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Inherited *TBX4* frameshifting variants predicted to escape nonsense mediated decay in two families with variable phenotypes, including lethal lung developmental disorders

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Abstract

Background Pathogenic variants involving the transcription factor *TBX4* gene have been associated with various skeletal and pulmonary abnormalities, including lethal lung developmental disorders (LLDD).

Methods Whole-genome sequencing (WGS) with AI-powered platform for variant detection and interpretation followed by Sanger sequencing targeted variant segregation analysis were used. Reverse transcription quantitative PCR (RT-qPCR) and immunohistochemistry (IHC) studies were performed to assess gene and protein expression levels, respectively.

Results We describe two unrelated families with intrafamilial variability in the *TBX4* phenotypic expressivity, including LLDDs. WGS analyses revealed two frameshift variants, c.1019del; p.(Arg340GlnfsTer40) in the penultimate exon and c.1167dup; p.(Arg390GlnfsTer30) in the last exon of *TBX4*, both predicted to escape nonsense mediated mRNA decay (NMD) and associated with highly variable phenotypes. RT-qPCR and IHC studies implied incomplete NMD in one family.

Conclusions Our data expand the phenotypic and genotypic landscape of *TBX4*–associated pulmonary disease to include asthma. We propose incompleteness and variability of NMD escape contributing to the observed wide phenotypic spectrum and increased risk of LLDDs.

Keywords Congenital alveolar dysplasia, Small patella syndrome, Variable expressivity, Premature stop codon-mediated decay, Loss-of-function, Gain-of-function

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Introduction

The *TBX4* gene (T-box transcription factor 4) on chromosome 17q23.2 plays a critical role in embryonic development, particularly in hindlimb formation [1] and lung organogenesis [2, 3] where it is expressed in the developing limb buds and pulmonary mesenchyme, respectively, and regulates pathways essential for morphogenesis and tissue differentiation [2, 4–7].

Pathogenic heterozygous variants in *TBX4* were first associated with ischiocoxopodopatellar syndrome with or without pulmonary arterial hypertension (ICPPS; OMIM #147891), also known as small patella syndrome (SPS), an autosomal dominant skeletal disorder characterized by patellar aplasia or hypoplasia, pelvic anomalies, foot abnormalities, and variable limb defects [1]. Subsequently, *TBX4* variants have been implicated in pulmonary arterial hypertension (PAH) [8], and more recently with lethal lung developmental disorders (LLDDs), including acinar dysplasia (AcDys), congenital alveolar dysplasia (CAD), and other unspecified pulmonary hypoplasias associated with intractable respiratory failure and severe PAH [3, 9–12]. *TBX4*-related pulmonary disease may present in infancy, childhood, or adulthood, with or without overt skeletal manifestations [3, 5, 13]. The reported phenotypic variability has been proposed to be influenced by genetic modifiers, spatial, temporal, and tissue-specific expression patterns of *TBX4*.

Nonsense mediated mRNA decay (NMD) is a conserved cellular quality surveillance control mechanism to degrade transcripts with premature termination codons (PTCs) and prevent the production of truncated proteins with potentially deleterious effects [14, 15].

Here, we report on frameshift variants involving *TBX4* segregating in two unrelated families with LLDDs. The affected individuals exhibit highly variable clinical presentations, underscoring the broad phenotypic spectrum and marked intra- and interfamilial variability associated with *TBX4* variants. Our findings add incomplete and variable NMD escape as a significant mechanism of *TBX4*-related phenotypic variation important for genetic counseling.

Patients

The study protocol was approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine (H-8712).

Family 1. The proband (AD149-III.2) was a female, the second child of a non-consanguineous couple, born at 38 2/7 weeks of gestation following a pregnancy complicated by mild preeclampsia, COVID-19 infection at 22 weeks and a prenatally detected cleft lip/palate. With the initial Apgar score of 8 at 1 min, she developed acute respiratory distress shortly after birth and died at 8 h of life despite maximal supportive interventions. Her mother (AD149-II.2), presently 31 years old, had a history of persistent pulmonary hypertension of neonatal onset that required management with conventional ventilation. Currently, she does not manifest any pulmonary issues. The couple had three other children, one (AD149-III.3) with a history of asthma and two apparently asymptomatic. The proband's maternal grandfather (AD149-I.1) also has a history of mild asthma (Fig. 1A, Additional file 2: Table S1).

