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CLINICAL REPORT

A novel pathogenic variant located just upstream of the C-terminal Ser423-X-Ser425 phosphorylation motif in *SMAD3* causing Loeys–Dietz syndrome

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Abstract

Objective: Loeys–Dietz syndrome (LDS) is a heritable disorder of connective tissue closely related to Marfan syndrome (MFS). LDS is caused by loss-of-function variants of genes that encode components of transforming growth factor-β (TGF-β) signaling; nevertheless, LDS type 1/2 caused by *TGFBR1/2* pathogenic variants is frequently found to have paradoxical increases in TGF-β signaling in the aneurysmal aortic wall. Here, we present a Japanese LDS family having a novel *SMAD3* variant.

Methods: The proband was tested via clinical, genetic, and histological analyses. In vitro analysis was performed for pathogenic evaluation.

Results: The novel heterozygous missense variant of *SMAD3* [c.1262G>A, p.(Cys421Tyr)], located just upstream of the C-terminal Ser423-X-Ser425 phosphorylation motif, was found in this instance of LDS type 3. This variant led to reduced phospho-SMAD3 (Ser423/Ser425) levels and transcription activity in vitro; however, a paradoxical upregulation of TGF-β signaling was evident in the aortic wall.

Conclusions: Our results revealed the presence of TGF-β paradox in this case with the novel loss-of-function *SMAD3* variant. The precise mechanism underlying the paradox is unknown, but further research is warranted to clarify the influence of the *SMAD3* variant type and location on the LDS3 phenotype as well as the molecular mechanism leading to LDS3 aortopathy.

KEYWORDS
aortic aneurysm, functional assay, genetic analysis, transforming growth factor-β

1 | INTRODUCTION

Aortic wall development and homeostasis depend heavily on Transforming growth factor-β (TGF-β) signaling, and dysregulated signaling has been linked to the formation of both hereditary and acquired aortic aneurysms and dissections (Lin & Yang, 2010). With several clinical characteristics resembling those of Marfan syndrome (MFS), Loeys–Dietz syndrome (LDS) is an autosomal-dominant heritable disease affecting connective tissue. It is distinguished by a triad of arterial tortuosity and aneurysm, widely spaced eyes (hypertelorism), and bifid uvula (MacCarrick et al., 2014). Pathogenic variants in genes that code for components of TGF-β signaling, such as *TGFBR1* (OMIM*190181), *TGFBR2* (OMIM*190182), *SMAD2* (OMIM*601366), *SMAD3* (OMIM*603109), *TGFB2* (OMIM*190220), and *TGFB3* (OMIM*190230), are the cause of LDS, which has a considerably more aggressive phenotype than MFS (MacCarrick et al., 2014). The TGF-β receptor complex, formed by two TGFBR1 and two TGFBR2 serine/threonine kinase receptors, directly phosphorylates R-SMAD (SMAD2/SMAD3) at the C-terminal Ser-X-Ser (SXS) motifs (Macias et al., 2015). The target gene expression is induced by the translocation of the activated R-SMAD/SMAD4 to the nucleus (Heldin et al., 1997). According to earlier publications, pathogenic variants of LDS are predicted to cause loss of function of the protein; however, overactivity of TGF-β signaling is demonstrated in the aortic wall of LDS patients and knock in mice (referred to as the TGF-β paradox), and the molecular mechanism involved remains unknown (Takeda et al., 2018). Here, we present a Japanese LDS family characterized by MFS-like systemic features and having a novel loss-of-function variant just upstream of the conserved C-terminal Ser423-X-Ser425 motif of SMAD3 (c.1262G>A, p.(Cys421Tyr); NM_005902.3), in which a paradoxical upregulation of TGF-β signaling was detected in the resected aortic wall.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The Institutional Ethics Committee of the University of Tokyo Hospital approved the genetic testing for hereditary thoracic aortic aneurysms and dissection (TAAD; Reference No. G-1538), and the patient provided written informed permission.

