Human Molecular Genetics, 2014, Vol. 23, No. 19 **5271–5282**
doi:10.1093/hmg/ddu224
Advance Access published on May 15, 2014

Rare variants in *FBN1* and *FBN2* are associated with severe adolescent idiopathic scoliosis

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Received February 10, 2014; Revised April 8, 2014; Accepted May 6, 2014

Adolescent idiopathic scoliosis (AIS) causes spinal deformity in 3% of children. Despite a strong genetic basis, few genes have been associated with AIS and the pathogenesis remains poorly understood. In a genome-wide rare variant burden analysis using exome sequence data, we identified *fibrillin-1* (*FBN1*) as the most significantly associated gene with AIS. Based on these results, *FBN1* and a related gene, *fibrillin-2* (*FBN2*), were sequenced in a total of 852 AIS cases and 669 controls. In individuals of European ancestry, rare variants in *FBN1* and *FBN2* were enriched in severely affected AIS cases (7.6%) compared with in-house controls (2.4%) (OR = 3.5, $P = 5.46 \times 10^{-4}$) and Exome Sequencing Project controls (2.3%) (OR = 3.5, $P = 1.48 \times 10^{-6}$). Scoliosis severity in AIS cases was associated with *FBN1* and *FBN2* rare variants ($P = 0.0012$) and replicated in an independent Han Chinese cohort ($P = 0.0376$), suggesting that rare variants may be useful as predictors of curve progression. Clinical evaluations revealed that the majority of AIS cases with rare *FBN1* variants do not meet diagnostic criteria for Marfan syndrome, though variants are associated with tall stature ($P = 0.0035$) and upregulation of the transforming growth factor beta pathway. Overall, these results expand our definition of fibrillin-related disorders to include AIS and open up new strategies for diagnosing and treating severe AIS.

INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a lateral curvature of the spine that presents in late childhood and occurs in up to 3% of all children. AIS causes scoliosis of unknown etiology and is diagnosed only after other causes of scoliosis, including neuromuscular disorders, congenital vertebral abnormalities, and other known conditions have been ruled out. AIS accounts for over 80% of all scoliosis. Although bracing is effective at limiting curve progression in some AIS patients (1), >18 000 children undergo major spinal surgery annually because of severe scoliosis (2).

Twin (3) and family (4–6) studies indicate that genetic factors play an important role in AIS etiology, but the genetic basis is likely complex with non-Mendelian inheritance and large genetic heterogeneity (7–11). Numerous candidate genes and loci have been identified (see review 12), but few associations have been replicated in independent populations. Genome-wide association studies have identified common genetic polymorphisms associated with AIS in neural cell adhesion genes (13), *ladybird homeobox 1* (*LBX1*) (14) and *G-protein coupled-receptor 126* (*GPR126*) (15); however, common polymorphisms account for only a small amount of AIS heritability and the genetic basis of AIS remains poorly understood.

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Rare variants are thought to have a greater contribution to disease risk because negative selection restricts variants of large genetic effect from rising to high frequency (16). We therefore hypothesized that rare genetic variants are a major contributor to severe AIS susceptibility. Although large-scale sequencing studies are needed to detect rare variants, such studies have recently become feasible due to emerging sequencing technologies and reduction in sequencing cost. Using exome sequencing, we first analyzed rare variants genome-wide in a small cohort of severe AIS cases and identified *FBN1* and *FBN2* as candidate genes for AIS, which we then sequenced in a larger case and control cohort using a newly described, cost-effective targeted capture method.

RESULTS

Exome sequencing screen identifies *FBN1* and *FBN2* as a candidate gene for severe AIS

To identify candidate genes for AIS, exome sequencing was performed on 91 AIS cases with severe scoliosis (spinal curves measuring $\geq 40^\circ$ or surgically treated). We restricted our analysis to AIS patients with severe scoliosis to reduce the likelihood of latent disease in the control population, thus increasing power of the association (17). Furthermore, identifying genetic variants associated with severe AIS may be clinically useful to predict curve progression. Cases in this cohort had an average spinal curve of 60.2° (SD = 15.2°) and 84% were female. The female bias is consistent with previous studies, where female-to-male ratios are estimated to be as high as 10:1 with severe deformity (18). Exome sequencing data were also generated for 337 controls. All cases and controls were unrelated and of European ancestry. Only coding variants that caused nonsense, splice-site, missense or insertion/deletion mutations which were rare (defined as being absent in dbSNP) were included in the analysis. This filtering strategy was selected to enrich in mutations that are very rare and most likely to be deleterious.

Using a gene-burden analysis, we compared the genome-wide frequency of rare coding variants in severe AIS cases and controls. Of nearly 13 000 genes identified with at least one rare variant, *FBN1* (encoding *fibrillin-1*) was the most significantly associated gene with AIS compared with controls ($P = 3.17 \times 10^{-4}$, Fisher's exact test). The collapsed minor allele frequency (cMAF) for all rare variants in *FBN1* was 0.044 in cases ($n = 91$), compared with 0.005 in controls ($n = 337$) (OR = 10.4, 95% CI = 2.7–39.5). This result was surprising, as mutations in *FBN1* are associated with Marfan syndrome (MIM 154700), an autosomal dominant and highly penetrant disorder that causes skeletal, ocular and cardiovascular phenotypes. Likely a result of the small sample size, the association did not reach exome-wide significance ($P < 2.5 \times 10^{-6}$). However, *FBN1* was a compelling candidate for AIS because 63% of Marfan syndrome patients develop scoliosis (19).

A related gene, *fibrillin-2* (*FBN2*), showed weak association with AIS in our exome sequencing screen (cMAF = 0.022) compared with controls (cMAF = 0.004) ($P = 0.0404$, Fisher's exact test). Autosomal dominant mutations in *FBN2* cause congenital contractural arachnodactyly (MIM 121050), a disorder that has large phenotypic overlap with Marfan syndrome and causes scoliosis in 50% of patients (20). Furthermore, a single-nucleotide polymorphism (SNP) in *FBN2* was identified among the top 100 SNPs associated with AIS in a recent genome-wide association study (13); therefore, we reasoned that rare *FBN2* variants could also be associated with AIS.

Rare coding variants in *FBN1* and *FBN2* are enriched in severe AIS

Based on the results of our exome sequencing screen, *FBN1* and *FBN2* were selected as candidate genes to study in a larger cohort. Both genes were sequenced in additional AIS patients and controls using Multiplex Direct Genomic Selection (MDiGS), a newly described targeted capture approach (21). In total, 323 severely affected AIS cases and 493 controls of European descent were analyzed for rare variants in *FBN1* or *FBN2*. All cases and controls had $\geq 95\%$ coverage at $\geq 8\times$ read depth to be included in the gene analysis. The average spinal curve in the AIS cohort was 57.4° (SD = 12.1°). As an independent control cohort, we also identified rare variants in *FBN1* and *FBN2* for 4300 individuals of European ancestry from the NHLBI GO Exome Sequencing Project (ESP) controls ($n = 4300$).