Family 2. Patient AD150.III.2 is a male, delivered at 39 2/7 weeks of gestation with an initial Apgar score of 8 at 1 min and birth weight of 3.6 kg (0.08 SD), total length of 54 cm (1.46 SD) and head circumference of 34.5 cm (−0.83 SD). Two hours after birth, he developed right-sided tension pneumothorax and on day two of life a large left-sided tension pneumothorax with subsequent respiratory decompensation requiring CPAP support. Chest CT and X ray performed on day two of

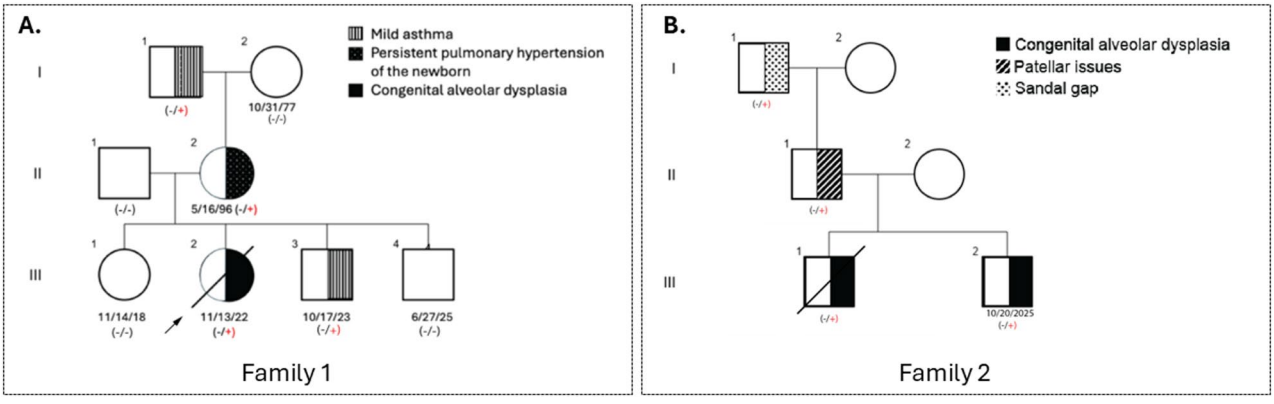

Fig. 1 **A** Three-generation pedigree of Family 1 showing the deceased proband (AD149-III.2) with congenital alveolar dysplasia (CAD) and her mother,who has a history of persistent pulmonary hypertension of the newborn; both carrying the TBX4 frameshifting variant c.1019del; p.(Arg340GlnfsTer40) inthe penultimate exon. Of note, the proband's sibling (AD149-III.3) and maternal grandfather (AD149-I.1) have a history of mild asthma. **B** Pedigree of twogenerationFamily 2 showing patient AD150-III.2 and his similarly affected, deceased sibling AD150-III.1, both presenting with CAD spectrum, and theirfather AD150-II.1, who has patellar abnormalities, all of whom carry the frameshift TBX4 variant, c.1167dup; p.(Arg390GlnfsTer30)

life showed bilateral pneumothorax (Fig. 2A) and underinflated lungs with right upper lobe atelectasis (Fig. 2B), respectively. Hypertensive changes of pulmonary arteries were not observed. He was hospitalized again on day 11 of life due to tachypnoea with intermittent episodes of increased work of breathing, including subcostal indrawing and occasional tracheal tug. At seven weeks, resolution of bilateral pneumothorax had occurred with clinical improvement off all support and only brief periods of tachypnoea. Of note, there is history his male sibling (AD150-III.1), born at 37 4/7 weeks of gestation, who had a similar phenotypic presentation and died of respiratory failure at approximately at 18 h of life (Fig. 1B). On autopsy, his lung showed deficient alveolarization with patchy hyaline membranes and severe acute congestion. Their father (AD150-II.1) has left patella subluxed laterally with dysplastic patellofemoral articular surfaces. The right femoral trochlear groove is also mildly flattened (Fig. 2C–E, Additional file 2: Table S1). Paternal grandfather (AD150-I.1) has a sandal gap. He also reported knee soreness, but has no history of other knee abnormalities, such as patellar dislocation, that would require knee imaging. MRI of his lumber spine revealed multilevel degenerative changes (L2–3 down to L5-S1). He had cervical spine surgeries (C6-C7 and C5-C6).

Materials and methods

Lung pathology and immunohistochemistry

Formalin fixed paraffin embedded (FFPE) lung autopsy specimens from the proband (AD149-III.2), and peripheral blood sample from her mother (AD149-II.2)i and FFPE lung biopsy specimen from patient AD150-III.2 and

