficiency of *FBN1*. Variable penetrance of the phenotype and negative genetic testing in MFS families should raise the possibility of deep intronic *FBN1* variants and the need for additional molecular studies.

Graphical Abstract



These results indicate that families with decreased penetrance of features of MFS and negative genetic testing should be assessed for deep intronic variants that lead to the insertion of a pseudoexon, and detection of these pseudoexons may be improved when the cells are treated with inhibitors of nonsense mediated decay.

Keywords

FBN1 ; Marfan syndrome; pseudoexon; heritable thoracic aortic disease

Pathogenic variants in *FBN1* cause Marfan syndrome (MFS), an autosomal dominant condition leading to highly penetrant thoracic aortic aneurysms and dissections associated with ocular and skeletal complications (Chen et al., 2021). Although diagnostic genetic testing (exon sequencing, splice sites, and deletion/duplication analyses) identifies a pathogenic *FBN1* variant in the majority of patients who meet the clinical diagnostic criteria for MFS, up to 8% of cases do not have a detectable variant in *FBN1*, *TGFBR1*, or *TGFBR2* (Baetens et al., 2011; Loeys et al., 2004). Deep intronic *FBN1* variants leading to introduction of pseudoexons in the transcript are a rare cause of MFS, but the overall frequency of such pathogenic non-coding variants is unknown (Gillis et al., 2014; Guo et al., 2008).

Multigene thoracic aortic disease panel testing in an affected family member of a five-generation family with variable penetrance of both thoracic aortic disease and systemic features of MFS failed to identify pathogenic variants in the known genes for heritable thoracic aortic disease (HTAD) (Figure 1A). The family consented for research approved by the Institutional Review Board at the University of Texas Health Science Center at Houston. The proband and three relatives with thoracic aortic disease underwent exome sequencing but no rare, shared variants were identified that could explain their phenotype. Subsequent linkage analysis using 8 samples from family members and the Illumina HumanCore-24

chip identified 5 loci with LOD scores > 2, including one encompassing *FBN1* with a LOD score of 2.7 (Figure 1B, supporting information). Using the linkage information, two distantly related family members with thoracic aortic disease underwent whole genome sequencing (WGS). Filtering for shared rare variants (gnomAD v3.1.2 global MAF < 10^{-4}) under linkage peaks (Figure 1b, supporting information), we identified a variant in intron 13 of *FBN1* (NM_000138.5), 15:48803583C>A (hg19), that segregated with thoracic aortic disease in the family and was absent from gnomAD v3.1.2. Sanger sequencing validated the variant (Figure 1C). Four computational splicing tools (SpliceAI, Human Splicing Finder, NetGene2, ESE Finder v3.0) predicted this deep intronic variant (c.1589–1217G>T) could lead to use of a new splice acceptor site within intron 13 of the *FBN1* transcript.

To assess this *FBN1* variant's effect on splicing, RNA was harvested from the proband's dermal fibroblasts. Using Sanger sequencing of the cDNA with primers in exons 13 and 14, we observed a novel pseudoexon, 202 bp in length, within intron 13 of the *FBN1* transcript (Figure 2A–C, supporting information). This pseudoexon introduced a premature termination codon (PTC) within the *FBN1* mRNA (p.Asp530Valfs*8) that was predicted to lead to nonsense mediated decay (NMD) (Figure 2A). *FBN1* transcript levels assayed by quantitative RT-PCR (qPCR) determined that the proband had a 50% reduction of total *FBN1* expression compared to controls when normalized by the expression of *GAPDH* (Figure 2D), suggestive of NMD. The level of mutant *FBN1* transcript was also significantly increased compared to control cells ($p < 0.01$). To confirm the reduction in expression was due to NMD, the proband's fibroblasts were treated with an NMD inhibitor, cyclohexamide (CHX), which inhibited degradation of mutant *FBN1* mRNA and significantly increased expression of mutant mRNA at hour 8 ($p < 0.01$); Figure 2E; supporting information)

RNA sequencing (RNA-seq) has emerged as a methodology to identify cryptic aberrant splicing in genes for Mendelian diseases (Saeidian et al., 2020). To determine if abnormal *FBN1* splicing and expression would be detected with this approach, bulk RNA-seq was performed from the proband's fibroblasts as previously described (Murdock et al., 2021). After optimizing pipeline parameters, a 37% reduction in *FBN1* expression was observed compared to controls along with 2.3% of reads containing the same pseudoexon detected before (Figure 2F, supporting information), consistent with NMD taking place. As expected, inhibition of NMD with CHX increased the number of reads spanning the pseudoexon to 12.7% with a slight increase in overall *FBN1* expression compared to the untreated sample (Figure 2F, supporting information).

