Activation of TGF-β signaling in an aortic aneurysm in a patient with Loeys-Dietz syndrome caused by a novel loss-of-function variant of TGFBR1

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Abstract

Loeys–Dietz syndrome (LDS) is caused by variants of transforming growth factor-β (TGF-β)-related genes and is characterized by aortic aneurysm and dissection. We report an LDS patient with a de novo missense variant of TGFBR1 [c.1126A>G, p.(Lys376Glu)] in which active TGF-β signaling was observed in the aorta, despite the in vitro demonstration that the loss-of-function mutation lies within the serine/threonine kinase domain. The mechanism underlying this TGF-β paradox in LDS aortopathy should be further investigated.

Loeys–Dietz syndrome (LDS) is an autosomal dominant genetic connective tissue disorder that is caused by pathogenic variants of genes that encode components of the transforming growth factor-β (TGF-β) signaling pathway. TGFBR1 and TGFBR2, which encode serine/threonine protein kinase (STK) receptors, are examples of such causative genes1,2. LDS patients have several features that overlap symptoms seen in Marfan syndrome (MFS) patients, including an increased prevalence of aortic aneurysm and dissection. However, LDS patients do not develop ectopia lentis, which is a hallmark feature of MFS patients, and are further characterized by the diagnostic clinical triad of arterial tortuosity and aneurysms, hypertelorism, and a bifid uvula. The TGF-β signaling pathway in the aortic wall is upregulated in LDS patients, although the causative variants of TGFBR1 and TGFBR2 are missense variants predicted to reduce their STK activities3.

To understand the crucial roles played by TGF-β signaling in LDS patients, in vitro and in vivo functional characterization of the genetic variants responsible for LDS is important. Here we report a case of an LDS patient with a de novo loss-of-function variant in the STK domain of TGFBR1 (c.1126A>G; NG_007461.1), in which a paradoxical upregulation of TGF-β signaling was observed in the resected aortic tissue.

The patient was a Japanese man with no apparent relevant family history of thoracic aortic aneurysm and/or dissection. It was thought that he suffered from an MFS-related disorder because of his tall stature, thin body habitus (height, 182 cm; weight, 43.6 kg), and scoliosis at the age of 15 years. Echocardiography revealed an enlargement of the sinus of Valsalva (36 mm; aortic root Z-score4, 5.87), for which he had been followed up at a local hospital. At the age of 27 years, his aortic root diameter was 41 mm (188 cm; 50 kg; Z-score, 5.17), and he was referred to the Marfan clinic at the University of Tokyo Hospital. He had a positive wrist and thumb sign and presented with pectus excavatum, a hindfoot deformity, lumbosacral dural ectasia, protrusio acetabuli, kyphoscoliosis, facial features (dolichocephaly, downslanting
palpebral fissures, and malar hypoplasia), skin striae, high myopia, and mitral valve prolapse (15 points, according to the 2010 revised Ghent nosology\(^5\)). Furthermore, he had a bifid uvula, hypertelorism, tortuous cerebral arteries, and cervical spine instability. At the age of 31, his aortic root diameter was 48 mm (Z-score, 8.08), and his aneurysm was treated using the David valve-sparing root replacement procedure. Extended histological examinations revealed elastin degradation, cystic medial necrosis, and increased SMAD2 phosphorylation, indicating active TGF-\(\beta\) signaling in the aortic wall (Fig. 1a, b).

Genetic analyses of thoracic aortic aneurysm-related genes (\(FBN1\), TGFBR1, TGFBR2, TGFB2, TGFB3, SMAD3, ACTA2, and MYH11\(^6,7\)) were conducted. A novel missense variant was identified within exon 6 of TGFBR1 [c.1126A>G, p.(Lys376Glu); Fig. 1c], and this variant was located in the evolutionarily well-conserved STK domain (data not shown). To examine the impact of the STK domain variant on TGFBR1 function, an in vitro functional assay was performed using wild-type and variant TGFBR1 expression vectors. A plasmid encoding human TGFBR1 with a C-terminal HA-tag (pCMV5-TBR1-HA, abbreviated as WT) was generated from a plasmid encoding a genetically altered human TGFBR1 mutant with intrinsic constitutive activity and a C-terminal HA-tag (pCMV5-TBR1-T204D-HA, abbreviated as CA; Addgene #19162, a gift from Joan Massague) by site-directed mutagenesis. Plasmids with the LDS missense variant (pCMV5-TBR1-LDS-HA and pCMV5-TBR1-T204D-LDS-HA, abbreviated as constructs LDS and LDS-CA, respectively) were generated from the WT and CA constructs, respectively, through site-directed mutagenesis. The plasmid-encoded wild-type and variant TGFBR1 proteins were expressed at uniformly high levels in HEK293T cells (Fig. 2a), and there was low endogenous TGFBR1 expression. Using these constructs, luciferase