For *FBN1*, rare variants were enriched in AIS cases (cMAF = 0.021) compared with both in-house controls (cMAF = 0.005) ($P = 0.0041$, Fisher's exact test) and ESP controls (cMAF = 0.005) ($P = 8.14 \times 10^{-5}$, Fisher's exact test) (Table 1). Thirteen AIS patients (4.2%, $n = 13/311$) and five in-house controls (1.0%, $N = 5/489$) were identified with a rare variant in *FBN1*. An estimated 1.0% ($n = 44/4300$) of ESP controls had a rare *FBN1* variant (assuming each individual harbored only one variant). All AIS variants were heterozygous and caused missense amino acid changes throughout the FBN1 protein (Fig. 1A, Table 2). Segregation was evaluated for nine families with DNA available from one or more additional family members (Supplementary Material, Fig. S1). *FBN1* variants segregated with AIS in some families. Two variants (p.G1217S and p.G1313S) were located in exons 24–32 of *FBN1*, which is occasionally associated with a more severe, early-onset form of Marfan syndrome (28,29). Three

Table 1. Rare variant frequencies for *FBN1* and *FBN2* in AIS cases and controls of European ancestry

	AIS cases ($\geq 40^\circ$)			Controls (this study)					Controls (ESP)				
	n	Variant alleles	cMAF	*n*	Variant alleles	cMAF	Odds ratio (95% CI)	*P*-value	*n*	Variant alleles	cMAF	Odds ratio (95% CI)	*P*-value
FBN1	311	13	0.021	489	5	0.005	4.2 (1.5–11.7)	0.0041	4300	44	0.005	4.2 (2.2–7.7)	8.14×10^{-5}
FBN2	316	11	0.017	427	5	0.006	3.0 (1.0–8.7)	0.0307	4300	56	0.007	2.7 (1.4–5.2)	0.0054
FBN1 or *FBN2*	304	24	0.039	425	10	0.012	3.5 (1.6–7.3)	5.46×10^{-4}	4300	100	0.012	3.5 (2.2–5.5)	1.48×10^{-6}

cMAF, collapsed minor allele frequency; ESP, NHLBI Exome Sequencing Project.

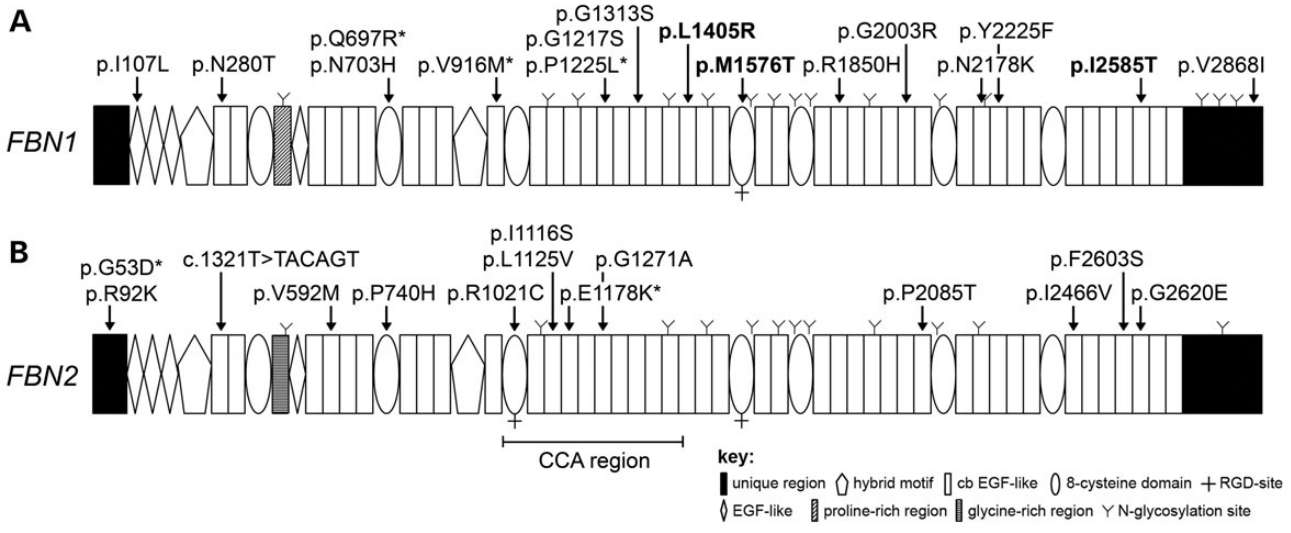

Figure 1. Rare *FBN1* and *FBN2* variants identified in AIS patients. Protein structure of FBN1 and FBN2 (redrawn from 22). (**A**) Missense variants were identified throughout FBN1 in AIS patients of European descent ($n = 417$) and other ancestries ($n = 47$). Variants previously associated with Marfan syndrome are in bold. (**B**) Missense and other coding changes were identified in FBN2 in AIS patients of European ($n = 423$) and other ancestries ($n = 47$). The region where congenital contractural arachnodactyly (CCA) mutations are primarily located is shown. Asterisk indicates variants identified in other ancestries.

variants were previously reported in Marfan syndrome [p.L1405R (23), p.M1576T (24,25) and p.I2585T (23,26,27)] and one of these variants (p.M1576T) was recurrent in three AIS patients (Table 2). Variants previously reported in Marfan syndrome were also identified in the ESP cohort [p.D1479E (23), p.E2019K (30), p.R2554W (31), p.R2694Q (32) and p.S2832G (32)], whereas none of the in-house control variants were previously reported as pathogenic.

Similarly, rare *FBN2* variants were also enriched in AIS cases (cMAF = 0.017) compared with in-house controls (cMAF = 0.006) ($P = 0.0307$, Fisher's exact test) and ESP controls (cMAF = 0.007) ($P = 0.0054$, Fisher's exact test) (Table 1). Eleven AIS patients (3.5%, $n = 11/316$), five in-house controls (1.2%, $n = 5/427$) and ~1.3% ($n = 56/4300$) of ESP controls were identified with a variant in *FBN2*. All AIS variants were heterozygous and caused missense mutations, except for a five base insertion present in one AIS case (Fig. 1B, Table 2). DNA was available for one or more additional family members in seven families. *FBN2* variants segregated with AIS in some families (Supplementary Material, Fig. S1). In congenital contractural arachnodactyly, pathogenic mutations are limited to exons 23–34 (33). In contrast, variants identified in AIS patients were located throughout the protein, including exons 23–34 (p.R1021C, p.I1116S, p.L1125V and p.G1271A). None of the variants identified in AIS cases or controls were previously reported in congenital contractural arachnodactyly.

