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First Genotype–Phenotype Study in *TBX4* Syndrome

Gain-of-Function Mutations Causative for Lung Disease

Matina Prapa^{1,2,*}, Mauro Lago-Docampo^{3,4,*}, Emilia M. Swietlik^{1,5,6}, David Montani⁷, Mélanie Eyries⁸, Marc Humbert⁷, Carrie L. Welch⁹, Wendy K. Chung^{9,10}, Rolf M. F. Berger¹¹, Harm Jan Bogaard¹², Olivier Danhaive^{13,14}, Pilar Escribano-Subías^{15,16}, Henning Gall¹¹, Barbara Girerd⁷, Ignacio Hernandez-Gonzalez¹⁷, Simon Holden¹⁸, David Hunt¹⁹, Samara M. A. Jansen¹², Wilhelmina Kerstjens-Frederikse²⁰, David G. Kiely^{21,22}, Pablo Lapunzina^{23,24,25}, John McDermott^{26,27}, Shahin Moledina²⁸, Joanna Pepke-Zaba⁶, Gary J. Polwarth⁶, Gwen Schotte¹², Jair Tenorio-Castaño^{23,24,25}, A. A. Roger Thompson^{21,22}, John Wharton²⁹, Stephen J. Wort²⁹, Karyn Megy^{1,5}, Rutendo Mapeta^{1,5}, Carmen M. Treacy¹, Jennifer M. Martin¹, Wei Li¹, Andrew J. Swift²¹, Paul D. Upton¹, Nicholas W. Morrell^{1,5,6,30}, Stefan Gräf^{1,30,31}, Diana Valverde^{3,4}, NIHR BioResource for Translational Research–Rare Diseases³², National Cohort Study of Idiopathic and Heritable PAH³³, and PAH Biobank Enrolling Centers' Investigators³³

Abstract

Rationale: Despite the increased recognition of *TBX4* (T-BOX transcription factor 4)-associated pulmonary arterial hypertension (PAH), genotype–phenotype associations are lacking and may provide important insights.

Objectives: To compile and functionally characterize all *TBX4* variants reported to date and undertake a comprehensive genotype-phenotype analysis.

Methods: We assembled a multicenter cohort of 137 patients harboring monoallelic *TBX4* variants and assessed the pathogenicity of missense variation ($n = 42$) using a novel luciferase reporter assay containing T-BOX binding motifs. We sought genotype–phenotype correlations and undertook a comparative analysis with patients with PAH with *BMPR2* (Bone Morphogenetic Protein Receptor type 2) causal variants ($n = 162$) or no identified variants in PAH-associated genes ($n = 741$) genotyped via the National Institute for Health Research BioResource–Rare Diseases.

Measurements and Main Results: Functional assessment of *TBX4* missense variants led to the novel finding of gain-of-function effects associated with older age at diagnosis of lung disease compared with loss-of-function effects ($P = 0.038$). Variants located in the T-BOX and nuclear localization domains were associated with earlier presentation ($P = 0.005$) and increased incidence of interstitial lung disease ($P = 0.003$). Event-free survival (death or transplantation) was shorter in the T-BOX group ($P = 0.022$), although age had a significant effect in the hazard model ($P = 0.0461$). Carriers of *TBX4* variants were diagnosed at a younger age ($P < 0.001$) and had worse baseline lung function (FEV₁, FVC) ($P = 0.009$) than the *BMPR2* and no identified causal variant groups.

Conclusions: We demonstrated that *TBX4* syndrome is not strictly the result of haploinsufficiency but can also be caused by gain of function. The pleiotropic effects of *TBX4* in lung disease may be in part explained by the differential effect of pathogenic mutations located in critical protein domains.

Keywords: pulmonary arterial hypertension; *TBX4*; interstitial lung disease; lung developmental disease; gain-of-function

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*These authors contributed equally to this work.

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Internet address: www.atsjournals.org

¹Department of Medicine and ³¹Department of Haematology, University of Cambridge, Cambridge Biomedical Campus, Cambridge, United Kingdom; ²St. George's University Hospitals National Health Service (NHS) Foundation Trust, London, United Kingdom; ³CINBIO, Universidade de Vigo, Vigo, Spain; ⁴Rare Diseases and Pediatric Medicine, Galicia Sur Health Research Institute (IIS Galicia Sur), SERGAS-UVIGO, Vigo, Spain; ⁵Addenbrooke's Hospital NHS Foundation Trust, Cambridge Biomedical Campus, Cambridge, United Kingdom; ⁶Royal Papworth Hospital NHS Foundation Trust, Cambridge Biomedical Campus, Cambridge, United Kingdom; ⁷Université Paris-Saclay, AP-HP, Service de Pneumologie, Centre de référence de l'hypertension pulmonaire, INSERM UMR_S 999, Hôpital Bicêtre, Le Kremlin-Bicêtre, Paris, France; ⁸Département de génétique, hôpital Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, and UMR_S 1166-ICAN, INSERM, UPMC Sorbonne Universités, Paris, France; ⁹Department of Pediatrics and ¹⁰Department of Medicine, Columbia University Irving Medical Center, New York, New York; ¹¹Centre for Congenital Heart Diseases, Pediatric Cardiology, Beatrix Children's Hospital, and ²⁰Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; ¹²Department of Pulmonary Medicine, Amsterdam University Medical Centre, Vrije Universiteit Amsterdam, Amsterdam Cardiovascular Sciences, the Netherlands; ¹³Division of Neonatology, St.-Luc University Hospital, Catholic University of Louvain, Brussels, Belgium; ¹⁴Department of Pediatrics, University of California San Francisco, San Francisco, California; ¹⁵Unidad Multidisciplinar de Hipertensión Pulmonar, Servicio de Cardiología, Hospital Universitario 12 de Octubre, Madrid, Spain; ¹⁶CIBERCV, Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares, ISCIII, Madrid, Spain; ¹⁷Departamento de Cardiología, Hospital Universitario Río Hortega, Valladolid, Spain; ¹⁸Department of Clinical Genetics, Cambridge University Hospitals NHS Foundation Trust, Cambridge, United Kingdom; ¹⁹Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, United Kingdom; ²¹Department of Infection, Immunity, and Cardiovascular Disease, University of Sheffield, Sheffield, United Kingdom; ²²Sheffield Pulmonary Vascular Disease Unit, Royal Hallamshire Hospital, Sheffield, United Kingdom; ²³Instituto de Genética Médica y Molecular (INGEMM)-IdiPAZ, Hospital Universitario La Paz-UAM, Madrid, Spain; ²⁴CIBERER, Centro de Investigación Biomédica en Red de Enfermedades Raras, ISCIII, Madrid, Spain; ²⁵ITHACA, European Reference Network on Rare Congenital Malformations and Rare Intellectual Disability, Brussels, Belgium; ²⁶Manchester Centre for Genomic Medicine, St. Mary's Hospital, Manchester University NHS Foundation Trust, Manchester, United Kingdom; ²⁷Division of Evolution and Genomic Sciences, School of Biological Sciences, University of Manchester, Manchester, United Kingdom; ²⁸Great Ormond Street Hospital, London, United Kingdom; ²⁹National Heart and Lung Institute, Imperial College London, London, United Kingdom; ³⁰National Institute of Health Research (NIHR) BioResource for Translational Research, Cambridge Biomedical Campus, Cambridge, United Kingdom; ³²www.ipahcohort.com; and ³³www.pahbiobank.org