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**Fig. 1** Histological and TGFBR1 genomic analyses. **a,** Histological analyses of surgically dissected aortic tissue samples from the proband with LDS and from a non-familial thoracic aortic aneurysm (TAA) in a 70-year-old patient. **a** Elastica van Gieson (EVG) staining revealed severely disorganized and fragmented elastic fibers (arrowhead) in the proband's aortic media with cyst-like lesions (cystic medial necrosis, arrow). Scale bars: 500 \(\mu\)m; 100 \(\mu\)m in the inset. **b** Immunohistochemical (IHC) staining against phosphorylated SMAD2 (Cell Signaling, Danvers, MA, USA) and smooth muscle alpha-actin (Sigma, St. Louis, MO, USA). Scale bars: 200 \(\mu\)m; 25 \(\mu\)m in the inset. **c** Genomic DNA sequencing revealed a heterozygous single-base substitution (c.1126A>G) in the proband.
assays were performed to assess the activity of the SMAD-responsive Smad-binding element (SBE) reporter in HEK293T cells co-transfected with the TGFBR1 expression vectors and either the pGL4.48 (luc2P/SBE/Hygro) vector (Promega, Madison, WI, USA) or the pRL-SV40 control vector (Promega, Madison, WI, USA). After treatment with recombinant human TGF-β (Wako, Osaka, Japan), the increased SBE luciferase activity seen in the WT-transfected HEK293T cells was abolished in the LDS-transfected cells (Fig. 2b). The high constitutive STK activity observed in the CA-transfected HEK293T cells was significantly inhibited in the LDS-CA-transfected cells (Fig. 2c). These results indicate that, at least in vitro, the Lys376Glu variant is a loss-of-function mutation. These results, we concluded that this variant could be classified as likely pathogenic without confirmation of paternity and maternity based on the classification guidelines of the American College of Medical Genetics and Genomics—Association for Molecular Pathology. The patient was finally diagnosed with LDS based on a constellation of clinical features and the results of the genetic analysis. The TGFBR1 variant data were submitted to the Leiden Open Variant Database (www.LOVD.nl/TGFBR1; Individual ID: #00151836). Here, we report a Japanese sporadic LDS patient characterized by aortic aneurysm and a heterozygous missense TGFBR1 variant (c.1126 A>G). In LDS patients, most of the TGFBR1/2 variants are missense mutations that have been verified and/or predicted to disrupt the activity of the wild-type proteins upon in vitro overexpression. As expected, overexpression of the missense variant TGFBR1 (c.1126 A>G) showed a loss-of-function phenotype in vitro; however, a paradoxical increase in TGF-β activity was observed in the aortic wall. The mechanism through which loss-of-function.
function TGFBR1/2 variants cause the paradoxical in vivo activation of TGF-β signaling in LDS aortopathy remains unknown. Heterozygous Tgfr1 and Tgfr2 knockout mice do not develop LDS phenotypes; however, complete deletion of Tgfr2 in postnatal smooth muscles (Myh11-CreERT2;Tgfr2^−/−), along with the resulting decrease in phospho-SMAD2 expression, induces rapidly progressing aortic aneurysms and dissections. These data indicate that basal TGF-β signaling in smooth muscles promotes postnatal aortic wall homeostasis and hinders aortic dilatation. Conversely, TGF-β signaling in LDS-associated aortic aneurysms is upregulated, as reported both in LDS patients and Tgfr1^N1138K/+ and Tgfr2^G857W/+ LDS knock-in mice, despite their loss-of-function phenotypes shown by in vitro experiments. Based on the following points, we (and others) speculate that augmented TGF-β signaling via the remaining homodimers of each TGFBR protein plays active roles in disease progression in LDS patients: (1) Expression of TGF-β ligands was increased in the impaired aortas of Tgfr1^N1138K/+ and Tgfr2^G857W/+ mice; (2) TGFBR1 and TGFBR2 act as homodimers on the plasma membrane, which allows TGF-β signaling to be transmitted through the wild-type/wild-type homodimers in vivo in LDS; (3) increased phosphorylation of SMAD2 was observed in in-vitro-cultured fibroblasts from LDS patients following TGF-β stimulation. Furthermore, cultured aortic vascular smooth muscle cells from Tgfr2^G857W/+ mice showed normal steady-state SMAD2 phosphorylation levels and in response to stimulation with 10 ng/ml of TGF-β1, but showed reduced activation capacity in response to stimulation with 1 ng/ml of TGF-β1, suggesting that excessive activation of TGF-β signaling plays a pivotal role in the pathogenesis of LDS aortopathy (Fig. 2d). On the other hand, we also suspect that the complete ablation of genes encoding TGF-β signaling molecules in Myh11-CreERT2;Tgfr2^−/− mice and in homozygous Smad3 knockout mice (Smad3^−/−) may cause aortic aneurysm via other mechanisms different from those underlying LDS, as the canonical SMAD target genes are generally not upregulated. Angiotensin II receptor signaling is also activated in LDS aortic walls, and the type I receptor blocker losartan inhibits SMAD2 phosphorylation and improves aortic root dilatation in Tgfr2^G857W/+ LDS knock-in mice; thus, further studies on the relationships between the TGF-β and angiotensin II receptor signaling pathways in LDS patients are also required.

In conclusion, we have described a patient with a sporadic case of LDS due to a novel TGFBR1 variant (c.1126A>G) who presented with Marfan-like habitus, a bifid uvula, hypertelorism, and an aortic aneurysm. The loss of STK activity in the missense variant protein caused a paradoxical increase in aortic TGF-β signaling; however, the precise mechanism remains to be elucidated. We suspect that secondarily activated TGF-β signaling via wild-type TGFBR1 homodimers underlies the discrepancy between the in vitro and in vivo STK activities. Further investigation is crucial to clarify the molecular mechanism underlying the “TGF-β paradox” in LDS aortopathy.

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