

ORIGINAL ARTICLE

TBX6 Null Variants and a Common Hypomorphic Allele in Congenital Scoliosis

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ABSTRACT

BACKGROUND

Congenital scoliosis is a common type of vertebral malformation. Genetic susceptibility has been implicated in congenital scoliosis.

METHODS

We evaluated 161 Han Chinese persons with sporadic congenital scoliosis, 166 Han Chinese controls, and 2 pedigrees, family members of which had a 16p11.2 deletion, using comparative genomic hybridization, quantitative polymerase-chain-reaction analysis, and DNA sequencing. We carried out tests of replication using an additional series of 76 Han Chinese persons with congenital scoliosis and a multicenter series of 42 persons with 16p11.2 deletions.

RESULTS

We identified a total of 17 heterozygous *TBX6* null mutations in the 161 persons with sporadic congenital scoliosis (11%); we did not observe any null mutations in *TBX6* in 166 controls ($P < 3.8 \times 10^{-6}$). These null alleles include copy-number variants (12 instances of a 16p11.2 deletion affecting *TBX6*) and single-nucleotide variants (1 nonsense and 4 frame-shift mutations). However, the discordant intrafamilial phenotypes of 16p11.2 deletion carriers suggest that heterozygous *TBX6* null mutation is insufficient to cause congenital scoliosis. We went on to identify a common *TBX6* haplotype as the second risk allele in all 17 carriers of *TBX6* null mutations ($P < 1.1 \times 10^{-6}$). Replication studies involving additional persons with congenital scoliosis who carried a deletion affecting *TBX6* confirmed this compound inheritance model. In vitro functional assays suggested that the risk haplotype is a hypomorphic allele. Hemivertebrae are characteristic of *TBX6*-associated congenital scoliosis.

CONCLUSIONS

Compound inheritance of a rare null mutation and a hypomorphic allele of *TBX6* accounted for up to 11% of congenital scoliosis cases in the series that we analyzed. (Funded by the National Basic Research Program of China and others.)

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CONGENITAL SCOLIOSIS, A FORM OF VERTEBRAL malformation, has an estimated prevalence of approximately 1 in 2000 live births.¹ It is manifested as a lateral curvature of the spine exceeding 10 degrees and results from defects in vertebra formation during embryogenesis.^{2,3}

Previous evidence in animal models suggested that genetic factors contribute to vertebral malformations.^{3,4} Genetic mutations have been implicated in human congenital scoliosis, but their low penetrance highlights the complex molecular basis of the disorder and hinders molecular diagnosis of and genetic counseling for congenital scoliosis.^{4,5}

The proximal 16p11.2 deletion in humans is rare but recurrent, with a population frequency of approximately 0.03% worldwide.⁶ It is a likely cause of common diseases, including autism and obesity.⁷⁻⁹ Vertebral malformations have been observed in a small proportion of persons with 16p11.2 copy-number variants (including deletion and duplication),¹⁰⁻¹² a finding that suggests the potential involvement of 16p11.2 copy-number variants (specifically, deletions)¹³ in congenital scoliosis.

METHODS

STUDY PARTICIPANTS

We enrolled 237 consecutive Han Chinese persons who received a diagnosis of congenital scoliosis between October 2010 and June 2014 at Peking Union Medical College Hospital (PUMCH). Ancestry was determined by self-report (see the Methods section in the Supplementary Appendix, available with the full text of this article at NEJM.org). The discovery set consisted of 161 persons with sporadic congenital scoliosis (series 1), and we tested for replication in an additional series of 76 persons with the disorder (series 2). A clinical diagnosis of congenital scoliosis was confirmed by radiologic imaging. We excluded persons with syndromic diseases.

A total of 166 unrelated Han Chinese persons with no evidence of congenital scoliosis or other malformations served as population controls. We also evaluated 2 Han Chinese pedigrees, family members of which carried the 16p11.2 deletion and had discordant intrafamilial phenotypes.

For a further replication study, 42 unrelated persons with 16p11.2 deletion (series 3) were en-

rolled from multiple centers in the United States and China. These persons were initially referred for clinical chromosomal microarray testing owing to various medical problems.¹³⁻¹⁵

CELL CULTURE AND INDUCED DIFFERENTIATION

The P19CL6 cell line (derived from a mouse embryonic carcinoma) was cultured and differentiated into cardiomyocytes, as described previously.¹⁶ The cells were harvested every 24 hours after treatment with dimethyl sulfoxide.¹⁷ We also used a human induced pluripotent stem cell (hiPSC) line that we derived from dermal fibroblasts by means of an efficient and integration-free method.¹⁸ The culture of this hiPSC line and its induced differentiation to mesodermal-cell and cardiac-cell lineages has been described previously.^{19,20} The cells were harvested 24 hours and 48 hours after differentiation with RPMI-B-27 medium without insulin.