ORCID IDs: 0000-0002-2717-5463 (M.P.); 0000-0002-8799-6079 (M.L.D.); 0000-0002-9358-6922 (D.M.); 0000-0003-0703-2892 (M.H.); 0000-0003-3521-4458 (C.L.W.); 0000-0003-3438-5685 (W.K.C.); 0000-0002-4385-5784 (R.M.F.B.); 0000-0001-5371-0346 (H.J.B.); 0000-0002-8208-3086 (O.D.); 0000-0002-6640-4839 (P.E.-S.); 0000-0001-7016-7373 (H.G.); 0000-0003-3493-3573 (S.M.A.J.); 0000-0003-2528-2203 (W.K.-F.); 0000-0002-6324-4825 (P.L.); 0000-0002-5220-8837 (J.M.); 0000-0003-0262-2340 (S.M.); 0000-0003-3764-3563 (J.P.-Z.); 0000-0003-0344-3088 (G.J.P.); 0000-0002-5308-2316 (J.T.-C.); 0000-0002-0717-4551 (A.A.R.T.); 0000-0001-8110-2575 (J.W.); 0000-0002-2826-3879 (K.M.); 0000-0002-1924-3120 (W.L.); 0000-0001-5700-9792 (N.W.M.); 0000-0002-1315-8873 (S.G.); 0000-0002-7024-1657 (D.V.).

Pulmonary arterial hypertension (PAH) is a rare progressive vasculopathy characterized by abnormal cell proliferation in the pulmonary arterioles leading to increased pulmonary artery pressure and, ultimately, right ventricular failure (1). It can occur idiopathically or in association with other medical conditions, such as congenital heart disease, or exposure to certain drugs and toxins. Since the landmark discovery of the *BMPR2* (Bone Morphogenetic Protein Receptor type 2) gene in 2000 as the main cause of heritable PAH (2), high-throughput sequencing has implicated more than 20 genes with identified causal variants in up to 25% of individuals diagnosed with idiopathic PAH (3–5). Monoallelic pathogenic variants in the *TBX4* (T-BOX transcription factor 4) gene are the second most common heritable cause of PAH, often enriched in pediatric cohorts (6–8). *TBX4* belongs to the T-BOX family of transcription factors, playing a critical role in early hindlimb development (9–11) and branching of the lungs (12) and regulating the

expression of *FGF10* (Fibroblast Growth Factor 10) together with *TBX5* (13, 14).

Transcriptome analysis suggests it continues to be active after organogenesis and plays an important role in the cellular homeostasis of adult lung fibroblasts (15).

TBX4 sequence variants and contiguous gene deletions, as part of the recurrent chromosome 17q23.2 microdeletion, were originally reported in association with small patella syndrome (SPS; MIM# 147891) (16–18). More recently, evidence is emerging that *TBX4* single-nucleotide variants and/or deletions are causative not only of PAH but also of a wide spectrum of developmental parenchymal lung disorders (19, 20). Partial or complete loss of a single *TBX4* functional allele is sufficient for the production of the phenotype, termed haploinsufficiency. Although the above disorders are dominantly inherited, both variable expressivity and reduced penetrance have been observed (6).

Herein, we assembled a large cohort of patients harboring *TBX4* sequence variants

to establish genotype–phenotype correlations. We developed *in vitro* assays to assess the pathogenicity of nontruncating variants. This led to the novel finding of functionally distinguishable missense variation, causing either gain of function (GoF) or loss of function (LoF).

Some of the results of these studies have been previously reported in the form of a preprint (<https://doi.org/10.1101/2022.02.06.22270467>).

Methods

Using the term “*TBX4*”, our PubMed search identified 22 publications limited to human studies dating from 2004 to 2021 (online supplement). Cases with the recurrent 17q23.2 deletion were excluded from this study's scope. We collected phenotypic information on 137 heterozygous carriers of *TBX4* sequence variants, including 15 novel cases from the National Institute for Health

Correspondence and requests for reprints should be addressed to Diana Valverde, Ph.D., CINBIO, Universidade de Vigo, Campus Universitario As Lagoas-Marcosende s/n, Vigo 36310, Spain. E-mail: dianaval@uvigo.es.

This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the

Subject: *TBX4* (T-BOX transcription factor 4) is the second most common gene culprit for development of pulmonary arterial hypertension, especially in pediatric-onset cases. A growing body of literature has expanded its phenotypic spectrum, which now includes developmental lung lesions, ranging from mild to lethal.

What This Study Adds to the

Field: Our study identifies genetic determinants of *TBX4* disease heterogeneity, inclusive of newly described gain-of-function missense variants associated with later-onset lung disease. It draws important conclusions on genotype–phenotype associations, informative to *TBX4* variant interpretation and genetic counseling of affected families.

Research BioResource–Rare Diseases (NBR) study (21), the UK National Cohort Study of Idiopathic and Heritable PAH (3, 5), the Spanish registry of PAH (22), the registry of the French PAH network (23, 24), and the DECIPHER database (25). Demographic and phenotypic information at the time of diagnosis was captured from relevant publications and databases (online supplement). Follow-up information was obtained, where available.

Annotation of variants was harmonized to the *TBX4* canonical transcript NM_018488.3 of the human reference genome assembly GRCh37/hg19 using the Mutalyzer and Ensembl Variant Effect Predictor web services. We assessed variant pathogenicity according to the American College of Medical Genetics and Genomics (ACMG) guidelines using the VarSome Clinical tool followed by manual curation (26). Nontruncating variants (missense, indels) and variants predicted to affect splicing were selected for functional characterization (online supplement). Truncating variants (frameshift, nonsense) were presumed to cause LoF; p.Tyr127Ter pathogenic variant was used as a positive control in functional experiments.