Combining rare variants from both *FBN1* and *FBN2* yielded a cMAF of 0.039 in AIS cases, 0.012 in controls ($P = 5.46 \times 10^{-4}$, Fisher's exact test) and 0.012 in ESP controls ($P = 1.48 \times 10^{-6}$, Fisher's exact test) (Table 1). Only one AIS case and none of the in-house controls had a variant in both *FBN1* and *FBN2*. Thus, variants in either *FBN1* or *FBN2* were identified in 7.6% ($n = 23/304$) of AIS cases, 2.4% ($n = 10/425$) of in-house controls and ~2.3% ($n = 100/4300$) of ESP controls.

In addition to AIS cases with severe scoliosis, we also sequenced 112 AIS cases of European ancestry with mild-to-moderate scoliosis (curve $<40^\circ$). The average spinal curve in this cohort was 28.1° (SD = 6.9°). Rare variants in *FBN1* and *FBN2* were identified in 1.9% ($n = 2/106$) and 0.9% ($n = 1/107$), respectively, of the mild to moderately affected AIS cases (Fig. 1A and B, Table 2). *FBN1* and *FBN2* rare variant frequencies were not significantly different in mild-to-moderate AIS cases compared with in-house controls ($P = 0.47$, Fisher's exact test) or ESP controls ($P = 0.42$, Fisher's exact test).

Distinct clinical features of AIS patients with rare fibrillin variants

To determine if AIS patients with rare fibrillin variants manifest unique clinical characteristics, we compared patients with variants in *FBN1* ($n = 15$) and *FBN2* ($n = 12$) to patients without rare variants in either gene ($n = 379$) (Table 3). Analysis was limited to individuals of European ancestry, but included all curve severities ($\geq 10^\circ$). The average spinal curve was 20° greater in AIS patients with an *FBN1* variant compared with other patients ($P = 6.91 \times 10^{-4}$, Mann–Whitney–Wilcoxon test). All 15 AIS patients with rare *FBN1* variants developed scoliosis with a Cobb angle of $\geq 35^\circ$ (Table 2), which would typically warrant bracing or surgery. *FBN1* variants were also associated with taller stature standardized to age and gender ($P = 0.0035$, Mann–Whitney–Wilcoxon test). Of all AIS patients in this cohort with height ≥90th percentile, 22.5% ($n = 9/40$) were found to have a rare *FBN1* variant. Rare *FBN1* variants were also associated with Marfan-associated features, which were quantified using the revised Ghent criteria (35) ($P = 0.0072$, Mann–Whitney–Wilcoxon test). Gender, weight, body mass index, family history of treated AIS and joint hypermobility were not associated with the presence of rare *FBN1* variants. There were no unique clinical characteristics in AIS patients with rare *FBN2* variants (Table 3). However, like *FBN1*, we noted a trend towards greater spinal curves in AIS patients with *FBN2* variants ($P = 0.10$).

Table 2. Rare *FBN1* and *FBN2* variants identified in AIS cases (curve ≥10°)

Case	Scoliosis curve	Ancestry	Genomic position (hg19)	Base change	Gene	GVS function	Amino acid change	ESP frequency[a]	Disease association
6128-001	84°	EUR	chr15:48 902 952	T>A	*FBN1*	Missense	p.Ile107Leu	–	–
6340-001	48°	EUR	chr15:48 826 300	T>G	*FBN1*	Missense	p.Asn280Thr	–	–
6390-001	60°	AFR	chr15:48 796 007	T>C	*FBN1*	Missense	p.Gln697Arg	–	–
6377-001	50°	EUR	chr15:48 795 990	T>G	*FBN1*	Missense	p.Asn703His	–	–
6016-001	36°	Other	chr15:48 784 766	C>T	*FBN1*	Missense	p.Val916Met	*N* = 1/6,494	–
1044	70°	EUR	chr15:48 777 634	C>T	*FBN1*	Missense	p.Gly1217Ser	–	–
6273-001	48°	AFR	chr15:48 777 609	G>A	*FBN1*	Missense	p.Pro1225Leu	–	–
1083	52°	EUR	chr15:48 773 879	C>T	*FBN1*	Missense	p.Gly1313Ser	–	–
6226-001	97°	EUR	chr15:48 764 870	A>C	*FBN1*	Missense	p.Leu1405Arg	–	MFS (23)
6272-001	90°	EUR	chr15:48 760 155	A>G	*FBN1*	Missense	p.Met1576Thr	–	MFS (24,25)
6418-001	65°	EUR							
6442-001	35°	EUR							
450-14600	38°	EUR	chr15:48 741 087	C>T	*FBN1*	Missense	p.Arg1850His	–	–
6320-001	66°	EUR	chr15:48 736 768	C>T	*FBN1*	Missense	p.Gly2003Arg	–	–
6368-001	100°	EUR	chr15:48 726 873	A>T	*FBN1*	Missense	p.Asn2178Lys	–	–
6111-001	95°	EUR	chr15:48 725 128	T>A	*FBN1*	Missense	p.Tyr2225Phe	–	–
6386-001	93°	EUR	chr15:48 712 949	A>G	*FBN1*	Missense	p.Ile2585Thr	–	MFS (23,26,27)
6005-001	55°	EUR	chr15:48 703 201	C>T	*FBN1*	Missense	p.Val2868Ile	–	–
6463-001	57°	AFR	chr5:127 873 139	C>T	*FBN2*	Missense	p.Gly53Asp	*N* = 2/6,489	–
6112-001	48°	EUR	chr5:127 872 157	C>T	*FBN2*	Missense	p.Arg92Lys	–	–
1157	45°	EUR	chr5:127 782 238	A>ACTGTA	*FBN2*	Frameshift	–	–	–
6124-001	75°	EUR	chr5:127 713 520	C>T	*FBN2*	Missense	p.Val592Met	–	–
6279-001	55°	EUR	chr5:127 704 904	G>T	*FBN2*	Missense	p.Pro740His	–	–
1022	25°	EUR	chr5:127 681 205	G>A	*FBN2*	Missense	p.Arg1021Cys	–	–
6191-001	90°	EUR	chr5:127 674 750	A>C	*FBN2*	Missense	p.Ile1116Ser	–	–
6129-001	80°	EUR	chr5:127 674 724	G>C	*FBN2*	Missense	p.Leu1125Val	–	–
6367-001	70°	Other	chr5:127 673 755	C>T	*FBN2*	Missense	p.Glu1178Lys	–	–
6229-001	65°	EUR	chr5:127 671 182	C>G	*FBN2*	Missense	p.Gly1271Ala	–	–
6206-001	50°	EUR	chr5:127 627 260	G>T	*FBN2*	Missense	p.Pro2085Thr	–	–
6383-001	43°	EUR	chr5:127 613 647	T>C	*FBN2*	Missense	p.Ile2466Val	*N* = 1/6,503	–
6418-001	65°	EUR	chr5:127 609 564	A>G	*FBN2*	Missense	p.Phe2603Ser	*N* = 2/6,503	–
6216-001	50°	EUR	chr5:127 607 792	C>T	*FBN2*	Missense	p.Gly2620Glu	–	–

NA, not available; EUR, European; AFR, African; ESP, NHLBI Exome Sequencing Project; MFS, Marfan syndrome.
[a]Frequency includes individuals of African and European ancestries.