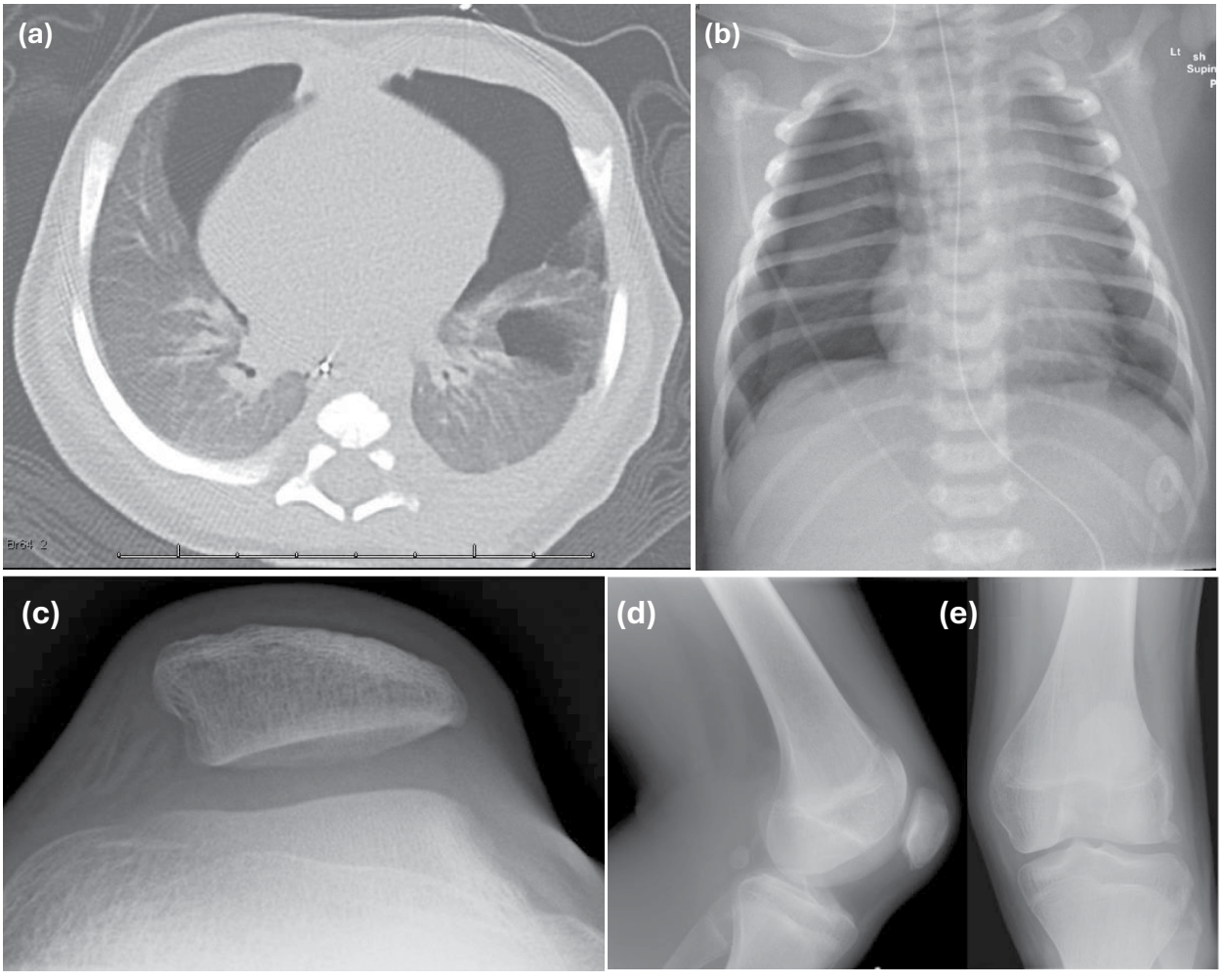

Fig. 2 **A** Computed tomography (CT) chest of patient AD150-III.2 performed at day 2 of life showing moderate right and large left-sided pneumothorax, with left-sided tension, as evidenced by shift of the mediastinal silhouette rightward, diffuse mild ground glass appearance of lungs and mild narrowing of the left pulmonary artery in the setting of a small patent ductus arteriosus and **B** Chest X ray taken at day two of life showing slightly underinflated lungs with right upper lobe atelectasis. **C** Radiographs of knee of individual 150.II.1 on skyline view showing hypoplastic patella with reduced overall ossification and lateral patellar subluxation, **D** Lateral view showing patellar hypoplasia and **E** Antero-posterior view showing hypoplastic patella and underdeveloped or flattened femoral condyle

FFPE lung autopsy specimen from his deceased sibling AD150-III.1 were collected. Histopathological studies were performed on these specimens from both families and stained with hematoxylin, eosin, and TMEM100 antibody (rabbit HPA055936, 1:50, Sigma-Aldrich, St. Louis, MO) as previously described [16].

Molecular analyses

DNA samples from the proband (AD149-III.2) and patient AD150.III.1 were extracted from lung autopsy FFPE block using Zymo Quick-DNA FFPE Kit (Zymo Research, Irvine, CA). Short read whole-genome sequencing (WGS) was carried out in the proband (AD149-III.2) using the TruSeq Nano DNA HT Library Preparation Kit (Illumina, San Diego, CA) and sequenced on the Illumina HiSeqX platform to an average depth of 30 × at Novogene Corporation (Sacramento, CA). Raw sequencing data were processed following previously published methods [12]. Variant analysis in short read WGS data was performed on FASTQ files using the AI-driven Lucid Genomics platform (Berlin, Germany, https://www.lucid-genomics.com/), an AI-assisted variant interpretation framework that integrates genomic data with phenotype-driven prioritization. Clinical phenotypic features from the affected individuals were incorporated into the analysis using standardized phenotype descriptors, which enabled phenotype-driven ranking of candidate genes associated with lung developmental and pulmonary vascular phenotypes. Variants were initially filtered based on standard criteria, including rarity in population databases, predicted functional impact and basic quality filters. The platform then prioritized candidate variants by integrating genotype data with phenotype similarity scores derived from known gene-disease associations. The detailed workflow of data processing on Lucid Genomics is provided in Additional file 1.