2.2 | Genetic analyses for hereditary thoracic aortic aneurysms and dissection

Hybridization capture-based gene panel testing for hereditary TAAD was provided by the Kazusa DNA Research Institute (Chiba, Japan) within the coverage of Japanese health insurance. This panel testing using the targeted next generation sequencing with the hybrid capture method contains the genes *FBN1* (OMIM*134797), *TGFBR1*, *TGFBR2*, *TGFB2*, *TGFB3*, *SMAD3*, *ACTA2* (OMIM*102620), *MYH11* (OMIM*160745), *MYLK* (OMIM*600922), and *COL3A1* (OMIM*120180) at the time of inspection (Takeda et al., 2021; Yagi et al., 2022). The GenBank reference sequences and version numbers were *FBN1* (NM_000138.4), *TGFBR1* (NM_004612.3), *TGFBR2* (NM_003242.5), *TGFB2* (NM_003238.3), *TGFB3* (NM_003239.4), *SMAD3* (NM_005902.3), *ACTA2* (NM_01613.4), *MYH11* (NM_002474.2), *MYLK* (NM_053025.3), and *COL3A1* (NM_00090.3).

2.3 | Human aortic tissue specimens

The Institutional Ethics Committee of the University of Tokyo Hospital approved the examinations of human aortic tissue (Reference No. 2233-9). Aortic root tissue was obtained from the proband (III-1, Figure 1a) undergoing elective aortic root surgery. After receiving written informed consent, control aortic tissue was obtained from a heart transplant recipient (MT11) with ischemic cardiomyopathy who was 45 years old.

2.4 | Histological analysis and immunohistochemical staining

Aortic tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5-μm thickness. Three serial sections were stained with Elastica van gieson, Masson's trichrome, anti-phospho-SMAD2 (Ser465/Ser467) antibody (Cell signaling, #3108, 1:200) and anti-phospho-SMAD3 (Ser423/Ser425) antibody (Abcam, ab52903, 1:200) using a VECTASTAIN ABC Kit (Vector Laboratories, PK-4001) and 3,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories, SK-4100).

