Spontaneous pneumothorax can be associated with *TGFBR2* mutation

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Conflict of Interest: None

Take home message: *TGFBR2* mutations that cause Loeys-Dietz Syndrome can present as pneumothorax. *In vitro* kinase assays can help confirm pathogenicity of novel variants.

Word count (excluding references and legend): 1,196

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To the Editor:

Primary pneumothorax affects 0.01% of the population. Ten per-cent of cases have a family history of pneumothorax, but in the majority a definitive genetic diagnosis is not made. We report a 26-year old white British woman who presented with left apical pneumothorax (Fig 1A). Previously, she had migraines, multiple stress fractures in her right foot, myopia, easy bruising, lumbar scoliosis and spontaneous dislocation of the right patella. She had no previous history of pneumothoraces or any other respiratory problems, and had never smoked.

On examination she was hypermobile (Beighton score 7/9), had facial milia, translucent hyperextensible skin, striae over her back, chest wall asymmetry, bilateral varicose veins and pes planus. Her uvula was bifid (Fig 1B), she had a high arched palate with dental crowding and her arm-span-to-height ratio was increased (1.14). In the ophthalmology clinic, lattice dystrophy (weakness in the peripheral retina predisposing to retinal detachment) was identified with no ocular features of Marfan syndrome. The patient's thoracic CT revealed apical blebs and her echocardiogram and CT showed aortic root dilatation (3.54 cm, Z-score > 2) (Fig 1C-D). Her 59-year old mother, who had not suffered pneumothoraces, was reviewed and found to have mild features of a connective tissue disorder: skin hyperextensibility, joint hypermobility with Beighton scale score of 5/9, high-arched palate, mild thoracic kyphosis, easy bruising, recurrent left shoulder dislocation, hiatus hernia, stress incontinence, and stress fractures of left foot.

These findings led to the clinical diagnosis of Loeys-Dietz Syndrome (LDS), an autosomal dominant disorder affecting the TGF-ß signalling pathway (1). LDS (LDS1-5; OMIM #609192, #610168, #613795, #614816, #615582) is characterized by vascular findings (cerebral, thoracic, and abdominal arterial aneurysms/dissections) and skeletal manifestations (pectus excavatum or pectus carinatum, scoliosis, joint laxity, arachnodactyly, talipes equinovarus). Approximately 75% of affected individuals have LDS type I with craniofacial manifestations (hypertelorism, bifid uvula/cleft palate, craniosynostosis); approximately 25% have LDS type II with systemic manifestations of LDSI but minimal or absent craniofacial features. LDSI and LDSII form a clinical continuum. The natural history of LDS is characterized by aggressive arterial aneurysms (mean age at death 26.1 years) and a high incidence of pregnancy-related complications, including death and uterine rupture. The diagnosis of LDS is based on characteristic clinical findings in the proband and family members and molecular genetic testing of *TGFBR1, TGFBR2, SMAD3, TGFB2* and *TGFB3*.

Other connective tissue disorders such as Marfan syndrome were not considered likely, especially with the clinical findings of facial milia and bifid uvula, as these are specific to LDS. Her *TGFBR1* and *TGFBR*2 genes were sequenced from leucocyte-derived DNA and she was found to be heterozygous for a novel missense variant of the *TGFBR2* gene (c.1262C>A; threonine 421 to asparagine: p.Thr421Asn; reference sequence NM_003242.5; Fig 1E). Subsequent testing found her mother to be a low-level mosaic for this variant.

The pathogenicity of novel missense variants of disease-associated genes is often

uncertain. The threonine at position 421 is a highly conserved residue within the protein kinase domain of TGFBRII. *In silico* analysis of the identified variant using SIFT and Polyphen suggested pathogenicity with SIFT predicting the change to affect protein function and Polyphen predicting the change to be damaging. This variant is neither listed on the HGMD database nor the ExAC (Exome Aggregation Consortium) database.