Among the T-BOX family members, *TBX4* has the highest similarity to *TBX5*

(27). Because no structure for *TBX4* has been reported, we analyzed the effect of variants using the crystal structure of the *TBX5* complex with DNA (Figure 3) (28). Variants were subgrouped by protein domains, including the highly conserved T-BOX domain (codons 71–251) containing the first nuclear localization segment (NLS1; 91–103), and the predicted NLS2 (338–351) and transactivating region (351–393) (29, 30).

We sought genotype–phenotype associations in carriers of likely pathogenic/pathogenic *TBX4* variants (excluded functionally assessed variants classed as benign/likely benign or of uncertain significance) and undertook a comparative analysis with genotyped patients with PAH recruited to the NBR study, termed in this paper as *BMRP2* ($n = 162$) and no identified causal variant ($n = 741$) groups (Table 1). The latter included patients with idiopathic PAH and likely pathogenic/pathogenic *BMRP2* variants or no identified likely pathogenic/pathogenic variants in PAH-associated genes (*BMRP2*, *TBX4*, *EIF2AK4*, *SMAD1/4/9*, *CAV1*, *KCNK3*, *ENG*, *ALK1*, *GDF2*, *AQP1*, *ATP13A3*, *SOX17*) (3). We additionally obtained computed tomography chest images from a subset of patients from the *BMRP2* ($n = 34$) and no identified causal variant ($n = 143$) groups and compared these to 11 *TBX4* cases from the NBR study. Details of the radiological substudy can be found in the online supplement. We assessed genotype–phenotype differences in the range of phenotypic features expected to differentiate the three selected genotypes. Because this analysis was preplanned and the results were consistent, we did not perform correction for multiple comparisons.

Statistical analysis was performed using R (www.r-project.org). The R package “survival” was used to compare event-free survival between different groups. Survival was estimated by the Kaplan–Meier method from the time of diagnosis to death or transplantation. To avoid immortal time bias, this was limited to a 10-year interval. Sex and age at diagnosis of lung disease were included as covariates in the semiparametric Cox proportional hazard models.

Results

Study Population

We identified 137 heterozygous carriers of *TBX4* variants, the majority of whom were

sporadic cases ($n = 127$, 93%). Out of four identified families, eight related individuals with available detailed phenotypic information were included in our analyses. Twenty-one cases had a primary phenotype of SPS. In the remaining 116 individuals presenting with lung disease, subsequent assessment for SPS was lacking in most, with only 29 cases (25%) reported having associated skeletal features. PAH was the predominant primary lung phenotype (Table 1). Median age at diagnosis of lung disease was 14 years (interquartile range [IQR], 2–47 yr). Fifty-three individuals (45%) presented in adulthood, 41 (36%) in childhood, and 22 (19%) in the perinatal period. In the overall patient cohort, there was an equal female-to-male ratio, with an observed female predominance (62%) in the lung disease group.

Spectrum and Functional Assessment of *TBX4* Variants

A total of 108 distinct *TBX4* variants were retrieved from the literature and aforementioned databases (Figure 1). Of these, 43 were missense, 39 frameshift, 15 nonsense, 6 indels including an additional deletion of whole exon 5, and 3 variants predicted to affect splicing (online supplement). A single case with a variant in the *TBX4* promoter region was excluded from our genotype–phenotype analyses (23).

We assessed the pathogenicity of all indels and 42/43 missense variants using a luciferase reporter assay (Figure 2). Variant c.1021G>C was assessed by a minigene assay instead as it was predicted to affect the same donor splice site as in c.1021 + 1G>A, which resulted in a double exon skipping. All indels and 23 missense variants caused LoF, with another 11 shown to be benign. Eight missense variants resulted in GoF with mean relative luciferase units of approximately twice the amount of the wild-type construct. We confirmed that GoF was not an artifact by checking *TBX4* protein expression amount of several constructs with different outcomes in the luciferase assay by quantitative PCR and western blot (see Figure E1 in the online supplement). Independent of their functional activity, all assessed variants translocated to the nucleus, replicating the translocation of wild-type and green fluorescent protein–tagged *TBX4* (Figures E2 and E3).

As per ACMG guidelines, our luciferase functional data altered the classification of the majority of respective variants, with a

Table 1. Demographic and Clinical Characteristics at Diagnosis of the Patient Population Included in the Genotype–Phenotype Dataset

	BMPR2 (n = 162)	No Causal Variant Identified (n = 741)	TBX4 (n = 98)	P Value	Available (Total n)
Primary diagnosis					1,001
1.1 Idiopathic PAH, including drug and toxin induced	107 (66.0)	741 (100)	58 (59.2)		
1.2 Heritable PAH	53 (32.7)	—	17 (17.3)		
1.4.1 PAH associated with connective tissue disease	1 (0.62)	—	2 (2.04)		
1.4.4 PAH associated with congenital heart disease	1 (0.62)	—	11 (11.2)		
1.6 PAH with overt features of venous/capillary (PVOD/PCH) involvement	—	—	1 (1.02)		
3.5 Developmental lung disorders	—	—	9 (9.18)		
Sex: female	107 (66.0)	530 (71.5)	62 (63.9)	0.156	1,000
Smoking history: past/current, adults only	53 (39.3)	293 (53.3)	10 (47.6)	0.039	706
Exposure to drug or toxins: yes	6 (3.70)	45 (6.07)	7 (19.4)	0.007	939
Ethnicity					966
European	137 (84.6)	630 (85.0)	53 (84.1)		
Finnish European	—	1 (0.13)	—		
African	2 (1.23)	20 (2.70)	5 (7.94)		
East Asian	2 (1.23)	6 (0.81)	1 (1.59)		
South Asian	6 (3.70)	48 (6.48)	1 (1.59)		
Other	15 (9.26)	36 (4.86)	3 (4.76)		
Age at diagnosis of lung disease, yr	39 (31–51)	51 (38–66)	14 (2–48)	<0.001	997
Age at transplantation or death, yr	52 (43–61)	67 (53–75)	64 (1–71)	<0.001	302
WHO functional class				0.277	918
I	2 (1.24)	15 (2.11)	2 (4.44)		
II	32 (19.9)	144 (20.2)	10 (22.2)		
III	96 (59.6)	466 (65.4)	27 (60.0)		
IV	31 (19.3)	87 (12.2)	6 (13.3)		
Exercise test					
Distance, m	350 (276–420)	330 (210–410)	371 (308–422)	0.028	810
Pretest saturation	96.0 (94.0–98.0)	96.0 (93.0–97.0)	97.5 (95.5–98.0)	0.006	738
Post-test saturation	94.0 (89.0–96.8)	91.0 (85.0–95.0)	95.5 (86.5–97.0)	0.001	683
Lung function, % predicted					
FEV ₁	91.0 (79.0–100)	85.0 (72.3–96.0)	82.0 (70.0–98.0)	0.001	719
FVC	99.7 (17.2)	93.2 (19.4)	88.0 (17.7)	<0.001	703
Kco	83.4 (74.2–96.5)	68.0 (49.0–83.0)	72.0 (59.8–89.8)	<0.001	516
TLC	96.0 (89.0–106)	94.0 (85.0–104)	104 (98.0–110)	0.044	511
Hemodynamics					
mPAP, mm Hg	57.0 (52.0–66.8)	52.0 (42.0–61.0)	60.5 (48.2–82.2)	<0.001	916
mPAWP, mm Hg	10.0 (7.00–12.0)	9.00 (7.00–12.0)	9.00 (7.00–11.0)	0.841	822
PVR, WU	14.5 (10.8–20.4)	10.3 (7.06–13.9)	12.8 (8.25–16.2)	<0.001	767
CO, L/min	3.30 (2.69–3.94)	4.04 (3.25–5.10)	3.65 (3.09–4.61)	<0.001	852
CI, L/min/m ²	1.90 (1.51–2.23)	2.30 (1.80–2.80)	2.63 (1.98–3.20)	<0.001	503
Vasoresponders	1 (1.28)	51 (17.7)	6 (10.2)	0.001	425
Increased BNP (>50 pg/ml) or NT-proBNP (>300 pg/ml)	34 (97.1)	140 (79.1)	5 (71.4)	0.011	219