Table 3. Clinical characteristics of AIS cases (curve ≥10°) of European ancestry with rare variants in *FBN1* and *FBN2*

	No variant	*FBN1*	*P*-value	*FBN2*	*P*-value
Female	86% (*n* = 379)	80% (*n* = 15)	0.35	83% (*n* = 12)	0.51
Spinal curve (avg ± s.d.)	49° ± 16 (*n* = 364)	69° ± 23 (*n* = 15)	6.91×10^{-4}	58° ± 18 (*n* = 12)	0.10
Height percentile (avg ± s.d.)	55th ± 30 (*n* = 217)	75th ± 31 (*n* = 15)	0.0035	56th ± 35 (*n* = 12)	0.41
Weight percentile (avg ± s.d.)	57th ± 31 (*n* = 217)	69th ± 30 (*n* = 15)	0.06	50th ± 33 (*n* = 12)	0.25
Body mass index (avg ± s.d.)	22.2 ± 5.1 (*n* = 217)	21.6 ± 4.0 (*n* = 15)	0.42	20.2 ± 3.6 (*n* = 12)	0.06
First degree relative with treated AIS	16% (*n* = 277)	33% (*n* = 12)	0.12	20% (*n* = 10)	0.50
Beighton joint hypermobility score (34) (avg ± s.d.)	1.4 ± 1.6 (*n* = 105)	2.5 ± 2.8 (*n* = 10)	0.09	1.0 ± 0.8 (*n* = 4)	0.45
Ghent systemic features score (35) (avg ± s.d.)	2.4 ± 1.4 (*n* = 105)	4.2 ± 2.6 (*n* = 10)	0.0072	3.5 ± 1.9 (*n* = 4)	0.10

avg, average; s.d., standard deviation.

Curve severity is associated with rare fibrillin variants in AIS cases of European and Han Chinese ancestries

AIS patients of European ancestry with a rare variant in either *FBN1* or *FBN2* (*N* = 26) developed an average spinal curve of 64.0°, compared with 49.0° in cases with no rare variants (*n* = 364) (*P* = 0.0012, Mann–Whitney–Wilcoxon test) (Fig. 2). Because predicting curve severity has important clinical implications, we performed a replication study in 370 Han Chinese AIS patients. Our replication cohort included all curve severities ≥10° (average = 34.1°, SD = 12.5°). Twenty-eight (7.6%, *n* = 28/370) Han Chinese cases were identified with a rare coding variant in *FBN1* or *FBN2*. The average spinal curve in these cases (37.2°, *n* = 28) was greater than cases with no rare variant in *FBN1* or *FBN2* (33.9°, *n* = 342) (*P* = 0.0376, Mann–Whitney–Wilcoxon test) (Fig. 2). Moreover, 11.9% (*n* = 12/101) of Han Chinese cases with severe spinal curves measuring ≥40° had a rare variant in *FBN1* or *FBN2*, compared with only 5.9% (*n* = 16/269) of cases with spinal curves <40° (*P* = 0.04826, Fisher's exact test).

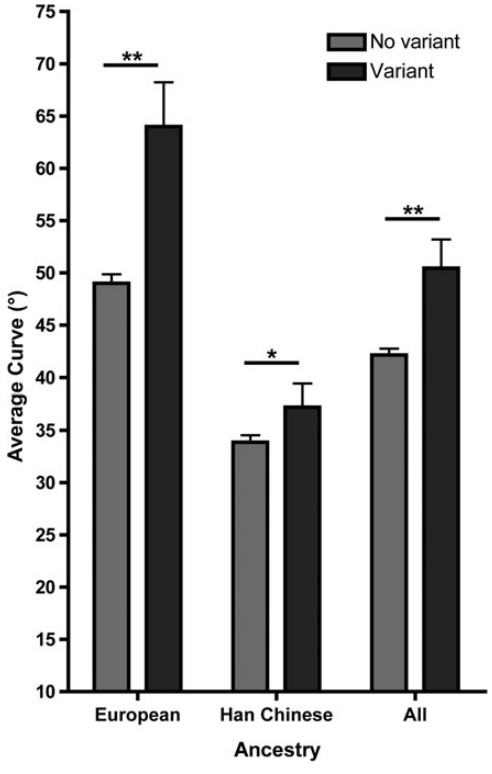

Figure 2. Rare *FBN1* and *FBN2* variants are associated with curve severity in AIS. The average spinal curve was determined for AIS cases with a rare variant in either *FBN1* or *FBN2* (Variant) and AIS cases without a rare variant in either gene (No variant). AIS cases of European ancestry ($n = 405$), Han Chinese ancestry ($n = 370$) and a combined analysis consisting of cases of European, Han Chinese and other ancestries ($n = 801$) consistently showed that patients with rare variants in *FBN1* or *FBN2* developed larger spinal curves. $^{*}P < 0.05$, $^{**}P < 0.01$.

AIS cases of other ancestral background ($n = 47$) were also sequenced in this study and an additional five rare variants in *FBN1* and *FBN2* were identified (Fig. 1A and B, Table 2). In all ancestries, the average spinal curve in AIS cases with a rare *FBN1* or *FBN2* variant was 50.5° ($n = 59$), compared with 42.1° in cases with no fibrillin variant ($n = 742$) ($P = 0.0026$, Mann–Whitney–Wilcoxon test) (Fig. 2).