Family trio short read WGS and exome sequencing (ES) including patient AD150.III.2 was performed at SOPHiA GENETICS (Boston, MA) followed by analysis and interpretation of the data. Trio-based long-read WGS was also performed on patient AD150.III.1 and both parents using Oxford Nanopore technology as part of the Rapidomics 2.0 study. Data from the ± 1 Mb genomic regions flanking the *TBX4* gene locus were extracted and variants within were merged across samples and filtered to retain those inherited maternally.

RNA was extracted from FFPE lung autopsy tissues from the proband (AD149-III.2) and family 2 deceased child (AD150-III.1), from FFPE lung biopsy specimen from patient AD150-III.2, and from FFPE lung autopsy tissue from two age-matched controls using the Zymo Quick-RNA FFPE MiniPrep kit (Zymo Research). Control 1 is a lung sample from a female (38-week gestation) who lived 4 days and died from complications of velamentous cord insertion with shearing of cord. Control 2 is a lung sample from a male (42.5 week-gestation) who lived 13 h and died from possible cord accident and complications of delivery. Traces of DNA were removed from RNA preps with Turbo DNA-*free* Kit (Thermo Fisher Scientific, Invitrogen, Waltham, MA). First-strand cDNA synthesis was performed with 5 ug total RNA using the SuperScript III First-Strand Synthesis kit (Thermo Fisher Scientific, Invitrogen) with random hexamer priming. Reverse transcription quantitative PCR (RT-qPCR) was performed using TaqMan probes specific to *TBX4* (Hs01057581_m1), *TMEM100* (Hs01039491_m1), and *ACTB* (Hs01060665_g1) (Thermo Fisher Scientific, Applied Biosystems). Reactions were run at least in triplicate on a CFX Connect Real-Time System (BioRad, Hercules, CA). Expression was normalized to *ACTB*, and relative quantification was calculated using the ΔΔCt method and with fold change determined relative to normal lung controls. Statistical significance was assessed using non-parametric Kruskal–Wallis ANOVA with multiple comparisons in GraphPad Prism, applied to $2^{\wedge -\Delta\Delta Ct}$ values.

From Family 1, DNA was extracted from peripheral blood from the mother (AD149-III.2) and saliva samples using DNA Genotek prepIT L2P (DNA Genotek Inc., Stittsville, ON, Canada) for members AD149.I.1, AD149.I.2, AD149.II.1, AD149.II.2, and AD149.III.1 were analyzed by Sanger sequencing. cDNA sample from family 2 siblings AD150.III.1 and DNA sample from individual AD150.III.2 were analyzed by Sanger sequencing using LA Taq DNA polymerase (Takara Bio, Kusatsu, Japan) using primer pair 5'-TCTTTCAGCAGACGGTACCC-3'/ 5'-CCACATGTTACAGCTCAGCG-3'.

Results

Pathology and immunohistochemistry

Pathology studies for the proband (AD149-III.2) were significant for a markedly decreased radial alveolar count and immature alveolar appearance, consistent with CAD. In addition, there was acute congestion with abundant squamous debris in terminal airspaces (Fig. 3A). Both siblings from family 2 (AD150-III.1, AD150-III.2) exhibited some degree of deficient alveolarization with the demised infant (AD150-III.1) being more severe, consistent with pulmonary hypoplasia in the spectrum of CAD (Fig. 3C, E).

Previous transcriptomic and immunohistochemistry (IHC) analyses in *FOXF1*, *TBX4*, and *FGF10* deficient lungs in patients with LLDD revealed significant down-regulation of the endothelial transmembrane protein 100 (*TMEM100*) gene. *TMEM100* expression in human is restricted mostly to fetal and mature adult lung, implying that it can be an important mediator of lung development and that loss of its function may