Definition of abbreviations: BMPR2 = Bone Morphogenetic Protein Receptor type 2; BNP = brain natriuretic peptide; CI = cardiac index; CO = cardiac output; mPAP = mean pulmonary artery pressure; mPAWP = mean pulmonary artery wedge pressure; NT-pro-BNP = N-terminal pro-brain natriuretic peptide; PCH = pulmonary capillary hemangiomatosis; PVOD = pulmonary venoocclusive disease; PVR = pulmonary vascular resistance; *TBX4* = T-BOX transcription factor 4; WHO = World Health Organization; WU = Wood units = mm Hg/L/min.

Data are presented as *n* (%) or median (interquartile range). Heterozygous carriers of *TBX4* variants shown to be benign by our functional studies were excluded.

total of 33/48 (67%) initially classified as variants of uncertain significance and an equivalent number (32/48) of likely pathogenic/pathogenic variants after application of the PS3 criterion (*in vitro* functional studies supportive of a damaging effect) where appropriate (Figure E4).

We evaluated the performance of *in silico* tool predictions for likely pathogenic/pathogenic *TBX4* missense variants, with SIFT (Sorting Intolerant From Tolerant) generating the highest overall percentage of correct calls (Figure E5). Overall, a total of 4/8 GoF and 22/23 LoF missense variants were classed as

likely pathogenic/pathogenic and included in the genotype–phenotype analyses (online supplement).

Finally, we assessed the functional impact of all previously reported *TBX4* variants predicted to affect splicing with recurrent variants c.702 + 1G>A and

Table 2. Radiological Features of Computed Tomography of the Chest in Pulmonary Arterial Hypertension Cases Analyzed as Part of the Imaging Substudy

	<i>BMPR2</i> (<i>n</i> = 34)	No Causal Variant Identified (<i>n</i> = 143)	<i>TBX4</i> (<i>n</i> = 8)	<i>P</i> Value	Available (Total <i>n</i>)
Vascular features					
Axial MPA diameter, mm	34.2 (30.9–37.4)	34.6 (31.4–38.3)	34.0 (30.7–38.5)	0.674	185
PA to Ao ratio	1.23 (1.12–1.40)	1.14 (0.99–1.29)	1.18 (0.94–1.32)	0.083	185
Neovascularity	7 (23.3)	10 (7.69)	—	0.048	167
Lung and mediastinal features					
Ground-glass opacities	18 (52.9)	62 (43.4)	—	0.016	185
Centrilobular pattern				0.088	185
None	17 (50.0)	90 (62.9)	8 (100)		
Subtle	4 (11.8)	21 (14.7)	—		
Present	13 (38.2)	32 (22.4)	—		
Nonspecific mosaic pattern				0.441	180
None	31 (91.2)	130 (90.9)	3 (100)		
Subtle	—	7 (4.90)	—		
Present	3 (8.82)	6 (4.20)	—		
Fibrosis				1.000	185
None	34 (100)	137 (95.8)	8 (100)		
Present	—	6 (4.2)	—		
Pleural effusion				1.000	185
None	33 (97.1)	132 (92.3)	8 (100)		
Subtle	—	3 (2.10)	—		
Present	1 (2.94)	8 (5.59)	—		
Interlobular septal thickening				0.157	179
None	27 (87.1)	122 (87.1)	5 (62.5)		
Subtle	3 (9.68)	9 (6.43)	1 (12.5)		
Present	1 (3.23)	9 (6.43)	2 (25.0)		
Mediastinal lymphadenopathy				0.721	185
None	29 (85.3)	122 (85.3)	6 (75.0)		
Present	5 (14.7)	21 (14.7)	2 (25.0)		
Airway/acinar features	11 (32.4)	48 (33.6)	7 (87.5)	0.009	185
Bronchial wall thickening				0.051	179
None	26 (83.9)	126 (90.0)	6 (75.0)		
Subtle	3 (9.68)	11 (7.86)	—		
Present	2 (6.45)	3 (2.14)	2 (25.0)		
Emphysema				0.026	185
None	32 (94.1)	120 (83.9)	5 (62.5)		
Subtle	—	13 (9.09)	—		
Present	2 (5.88)	10 (6.99)	3 (37.5)		
Air trapping				0.040	185
None	28 (82.4)	121 (84.6)	4 (50.0)		
Subtle	4 (11.8)	7 (4.90)	1 (12.5)		
Present	2 (5.88)	15 (10.5)	3 (37.5)		
Suspected PVOD	1 (3.03)	8 (5.59)	1 (12.5)	0.500	184

Definition of abbreviations: Ao = aortic; MPA = main pulmonary artery; PA = pulmonary artery; PVOD = pulmonary venoocclusive disease; *TBX4* = T-BOX transcription factor 4.

Data are presented as *n* (%) or median (interquartile range).

c.1021 + 1G>A inducing exon skipping events (Figure E6). Key findings of the structural variant analysis are summarized in Figure 3.

Genotype–Phenotype Associations

Patients with lung disease and variants within the DNA binding T-BOX domain presented at a younger age (median [IQR], 7.5 [1–18.5] yr) than carriers of variants outside this domain (18 [3–51.5] yr; *P* = 0.028). This remained true for sequence variants

located in the T-BOX domain containing either NLS1 or NLS2 at the C terminus (*P* = 0.005; Figure 4). Individuals with LoF variants (missense, indels) were also diagnosed at a younger age than GoF variant carriers (14 [5–31] yr vs. 57 [42.5–59.5] yr; *P* = 0.038; Figure 4).