Evaluation of Marfan syndrome in AIS patients with *FBN1* variants

Pathogenic mutations in Marfan syndrome result in progressive dilation of the aorta, which predisposes individuals to aortic aneurysms and dissections (35). Because the life-threatening cardiovascular complications of Marfan syndrome are preventable, it is currently recommended that known or expected pathogenic mutations identified incidentally during clinical sequencing are reported (36). Therefore, AIS patients with rare *FBN1* variants were referred for additional evaluations assessing potential Marfan syndrome. Nine patients agreed to additional evaluations and were subsequently assessed by a clinical geneticist (Table 4). Three AIS patients had Marfan syndrome-associated mutations (p.L1405R, p.M1576T and p.I2585T) and six patients had variants of unknown significance (p.N280T, p.Q697R, p.N703H, p.N2178K, p.Y2225F and p.V2868I). The revised Ghent nosology (35) was employed. AIS patients were between 10 and 24 years old at time of evaluation. Seven patients had imaging studies for aortic root dilation and five patients received a dilated eye exam. Two AIS patients received physical examinations but no cardiac imaging studies. Familial DNA was available for genotyping in seven cases. The *FBN1* variant was inherited in six cases and one family (6005-001) was inconclusive (Supplementary Material, Fig. S1). None of the AIS patients reported significant features suggestive of Marfan syndrome (ectopia lentis, unexplained death, aortic dissection or aortic aneurysm) in any family members. Nonspecific features of Marfan syndrome (e.g. pectus deformity, hypermobility) were present in some family members. Only one of the nine patients that received additional evaluation met the diagnostic criteria for Marfan syndrome. This patient (6386-001) had aortic root dilation (z-score = 2.6) and significant Marfan-associated systemic features (Ghent systemic features score = 10) (Table 4). Of all patients of European descent evaluated for systemic features ($n = 118$), 6386-001 was the only patient that scored ≥ 7, which is considered clinically significant (35). The mutation identified in 6386-001 (p.I2585T) was previously described in multiple individuals with Marfan syndrome (23,26,27).

Increased phosphorylation of SMAD2 in paraspinous muscle of AIS patients with rare *FBN1* variants

Previous studies have shown that pathogenic *FBN1* mutations causing Marfan syndrome result in upregulated transforming growth factor beta (TGF-β) signaling that can be measured in plasma or indirectly measured through activation of downstream targets, such as phosphorylation of SMAD2 (pSMAD2) (37–40). To determine if AIS patients with rare *FBN1* and *FBN2* variants also have upregulated TGF-β, we used western blotting to examine pSMAD2 levels in paraspinous muscle from three AIS cases harboring a variant in *FBN1* (p.P1225L, p.M1576T and p.G2003R) and one AIS case with a rare variant in *FBN2* (p.P740H) (Fig. 3). Paraspinous muscle was also collected for a patient diagnosed with Marfan syndrome (positive control) and an unaffected individual who did not have scoliosis (negative control). Compared with the unaffected control, pSMAD2 was elevated in all AIS patients with rare *FBN1* variants and in the Marfan syndrome patient. We observed similar pSMAD2 levels the AIS patient with a rare *FBN2* variant compared with the unaffected control.

DISCUSSION

Pathogenic mutations in *FBN1* are most frequently associated with Marfan syndrome (41) where over 1000 mutations have been described (42), but mutations in *FBN1* have also been reported in ectopia lentis syndrome (43), isolated aortic aneurysm (44–47), Shprintzen–Goldberg syndrome (48), Weill–Marchesani syndrome (49), geleophysic and acromicric dysplasia (50), stiff skin syndrome (51) and MASS phenotype (myopia, mitral valve prolapse, borderline aortic root dilation, skeletal and skin findings) (52). Mutations in *FBN2* have previously been associated only with congenital contractural arachnodactyly (53). In the current study, we demonstrate an important role for *FBN1* and *FBN2* in the pathogenesis of AIS, broadening the spectrum of fibrillin-related disorders.

Table 4. Clinical features of AIS patients evaluated for Marfan syndrome

Case	6340-001	6390-001	6377-001	6226-001	6418-001	6368-001	6111-001	6386-001	6005-001
General patient information									
Ancestry	EUR	AFR	EUR	EUR	EUR	EUR	EUR	EUR	EUR
Gender	M	F	F	F	F	F	M	F	F
Age (years)	13	14	17	18	14	10	13	15	24
Height (percentile)	>99th	70th	57th	91st	>99th	>99th	93rd	98th	91st
Weight (percentile)	99th	96th	62nd	85th	87th	88th	86th	92nd	95th
Spinal curve	48°	60°	50°	97°	65°	100°	95°	93°	55°
Curve type	R–T	R–T	R–TC	R–T	R–T	R–T	L–T	R–T	R–T
Treatment	None	Surgery	Surgery	Surgery	Surgery	Surgery	Surgery	Surgery	Surgery
FBN1									
FBN1 variant	p.N280T	p.Q697R	p.N703H	p.L1405R	p.M1576T	p.N2178K	p.Y2225F	p.I2585T	p.V2868I
Variant reported in MFS	No	No	No	Yes	Yes	No	No	Yes	No
Marfan syndrome evaluation									
MFS diagnosis	No	No	No	No	No	No	No	Yes	No
Aortic root dilation (*z*-score)	− (−1.1)	NA	NA	− ([a])	− (−1.5)	− (−0.8)	− (0.07)	+ (2.6)	− (−0.96)
Ectopia lentis	−	NA	NA	NA	−	NA	−	−	−
Systemic features score	3/22	3/17	1/17	2/20	4/18	2/22	5/18	10/22	6/18
Pectus deformity	−	−	−	−	−	−	−	−	−
Hindfoot deformity	−	−	−	−	−	−	−	+	+
Pes planus	−	+	−	−	+	−	+	+	+
Wrist/thumb sign	−	−	−	−	−	−	−	+	−
Upper/lower segment	1.17	0.93	0.97	1.00	0.95	0.88	0.85	0.76	0.99
Arm span/height	1.03	1.08	0.98	1.01	1.01	1.03	1.07	1.09	1.02
Reduced elbow extension	−	−	−	−	+	−	−	−	−
Dural ectasia	−	NA	NA	−	NA	−	NA	−	NA
Protrusio acetabuli	−	NA	NA	NA	NA	−	NA	−	NA
Skin striae	+	+	−	+	+	−	+	+	+
Facial features (3/5)	−	−	−	−	−	−	−	−	−
Myopia	+	−	−	−	−	−	+	+	+
Pneumothorax	−	−	−	−	−	−	−	−	−
Mitral valve prolapse	−	NA	NA	−	−	−	−	−	−
Hypermobility evaluation									
Hypermobility score	0/9	0/9	1/8	4/8	1/9	2/9	2/8	2/9	9/9
Hands flat on floor	−	−	−	NA	−	−	−	−	+
L knee overextends	−	−	−	−	−	−	+	−	+
R knee overextends	−	−	−	−	−	−	+	−	+
L elbow bends >10°	−	−	−	−	−	−	−	−	+
R elbow bends >10°	−	−	−	−	−	−	−	−	+
L thumb touching forearm	−	−	+	+	−	+	−	+	+
R thumb touching forearm	−	−	NA	+	+	+	NA	+	+
L little finger >90°	−	−	−	+	−	−	−	−	+
R little finger >90°	−	−	−	+	−	−	−	−	+

+, present; −, absent; L, left; R, right; T, thoracic; TC, thoracolumbar; EUR, European; AFR, African; MFS, Marfan syndrome; NA, not available.
[a]2.7 cm at the sinuses of Valsalva by MRI (normal for BSA, age per gender).