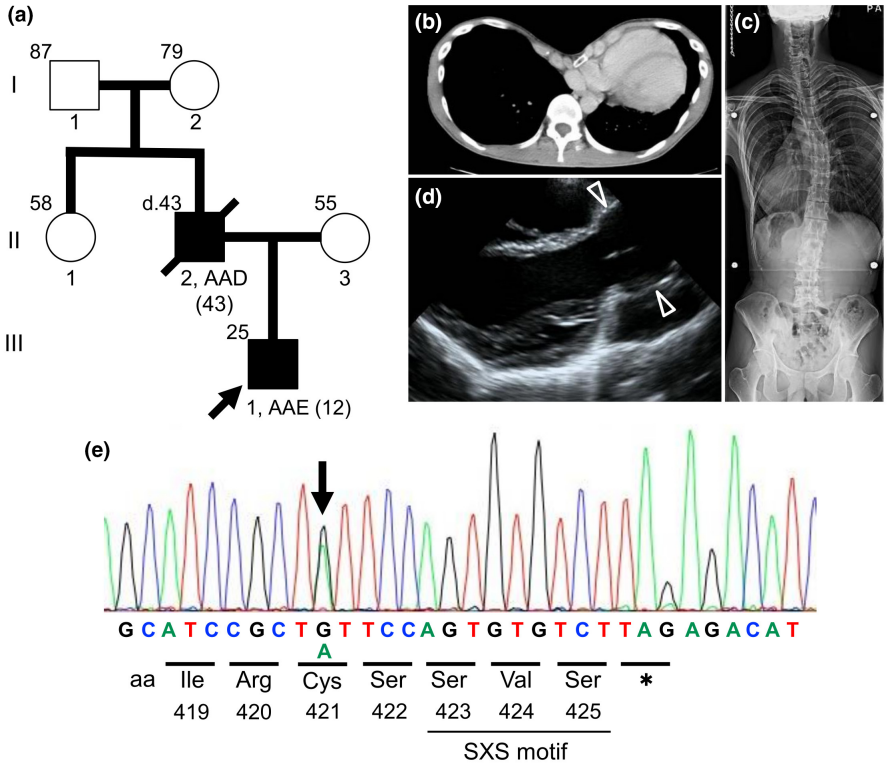

FIGURE 1 Japanese family case of LDS presenting with aortic diseases. (a) The family tree of this LDS case with clinical characteristics. The age is shown in the upper left corner, and "d" denotes the age at death. The age at diagnosis for acute aortic dissection (AAD) or annuloaortic ectasia (AAE) is shown in parentheses. Symbols: arrow (proband); square (male); circle (female); open (unaffected); filled (aortic aneurysm or dissection affected); and died on a diagonal line. (b–d) Clinical features of the proband at the age of 25. Funnel chest (b), scoliosis (c), and aortic root dilatation at the sinus of Valsalva (d, arrowheads) were discovered by computed tomography, roentgenogram, and echocardiography, respectively. (e) Sequencing analysis of genomic DNA identified a heterozygous single-base substitution (c.1262G>A, arrow) in the *SMAD3* gene. The variant was located just upstream of the C-terminal SXS motif of SMAD3. The GenBank reference sequence and version number for *SMAD3* was NM_005902.3. aa, the amino acid position of SMAD3; *, translation stop codon.

2.5 | In vitro analysis

2.5.1 | Generation of the SMAD3 expression constructs

A plasmid encoding a human SMAD3 variant with an N-terminal Flag-tag (pRK5F-Smad3-Cys421Tyr, abbreviated as C421Y) was produced from the plasmid encoding a human SMAD3 with an N-terminal Flag-tag (pRK5F-Smad3, abbreviated as WT; Addgene plasmid #12625, a gift from Rik Derynck; Zhang et al., 1998) by site-directed mutagenesis.

2.5.2 | Dual luciferase assay

HEK293T cells were co-transfected with 600 ng/mL SMAD3:SMAD4-inducible firefly luciferase reporter plasmid (pGL4.48 (luc2P/SBE/Hygro), Promega), 200 ng/mL constitutively active renilla luciferase plasmid (pRL-SV40, Promega), 600 ng/mL pCMV-TGFBR1 vector (Fujiwara et al., 2018), and one of either 10 ng/mL pRK5F-Smad3-WT, pRK5F-Smad3-C421Y, or pRK5F-LacZ, using Lipofectamine 2000 reagent (Thermo Fisher Scientific; Fujiwara et al., 2018; Hara et al., 2019). Transfected HEK293T cells were cultured for 36 h, and starved in a medium devoid of fetal calf serum (FCS) for 4 h. Then, the cells were treated with recombinant TGF-β1 (209–16544; Wako) at 5 ng/mL for 8 h, as previously reported (Fujiwara et al., 2018; Hara et al., 2019). Using a Dual-Glo Luciferase Assay System (Promega) and GloMax-Multi Luminescence System (Promega), luciferase fluorescence intensities were examined. Reporter activities were normalized to match the renilla luciferase activity of pRL-SV40. Experiments were conducted in triplicate and repeated three times.

2.5.3 | Western blot analysis

Using Lipofectamine 2000 reagent, HEK293T cells were transfected with a 600 ng/mL pCMV-TGFBR1 vector, and

one of either 20 ng/mL pRK5F-Smad3-WT or pRK5F-Smad3-C421Y. Transfected HEK293T cells were cultured for 30 h, and starved for 16 h in a medium devoid of FCS. Then, the cells were treated with recombinant TGF-β1 (209–16544; Wako) at 5 ng/mL for 2 h. Given that TGF-β-inducible phosphorylation of SMAD3 is a rapid and transient process, we adopted regiment for starvation and TGF-β stimulation that is different from one used in luciferase assay (Cohen-Solal et al., 2011; Liang et al., 2021; Wang et al., 2009). Western blotting was used to examine the proteins that had been extracted from the cell lysates using specific antibodies: SMAD3 (Abcam, ab40854) diluted at 1:15,000 in 5% bovine serum albumin (BSA), pSMAD3 (phospho-Ser423/Ser425; Abcam, ab52903) diluted at 1:7500 in 5% BSA, and actin (Thermo Fisher, MA5-11869) diluted at 1:3000 in 5% non-fat dry milk. As secondary antibodies, anti-rabbit IgG antibody (7074S; Cell signaling technology) was used at dilution 1:5000 for pSMAD3 or at 1:15,000 for SMAD3, and anti-mouse IgG antibody was used at 1:3000 (NA931; Cytiva). Densitometric analysis was conducted with ImageJ software (NIH) and normalized to actin. Western blots were repeated at three times.