Based on the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines, the following evidence indicates that the chr15:48803583 C>A *FBN1* deep intronic variant (c.1589–1217G>T, p.Asp530Valfs*8) is pathogenic: (1) nonsense or frameshift variant in a gene where loss of function (LoF) is a known mechanism of disease (PVS1); (2) co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease (PP1_strong); (3) low minor allele frequency in the population (PM2); and (4) Marfan-like systemic features in cases (PP4). Nine living family members were confirmed to be heterozygous for the *FBN1* deep intronic variant and four were obligate heterozygotes (Table S1). Three deceased individuals were at 50% risk to have the variant, one of which had an autopsy-proven dissection (died at age 19

years), and the other two died suddenly of unknown causes (died at ages 36 and 43 years; supporting information). Data was obtained for these 16 individuals on aortic events (defined as an aortic dissection or surgical repair of an aortic aneurysm) and MFS systemic features, along with aortic root diameters from individuals who had not had an event (Loeys et al., 2010). In the 13 confirmed and obligate heterozygotes with the *FBN1* variant, there were three aortic surgical repairs, two of which occurred at aortic root diameters of 6.5 and 6.9 cm, respectively. Aortic root enlargement (Z score > 2) was present in seven individuals, with initial enlargement noted at ages ranging from 5 to 58 years. Two individuals with the variant remain without aortic root enlargement at ages 27 and 59 years, respectively (Table S2). Thus, the cumulative risk for aortic events in this family appears to be lower than previously reported for *FBN1* haploinsufficiency variants (Figure 1D, supporting information) (Arnaud, Morel, et al., 2021). The MFS systemic scores for nine family members who were assessed by a physician ranged from 1 to 9 with a median score of 7 (supporting information). None of the affected individuals had history of ectopia lentis, mitral valve prolapse, dural ectasia, protrusio acetabuli, or spontaneous pneumothorax.

Non-coding variants that lead to pseudoexon formation, intron retention, and regulatory changes are not typically identified by exome sequencing or gene panels that focus on coding and consensus splice site regions. Similarly, synonymous variants that may affect splicing are difficult to fully assess. The deep intronic *FBN1* variant in the family reported here leads to the insertion of an out-of-frame pseudoexon in the *FBN1* transcript and resultant haploinsufficiency via NMD. Notably, the rate of aortic events in this family was less than that reported for individuals with *FBN1* haploinsufficiency variants (Arnaud, Milleron, et al., 2021). One possible explanation for the reduced penetrance in the family reported here is decreased efficiency of insertion of the pseudoexon into the *FBN1* transcript, which would lead to higher levels of the normal transcript in individuals with less penetrant disease.

In conclusion, these data indicate that non-coding variants disrupting the *FBN1* transcript need to be considered in families with MFS, particularly if the clinical features are less penetrant or later onset than typically seen in MFS. As demonstrated here, such non-coding variants require additional molecular analyses, which can include WGS and RNA-seq, to identify and fully assess a functional effect on the transcript.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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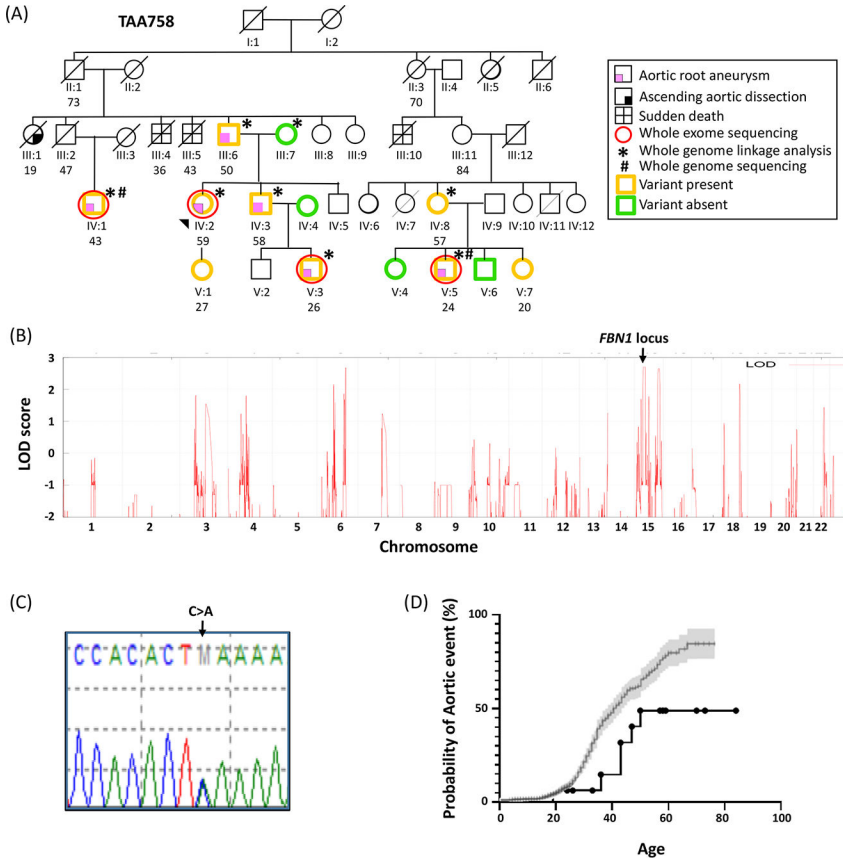

FIGURE 1.

