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Comprehensive genetic screening for vascular Ehlers–Danlos syndrome through an amplification-based next-generation sequencing system

Tomomi Yamaguchi[1,2,3] | Shujiro Hayashi[4] | Daisuke Hayashi[5] | Takeshi Matsuyama[6] | Norimichi Koitabashi[7] | Kenichi Ogiwara[8] | Masaaki Noda[9,10] | Chiai Nakada[11] | Shinya Fujiki[12,13] | Akira Furutachi[14] | Yasuhiko Tanabe[15] | Michiko Yamanaka[16] | Aki Ishikawa[17] | Miyako Mizukami[17,18] | Asako Mizuguchi[19] | Kazumitsu Sugiura[20] | Makoto Sumi[21] | Hirokuni Yamazawa[22,23] | Atsushi Izawa[24,25] | Yuko Wada[26] | Tomomi Fujikawa[3] | Yuri Takiguchi[3] | Keiko Wakui[1,2] | Kyoko Takano[1,2] | Shin-Ya Nishio[27] | Tomoki Kosho[1,2,3,28]

[1]Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto, Japan

[2]Center for Medical Genetics, Shinshu University Hospital, Matsumoto, Japan

[3]Division of Clinical Sequencing, Shinshu University School of Medicine, Matsumoto, Japan

[4]Department of Dermatology, Dokkyo Medical University, Mibu, Japan

[5]Department of Dermatology, Osaka Metropolitan University, Osaka, Japan

[6]Department of Pediatrics, Fussa Hospital, Tokyo, Japan

[7]Department of Cardiovascular Medicine, Gunma University Graduate School of Medicine, Maebashi, Japan

[8]Department of Pediatrics, Nara Medical University, Nara, Japan

[9]Department of Hematology, Hiroshima City Hiroshima Citizens Hospital, Hiroshima, Japan

[10]Noda Family Clinic, Hiroshima, Japan

[11]Division of Rheumatology, Yuuai Medical Center, Tomigusuku, Japan

[12]Division of Cardiology, Department of Medicine, Tsuruoka Municipal Shonai Hospital, Tsuruoka, Japan

[13]Department of Cardiovascular Medicine, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

[14]Department of Thoracic and Cardiovascular Surgery, National Hospital Organization Ureshino Medical Center, Saga, Japan

[15]Department of Cardiology, Niigata Prefectural Shibata Hospital, Shibata, Japan

[16]Center for Medical Genetics, St. Luke's International Hospital, Tokyo, Japan

[17]Department of Medical Genetics, Sapporo Medical University School of Medicine, Sapporo, Japan

[18]Department of Pediatrics, Sapporo Maternity Women's Hospital, Sapporo, Japan

[19]Department of Neurosurgery, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[20]Department of Dermatology, Fujita Health University School of Medicine, Toyoake, Japan

[21]Department of Cardiovascular Surgery, Saitama Cardiovascular and Respiratory Center, Saitama, Japan

[22]Department of Pediatrics, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

[23]Division of Clinical Genetics, Hokkaido University Hospital, Sapporo, Japan

[24]Department of Cardiovascular Medicine, Shinshu University School of Medicine, Matsumoto, Japan

[25]School of Health Sciences, Shinshu University, Matsumoto, Japan

[26]Division of Cardiovascular Surgery, Department of Surgery, Shinshu University School of Medicine, Matsumoto, Japan

[27]Department of Hearing Implant Science, Shinshu University School of Medicine, Matsumoto, Japan

[28]Research Center for Supports to Advanced Science, Shinshu University, Matsumoto, Japan

Correspondence
Tomoki Kosho, Department of Medical Genetics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
Email: ktomoki@shinshu-u.ac.jp

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Abstract

Vascular Ehlers–Danlos syndrome (vEDS) is a hereditary connective tissue disorder (HCTD) characterized by arterial dissection/aneurysm/rupture, sigmoid colon rupture, or uterine rupture. Diagnosis is confirmed by detecting heterozygous variants in *COL3A1*. This is the largest Asian case series and the first to apply an amplification-based next-generation sequencing through custom panels of causative genes for HCTDs, including a specific method of evaluating copy number variations. Among 429 patients with suspected HCTDs analyzed, 101 were suspected to have vEDS, and 33 of them (32.4%) were found to have *COL3A1* variants. Two patients with a clinical diagnosis of Loeys–Dietz syndrome and/or familial thoracic aortic aneurysm and dissection were also found to have *COL3A1* variants. Twenty cases (57.1%) had missense variants leading to glycine (Gly) substitutions in the triple helical domain, one (2.9%) had a missense variant leading to non-Gly substitution in this domain, eight (22.9%) had splice site alterations, three (8.6%) had nonsense variants, two (5.7%) had in-frame deletions, and one (2.9%) had a multi-exon deletion, including two deceased patients analyzed with formalin-fixed and paraffin-embedded samples. This is a clinically useful system to detect a wide spectrum of variants from various types of samples.