Baseline clinical features and hemodynamic parameters did not differ significantly between protein domain groups, with the exception of interstitial lung disease, which was more frequently reported in carriers

of *TBX4* variants located in the T-BOX domain alone (75% vs. 21%; *P* = 0.003) or in combination with NLS2 variants (*P* = 0.001). Similarly, no significant differences were observed between LoF and GoF variation, and this was consistent when protein-truncating variants were added to the LoF group.

The observed primary phenotypes varied significantly between protein domains, with a greater frequency of developmental lung disorders (including acinar dysplasia and congenital alveolar dysplasia) in the T-BOX

Table 3. Clinical Features of Individuals Heterozygous for *TBX4* Gain-of-Function Missense Variants

Variant	Demographics	Primary Lung Phenotype; Age at Diagnosis	Details of Lung Disease and Comorbidities	Skeletal Features	Follow-Up	Pedigree Information	ACMG Classification; Population Frequency (gnomAD Exomes)
c.104C>T; p.Ala35Val	Dutch PAH patient cohort (40)	PAH; adult onset	N/A	N/A	Deceased (unavailable details)	N/A	Likely benign; 0.009
c.432G>T; p.Met144Ile	Female, White Hispanic (22)	IPAH; 28 yr	Clinical diagnosis of possible PVOD due to radiological findings and reduced diffusion capacity. Histopathological findings (explanted lung tissue): typical PAH features, no evidence of PVOD or ILD. Unknown smoking status.	Absent	Clinical deterioration with bilateral lung transplantation 9 yr after diagnosis	Maternally inherited variant, PAH ruled out at 67 yr	Pathogenic; 3.98×10^{-6}
c.432G>T; p.Met144Ile	Male, White Hispanic (22)	PVOD; 62 yr	Radiological findings typical of PVOD. Signs of heart failure, severe respiratory insufficiency. Not eligible for lung transplant because of advanced age and comorbidities. Unknown smoking status.	Absent	Deceased (26 mo after diagnosis)	No known family history of PAH/SPS. Deceased parents before PAH diagnosis. Genetic counseling provided to the rest of the family (declined genetic testing)	Pathogenic; 3.98×10^{-6}
c.652G>A; p.Val218Met	Female, White European (24)	chILD; 5 mo	Lung biopsy at 7 yr: diffuse alveolar simplification, moderate thickened PA muscular wall, and PA fibrointimal proliferation. PFO, microcephaly, hearing loss.	Short stature, no SPS features	10 yr: chILD, moderate pulmonary hypertension	Parents not tested	VUS; 1.23×10^{-4}
c.743G>T; p.Gly248Val	Dutch family (16, 40)	—	Family members screened for PAH by echocardiography with none fulfilling diagnostic criteria (unavailable details).	Classical SPS phenotype, including patellar and pelvic anomalies	N/A	Three-generation pedigree (family A) (16)	Pathogenic; not reported
c.809T>G; p.Ile270Ser	Female, White European (37)	IPAH; 57 yr	Functional class III at diagnosis. Asthma, HTN, obesity, sleep apnea. Never smoked.	N/A	Alive (1-yr follow-up)	N/A	Likely pathogenic; 4.00×10^{-6}
c.1070C>T; p.Ala357Val	Female, White British (3)	IPAH; 81 yr	Functional class III at diagnosis. Radiological findings included minor bronchial wall thickening, no evidence of PVOD except mediastinal nodes, no interstitial lung disease. Hypothyroidism, IHD, T2DM, HTN, breast cancer. Never smoked.	N/A	Died in hospice 7 mo after diagnosis (progressive heart failure)	N/A	VUS; 1.99×10^{-5}
c.1102C>T; p.Arg368Cys	Female, African Caribbean (3)	IPAH; 39 yr	Functional class III at diagnosis. Radiological findings are typical of PAH, no lung disease, PFO, hypothyroidism, chronic subdural hematoma. Unknown smoking status.	N/A	Deceased (50 yr)	N/A	VUS; 5.58×10^{-5}
c.1592A>G; p.Gln531Arg	Dutch family (16, 40)	—	Family members screened for PAH by echocardiography with none fulfilling diagnostic criteria (unavailable details)	Classical SPS phenotype, including patellar and pelvic anomalies	N/A	Three-generation pedigree (family C) (16)	Likely pathogenic; not reported

Definition of abbreviations: ACMG = American College of Medical Genetics and Genomics; chILD = childhood-onset interstitial lung disease; HTN = systemic hypertension; IHD = ischemic heart disease; ILD = interstitial lung disease; IPAH = idiopathic pulmonary arterial hypertension; N/A = not available; PA = pulmonary artery; PAH = pulmonary arterial hypertension; PFO = patent foramen ovale; PVOD = pulmonary venoocclusive disease; SPS = small patella syndrome; T2DM = type 2 diabetes mellitus; *TBX4* = T-BOX transcription factor 4; VUS = variant of uncertain clinical significance.

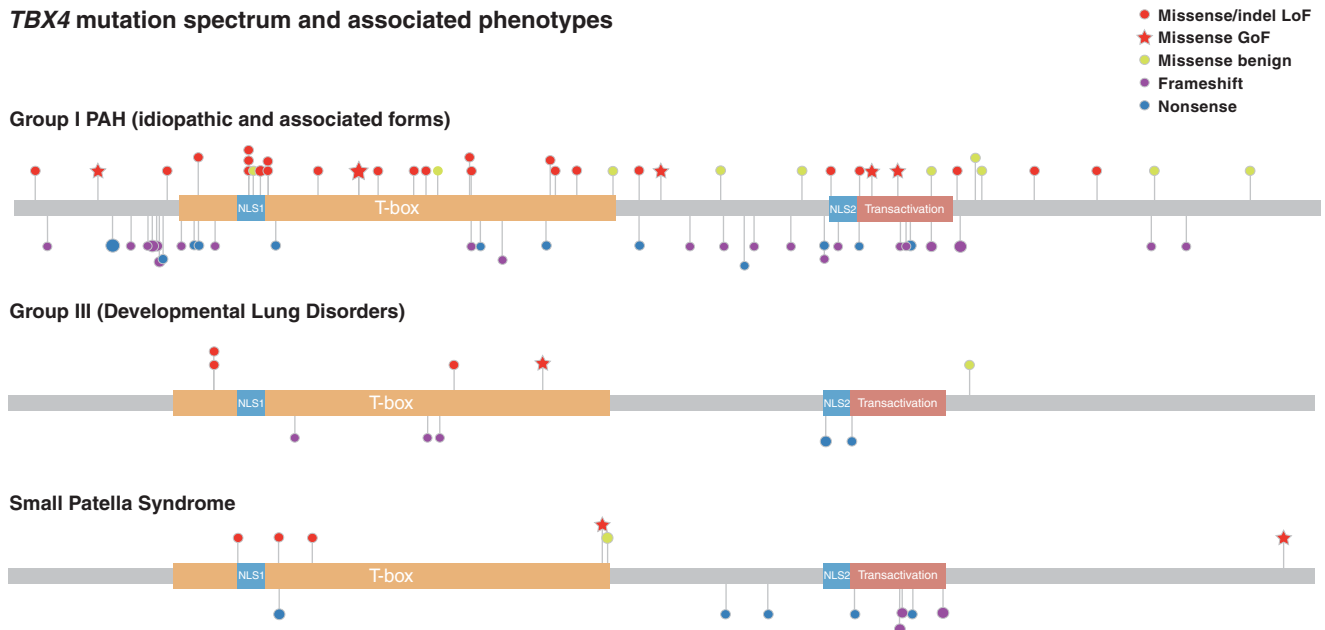
TBX4 mutation spectrum and associated phenotypes