ANALYSIS OF GENOTYPE-PHENOTYPE CORRELATION

We reviewed the medical records and spinal radiographs of all 237 persons with sporadic congenital scoliosis. Vertebral malformations were classified as hemivertebra or hypoplasia, segmentation defect, or butterfly vertebra, as defined in a previous report.²¹ Vertebral malformations at more than one site in a patient were classified and counted independently according to location.

STUDY OVERSIGHT

This study was approved by the institutional review boards of the PUMCH, Fudan University, the Capital Institute of Pediatrics, and the other participating institutions. We obtained written informed consent from the participants (those who were ≥ 18 years of age at the time of enrollment) or their guardians (for participants who were < 18 years of age).

STATISTICAL ANALYSIS

Fisher's exact test was used to assess the difference in *TBX6* mutation or variant frequencies between the persons with congenital scoliosis and controls. The unpaired t-test was used for statistical analysis of the luciferase reporter assays. Pearson's chi-square test was used to assess the difference in the prevalence of vertebral malformations between persons with *TBX6*-associated congenital scoliosis and those with congenital scoliosis but no *TBX6* nonsynonymous mutation. Odds ratios and

95% confidence intervals were calculated. The statistical analysis was performed with the use of SPSS software, version 15.0 (SPSS). A two-sided P value of less than 0.05 was considered to indicate statistical significance.

Further details of the study participants and of assays using comparative genomic hybridization (CGH), quantitative polymerase chain reaction, DNA sequencing of *TBX6*, haplotyping of *TBX6* variants, and luciferase assays can be found in the Supplementary Appendix.

RESULTS

PREVALENCE OF 16P11.2 DELETIONS IN PERSONS WITH CONGENITAL SCOLIOSIS

A genomewide analysis of copy-number variants was performed in 20 trios (consisting of a person with sporadic congenital scoliosis and two healthy parents) with the use of Agilent 1×1M CGH microarrays. We identified recurrent deletions in proximal 16p11.2 (Fig. 1A) in two persons (Patient XH004 and Patient XH042) (Fig. S1 in the Supplementary Appendix). Both of these heterozygous deletions were de novo (i.e., absent in the patient's parents).

Of the remaining 141 persons with sporadic congenital scoliosis in series 1, 10 had a heterozygous 16p11.2 deletion (Table 1), whereas none of the 166 Han Chinese controls had the deletion (12 of 161 persons with congenital scoliosis vs. 0 of 166 controls, $P < 0.001$ by Fisher's exact test). These findings are consistent with the rarity of the 16p11.2 deletion in human populations (0.03%).^{22,23} Remarkably, the prevalence of the deletion among persons with sporadic congenital scoliosis (12 of 161 persons, 7.5%) was much higher than its reported prevalence among persons with autism (0.4 to 0.8%)^{6,7} or obesity (0.7%),^{8,9} two phenotypes that it is known to cause. None of our patients with congenital scoliosis were obese. Their detailed neurodevelopmental records were not available for us to evaluate symptoms of autism.

ASSOCIATION OF *TBX6* LOSS-OF-FUNCTION MUTATIONS WITH CONGENITAL SCOLIOSIS

The association of the 16p11.2 deletion with congenital scoliosis suggested that a gene related to the disorder may be located in the segment of DNA that is deleted: of these genes, *TBX6* was considered to be the best candidate (Fig. 1B).²⁴⁻²⁶