2.6 | Statistical analysis

All data are expressed as mean ± standard deviation. For multiple comparisons, one-way ANOVA with Tukey's multiple comparisons test was used to compare each group. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Case report

A 12-year-old Japanese boy (III-1) visited our Marfan syndrome center because his father (II-2) had abruptly passed away at age 43 from an acute type-A aortic dissection (Figure 1a). The father was 181 cm tall and had a history of pneumothorax. The patient's height was 170.1 cm and his weight was 42 kg. He has a funnel-shaped chest, long fingers and arms, a high-arched palate, and pes planus. Echocardiography demonstrated enlargement of the sinus of Valsalva (32 mm; aortic root *Z*-score [Roman et al., 1989], 4.2). At that time, MFS was suspected, and he was followed up with atenolol and/or losartan treatment at a different hospital. He revisited our clinic at the age of 18 years, and his height and weight were 190.1 cm and 59.2 kg, respectively. His kyphoscoliosis had advanced, and the aortic root diameter was 37.6 mm (*Z*-score, 5.2), and his systemic score for the diagnosis of MFS (Loeys et al., 2010) reached a score of 8 points with wrist and thumb sign, increased arm span, pectus excavatum, scoliosis, and hindfoot deformity (Figure 1b,c). He was clinically diagnosed with MFS but lacked arterial tortuosity, osteoarthritis, and LDS-specific craniofacial features like hypertelorism or bifid uvula. At the age of 25 years, the aortic root diameter reached 43 mm (*Z*-score, 5.55; Figure 1d). A David valve-sparing root replacement procedure and tricuspid annuloplasty for tricuspid regurgitation were carried out concomitantly with Ravitch intervention for pectus treatment.

Using hereditary TAAD multigene panel testing for 10 candidate genes, we conducted a genomic study. A novel missense variant was detected in exon 9 of *SMAD3* [c.1262G>A, p.(Cys421Tyr)] (Figure 1e), and the variant was located just upstream of the conserved C-terminal Ser423-X-Ser425 (SXS) motif. The mother (II-3) did not have the variant, according to Sanger sequencing data, and in silico analysis of the variant predicted it to be damaging (Polyphen-2) or deleterious (SIFT), with a CADD score of 32 (highly pathogenic). The variant was absent from controls in ExAc database (Lek et al., 2016).

3.2 | Functional analysis of c.1262G>A variant in *SMAD3*

To investigate the impact of the c.1262G>A variant on Smad-binding element (SBE) luciferase reporter, HEK293 cells were transfected with a vector expressing WT or C421Y SMAD3 (Figure 2a,b). Exogenous WT SMAD3 boosted the basal SBE luciferase activity, which was further enhanced after treatment with TGF-β1. C421Y-transfected cells failed to exhibit the enhanced basal and inducible SBE luciferase activity seen in WT-transfected cells (Figure 2c). The phosphorylation level of Ser423/425 residues of SMAD3 was significantly reduced in C421Y-transfected cells, compared to WT-transfected cells (Figure 2d). These results suggested that the mutant C421Y protein exerts loss-of-function effects at least in this in vitro condition, while extended histological examinations revealed increased phospho-SMAD3 (Ser423/Ser425) and phospho-SMAD2 (Ser465/Ser467) levels in the aortic wall, indicating active TGF-β signaling in vivo (Figure 2e). Based on a configuration of his clinical characteristics and the genetic analysis results, and the presence of TGF-β paradoxical findings in the current instance, we ultimately classified the patient as having LDS with a *SMAD3* variant (LDS type 3: LDS3).