Figure 1. Lollipop depicting *TBX4* (T-BOX transcription factor 4) mutation spectrum. Recurrently mutated positions are represented by a proportionally sized lollipop. Critical protein domains are highlighted, inclusive of the DNA binding T-BOX, nuclear localization segments (NLS1 and NLS2), and transactivation domains. Variants are grouped by primary associated phenotype and color-coded, taking into account the functional assessment of missense and inframe insertion/deletions (indels). GoF = gain-of-function; LoF = loss-of-function; PAH = pulmonary arterial hypertension.

group (15.4% vs. 3.8%; overall $P = 0.046$); when combined with variants in the NLS2 domain, this remained statistically significant (overall $P = 0.003$). Lung histology was available in 17 previously published *TBX4* cases (see online supplement for details). A greater frequency of a confirmed secondary phenotype of SPS was observed in carriers of variants located outside the T-BOX and NLS2 domains (29.7% vs. 11.4%; $P = 0.038$) and was more prevalent in protein-truncating versus missense variation (27.8% vs. 7.1%; $P = 0.044$). Variant exon location did not appear to have any phenotypic impact (data not shown).

As previously reported (31), patients with *TBX4*-associated lung disease presented at a younger age (median [IQR], 14 [2–48] yr) than patients in the *BMP2* (39 [31–51] yr) and no identified causal variant (51 [38–66] yr; $P < 0.001$) groups. They performed better at the 6-minute-walk test, with no significant differences in functional class at presentation (Table 1). They also had worse baseline lung function (FEV₁, FVC), despite presentation at a younger age (Table 1). The frequency of airway and/or acinar abnormalities was significantly greater in the *TBX4* (87.5%)

versus the *BMP2* (32.4%) and no identified causal variant groups (33.6%; $P = 0.009$) (Table 2).

Impact of Genotype on Survival

Clinical outcomes were available for 89/115 *TBX4* cases presenting with lung disease, with a median follow-up of 8 years (IQR, 3–10 yr). Event-free survival was shorter in the T-BOX domain variant group ($P = 0.022$ for log-rank test), although age at diagnosis also had a significant effect ($P = 0.0461$ for Cox proportional hazard model; Figure 5). There were no observed differences in outcomes between GoF and LoF variants or missense versus protein-truncating variants. Overall, event-free survival was longer in the *TBX4* group compared with *BMP2* and no identified causal variants groups ($P = 0.0025$). Pairwise comparisons showed no significant differences between *TBX4* and *BMP2* variant carriers ($P = 0.69$). Compared with individuals with no causal sequence variants, both patients with *TBX4* ($P = 0.035$) and *BMP2* variants ($P = 0.016$) had higher event-free survival rates. However, genotype did not have a significant effect on survival after correction for age and sex; both male sex ($P = 0.0002$) and older age at the time of

diagnosis ($P < 2 \times 10^{-16}$) were associated with shorter survival in the Cox proportional hazard model.

Discussion

Distinct Mutational Mechanisms Disrupt *TBX4* Function

A wide range of *TBX4* mutations can result in human disease. Intragenic sequence variants occur throughout the gene and are mostly private. The functional impact of *TBX4* variants was not previously investigated, and a haploinsufficient effect was assumed. Our assessment of *TBX4* missense variants reported to date led to the discovery of two distinct functional classes of variants, GoF or LoF. Although this constitutes a novel finding, GoF variants have been reported in other members of the T-BOX gene family in association with a similar phenotypic spectrum caused by LoF. This includes carriers of missense variants in the *TBX1* gene with velocardiofacial syndrome, a single *TBX5* variant in a family with Holt-Oram syndrome, and a *TBX20* variant in a family with atrial septal defects and valvular disease (32–34). All of the above