There is no crystal structure available for the TGFBRII protein; however, based on a structure of the homologous activin receptor ActRIIB (47.57% identity within their kinase domains: TGFBRII residues 244-544 and ActRIIB residues 190-480), we assessed the interactions in this highly-conserved region. This predicted the new mutation would disrupt an intramolecular hydrogen bond stabilising the highly conserved kinase activation loop and so affect activity (Fig 1F). Effects upon folding could not be excluded by this approach though we considered this unlikely. We therefore assessed the folding and function of the new variant. Human Embryonic Kidney (HEK) 293T cells were transiently transfected with expression vectors encoding TGFBRII proteins: wild-type (WT), the new T421N mutant, and a known kinase-dead R528C mutant (2). Protein lysates were either left untreated or digested with EndoH, which digests immature glycans on proteins within the endoplasmic reticulum, or PNGase, which removes all N-linked glycans regardless of their complexity. Lysates were then subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted for TGFBRII. The T421N variant expressed well in cultured mammalian cells and, similar to the known inactive R528C mutant, displayed a mature glycosylation pattern suggesting it was well folded and had exited the endoplasmic reticulum (Fig 1G).

It is known that mutants of TGFBRII exist that lack normal kinase activity but traffic normally (2). We therefore purified TGFBRII wild-type protein, the T421N variant and the kinase-deficient variant, R528C. HEK293T cells were transiently transfected with expression vectors encoding TGFBRII proteins tagged on their C-termini with FLAG: wild-type (WT), T421N, R528C, and a weak hypomorphic variant V387M (our unpublished observation). Cells were lysed and FLAG-tagged proteins purified by immunoprecipitation. After elution from the antibody using FLAG peptide, one third of the material was incubated with [γ^{32}P]-ATP for 30 minutes at 30°C then separated by SDS-PAGE and exposed to a phosphorimager plate. As expected, the positive control wild-type TGFBRII autophosphorylated strongly, while the negative control R528C did not (upper panel Fig 1H). The novel T421N mutant also showed impaired autophosophorylation, indicating it to be pathogenic and confirming the clinical diagnosis of Loeys-Dietz syndrome. Since radiolabelling does not lend itself well to clinical laboratory practice, we also examined unlabelled proteins by Phos-tag gel followed by immunoblotting. This technique separates proteins both by their size and by degree of phosphorylation (3). Once again, the positive and negative controls separated well and the T421N mutant showed a clear reduction of autophosphorylation (lower panel Fig 1H).

The precise mechanism by which haploinsufficiency for TGFBR2 leads to disease is unclear, but in mice, deficiency of TGFBR2 in cranial neural crest cells leads to elevated expression of TGF-ß2 and TGF-ß receptor III (4). This increases SMAD-independent downstream signalling leading to defective proliferation of palatal

mesenchyme and may account for the craniofacial defects observed in LDS.

Although one review has noted pneumothoraces in cases of known LDS (5), to our knowledge, this is the first documented case of LDS to present with pneumothorax. We have confirmed the causative mutation to be a kinase-dead allele of *TGFBR2* by developing both radioactive and non-radioactive assays for TGFRII autokinase activity. As the identification of multiple variants, either through targeted gene analysis or through panel testing of large numbers of genes, becomes routine, such *in vitro* techniques for the functional characterisation and validation of putative disease-causing mutations will be increasingly important. LDS should be considered as a monogenic cause of pneumothorax in addition to the more widely appreciated causes: Marfan Syndrome, vascular Ehlers-Danlos Syndrome, Tuberous Sclerosis/ Lymphangioleiomyomatosis (LAM) and Birt-Hogg-Dube Syndrome (6).

Our patient's pneumothorax was treated conservatively and resolved. There have been no further pneumothoraces during the subsequent 3 years. She was commenced on losartan and propranolol as aortic protection and we continue to monitor her aortic root diameter. In conclusion, we describe a case of LDS presenting as spontaneous pneumothorax, which illustrates the growing challenges of functional validation in the genomic era, as well as enriching the phenotypic spectrum of known genetic disorders and demonstrating overlap with related disorders.