4 | DISCUSSION

A novel heterozygous *SMAD3* missense variant [c.1262G>A, p.(Cys421Tyr)] was found in a family case

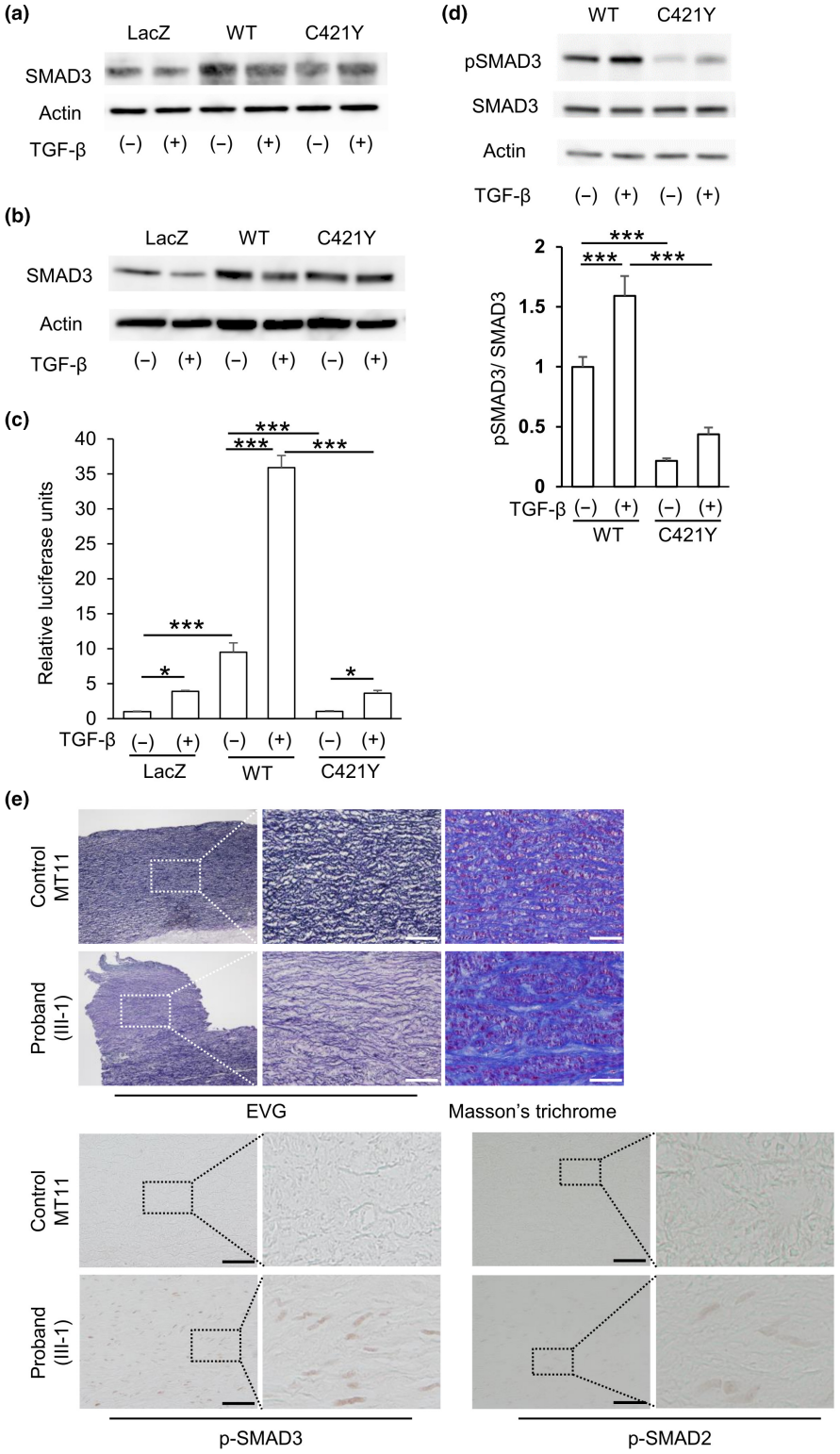

FIGURE 2 Functional analysis of Cys421Tyr variant in *SMAD3* gene. (a–c) Evaluation of TGF-β signaling activity using dual luciferase assay. (a) Western blot evaluation of the produced constructs for SMAD3 expression. (b) Western blot evaluation for SMAD3 expression in the cell lysates used for luciferase assay. (c) Activity of SBE-firefly luciferase in HEK293T cells. HEK293T cells transfected with the indicated constructs were cultured for 36 h and starved for 4 h in an FCS-free medium. The cells were then treated for 8 h with recombinant human TGF-β1 at a concentration of 5 ng/mL. Reporter activities are normalized to the renilla luciferase activity of pRL-SV40. Experiments were conducted in triplicate and repeated three times. (d) Representative western blot analysis and quantification of the indicated proteins. HEK293T cells transfected with the indicated constructs were cultured for 30 h and starved for 16 h in an FCS-free medium. Then, the cells were treated with recombinant TGF-β1 at 5 ng/mL for 2 h. $n = 3$. (e) A control patient #MT 11 with ischemic cardiomyopathy who underwent heart transplantation at the age of 45 and the proband's surgically dissected aortic tissue were both analyzed histologically (sinus of Valsalva, 35 mm; aortic root *Z*-score, 0.95). Scale bars: 50 μm. ***$p < 0.001$, *$p < 0.05$ by one-way ANOVA with Tukey's multiple comparisons test.

of LDS3 that we have reported here. This variant should be classified as pathogenic, based on the American College of Medical Genetics and Genomics—Association for Molecular Pathology classification guidelines (PS3 + PM1 + PM2 + PP3 + PP4; Richards et al., 2015). In LDS3, approximately 60% of the *SMAD3* variants were missense and resided in the MH2 domain, a well-conserved region responsible for receptor recognition, interaction with transcription factors, and oligomerization among R-SMADs (SMAD2/SMAD3) and co-SMAD (SMAD4; Macias et al., 2015; Schepers et al., 2018). In the MH2 domain, the current variant was discovered just upstream of the C-terminal SXS motif. The C-terminal SXS motif is a critical and well-established functional domain in that