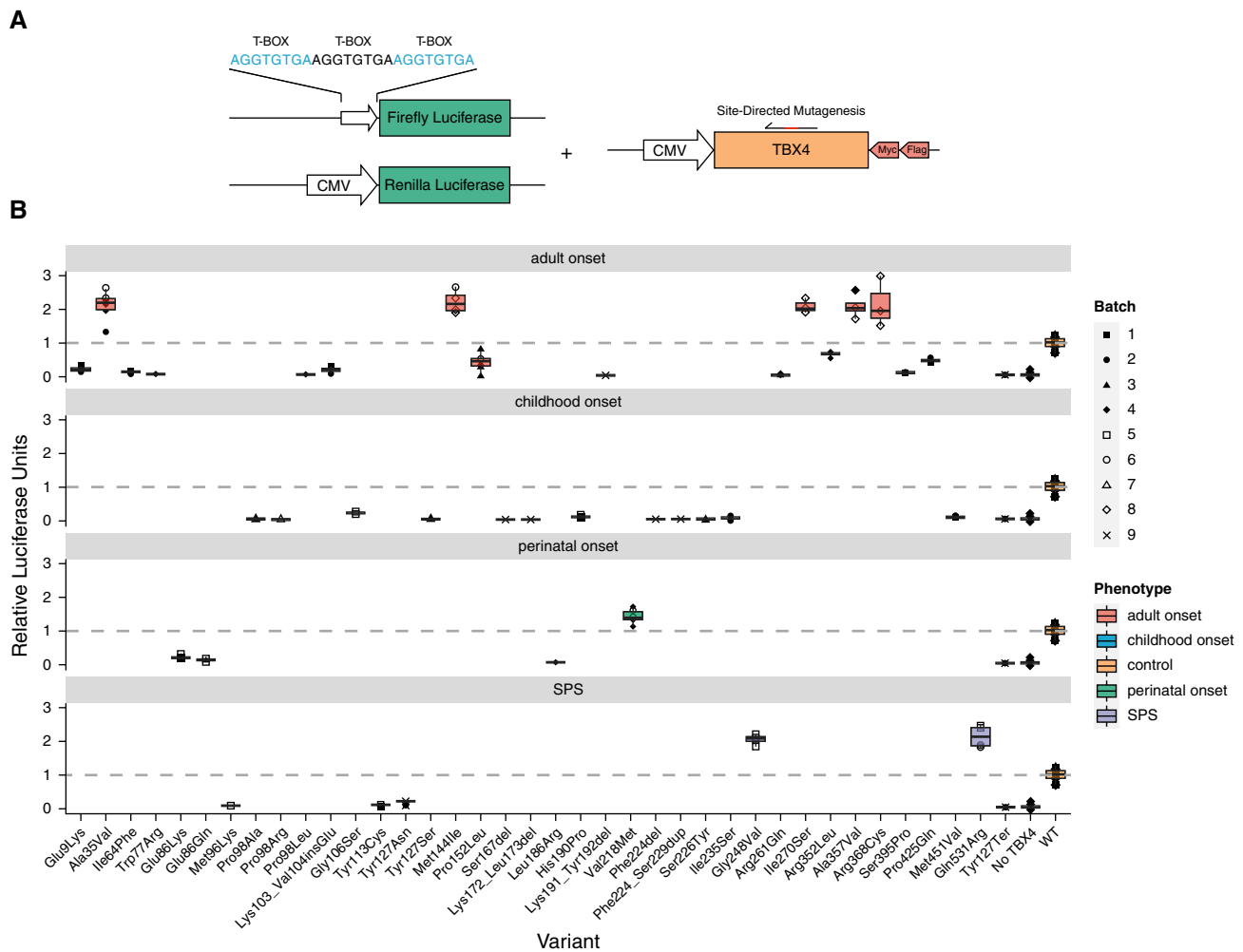


Figure 2. Functionally assessed *TBX4* (T-BOX transcription factor 4) variants by luciferase assay inducing gain or loss of function. (A) Schematic representation of the *in vitro* dual-luciferase reporter assay. We cotransfected three different plasmids: Firefly luciferase with x3 T-BOX motifs as the promoter, Renilla luciferase, and *TBX4* overexpression plasmid (wild type/mutated). (B) Variants inducing gain or loss of function grouped by primary phenotype; lung disease (perinatal-, childhood-, and adult-onset) or small patella syndrome (SPS). The y-axis represents the relative luciferase units; the dashed line marks the level of the wild type. Data are shown as box plots representing median \pm quartiles. Dots represent biological replicates with corresponding batches in different shapes. CMV = Cytomegalovirus promoter; Flag = Flag tag; Myc = partial cMyc tag.

were located in the highly conserved T-BOX domain, with the exception of a single *TBX1* variant in exon 8 of 9 (c.928G>A). Structural analyses were suggestive of increased protein stability; our respective analysis was indicative of the detrimental effects of LoF variants, with no simple explanation for GoF variation (Figure 3).

Out of 31 pathogenic missense *TBX4* variants reported in our study, 8 resulted in GoF, including the recurrent variant c.432G>T (22). These were located across the gene, with three in the T-BOX region and two in the transactivation domain (Figure 1). Two variants (c.743G>T and c.1592A>G)

were originally reported in 2004 in two Dutch families with classic SPS and constituted one of the first reports implicating *TBX4* in this phenotype (16). The remaining variants were described in association with adult-onset lung disease diagnosed as late as 81 years, with the exception of a neonate presenting with interstitial lung disease (Table 3). Notably, six of eight GoF variants were present in control populations (gnomAD database) including variant c.104C>T, with the highest overall population frequency in the cumulative *TBX4* variant dataset. As a result, many of these were classified

as likely benign or of uncertain clinical significance and cannot be considered as responsible for the underlying phenotype at present (Table 3).

It remains to be seen whether these GoF variants are purely hypermorphic, increasing the protein's function, or neomorphic, causing ectopic expression or acquisition of a new function. This may in turn influence their exerted phenotypic effects. From a mechanistic perspective, our knowledge of the consequences of *TBX4* overexpression during embryogenesis and/or after organogenesis is lacking. Transcription can be affected not only by decreased but also by

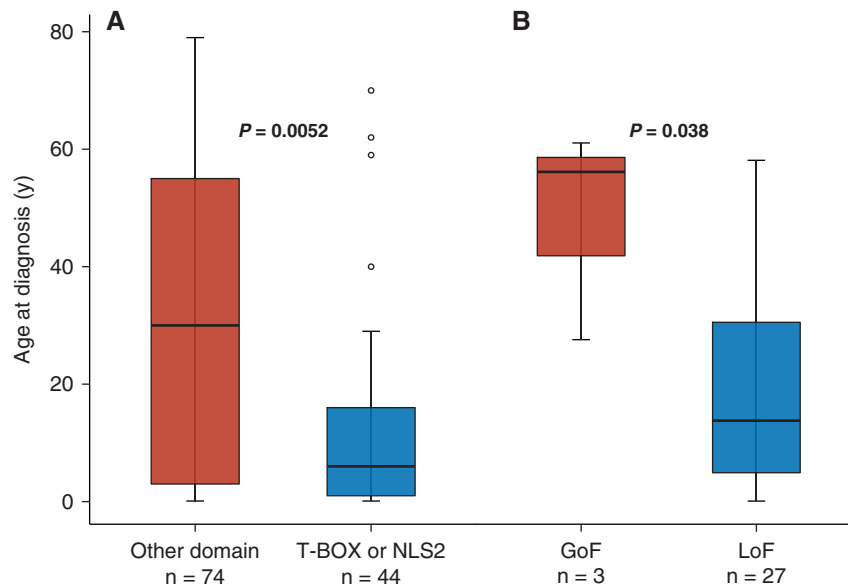


Figure 4. Age at diagnosis of lung disease by *TBX4* (T-BOX transcription factor 4) genotype. (A) Variants in the T-BOX and second nuclear localization segment (NLS2) versus other domains. (B) Likely pathogenic/pathogenic gain-of-function (GoF) versus loss-of-function (LoF) missense variants.

with evidence of possible disrupted alveolarization on computed tomography imaging suggestive of underlying developmental lung lesions. It can be postulated that this partly accounts for the variable age of onset of *TBX4*-associated pulmonary hypertension with less pulmonary reserve and increased susceptibility to external insults acting as environmental modifiers of penetrance. There are insufficient data at present to suggest a specific PAH treatment approach, distinct from other etiologies, for *TBX4* disease. However, at the point of diagnosis, it is important to evaluate the potential impact of any parenchymal abnormalities versus pure vascular disease to adapt treatment accordingly.