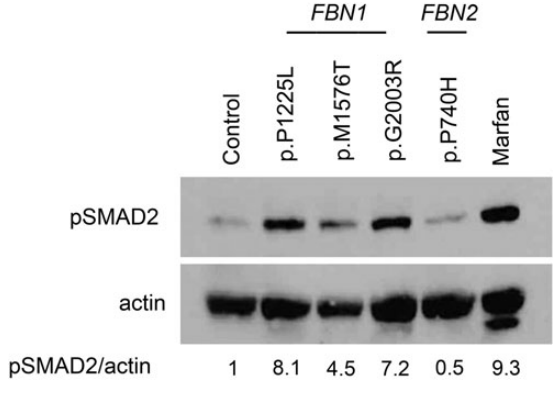

Figure 3. Elevated pSMAD2 in AIS patients with rare *FBN1* variants. Paraspinous muscle from an unaffected control, AIS cases with rare variants in *FBN1* or *FBN2* and a Marfan syndrome patient were immunostained for pSMAD2. pSMAD2 was elevated in AIS cases with rare *FBN1* variants at levels similar to Marfan syndrome. pSMAD2/actin ratio is shown normalized to the unaffected control.

Until now, the majority of *FBN1* and *FBN2* sequencing studies have been limited to select cohorts of patients with suspected Marfan syndrome and congenital contractural arachnodactyly, particularly because the cost of sequencing *FBN1* and *FBN2*, each consisting of ∼11 kb of coding sequence spread over 65 exons, is prohibitively high in large cohorts. However, *FBN1* mutations were previously described in a family with isolated kyphoscoliosis (54) and a family with isolated skeletal features including scoliosis (55), suggesting that fibrillin might contribute to isolated skeletal phenotypes, such as AIS. In addition, earlier investigations revealed fibrillin abnormalities in the fibroblasts of AIS patients (56), but linkage analysis did not support segregation of the *FBN1* locus in large AIS pedigrees (57) and further genetic studies in AIS were not pursued.

Using exome sequencing and a cost-effective targeted capture approach to sequence *FBN1* and *FBN2*, we found that the burden of *FBN1* and *FBN2* rare variants is relatively high in severely affected AIS patients, with 7.6% of patients of European

ancestry having a rare variant in either gene, over three times the frequency of controls (2.4%). Curve severity was the most striking clinical finding that distinguished patients with rare fibrillin variants, leading to spinal curves that were on average 15° larger than observed in other AIS patients. Our findings indicate that *FBN1* and *FBN2* variants could serve as prognostic genetic markers to predict scoliosis progression. This has important clinical implications because of the potential to develop personalized prevention strategies for those at high risk of severe deformity and to eliminate screening and treatment for patients at low risk (1,58,59). Interestingly, scoliosis is common in patients with Marfan syndrome (19) and congenital contractural arachnodactyly (20) and, like the AIS cases studied here, often progresses to severe curves (35,60,61). However, there are likely additional genetic and environmental factors contributing to this complex phenotype, so additional studies will be needed to address the sensitivity and specificity of predicting curve severity based on the presence of rare fibrillin variants.

In addition to spinal curve progression, we found that rare *FBN1* variants are also associated with tall stature in the AIS population. Because nearly one-quarter of AIS cases in our study with height ≥90th percentile had a rare *FBN1* variant, this easily obtainable clinical measure can identify patients who are particularly likely to harbor these genetic variants and may benefit from *FBN1* sequencing studies. We also found that AIS patients with *FBN1* variants collectively scored higher using the Ghent systemic features scoring system (35), which evaluates several nonspecific physical traits that are common in Marfan syndrome. Although myopia, skin striae, pes planus, joint hypermobility and other nonspecific Marfan-associated features were noted in some AIS patients, the overall difference in the systemic feature score was small and this score is unlikely to be useful for identifying individual AIS patients with rare *FBN1* variants. Despite this, our data confirm that the systemic features scores do have utility as a screen for unrecognized Marfan syndrome in the AIS population, as the only patient with a clinically significant systemic feature score (≥7) was identified with an *FBN1* mutation and subsequently diagnosed with Marfan syndrome after additional clinical testing as a part of this study.

While *FBN1* mutations causing Marfan syndrome are associated with life threatening cardiovascular complications, our data suggest that the majority of AIS-associated *FBN1* variants are associated with a more benign course. First, most AIS patients ($n = 8/9$) did not have significant systemic features of Marfan syndrome based on the current diagnostic criteria (Table 4) (35). Only mild, nonspecific physical findings (myopia, skin striae, pes planus and hypermobility) were observed in some AIS patients. Although the presence of these features suggests *FBN1* variants contribute to other mild features beyond scoliosis, it is of our opinion that, had these AIS patients been evaluated without knowledge of *FBN1* mutation status, Marfan syndrome would not have been suspected and *FBN1* testing would not have been recommended based on physical evaluation alone. Second, the majority of patients who received imaging studies of the aorta showed no evidence of aortic dilation ($n = 6/7$), which is the cardinal feature of Marfan syndrome. Third, family histories did not endorse Marfan syndrome. Rare *FBN1* variants were inherited from a parent in at least eight families, but no patient reported family members with features suggestive of Marfan syndrome, including aortic dilation, aortic aneurysm or dissection, ectopia lentis or unexplained death. Finally, over 60% of missense mutations causing Marfan syndrome disrupt a cysteine residue (29), but none of the missense mutations in AIS patients affected cysteine residues. In Marfan syndrome, patients with mutations creating or removing a cysteine are more likely to develop ectopia lentis compared with other missense mutations (29). This finding may explain why significant ocular phenotypes were not present in AIS patients or reported in family members.

Rare *FBN1* and *FBN2* variants segregated with AIS in some tested families with incomplete penetrance, but not all. This mode of transmission is consistent with a complex genetic model in which there are additional genetic and environmental risk factors. However, interpretation of segregation was hindered by the lack of DNA from all affected family members and the possibility of phenocopies within a family, which seems most plausible in cases where a variant failed to segregate with a mildly affected relative. Interestingly, other nonspecific phenotypes observed in Marfan syndrome and congenital contractural arachnodactyly, such as pectus carinatum, were also reported in some family members, although further studies are needed to determine if these features have any association with rare fibrillin variants.