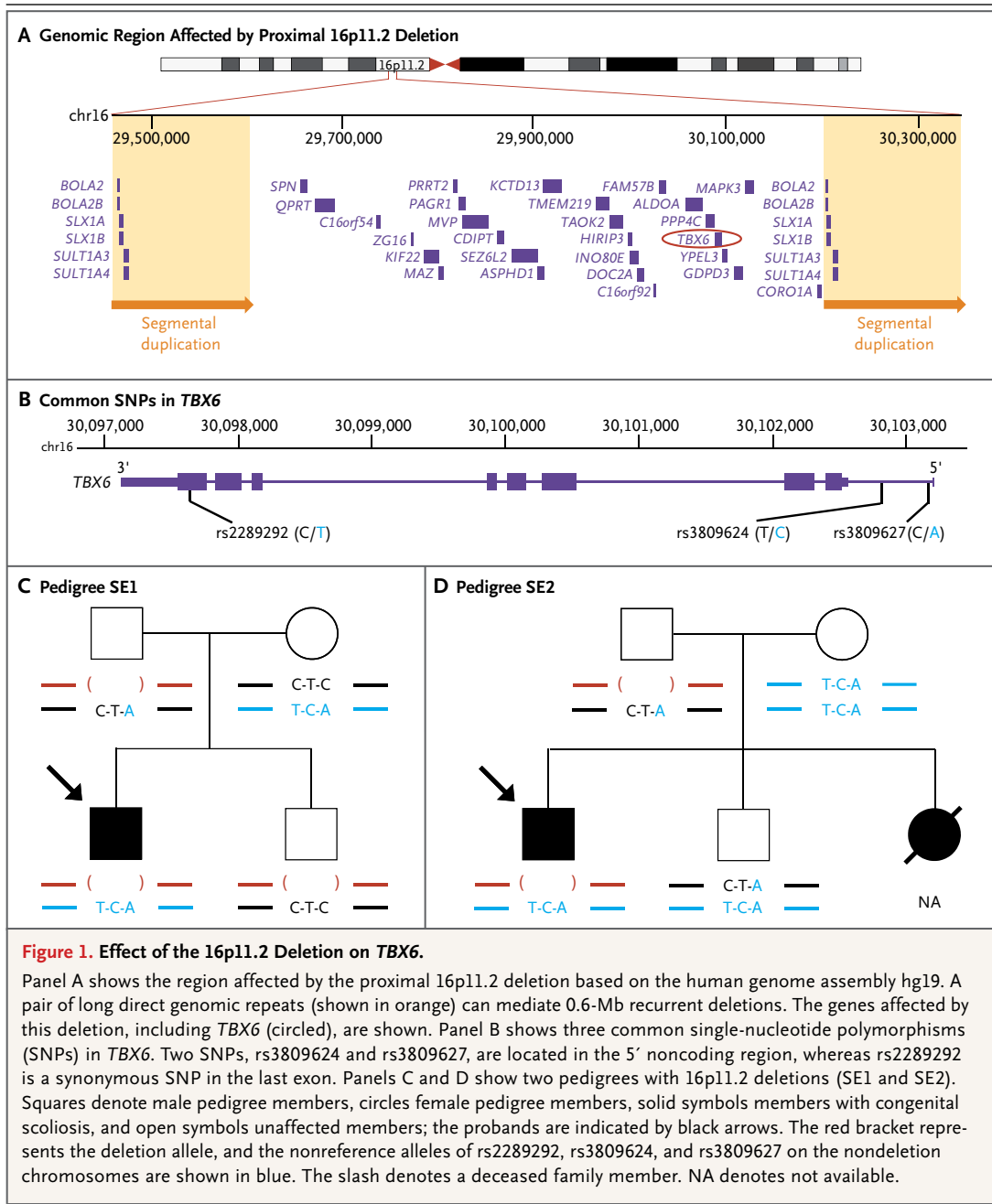
Accordingly, we hypothesized that other null or loss-of-function mutations (e.g., nonsense, frame-shift, and essential splice-site variants)²⁷ of *TBX6* cause congenital scoliosis. One heterozygous nonsense and four heterozygous frame-shift mutations of *TBX6* were identified among the patients with congenital scoliosis in series 1 (Table 1, and Fig. S2 in the Supplementary Appendix). The frame-shift mutation in Patient XH122 was de novo, whereas the status (i.e., de novo or inherited) of the other four null mutations is unknown. In contrast, we did not observe any null mutations in the controls (5 of 161 persons with congenital scoliosis vs. 0 of 166 controls, $P = 0.03$ by Fisher's exact test). These findings suggest an association of *TBX6* null mutations with congenital scoliosis.

Furthermore, we identified *TBX6* missense mutations in 7 of 161 persons with congenital scoliosis in series 1, a prevalence higher than that among the controls (3 of 166 persons) and among Han Chinese populations in Beijing and in southern China (CHB and CHS populations) in the 1000 Genomes Project (1 of 197 persons) (Fig. S3 and Tables S1 through S4 in the Supplementary Appendix).²² In a combined analysis, the association of *TBX6* missense mutations with congenital scoliosis was significant (7 of 161 persons with congenital scoliosis vs. 4 of 363 controls, $P = 0.04$ by Fisher's exact test). Because missense mutations could be benign or even confer a gain of function, we did not include them in additional analyses, although we cannot exclude the possibility of a role for them in congenital scoliosis.