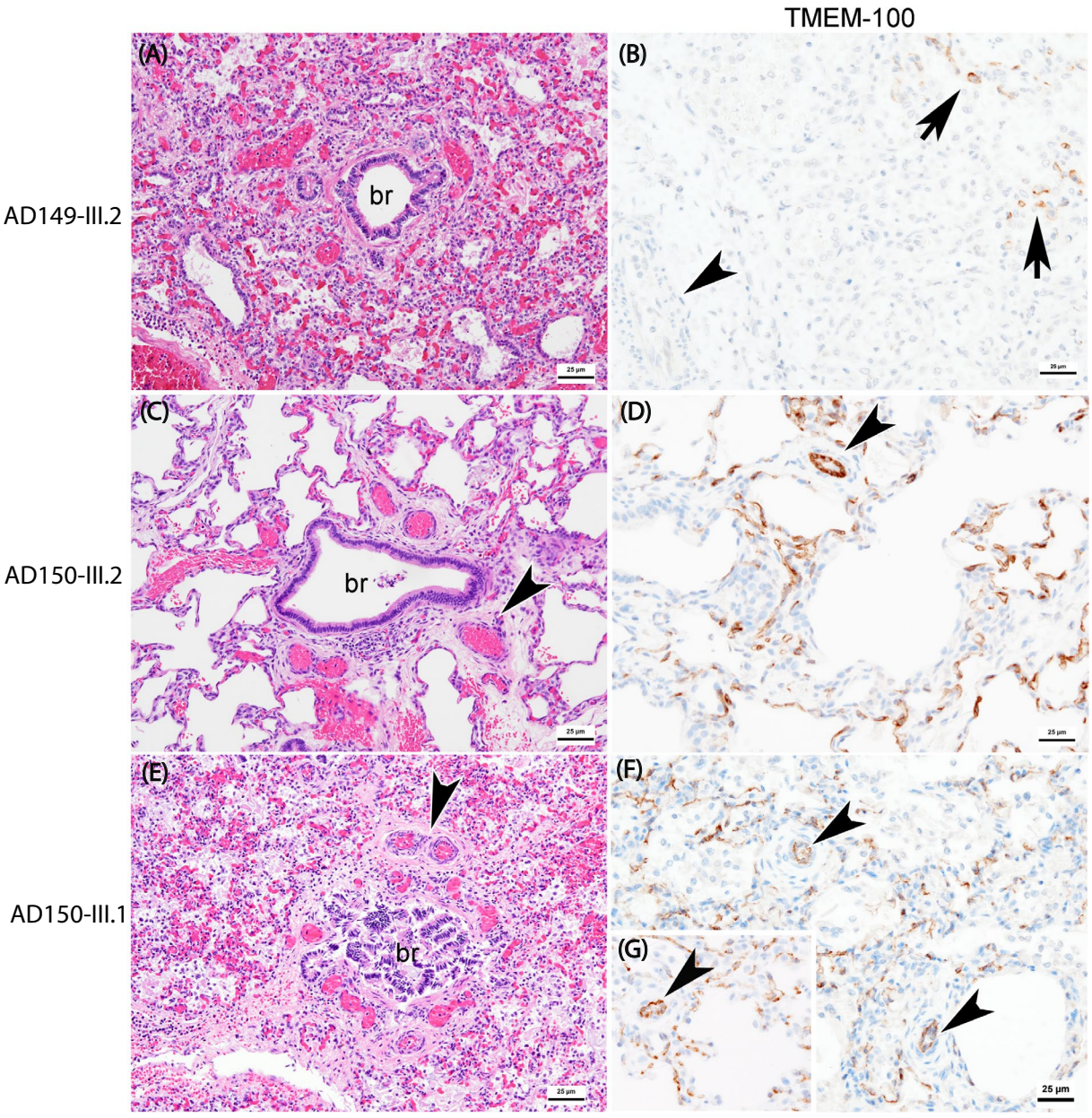

Fig. 3 Lung histopathology and TMEM100 expression in family 1 (AD149-III.2) and 2 (AD150-III.1, AD150-III.2). **A** The postmortem lung of the proband shows a significant arrest of lung development for a term gestation with no normal alveolar structures. **B** Marked diminished expression for TMEM100 is present with positivity limited to small foci of capillaries (arrows). **C** The lung biopsy in patient AD150-III.2 shows alveolar simplification for age with no evidence of pulmonary arterial hypertension. **D** Normal expression for TMEM100 is present in the arterial vascular endothelium. **E** The demised sibling (AD150-III.1) lung shows more advanced growth arrest/pulmonary hypoplasia (**F**) but relatively normal expression for TMEM100 in the endothelium compared to term control autopsy lung (**G**). Arrowheads denote arteries

directly contribute to LLDDs [16–19]. In the proband (AD149-III.2), TMEM100 was significantly decreased in the vasculature with only patchy capillary staining in IHC analysis (Fig. 3B). IHC for TMEM100 in patient AD150-III.2 lung biopsy showed normal endothelial expression (Fig. 3D) within the arteries and capillaries, which was also observed in his demised sibling (AD150-III.1) (Fig. 3F).

Molecular analyses

Using AI-driven Lucid Genomics software to analyze WGS data in the proband (AD149-III.2), we have identified a novel frameshift variant, chr17:61480316:CG>C (GRCh38) c.1019del; p.(Arg340GlnfsTer40) in exon 8 of *TBX4* (NM_001321120.2). This variant is not found in gnomAD v4.1 and on ClinVar. Targeted Sanger sequencing confirmed the variant was inherited from the mother

(AD149-II.2) and proband's maternal grandfather (AD149-I.1). Its CADD score is 28.7. However, it is predicted by MutationTaster as benign. According to American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) standards and guidelines [20], this variant is classified as pathogenic [PVS1 (Null variant where loss of function is a known mechanism of disease) + PM2 (Absent in gnomAD or very rare in other population databases) + PP4 (Highly specific genetic etiology matching patient)].

In patient AD150-III.2, WGS and ES analyses revealed a frameshift variant chr17:61483037:A>AC (GRCh38), c.1167dup; p.(Arg390GlnfsTer30) in exon 9 of *TBX4* (NM_001321120.2) inherited from the father and the paternal grandfather. Sanger sequencing identified this mutation also in his deceased sibling AD150-III.1 (Additional file 1: Figure S1). The CADD score is 21.9 and is predicted deleterious by MutationTaster and is listed as pathogenic on ClinVar (ID: 1341420). Of note, it was previously reported by Vanlerberghe et al. [21] and Kerstjens-Frederikse et al. [8]. Its allele frequency in gnomAD v4.1 is 0.000001248. According to the ACMG/AMP guidelines, this variant is classified as pathogenic [PVS1 + PM2 + PP1 + PP3 (Multiple in silico predictions support pathogenicity) + PP4 + PP5 (Reputable source recently reports variant to be pathogenic)].