Gene-Specific Variant Classification and Prognostic Value

Inconsistent variant interpretation can not only lead to misdiagnosis of individual patients but also have significant consequences for at-risk relatives through inappropriate predictive testing. Gene-specific knowledge overcomes some of these pitfalls, especially when semiautomated impact analysis tools are used for variant classification. *In silico* predictions did not reliably reflect the true effects of *TBX4* missense variants on gene activity (Figure E4). Structural analysis was also not capable of discerning *TBX4* GoF variants. In light of these limitations, an integrated

pipeline incorporating molecular testing and functional assessment of novel *TBX4* missense variants by standardized assays would be of high diagnostic value.

Our results indicate that both protein-truncating and missense variants contribute to *TBX4* disease, with the majority of the latter (26/42) classified as likely pathogenic/pathogenic after functional assessment. In light of this, we removed the ACMG BP1 criterion (missense variant in a gene for which primarily truncating variants are known to cause disease) from *TBX4* variant annotation. In addition, the PM1 criterion (variant located in critical or well-established functional domain) could be expanded to include not only variants located in the T-BOX domain but also the predicted nuclear localization segments (NLS1 and NLS2), as our study was suggestive of an equivalent effect on produced phenotypes, including a higher rate of developmental (early-onset) lung disorders.

Estimating the true penetrance of *TBX4* disease remains a high priority and impacts variant interpretation as well as genetic counseling of respective families. A characteristic example exhibiting inter- and intrafamilial variability is that of splice variant c.1021 + 1G>A reported by three independent studies (online supplement); index cases had adult-onset lung disease with positive family history, including first-degree

relatives with severe PAH resulting in death in infancy or childhood or absent patella only (22, 36, 37). Leaky splicing variants (reducing but not completely abolishing the production of normal transcripts) can result in reduced penetrance, although this phenomenon would still not explain the variable phenotypes observed in association with other types of recurrent variants (online supplement). We applied the ACMG BS1 criterion (allele frequency is greater than expected for the disorder) using a maximum tolerated population frequency of 5.00×10^{-8} arising from a generous estimate of 50% for lung disease penetrance (38); out of 17/42 missense *TBX4* variants present in gnomAD, several GoF and LoF variants remained of uncertain clinical significance (online supplement). Where there is enough evidence to support (likely) pathogenicity for missense variation in critical protein domains, important prognostic information can be inferred based on our genotype–phenotype analysis.

Limitations

We were limited by the multicenter nature of this study including retrospective data collection with variable follow-up duration. This methodology introduced selection bias, because the vast majority of cases were previously published, a proportion of which consisted of severe cases with lung histopathology from postmortem

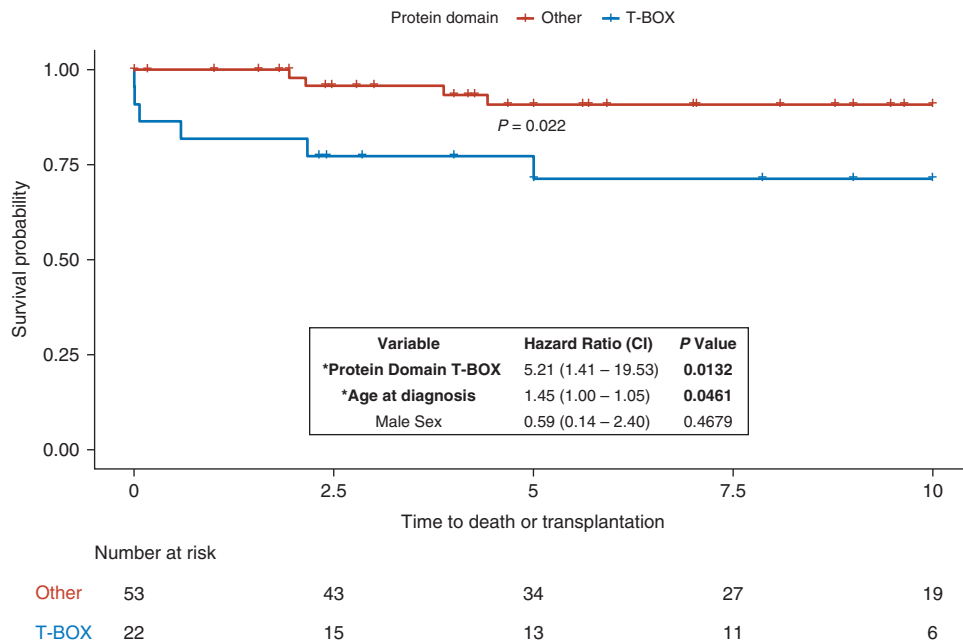


Figure 5. Time to death or lung transplantation (years) by *TBX4* (T-BOX transcription factor 4) protein domain genotype. Event-free survival was shorter in the T-BOX domain variant group, although age had a significant effect in the hazard model. CI = confidence interval; *Variables with $P < 0.05$.

examination or explants at the time of lung transplantation. Published cases may not be truly representative of the natural history of *TBX4* disease at large, which is yet to be elucidated via recall-by-genotype studies. We were unable to account for missing phenotypic information (presence or absence of SPS features in individuals with a primary lung phenotype), although estimation of *TBX4* disease penetrance was not the focus of this work. The main limitation of our functional assay is that we used overexpression plasmids, forcing the expression of variants whose effect could be lowered by post-transcriptional regulation. Our reporter system was designed using the standard T-BOX motif and *FGF10* promoter sequences. A recent report of *TBX4* chromatin immunoprecipitation in human

fetal lung fibroblasts identified several other potential genome-wide target sites whose effects we have not tested (39). Therefore, a variant shown to have a modest or benign effect by our luciferase assay may still have significant damaging effects on interactions with other crucial binding sites in biologically relevant tissues.

Conclusions

We combined functional and phenotypic characterization of all reported *TBX4* sequence variants to date to determine the hallmarks of *TBX4*-mediated lung disease. We used *in vitro* analyses to assess the pathogenicity of missense, indel, and splice variants, resulting in either LoF or GoF effects with phenotypic and prognostic implications when also taking into account variant location

in functional domains. Our knowledge of *TBX4* genotype–phenotype associations can only be furthered by active collaborations between molecular scientists and clinicians, requiring both an in-depth understanding of the biological aspects of the disease and a systematic approach to phenotyping. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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