A COMMON RISK HAPLOTYPE IN NULL MUTATION CARRIERS

Each person with congenital scoliosis in series 1 who carried a *TBX6* mutation was found to have only one null mutation (Table 1), which, at face value, suggests a simple dominant mode of inheritance. However, dominant inheritance is not supported by the low penetrance of congenital scoliosis in carriers of the 16p11.2 deletion affecting *TBX6*.¹⁰⁻¹² We therefore hypothesized that an additional genetic modifier acts in concert with *TBX6* null mutations to cause congenital scoliosis.

To test this hypothesis, we analyzed two pedigrees with an affected member and unaffected members who had the same 16p11.2 deletion. In Pedigree SE1 (Fig. 1C), the proband had congenital scoliosis with hemivertebra, whereas



the father and sibling had the same deletion but did not have congenital scoliosis.¹² In Pedigree SE2 (Fig. 1D), the deletion in the proband was paternally inherited. The radiograph revealed a wedge-shaped vertebral malformation in the proband (Fig. 2); congenital scoliosis was not observed in the father.

We did not identify a rare mutation in *TBX6*,

other than the deletion itself, in any of the family members. However, we did observe a common *TBX6* haplotype of T-C-A (defined by the nonreference alleles of three common single-nucleotide polymorphisms [SNPs] — rs2289292, rs3809624, and rs3809627, respectively) that cosegregated with congenital scoliosis in both pedigrees (Fig. 1). Therefore, we further evaluated the 17

Table 1. Genetic and Clinical Characteristics of Patients with *TBX6*-Associated Congenital Scoliosis.

Patient No.	Sex	Age (yr)	<i>TBX6</i> Allele		Vertebral Malformation*	Other Congenital Malformation
			Null Mutation at First Allele	Risk Haplotype at Second Allele†		
Congenital scoliosis series 1 (discovery)						
XH004	F	4	16p11.2 del	T-C-A	L1 hemivertebral (left)	None
XH025	M	3	16p11.2 del	T-C-A	T11 hemivertebral (left)	Rib abnormality (missing left 11th rib)
XH042	M	6	16p11.2 del	T-C-A	T9 hemivertebral (left)	Patent foramen ovale and rib abnormalities (missing right 9th and left 12th ribs)
XH141	F	18	16p11.2 del	T-C-A	T4 hemivertebral (left)	Syringomyelia, spina bifida, and rib abnormalities (fused left 3rd through 6th ribs)
XH149	F	7	16p11.2 del	T-C-A	T12 hemivertebral (right)	Rib abnormality (missing left 12th rib)
XH186	M	4	16p11.2 del	T-C-A	T11 hemivertebral (left)	Rib abnormality (missing right 11th rib)
XH237	M	13	16p11.2 del	T-C-A	T12 hemivertebral (left) and L3 butterfly vertebra	Rib abnormality (missing right 12th rib)
XH265	M	4	16p11.2 del	T-C-A	L1 hemivertebral (right)	None
XH270	M	14	16p11.2 del	T-C-A	T11 and T12 hemivertebrae (left)	Rib abnormalities (missing right 12th rib and bifurcation of right 2nd rib)
XH292	F	11	16p11.2 del	T-C-A	T6 hemivertebral (left) and T12 wedge vertebra	Rib abnormality (missing right 6th rib)
XH300	M	3	16p11.2 del	T-C-A	T10 hemivertebral (left)	Rib abnormalities (missing right 10th rib and bifurcation of right 9th rib)
XH303	M	5	16p11.2 del	T-C-A	T7 butterfly vertebra and T12 hemivertebral (left)	Rib abnormality (missing left 12th rib)
XH101	F	7	c.1250_1251insT frame shift	T-C-A	L2 hemivertebral (left)	Atrial septal defect and rib abnormalities (missing bilateral 12th ribs)
XH122	M	5	c.266_267insC frame shift	T-C-A	Hemivertebral (left) between T12 and L1	Rib abnormality (missing right 12th rib)
XH170	M	5	c.704_705insG frame shift	T-C-A	T12 hemivertebral (left)	Rib abnormality (missing right 12th rib)
XH286	M	2	c.1169_1170insC frame shift	T-C-A	L2 hemivertebral (left) and T8 butterfly vertebra	None
XH148	F	16	c.844C→T (p.R282X)	T-C-A	T1 and L3 butterfly vertebrae, T4 hemivertebral (right), and T7 hemivertebral (left)	None
Congenital scoliosis series 2 (replication)						
XR345	F	13	16p11.2 del	T-C-A	Hemivertebral (left) between T12 and L1	None
XR353	M	11	16p11.2 del	T-C-A	T11 hemivertebral (right)	Rib abnormality (missing left 11th rib)
XR402	M	11	16p11.2 del	T-C-A	C5 hemivertebral (left), T5 hemivertebral (right), and T8 hemivertebral (left)	Rib abnormalities (missing left 5th and right 8th ribs)
XR434	F	16	16p11.2 del	T-C-A	T12 hemivertebral (left) and T9 butterfly vertebra	None
XR439	F	7	16p11.2 del	T-C-A	L3 hemivertebral (right)	None
XR341	M	2	c.1179_1180delAG frame shift	T-C-A	T12 hemivertebral (right)	Rib abnormality (missing left 12th rib)

* C denotes cervical, L lumbar, and T thoracic.