Both frameshift variants are located distal to approximately residues 323–325 of TBX4 [5] and thus are predicted to escape NMD and generate aberrant C-terminal neopeptide sequences (Additional file 1: Figure S2).

Long-read WGS analysis revealed in the deceased patient AD150-III.1 a total of 405 maternally inherited variants within 1 Mb regions flanking *TBX4,* 38 of which are absent in the gnomAD v4.1 database (Additional file 3: Table S2).

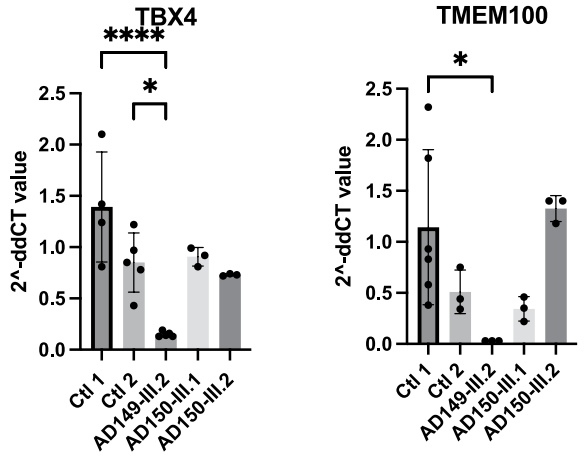

Fig. 4 Relative expression of *TBX4* and *TMEM100* in the proband AD149-III.2 and patients AD150-III.1 and AD150-III.2 determined by RT-qPCR ran at least in triplicate. cDNA analysis showed significant downregulation of *TBX4* and *TMEM100* for the proband (AD149-III.2). However, there was no significant difference for family 2 deceased sibling AD150-III.1 and AD150-III.2 compared to lung controls. Graph demonstrates relative fold change normalized to *ACTB* relative to controls *TBX4* and *TMEM100* expression. Error bars represent SD. (*$p < 0.05$, **$p < 0.01$)

RT-qPCR in RNA samples from the proband (AD149-III.2) showed significantly decreased *TBX4* and *TMEM100* transcript levels compared to normal lung controls (Fig. 4). In contrast, patient AD150-III.2 and his deceased sibling AD150-III.1 showed no statistically significant difference in *TBX4* and *TMEM100* levels compared to lung controls (Fig. 4). Corroboratively, Sanger sequencing identified the c.1167dup; p.(Arg390GlnfsTer30) mutation in in patient AD150-III.2 cDNA, demonstrating that the mutant transcript is not completely degraded (Additional file 1: Figure S1).

Discussion

Haploinsufficiency was initially presumed to be the main mechanism for the *TBX4*-related disorders; however, recent genotype–phenotype studies indicated missense variants can also result in gain-of-function effects [13] or exert dominant negative effect [13]. Karolak et al. [5] reported that pathogenic variants affecting the T-box DNA-binding domain are associated with earlier disease onset and more severe clinical manifestations. Moreover, they also noted that even variants clustered within the same functional residue can give rise to a wide range of phenotypes, including LLDDs, SPS, or a combination of lung and skeletal phenotypes, thereby underscoring the pleiotropic nature, variable expressivity, and incomplete penetrance of the *TBX4* variants. Karolak et al. [5, 11] proposed a complex compound inheritance model in individuals with LLDDs, in which rare noncoding variants located within a lung-specific super-enhancer act in *trans* with pathogenic *TBX4* coding variants. These regulatory variants, absent in unaffected controls, are thought to function as hypomorphic alleles that further reduce *TBX4* activity and contribute to disease severity [5, 11].

NMD is an mRNA surveillance mechanism that regulates around 10% of the normal transcriptome [23]. It degrades transcripts harboring PTCs arising as a consequence of frameshifting or nonsense variants, alternative or aberrant mRNA splicing, errors in transcription, and programmed gene rearrangements [24]. NMD efficiency is influenced by the PTC position. In particular, it is often less effective for the variants located in the last 50–55 nucleotides of the penultimate coding exon [25, 26]. Incomplete or variable NMD efficacy has been estimated to occur for ~ 30% of frameshift or nonsense variants and is recognized as an important factor influencing disease severity and clinical phenotype [27–29]. Moreover, studies have also indicated that differences in NMD efficiency may contribute to divergent clinical phenotypes among individuals harboring the same PTC variants [30–32].