A large HTAD family for whole genome linkage, whole exome, and whole genome sequencing assays. (A) Pedigree of TAA758 showing segregation of a deep intronic variant, that leads to an *FBN1* pseudoexon, with HTAD in the family. The age at diagnosis or death in years is shown below the individual symbols. The legend shows the symbols representing the phenotypic features and genetic testing in the family members. (B) Whole genome linkage analysis identified a linkage peak covering *FBN1*. (C) Sanger sequencing validated the variant that causes the *FBN1* pseudoexon. (D) Kaplan-Meier analyses depicting the probability of aortic events in TAA758 family members who carry the *FBN1* variant and in Marfan syndrome patients with *FBN1* premature codon termination (PTC) variants (gray line) (Arnaud, Milleron, et al., 2021).

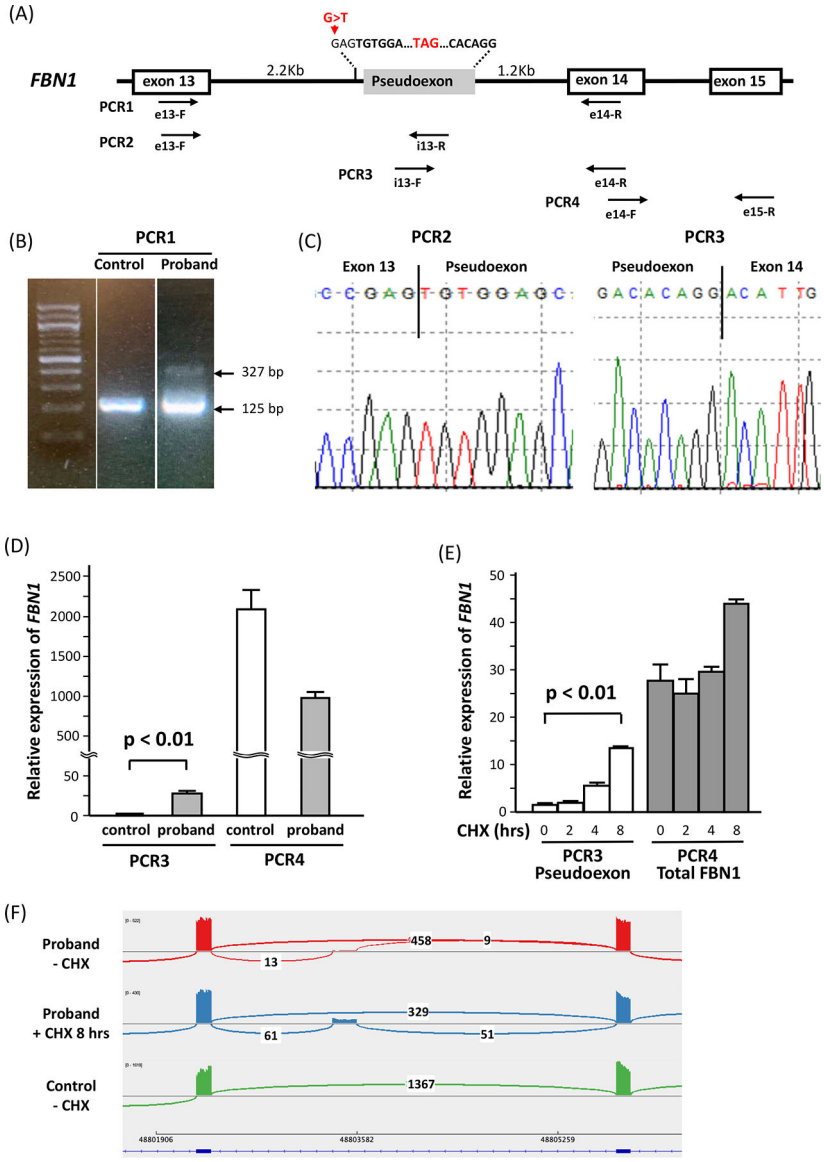

FIGURE 2.

Molecular biological analysis confirmed that the *FBN1* pseudoexon led to HTAD in the family. (A) Schematic diagram of *FBN1* pseudoexon and the location of primers for RT-PCR assays. (B) RT-PCR assay on mRNA extracted from fibroblast cells of the proband and an age-matched control, consistent with the 202 bp *FBN1* pseudoexon in the proband. These RT-PCR products were assayed on the same agarose gel (Original image, Supporting Figure 2). (C) Sanger sequencing on RT-PCR products identified the boundaries of the pseudoexon. (D) q-PCR assay showed that in the proband, the mRNA level of q-PCR assay showed that in the proband, the mRNA level of *FBN1* was reduced and the pseudoexon *FBN1* was significantly increased compared to that of the control. (E) Fibroblast cells from the proband treated with CHX could increase the mRNA level of pseudoexon *FBN1*. (F) RNA-seq results of the *FBN1* region on RNA extracted from fibroblast cells of proband with or without

8 hours of CHX treatment showed that the *FBN1* pseudoexon can only be detected on fibroblast cells with CHX treatment.