† T-C-A represents the haplotype defined by three common *TBX6* single-nucleotide polymorphisms (reference/nonreference): rs2289292 (C/T), rs3809624 (T/C), and rs3809627 (C/A).

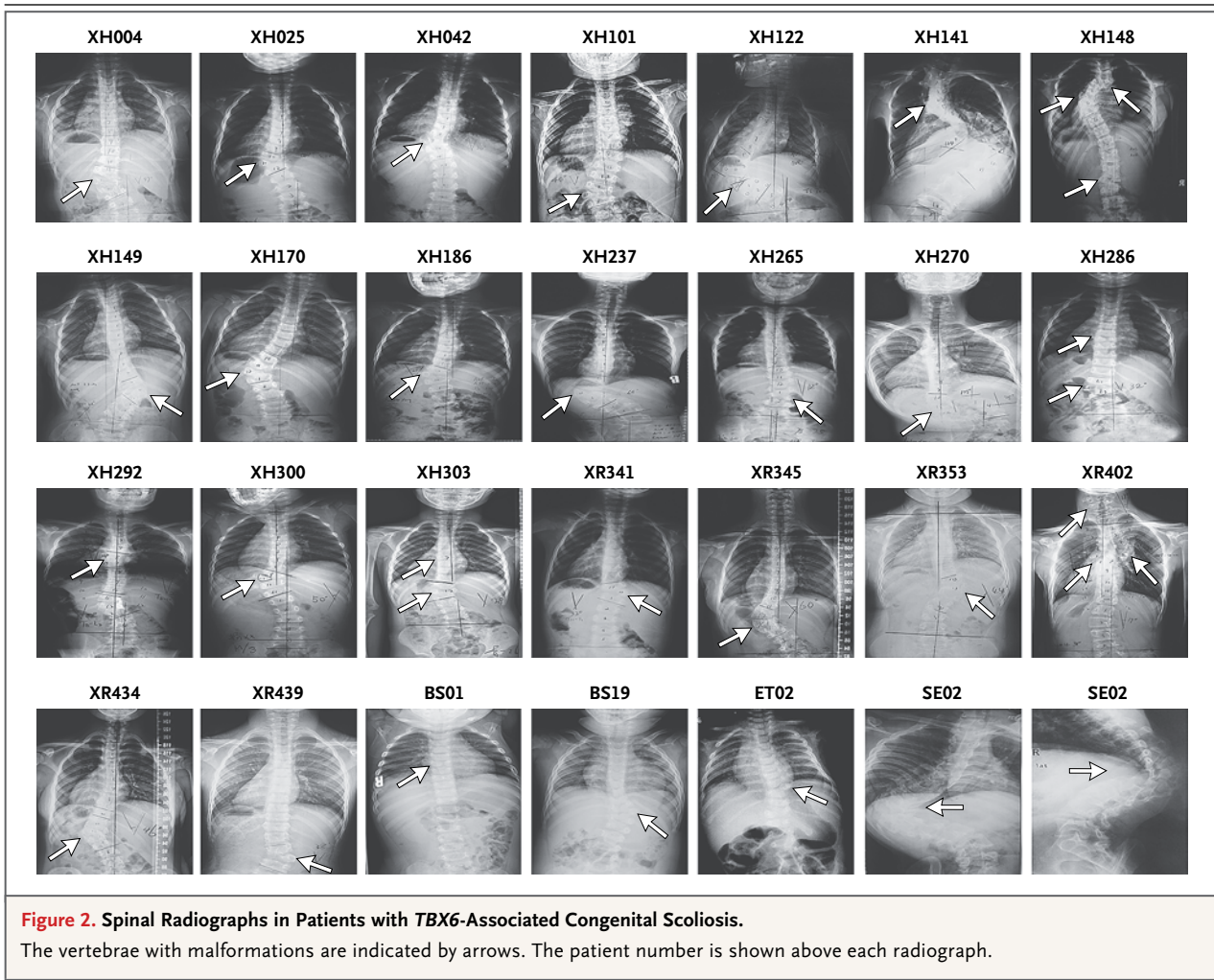


Figure 2. Spinal Radiographs in Patients with *TBX6*-Associated Congenital Scoliosis.