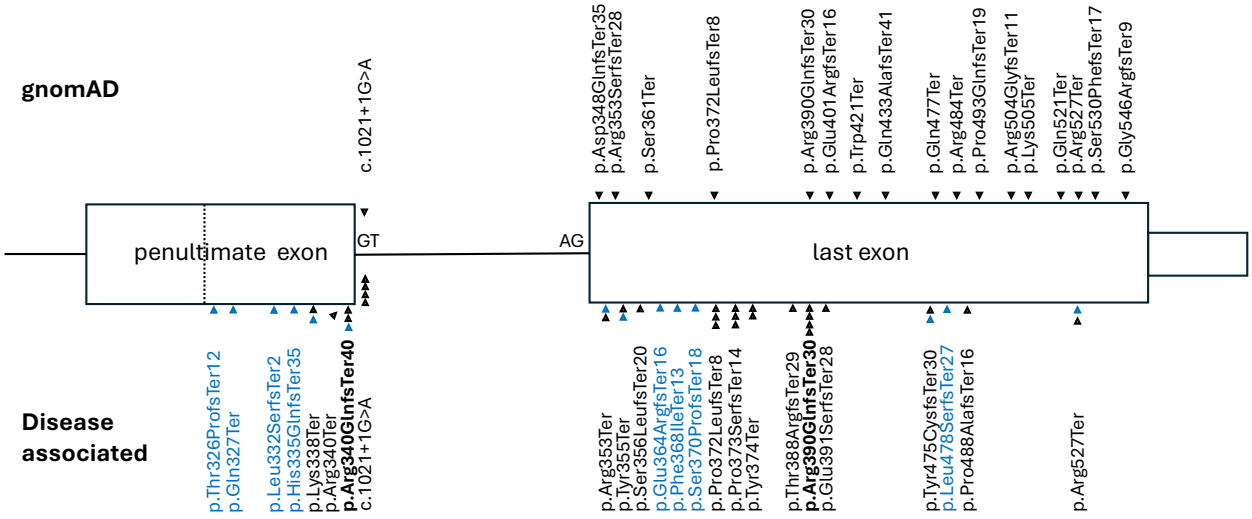

Fig. 5 Schematic representation of distribution of the *TBX4* truncating variants predicted to escape NMD in the penultimate and last exon reported as disease associated (lower panel) in reported patients (in bold), ClinVar (in blue), and in the literature (Karolak et al. 2023) and identified in apparently unaffected individuals (gnomAD v4.1.0) (upper panel) (harmonized to transcript NM_001321120.2). The dashed vertical line depicts NMD border based on the 50–55 rule

The two identified truncating *TBX4* variants in our study, c.1019del; p.(Arg340GlnfsTer40) and c.1167dup; p.(Arg390GlnfsTer30), fall within the penultimate and last exon of *TBX4,* respectively, the reported NMD-escape region distal to amino acid residues 323–325, and therefore are expected to evade NMD [5].

Both the proband (AD149-III.2) and patient AD150-III.2 presented with histopathologically diagnosed CAD spectrum, whereas their carrier parents, grandparents, and sibling exhibited milder and distinct phenotypes. The mother of the proband AD149-II.2 has a history of persistent pulmonary hypertension of the newborn and the maternal grandfather (AD149-I.1) and sibling (AD149-III.3) have asthma, whereas the father of patient AD150-III.2 (AD150-II.1) has patellar abnormalities and the paternal grandfather (AD150-I.1) has sandal gap, a feature previoulsy associated with variants in *TBX4*. Interestingly, the c.1167dup; p.(Arg390GlnfsTer30) variant was previously reported in a child with pediatric onset PAH and ICPPS who inherited it from the father with ICPSS [8], two siblings with ICPSS who inherited it from their father with feet abnormalities [21], and in a patient with hereditary PAH [33]. Moreover, it was also identified in an apparently asymptomatic individual from the gnomAD database (Fig. 5, Additional file 4: Table S3). This striking inter- and intrafamilial phenotypic variability highlights that these alleles do not act as simple nulls and suggests variable or incomplete escape from NMD with a likely interference of noncoding modifying factors.

Interestingly, Yıldız Bölükbaşı et al. [34] reported a similar pattern for a closely located C-terminal frameshift variant c.1115dup; p.(Pro373SerfsTer14), mapping to the last exon of *TBX4* that was also predicted to evade NMD. This variant segregated with a broad clinical spectrum, ranging from LLDDs to milder pulmonary phenotypes (mild interstitial lung disease, bronchiolitis obliterans, recurrent pneumothorax), ICPPS, and was even found in reportedly unaffected carriers [21, 34, 35].

Another example of intra- and inter-familial variability is the recurrent splice-site variant c.1021 + 1G>A involving the 5' splice donor site in the last intron also predicted to escape NMD. It was described in unrelated families with strikingly different clinical outcomes, ranging from adult-onset lung disease with a positive family history to severe, early-onset pulmonary arterial hypertension leading to death in infancy or childhood, as well as isolated skeletal features such as patellar agenesis [36–38]. Prapa et al. [13] investigated this variant using a minigene splicing assay and reported double exon skipping, resulting in a frameshift. It is also present in the gnomAD database (Fig. 5, Additional file 4: Table S3).