KEYWORDS
amplification-based next-generation sequencing, COL3A1, copy number variations, formalin-fixed and paraffin-embedded (FFPE) samples, hereditary connective tissue disorders (HCTDs), vascular Ehlers–Danlos syndrome (vEDS)

1 | INTRODUCTION

The Ehlers–Danlos syndromes (EDS) are a group of hereditary connective tissue disorders (HCTDs) characterized by skin hyperextensibility, joint hypermobility, and tissue fragility (Malfait et al., 2020). They comprise 13 subtypes based on symptoms and causative genes according to the 2017 International Classification (Malfait et al., 2017). Vascular Ehlers–Danlos syndrome (vEDS) (OMIM#130030) is an autosomal dominant disorder caused by pathogenic variants in the *COL3A1* gene encoding the alpha 1 chain of type III procollagen, with an estimated prevalence of 1/50,000–1/200,000 (Byers et al., 2017). The disorder is recognized as the most serious type of EDS. The major criteria include a family history of vEDS with a documented causative variant in *COL3A1*, an arterial rupture at a young age, a spontaneous sigmoid colon perforation in the absence of known diverticular disease or other bowel pathology, a uterine rupture during the third trimester in the absence of previous cesarean section and/or severe peripartum perineum tears, and carotid-cavernous sinus fistula formation in the absence of trauma. Diagnosis is confirmed through genetic testing (Malfait et al., 2017), which enables the distinction of vEDS from clinically overlapping disorders, including other types of EDS and other HCTDs. Accurate diagnosis is critical, allowing the appropriate surveillance for arterial complications (Byers et al., 2017, 2019) and optimal intervention, including celiprolol therapy (Frank et al., 2019; Ong et al., 2010).

Most pathogenic variants in *COL3A1* are missense variants leading to glycine (Gly) substitutions and in-frame splice site alterations in the triple helical domain, resulting in unstable assembly of type III procollagen homotrimers (Byers et al., 2017, 2019). Biochemical analysis or direct sequencing of mRNA using cultured skin fibroblasts have long been performed as standard procedures for genetic testing of vEDS, and several case series have been published with pathogenic variants in *COL3A1*, including a report of 23 cases by Pope et al. (1996), 33 cases by Schwarze et al. (1997), 22 cases by Smith et al. (1997), 17 cases by Kerwin et al. (2008), 20 cases by Shimaoka et al. (2010), 22 cases by Drera et al. (2011), 19 cases by Leistritz et al. (2011), 17 cases by Ferré et al. (2012), 41 cases by Morissette et al. (2014), 572 cases by Pepin et al. (2014), 68 cases by

Shalhub et al. (2014), and 126 cases by Frank et al. (2015). In 2016, Weerakkody et al. published the first report of next-generation sequencing (NGS) panel-based screening for EDS and other HCTDs; they described a patient clinically suspected as having vEDS but found to have a pathogenic variant in *COL5A1* (i.e., classical EDS [cEDS]), and a patient clinically suspected as having cEDS but found to have a pathogenic variant in *COL3A1* (i.e., vEDS). To our knowledge, only a case series by Ghali et al. (2019) has been published on patients with vEDS investigating pathogenic variants in *COL3A1* using NGS; they described seven index patients and 11 affected relatives clinically suspected as having cEDS but found to have missense variants in *COL3A1* leading to non-Gly (glutamic acid to lysine [Glu>Lys]) substitutions in the triple helical domain.

Here, we describe a novel series of patients with vEDS, identified through an amplification-based NGS with custom panels of causative genes for HCTDs, including a copy number variation (CNV) analysis.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

This study was approved by the Ethics Committee of Shinshu University School of Medicine (#435, #628). Written consent was obtained from all participants before participation in this study.

2.2 | Study subjects

In total, 429 unrelated patients who were clinically suspected to have HCTDs were recruited from the Center for Medical Genetics, Shinshu University Hospital and 100 institutes in Japan, and genetic investigation was performed between April 2013 and December 2020.

2.3 | NGS panel-based analysis

Genomic DNA was extracted from peripheral blood using Gentra Puregene Blood Kit or QIAamp DNA Blood Mini Kit on QIAcube (Qiagen). Target resequencing was performed on the Ion Torrent system (Ion PGM system, Ion PGM Dx system or Ion GeneStudio S5 system) (Thermo Fisher Scientific) using Ion AmpliSeq panels that were designed by Ion AmpliSeq Designer (https://ampliseq.com/browse.action) for 17 genes (HCTDs version 1 panel), 54 genes (HCTDs version 2 panel), 52 genes (HCTDs version 3 panel), 71 genes (HCTDs version 4 panel), and 52 genes (HCTDs version 5 panel) associated with various HCTDs, including EDS, Marfan syndrome (MFS), Loeys–Dietz syndrome (LDS), familial thoracic aortic aneurysm and dissection (FTAAD), and osteogenesis imperfecta. The gene lists and analyzed patients in each panel are shown in Table S1. The sequencing data were mapped to human genome hg19 using Torrent Suite software (Thermo Fisher Scientific), and single nucleotide variants (SNVs) and small insertions/deletions (indels) were detected from the mapped data using the Torrent Variant Caller plug-in.