The vertebrae with malformations are indicated by arrows. The patient number is shown above each radiograph.

persons with congenital scoliosis in series 1 who had the *TBX6* null mutations. All 17 persons carried the T-C-A haplotype at the second *TBX6* allele (Table 1). The probability of this coinheritance is $P=(44\%)^{17}$, which is equivalent to $P<1.1\times 10^{-6}$, where 44% is the frequency of the T-C-A haplotype among Han Chinese persons in the 1000 Genomes Project (Table S5 in the Supplementary Appendix).²² Our observations suggest a genetic model of *TBX6* compound inheritance involving a combination of rare null and common risk alleles in the causation of congenital scoliosis. This model accounts for 17 of 161 cases of congenital scoliosis (11%) in series 1 (Table 2).

REPLICATION STUDIES

We recruited an additional 76 persons with congenital scoliosis (series 2) and identified 6 *TBX6* null mutations (Table 1). Again, we observed the

T-C-A risk haplotype at the second allele in all persons with these mutations. The *TBX6* compound inheritance model accounts for 6 of 76 cases of congenital scoliosis (8%) in series 2 (Table 2).

Furthermore, we enrolled 42 persons with the 16p11.2 deletion (series 3) from multiple centers (Table S6 in the Supplementary Appendix). These persons were categorized into three subgroups: those with congenital scoliosis (vertebral malformations), those who had scoliosis without vertebral malformations, and those with no evidence of scoliosis. This made it feasible to investigate whether the hemizygous T-C-A haplotype is associated with congenital scoliosis in 16p11.2 deletion carriers. In the congenital scoliosis subgroup, we identified 5 of 6 persons (83%) as having the hemizygous T-C-A haplotype (Table 2). The only exception (Patient PT03) was of African ancestry,¹³ and the T-C-A haplotype is rare among such persons (prevalence, <1% among Africans, as com-

Table 2. Distribution of Cases of TBX6-Associated Congenital Scoliosis.

Series	No. of Patients	Cases of Congenital Scoliosis Explained by TBX6 Compound Inheritance	P Value
		percent	
Series 1: patients with congenital scoliosis	161	11	$<3.8 \times 10^{-6*}$
Series 2: patients with congenital scoliosis	76	8	$<8.4 \times 10^{-4\dagger}$
Series 3: patients with 16p11.2 deletion	42‡	83	0.004§

* The P value was calculated on the basis of 17 of 161 patients with congenital scoliosis versus 0 of 166 controls.

† The P value was calculated on the basis of 6 of 76 patients with congenital scoliosis versus 0 of 166 controls.

‡ Six of 42 patients with 16p11.2 deletion had a congenital scoliosis phenotype.

§ The P value was calculated on the basis of 5 of 6 deletion carriers with congenital scoliosis versus 5 of 30 deletion carriers without scoliosis.

pared with 44% among Asians and 33% among persons of European descent) (Table S5 in the Supplementary Appendix). Patient PT03 had the nonreference allele of SNP rs111939223 (present in 4% of Yoruba Africans but absent in Asians and Europeans), which is located at a predicted messenger RNA polyadenylation site of *TBX6*.²⁸ Therefore, we cannot exclude the possibility of compound inheritance of a *TBX6* null mutation and an alternative risk haplotype among persons of African ancestry in Patient PT03.

In the no-scoliosis subgroup, the T-C-A haplotype was present in only 5 of 30 persons (17%) (Table S7 in the Supplementary Appendix). The T-C-A risk haplotype was a significant risk factor for congenital scoliosis in series 3 (5 of 6 persons with congenital scoliosis vs. 5 of 30 persons without scoliosis, $P=0.004$ by Fisher's exact test), which further supports the *TBX6* compound inheritance model of the disorder.

THE HYPOMORPHIC RISK HAPLOTYPE IN VITRO

The *TBX6* SNPs defining the haplotype for risk of congenital scoliosis could be functional or, alternatively, could be a composite genetic marker that is linked with the true functional variants. We conducted a linkage-disequilibrium analysis using common human SNPs²² and found that the linkage-disequilibrium block of *TBX6* is short (5 kb by the four-gamete rule) (Fig. S4 in the Supplementary Appendix). We identified no rare mutation in this block. The most parsimonious explanation for this observation is that the common *TBX6* SNPs defining the risk haplotype are functional.