Of note, we identified 18 truncating variants within the last exon of *TBX4* in the apparently unaffected individuals from the gnomAD database (v4.1.0). Some of these variants, c.1115del; p.(Pro372LeufsTer8) and c.1579C>T, p.(Arg527Ter), have been also previously disease associated. For example, c.1115del; p.(Pro372LeufsTer8) was described in a patient with early PAH, asthma, and multiple tracheal and bronchial anomalies, in another individual with sporadic tracheal and bronchial diverticulosis, and irregular bronchial walls, as well as in an unaffected carrier [39] (Fig. 5).

In family 149, individuals 149-I.I and 149-III.3 with the variant c.1019del; p.(Arg340GlnfsTer40) have mild asthma with no other skeletal/patellar phenotypes.

Thus, we propose that *TBX4* variants can be associated also with asthma.

The two variants identified in our study are inherited frameshift mutations located in regions predicted to be less susceptible to NMD based on their position within the penultimate and final exons, raising the possibility that they may persist and potentially give rise to abnormal TBX4 proteins. RT-PCR analysis in patient AD150-III.2 detected relatively normal *TBX4* transcript; however, this approach does not distinguish between wild-type and mutant alleles and therefore cannot be interpreted as direct evidence of NMD escape. Moreover, these experiments are not designed to quantify transcript abundance or assess the stability or functional consequences of any resulting protein products, one potential explanation is variable or incomplete NMD. This has been reported for transcripts with premature termination codons near the 3' end of genes and could lead to differing levels of residual transcript across individuals in this family. However, our data do not directly demonstrate the presence of the altered TBX4 protein. Alternatively, if truncated transcripts completely escape NMD, the resulting proteins could retain partial activity, effectively acting as hypomorphic alleles. In line with the ACMG/AMP guidelines, when NMD is not predicted to occur, the potential impact of a variant depends on whether the truncated C-terminal region is critical to protein function [40]. Notably, truncating variants that remove a substantial portion of the protein (e.g., > 10%) are more likely to have functional consequences [40]. Finally, genetic modifiers within the *TBX4* locus or in interacting developmental pathways may further contribute to the spectrum of phenotypes observed in carriers of *TBX4* variants. Taken together, these considerations are compatible with a model in which *TBX4*-associated disease may not be driven solely by reduced gene dosage, but could also involve incomplete or variable NMD, permitting the expression of truncated proteins. In such a scenario, these proteins might exert dominant-negative or gain-of-function effects; however, this remains speculative.

In support of this notion, Yıldız Bölükbaşı et al., [34] provided direct functional evidence that coding truncations alone might be insufficient to cause severe lung disease. In two neonates with LLDD, a noncoding hypomorphic SNV (rs62069651-C) located in a lung-specific enhancer upstream of *TBX4* was present in *trans* to the frameshift allele and reduced *TBX4* promoter activity by 63% in *vitro*, whereas relatives carrying only the coding variant had milder or no lung disease [34]. Most recently, Szafranski et al., [41] reported a three-generation family in which three neonates died from histopathologically confirmed LLDDs and were found to carry an ~ 108 kb deletion encompassing *TBX4*, which was also present in their mildly affected mother with pulmonary findings and minimally affected maternal grandfather with skeletal issues. Lung tissue analysis demonstrated a > 50% reduction in *TBX4* transcript levels, and computational interrogation of the *TBX4* super-enhancer identified multiple noncoding hypomorphic variants inherited from the father [41]. Interestingly, using long-read WGS, we have identified a few coding and noncoding variants that may act as potential modifiers. Out of 405 total variants inherited from mother in the *TBX4* locus, 38 are not found in gnomAD and could potentially act as hypomorphic variants (Additional file 3:Table S2).

In conclusion, our findings expand the genotype–phenotype correlation spectrum of *TBX4*-associated lung developmental disorders by identifying two C-terminal frameshift variants predicted to escape NMD, providing a plausible molecular basis for the marked phenotypic variability, including asthma, observed across and within families. Our data do not directly demonstrate the presence, stability, or functional impact of the truncated TBX4 proteins and further studies are needed to determine the precise molecular mechanism. The variability, ranging from LLDD to mild or isolated skeletal and pulmonary features has important implications for recurrence risk assessment and genetic counseling. The wide spectrum of expressivity and the influence of modifier variants make clinical outcomes challenging for prediction of future pregnancies. Comprehensive genetic evaluation, cascade testing of at-risk relatives, and consideration of prenatal or preimplantation genetic testing are essential to support informed reproductive decision-making in affected families.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40246-026-00974-3.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

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Author contributions

S.A.P. and H.C.J. contributed to data analysis, writing the manuscript, and preparing the figures P.Sz. contributed to data analysis and manuscript reviewing; M.Wa., J.M.F., and T.G. contributed in data generation and analysis; M.Wr., N.A., and C.B. contributed to patient evaluation, patient referral and radiodiagnosis; Q.W., J.S., N.C.S., G.D., contributed to pathology and IHC analyses; P.S. contributed to patient recruitment, analysis, interpretation of the genomic testing, planning and conceptualizing the manuscript and overall supervision.

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Data availability
gnomAD database

Declarations

Competing interests
The authors declare no competing interests.

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