TGF-β-induced phosphorylation at the site works as the switch that enables R-SMADs to interact with SMAD4, accumulate in the nucleus, and bind to DNA and transcription cofactors (Xu, 2006). Our findings demonstrated that the Cys421Tyr variant protein exerts a loss-of-function impact by lowering the phosphorylation levels of Ser423/425 residues in vitro experiment; however, a paradoxical increase in phospho-SMAD3 (Ser423/Ser425) levels was detected in the aortic wall.

The relationship between the phenotype and genotype of LDS3 is gradually being understood. Hostetler et al. reported that patients with *SMAD3* missense variants in the MH2 domain who experienced aortic events were younger than those with haploinsufficient (HI) variants, while individuals with alterations of the MH1 domain presented at later ages, indicating that pathogenic variants in the MH2 domain are more severe than HI or variants of the MH1 domain (Hostetler et al., 2019). While LDS4/LDS5 caused by *TGFB2/3* pathogenic variants indicates milder cardiovascular phenotypes, LDS3 is claimed to have similarities to typical LDS characteristics induced by *TGFBR1/2* pathogenic variants (LDS1/2) in terms of the aortic phenotype (Schepers et al., 2018). Recent reports have shown that the *SMAD3* group had a higher incidence of type A dissection and fewer elective aneurysm surgeries compared to other LDS genes. And patients with *SMAD3* variants had fewer LDS nonvascular features and instead had atypical complications not seen with other LDS genes, such as early onset osteoarthritis and age-related Charcot–Marie-Tooth-like neuropathy. This report suggests that the lack of syndromic features delayed the diagnosis of hereditary TAAD and led to aortic dissection (Regalado et al., 2022). In our case, the physical findings of MFS were remarkable, making it possible to perform appropriate management and preventive aortic surgery prior to the onset of aortic dissection. Further research is essential to elucidate the connection between phenotype and genotype as well as the underlying mechanisms.