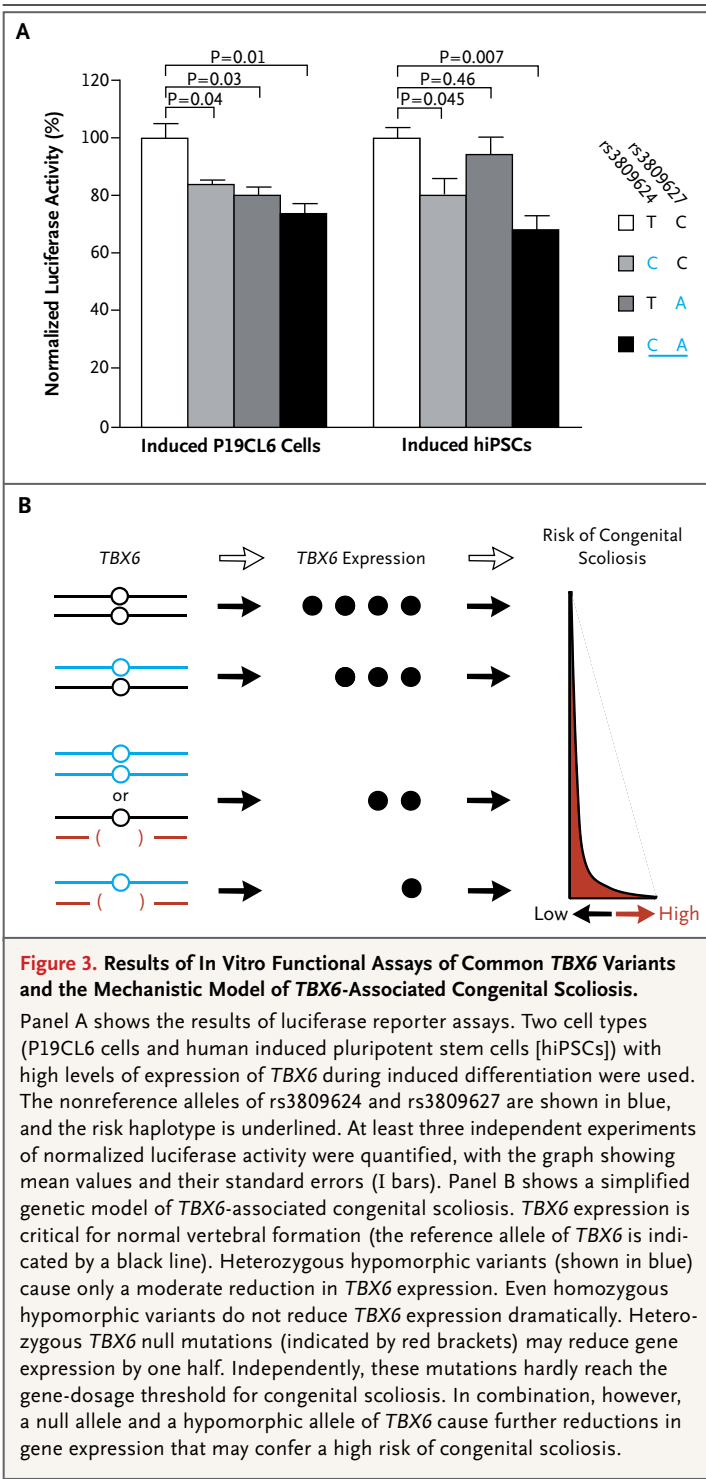
The SNPs rs3809624 and rs3809627 are located in the 5' noncoding region of *TBX6* (Fig. 1B), potentially regulating *TBX6* expression.²⁹

Data from the Encyclopedia of DNA Elements (ENCODE) Project suggest the possible involvement of these two noncoding SNPs in the regulation of gene expression.³⁰ For example, both SNPs are located in the same binding site of upstream stimulatory factor 1 (USF1), a transcription factor potentially involved in somitogenesis.^{31,32}

We used in vitro luciferase assays to study the effects of rs3809624 and rs3809627 on gene expression. Preliminary analyses in three human cell lines (HEK293, HepG2, and HeLa) showed significant reductions in the expression of the reporter gene when linked to the portion of *TBX6* harboring nonreference variants at both rs3809624 and rs3809627 (Fig. S5 and S6 in the Supplementary Appendix). We carried out the same experiment using the multipotent P19CL6 cells^{16,17} and hiPSCs. *TBX6* was strongly expressed in these stem cells when they differentiated into mesodermal-cell lineages (Fig. S5 in the Supplementary Appendix). We observed that P19CL6 cells and hiPSCs carrying the luciferase reporter construct with nonreference alleles at both rs3809624 and rs3809627 expressed significantly less reporter protein on differentiation into mesodermal and cardiac cells (Fig. 3A). These findings consistently support the hypomorphic effects of the combination of nonreference alleles at rs3809624 and rs3809627 on *TBX6* expression.