Legend:
(A) Chest radiograph demonstrating left apical pneumothorax.
(B) Photograph of patient's uvula.
(C) Coronal reformat of CT chest (lung windows) black arrows showing numerous subpleural blebs at both apices
(D) Sagittal reformat of contrast enhanced CT scan of thoracic aorta. Black lines shows maximal dimensions of the aortic root at the Sinuses of Valsalva (3.54 cm) confirmed by ecocardiography and sinotubular junction (2.93 cm).
(E) Chromatogram of sequence in the region of the c.1262C>A; p.Thr421Asn mutation (arrowhead).
(F) Mutation site (zoomed) modelled on crystal structure (PDB ID 2QLU) of activin receptor 2b (ActR2B). Whole domain and zoom on mutation site. Residue numbering given in form ActR2B/TGFBR2. The mutation site occurs within the activation loop (orange) of the molecule that is critical for regulation of substrate binding and kinase activity. Thr361/421 forms a hydrogen bond (dashed line) with Lys323/381. Its perturbation would therefore destabilise the conformational behaviour of the activation loop.
(G) Protein glycosylation. Endo H and PNGase digestion of TGFBRII protein. Human Embryonic Kidney (HEK) 293T cells were transiently transfected with expression vectors encoding TGFBR2 proteins, wild-type (WT), the new T421N

mutant, and a known kinase-dead R528C mutant. Protein lysate was either left untreated or digested with EndoH or PNGase. Mature glycosylation is indicated by arrow 1; immature glycosylation (endoplasmic reticulum form) by arrow 2; fully deglycosylated form arrow 3. Note the majority of each protein is resistant to Endo H digestion indicating glycan processing within the Golgi apparatus.

(H) Protein autophosphorylation. HEK293T cells were transiently transfected with expression vectors encoding FLAG-tagged TGFBRII proteins: wild-type (WT), T421N, R528C, and V387M.   Cells were lysed and FLAG proteins purified by immunoprecipitation. After elution with FLAG peptide, one third of the material was incubated with 1µCi of [γ^{32}P]-ATP for 30 minutes at 30˚C then separated by SDS-PAGE. Equal amounts of remaining eluate were subjected to SDS-PAGE or phos-tag SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted for FLAG. Arrow 4 identifies a slowly migrating phosphorylated species, while arrow 5 indicates a rapidly migrating poorly-phosphorylated species.

References:

1.   Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, et al. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. Nat Genet. 2005;37(3):275-81.
2.   Horbelt D, Guo G, Robinson PN, Knaus P. Quantitative analysis of TGFBR2 mutations in Marfan-syndrome-related disorders suggests a correlation between phenotypic severity and Smad signaling activity. J Cell Sci. 2010;123(Pt 24):4340-50.
3.   Kinoshita E, Kinoshita-Kikuta E, Koike T. Phos-tag SDS-PAGE systems for phosphorylation profiling of proteins with a wide range of molecular masses under neutral pH conditions. Proteomics. 2012;12(2):192-202.
4.   Iwata J, Hacia JG, Suzuki A, Sanchez-Lara PA, Urata M, Chai Y. Modulation of noncanonical TGF-beta signaling prevents cleft palate in Tgfbr2 mutant mice. J Clin Invest. 2012;122(3):873-85.
5.   MacCarrick G, Black JH, 3rd, Bowdin S, El-Hamamsy I, Frischmeyer-Guerrerio PA, Guerrerio AL, et al. Loeys-Dietz syndrome: a primer for diagnosis and management. Genetics in medicine : official journal of the American College of Medical Genetics. 2014;16(8):576-87.
6.   Hopkins TG, Maher ER, Reid E, Marciniak SJ. Recurrent pneumothorax. Lancet. 2011;377(9777):1624.