The comprehensive protein functional analysis of *SMAD3* variants causing LDS3 has not been reported previously; however, SMAD3 variants artificially generated at putative protein-binding amino acid residues of the MH2 domain have been reported to reduce binding to various subsets of interacting proteins, and make a significant change in the expression of SMAD3 target genes (Schiro et al., 2011). In the present variant, the resultant mutant C421Y protein exhibits a loss-of-function effect in vitro, despite the overactive TGF-β signaling in vivo aortic aneurysmal wall, and this paradoxical result is commonly found in LDS1/2 caused by pathogenic *TGFBR1/2* variants (Cardoso et al., 2012; Fujiwara et al., 2018; Hara et al., 2019; Horbelt et al., 2010). However, it needs to be emphasized that our in vitro functional study was performed using not vascular cells but HEK-293T cells. In order to elucidate the mechanism of TGF-β paradox caused by *SMAD3* variants, further in vitro study using vascular cells is needed.

Cardiac neural crest (CNC) and secondary heart field (SHF) cells, which have different origins in the ascending aorta, are present separately on the intima and adventitia, respectively, whereas these cells are mixed in the aortic root (Sawada et al., 2017). In vitro evaluation of the differences between CNC- and SHF-derived vascular smooth muscle cells (vSMCs) in the aortic root of *Tgfbr1*$^{M318R/+}$ mice revealed that SHF-derived vSMCs overexpressed the AT_1 receptor and secreted more TGF-β1 ligand but were less responsive to TGF-β stimulation. On the other hand, CNC-derived vSMC did not overexpress AT_1 receptors but maintained responsiveness to TGF-β stimulation. Consequently, it was assumed that the TGF-β1 ligand associated with the activation of AT_1 receptor signaling in SHF-derived vSMC acted on CNC-derived vSMC mixed at the aortic root to activate the TGF-β signaling pathway (in vivo; MacFarlane et al., 2019). Furthermore, Gong J et al. generated human-induced pluripotent stem cells with a frameshift mutation (c.652delA) in *SMAD3*, induced differentiation, and created lineage-specific vSMCs. In SHF-derived vSMCs, *SMAD3* deletion significantly disrupted canonical TGF-β signaling and decreased gene expression of vSMC markers. In CNC-derived vSMCs, *SMAD3* deficiency did not significantly affect the vSMC differentiation but increased phosphorylated SMAD2 (Gong et al., 2020). These reports suggested that the different nature of lineage-specific vSMCs causes discrepancies in the analysis of TGF-β signaling in vivo and in vitro.

Given that heterozygous loss-of-function mutations cause human LDS and homozygous ablation of TGF-β-related genes in mice (e.g., *Tgfbr2* [Hu et al., 2015; Li et al., 2014], *Smad3* [van der Pluijm et al., 2016]) develops aortic aneurysm without upregulation of downstream TGF-β-activated target genes, loss-of-function mutations, and homozygous ablation of TGF-β-related genes might contribute to aortic aneurysm formation via different mechanisms. Heterozygous *Tgfb2* knockout mice develop aortic aneurysms with increased phosphorylation levels of SMAD2 and SMAD3 compared to wild-type mice, and we hypothesize that secondarily activated TGF-β signal via intact (wild-type) TGF-β signal transduction molecule may promote the aortic aneurysmal formation in LDS (Takeda et al., 2018).

We reported a Japanese familial case of LDS3 with a novel *SMAD3* pathogenic variant [c.1262G>A, p.(Cys421Tyr)]. Despite the fact that this variant decreased the phospho-SMAD3 (Ser423/Ser425) levels and transcription activity in vitro, a paradoxical upregulation of TGF-β signaling was evident in the aortic wall. It is necessary to conduct more research to determine how the kind and

location of the SMAD3 mutation affect LDS characteristics and the underlying molecular mechanisms.

AUTHOR CONTRIBUTIONS

Hiroki Yagi, Norifumi Takeda, Masahiko Ando, Haruo Yamauchi, Ryo Inuzuka, and Yuki Taniguchi directly contributed to the management of the cases. Satoshi Ishii and Takayuki Fujiwara were directly involved in the variant analysis. Norifumi Takeda, Masaru Hatano, and Issei Komuro revised the article critically for important intellectual content. All authors approved the content of the article and confirmed the accuracy or integrity of any part of the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the University of Tokyo Hospital (Reference No. G-1538 and 2233-9). Written informed consent was obtained from the proband.

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