HEMIVERTEBRAE IN PERSONS WITH TBX6-ASSOCIATED CONGENITAL SCOLIOSIS

To assess the influence of *TBX6* disruption on the morphologic features of vertebral malformations, we evaluated the types of vertebral malformations in 23 persons with sporadic congenital scoliosis and *TBX6* null mutations and in 204 persons with sporadic congenital scoliosis and no detected *TBX6*



tal scoliosis than in those with congenital scoliosis but no apparent *TBX6* mutation ($P < 0.001$) (Table S8 in the Supplementary Appendix).

DISCUSSION

The genetic basis for the risk of congenital scoliosis is poorly understood. The involvement of *Tbx6* in vertebral malformations in mice has been suggested.^{4,25,33} However, a previous study involving 50 persons with congenital vertebral malformations identified no *TBX6* mutations,⁵ and only a weak association between common *TBX6* SNPs and congenital scoliosis in the Han Chinese population was reported previously.²⁶ Our data show that *TBX6* null mutations are associated with sporadic congenital scoliosis, and in conjunction with the *TBX6* haplotype of T-C-A, these mutations accounted for 8 to 11% of congenital scoliosis cases in the study population with the disorder (Table 2). *TBX6* null mutations and noncoding variants therefore contribute substantively to the complex trait of sporadic congenital scoliosis.

Generally, the molecular mechanism of human mendelian disorders associated with heterozygous gene deletions or null mutations is haploinsufficiency, in which half the gene dosage is not sufficient for normal function.^{34,35} In contrast, the effect of diminished *TBX6* dosage in congenital scoliosis is complex. We found that an additional *TBX6* hypomorphic allele is required to cause a further decrement in gene-expression dosage beyond haploinsufficiency for penetrance of the congenital scoliosis phenotype (Fig. 3B).

A precedent for the mechanism of compound inheritance was provided by a recent study of human thrombocytopenia with absent radii (TAR) associated with the gene *RBM8A* in 1q21.1.³⁶ The compound inheritance of *TBX6* in congenital scoliosis, like that of *RBM8A* in TAR and analogous to a recessive trait, requires two mutant alleles at a genetic locus for trait manifestation. This model may also pertain to phenotypic variation and penetrance of particular endophenotypes manifested in other genomic disorders that are associated with large-scale copy-number variants.³⁷⁻³⁹

The phenotypes of sporadic congenital scoliosis can be dramatically different. However, the 23 persons whom we found to have *TBX6*-associated congenital scoliosis were phenotypically consistent with one another, having one or more hemivertebrae. A heterozygous *TBX6* stop-loss mutation (p.*437Cext*81) was reported in a pedigree

nonsynonymous mutations. All 23 persons with *TBX6*-associated congenital scoliosis had one or more hemivertebrae (Table 1 and Fig. 2). The number of hemivertebrae was significantly higher in the persons with *TBX6*-associated congeni-

with dominantly inherited spondylocostal dysostosis, a specific group of vertebral abnormalities characterized by multiple segmentation defects of the vertebrae, malalignment of the ribs (though often with a symmetric thoracic cage), and non-progressive scoliosis.^{40,41} Functional studies showed that this mutation caused partial reduction in the ability of TBX6 to activate transcription.⁴¹ We further analyzed this previously reported pedigree and found that this TBX6 stop-loss mutation was located within the T-C-A risk haplotype. The combined reduction in TBX6 expression and transcriptional activation might therefore account for spondylocostal dysostosis in this family. Alternatively, the 81 amino acids added to the C-terminal may affect the ability of TBX6 to interact with other proteins that are critical in vertebral formation, such as MESP2 and RIPPLY2.^{42,43}

Environmental factors could also contribute to the variety of phenotypes of TBX6-associated congenital scoliosis. Mouse embryos are susceptible to gestational hypoxia, and developmental processes such as somitogenesis are affected by cellular hypoxia.^{4,44} Moreover, gene–environment interactions can affect the penetrance of vertebral phenotypes in mouse embryos that are geneti-

cally predisposed to vertebral defects. Similar mechanisms could affect the phenotypes of persons with variants in and deletions of TBX6.⁴

Our data suggest that TBX6 mutations are a common genetic cause of congenital scoliosis in the populations from which we drew our samples. Our finding that compound inheritance of a rare TBX6 null mutation and a common risk haplotype causes congenital scoliosis will enable the molecular diagnosis of the disorder and genetic counseling for some persons who have or are at risk for congenital scoliosis.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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APPENDIX

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