Despite little evidence supporting a diagnosis of Marfan syndrome in most AIS patients with rare *FBN1* variants, one AIS patient not previously suspected of the disorder was diagnosed as a direct result of this study. Because there are treatments available to prevent the life-threatening aortic complications of Marfan syndrome (e.g. losartan) (37,62–64), recognition of the disorder can significantly affect patient outcomes. Unfortunately, for AIS patients with *FBN1* variants who did not fulfill the diagnostic criteria at the time of examination, the absence of aortic dilation does not conclusively rule out Marfan syndrome and aortic dilation may develop later in life (35). Ongoing screening of all AIS patients with rare *FBN1* variants is therefore warranted and is consistent with the current recommendations for patients with *FBN1* mutations who do not fulfill diagnostic criteria (30,65). Regardless of the long-term risks associated with rare *FBN1* variants, *FBN1* sequencing in the AIS population is likely to have clinical value, either as an early indicator of potential Marfan syndrome or as a predictor of progressive AIS.

Congenital contractural arachnodactyly does not appear to be associated with the same risk of life-threatening cardiovascular complications as Marfan syndrome; however, aortic dilation has been described in several patients and echocardiographic evaluations are currently recommended (33). AIS patients with rare *FBN2* variants did not receive additional clinical evaluation, but patient histories did not indicate 'crumpled' ears, muscular hypoplasia, contractures of the major joints or other features that would be suggestive of congenital contractural arachnodactyly. Moreover, *FBN2* mutations causing congenital contractural are limited to exons 23–34 (33), whereas many rare *FBN2* variants identified in AIS patients were located outside this region, providing further evidence that unrecognized congenital contractural arachnodactyly is unlikely in these patients.

Although our findings suggest that rare variants in *FBN1* and *FBN2* are associated with AIS rather than unrecognized Marfan syndrome or congenital contractural arachnodactyly, additional long-term studies of AIS patients with fibrillin abnormalities are needed. Such studies would clarify the potential future

complications, if any, associated with rare fibrillin variants and inform the interpretation of incidentally identified *FBN1* and *FBN2* variants in future diagnostic or research studies.

FBN1 and *FBN2* are expressed with significantly overlapping patterns beginning during early development (see review 66) and the biological mechanism leading to scoliosis when *FBN1* and *FBN2* are mutated remains unclear, even in cases of Marfan syndrome and congenital contractural arachnodactyly. Aberrant activation of the TGF-β pathway has been observed in Marfan syndrome (39) and may be responsible for some of the skeletal features, such as tall stature (67). We found that AIS patients with *FBN1* variants were also tall, which suggests that these protein changes also alter TGF-β signaling. We showed upregulation of the TGF-β pathway in paraspinous muscle of three AIS patients with rare *FBN1* variants, confirming that at least some of the variants identified in AIS have functional effects. Recent studies have shown that *FBN2* also regulates TGF-β signaling (68), but we did not observe upregulation of TGF-β in muscle from an AIS patient with a rare *FBN2* variant, suggesting that the variant may not have an effect on this aspect of *FBN2* function. Unfortunately, we did not have access to muscle on a larger cohort of AIS patients and further studies will be required to demonstrate more definitively that these gene variants activate the TGF-β pathway. Interestingly, the TGF-β pathway is also upregulated in patients with Loeys–Dietz syndrome (69,70), a disorder that has many overlapping features with Marfan syndrome and congenital arachnodactyly and is highly associated with scoliosis (71). Because scoliosis is a common feature of several Mendelian disorders involving the TGF-β signaling pathway, other genes in the TGF-β pathway may be good candidates for AIS, which is supported by a recent study that identified *TGFβ-1* as a novel susceptibility gene for AIS (72). Moreover, an association of TGF-β signaling with AIS could explain why other Marfan-associated features are observed more frequently in AIS patients than in the general population, including tall stature (73), joint hypermobility (74), pectus excavatum (75,76) and dural ectasia (77). Recent studies in mouse models and humans with Marfan syndrome have shown promising developments in therapeutic strategies to reduce the hyper-activated TGF-β pathway, including TGF-β neutralizing antibody and the angiotensin II type 1 receptor blocker, losartan (37,62–64). The potential link between AIS and the TGF-β signaling pathway suggests the possibility that drugs like losartan may someday be useful to treat AIS cases in which the TGF-β pathway is activated.

In summary, we demonstrate an important role for *FBN1* and *FBN2* in AIS pathogenesis. We show that rare variants are enriched in severely affected AIS patients and are significantly associated with curve severity. Because reliable methods for predicting scoliosis progression are not currently available, these results may be useful for patients and families. Furthermore, we show altered TGF-β signaling in AIS patients with fibrillin mutations, which opens up the possibility of novel treatments that have not previously been considered for AIS.

MATERIALS AND METHODS

Patient samples and controls

AIS patients were recruited from St. Louis Children's Hospital, St. Louis Shriners Hospital for Children, the University of Iowa, the University of Colorado and the Chinese University of Hong Kong. All patients had scoliosis of unknown etiology with spinal curves measuring $\geq 10^{\circ}$. Curve measurements are reported for the largest lateral spinal curve using the Cobb method (78). Patients with developmental delay, multiple congenital anomalies or known underlying medical disorders (e.g. Ehlers–Danlos syndrome, Marfan syndrome) were excluded. We selected 91 unrelated AIS cases of European ancestry with severe deformity (spinal curves measuring $\geq 40^{\circ}$ or requiring surgery) for the exome sequencing screen. Additional AIS cases of European ($n = 344$), Han Chinese ($n = 370$) and other ancestries ($n = 47$) were included in subsequent analyses. DNA was collected from affected probands after obtaining informed consent. Growth parameters were calculated using the National Center for Health Statistics (http://www.cdc.gov/nchs). Systemic features of Marfan syndrome and joint hypermobility were quantified according to the revised Ghent scoring system (35), and the Beighton scoring system (34), respectively, and were obtained in patients without knowledge of genetic sequencing results. A subset of AIS cases were recontacted after *FBN1* variants previously identified in individuals with Marfan syndrome or novel variants of uncertain significance were identified as a part of this research study. These cases were referred to clinical geneticists at St. Louis Children's Hospital and St. Louis Shriners Hospital for Children for evaluation of possible Marfan syndrome.

Controls for this study consisted of unrelated healthy individuals or patients with neurological (e.g. Alzheimer's disease) or other musculoskeletal disorders without spine involvement (e.g. limb deformity). For the exome sequencing screen, we selected 337 controls of European ancestry. Additional in-house controls of European ($n = 249$) or Han Chinese ($n = 83$) ancestry were included in subsequent analyses. Individuals of European ancestry ($n = 4300$) were included from the Exome Variant Server, NHLBI GO ESP (http://eversusgs.washington.edu/EVS) public database as an additional control cohort (ESP6500SI-V2). Although controls were unselected for presence of AIS, the frequency of severe AIS (curve $>40^{\circ}$) is expected to be low ($<0.1\%$) in the general population (79).

Exon enrichment and sequencing

Exon enrichment was performed using the SureSelect Human All Exon 38 and 50 Mb kits (Agilent Technologies, Santa Clara, CA, USA) or the TruSeq Exome Enrichment kit (Illumina, San Diego, CA, USA). Exome data were extracted from whole genome sequencing in a subset of in-house controls. Samples were sequenced on a Genome Analyzer IIx or HiSeq 2000 sequencer (Illumina) by the Washington University Genome Technology Access Center.

Targeted capture of *FBN1* and *FBN2*

Target enrichment of *FBN1* and *FBN2* was performed using the MDiGS method (21), which utilizes multiplexed capture (80) with targeted selection using bacterial artificial chromosomes (BACs) (81) and next-generation sequencing. DNA samples (300 ng) were sonicated using a Covaris E210 focus-ultrasonicator (Covaris Inc., Woburn, MA, USA). Samples were indexed using previously described adapter sequences (80). Indexed samples were pooled in batches of 48–96 prior to capture. Four BACs

spanning the entire coding regions of *FBN1* (RP11-1144G24 [chr15:48 676 783–48 822 143] and RP11-147E14 [chr15: 48 822 351–48 985 158]) and *FBN2* (RP11-909P14 [chr5:127 567 172–127 756 046] and RP11-351A8 [chr5:127 752 763–127 906 815]) (BACPAC Resources Center, Oakland, CA, USA) were used as baits. BACs were biotinylated using nick translation with biotin-16-dUTP (Roche Applied Science, Penzberg, Germany). Pooled samples were hybridized with biotinylated BACs. Dynabeads M-280 Streptavidin (Life Technologies Co., Carlsbad, CA, USA) were used to isolate BAC-associated DNA. Postcapture, pooled samples were sequenced using a MiSeq Personal Sequencer (Illumina).

Alignment and variant calling

Next generation sequencing reads were aligned to hg19 human reference sequence (Genome Reference Consortium Human Build 37) using Novoalign software (Novocraft Technologies, Selangor, Malaysia). Variant calling and annotation were completed using SAMtools (82) and SeattleSeq Annotation 131 (http://snp.gs.washington.edu/SeattleSeqAnnotation131), respectively. All AIS cases and in-house controls were analyzed using an identical methodology.

Quality control and gene burden analysis

For all analyses, variants were defined as rare when absent from the dbSNP database (build 137). Analysis was restricted to variants altering the coding sequence (nonsense, splice-site, missense and insertion/deletion mutations). We used a collapsing approach to measure gene burden (83). Rare variant burden was quantified as the sum of all variant alleles for each gene divided by the total alleles in the population and is expressed as the cMAF.

For the exome sequencing screen, only AIS cases and in-house controls with $\geq$90% of the exome covered at $\geq 8\times$ coverage were included in the analysis. Variant calls were merged at all sites and filtered for sites called in $>$80% of individuals. Gene burden analysis was performed exome-wide for each gene with at least one variant site.

For gene burden analysis of *FBN1* and *FBN2* in the larger dataset, more conservative quality thresholds were applied. Only individual samples with $\geq$95% of *FBN1* (NM_000138) or *FBN2* (NM_001999) covered at $\geq 8\times$ read depth were included. Low-quality variants (phred-scaled quality score $<$30 or genotype quality score $<$75) were removed. Controls from the ESP were similarly analyzed using build 137 of the dbSNP database as a filter, which does not include individuals from this dataset. In the Han Chinese patient cohort, variants previously identified in dbSNP were excluded from analysis. Variants were also excluded from analysis if identified in Han Chinese controls ($n = 83$). All rare variants in AIS cases were validated by Sequenom MassARRAY (Sequenom, San Diego, CA, USA) by the Washington University Human Genetics Division of Genotyping Core or by Sanger sequencing using an ABI 3730 Sequencer (Life Technologies). All genotyped variants ($n = 32$) validated.

Statistical analysis

One-tailed *P*-values of <0.05 were considered statistically significant. A Fisher's exact test was used for all gene burden analyses. The Shapiro–Wilk test demonstrated non-normally distributed clinical characteristics ($P < 0.05$); therefore, the Mann–Whitney–Wilcoxon test was used to compare clinical features, except gender and AIS family history, which were compared using a Fisher's exact test. Statistical tests were performed with GraphPad Prism software (http://www.graphpad.com/scientific-software/prism).

Protein immunoblotting

Immunoblotting was performed using paraspinous muscle collected during surgery for scoliosis (AIS and Marfan syndrome) or for a ruptured spinal disc in a patient without scoliosis (unaffected control). All samples were collected from females between 9 and 18 years old. Denatured protein (200 μg) was separated on a 7.5% gel by SDS–PAGE and transferred to a nitrocellulose membrane. Rabbit polyclonal antibody to pSmad2 (1:1000; EMD Millipore Co.) and mouse monoclonal antibody to actin (AC1-20.4.2; 1:2000; Sigma-Aldrich Co.) were used. Chemiluminescent signals in AIS cases and Marfan syndrome were quantified using ImageJ (84). Phospho-SMAD2 signal was normalized to actin and the unaffected control.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank the patients and their families for their role in this work. We also thank Anne Bowcock for helpful discussions. We thank Drs Scott Luhmann, Lawrence Lenke, and Keith Bridwell for allowing us to recruit patients from their clinics at Washington University. We thank the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine for help with genomic analysis. The Center is partially supported by NCI Cancer Center Support Grant #P30 CA91842 to the Siteman Cancer Center and by ICTS/CTSA Grant #UL1RR024992 from the National Center for Research Resources (NCRR), a component of the NIH and NIH Roadmap for Medical Research. The authors thank the NHLBI GO ESP and its ongoing studies which produced and provided exome variant calls for comparison: the Lung GO Sequencing Project (HL-102923), the WHI Sequencing Project (HL-102924), the Broad GO Sequencing Project (HL-102925), the Seattle GO Sequencing Project (HL-102926) and the Heart GO Sequencing Project (HL-103010). This publication is solely the responsibility of the authors and does not necessarily represent the official view of NCRR or NIH.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by Shriners Hospital for Children (85200-STL to M.B.D.), the Children's Discovery Institute of Washington University and St. Louis Children's Hospital (MD-II-2012-179 to C.A.G.); the National Institute of Health (K12 HD001459-08 to C.A.G., K08NS075094 to M.B.H., R01AG044546 to C.C.); and the Alzheimer Association (NIRG-11-200110 to C.C.).

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