Detected variants were annotated by SnpEff and SnpSift (http://snpeff.sourceforge.net) using the processed vcf file of the Genome Aggregation Database (gnomAD) v2.1.1 (https://gnomad.broadinstitute.org/downloads), ToMMo 8.3KJPN Genotype Frequency Panel (v20200831) (https://jmorp.megabank.tohoku.ac.jp/202008/downloads#variant) (Tadaka et al., 2019), ClinVar (ftp://ftp.ncbi.nlm.nih.gov/pub/clinvar/vcf_GRCh37/clinvar_20210927), dbNSFP3.4c, and dbscSNV1.1 (https://sites.google.com/site/jpopgen/dbNSFP). Additionally, splice site alterations were also analyzed using MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html; http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html). We evaluated detected variants through the Human Gene Mutation Database (HGMD) professional 2020.1 (Qiagen), ClinVar (https://www.ncbi.nlm.nih.gov), and the Ehlers–Danlos Syndrome Variant Database in the Leiden Open Variation Database (https://eds.gene.le.ac.uk/home.php), and if not registered, we evaluated them according to the 2015 American College of Medical Genetics and Genomics/the Association for Molecular Pathology guidelines (2015 ACMG/AMP guidelines) (Richards et al., 2015) and the ClinGen Sequence Variant Interpretation Working Group recommendations (SVI recommendations). The exon numbering in *COL3A1* was based on the reference sequence NM_000090.3 with 51 exons (exons 4 and 5 were fused as a single exon named exon 4/5), and the variant nomenclature was used according to the Human Genome Variation Society recommendations.

When no variant was identified, CNV in *COL3A1* was analyzed using the CNV visualization method for Ion AmpliSeq data, established by Nishio et al. (2018). In brief, using the feature that Ion AmpliSeq data from multiplex PCR products are uniform in length and position, the depth of coverage of each amplicon was evaluated. The ratio of the coverage depth of each amplicon, normalized by the mean depth of the entire panel, to the average of all samples in the same sequencing run was calculated.

2.4 | Validation of the candidate variants detected by NGS

SNVs and small indels were confirmed by standard Sanger sequencing. Sanger sequencing was performed on an ABI 3130xl genetic analyzer using a BigDye Direct Cycle Sequencing Kit with M13 tailed primers and a BigDye XTerminator Purification Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

CNVs were confirmed by Multiplex Ligation-dependent Probe Amplification (MLPA) using a SALSA MLPA Kit P155-D2 for the *COL3A1* gene, according to the manufacturer's instructions (MRC-Holland). Electrophoresis was conducted using an ABI 3130xl genetic analyzer (Thermo Fisher Scientific) and the data were analyzed with Coffalyzer.Net (MRC-Holland).

2.5 | FFPE samples

For Patient 3, genomic DNA was extracted from formalin-fixed and paraffin-embedded (FFPE) spleen tissue at Filgen. For Patient 12, genomic DNA was extracted from FFPE liver tissue using NucleoSpin DNA FFPE XS (TaKaRa Bio) at TaKaRa Bio. Uracil DNA Glycosylase (UDG) (Thermo Fisher Scientific) treatment was performed before Sanger sequencing for Patient 3 and before NGS and Sanger sequencing for Patient 12 to remove uracil bases in the template DNA produced by cytosine deamination.

2.6 | cDNA-based sanger sequencing

Total RNA from Patient 19 was extracted from skin fibroblasts using a QIAamp RNA Blood Mini Kit and was treated with RNase-Free DNase Set (Qiagen). Total RNA was reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa Bio). Gene-specific primer pairs with M13 tailed primers were designed as follows: the forward primer at exon 19, 5′-GGGCACCCCTTTCTCCTG-3′; and the reverse primer spanning exons 21 and 22, 5′-AGGACCAGCCGGTGCTAAT-3′ for *COL3A1*. Sanger sequencing was performed on an ABI 3130xl genetic analyzer using a BigDye Direct Cycle Sequencing Kit and a BigDye XTerminator Purification Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

2.7 | Type III collagen production analysis

Type III collagen production was analyzed as described previously (Shimaoka et al., 2010). In brief, skin fibroblasts were cultured with ^{3}H-proline (Amersham Biosciences), and the radiolabeled collagenous proteins were analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fluorography. We performed densitometric scans of the bands of type III collagen and type I collagen [α1(I) + α2(I)] produced by skin fibroblasts obtained from the patients and from age- and sex-matched controls. The level of type III collagen production was normalized to the level of type I collagen production, and the type III collagen production levels of the patients were calculated as percentages of the levels of the controls.

2.8 | Electron microscopic investigation

Skin samples, collected from the unexposed areas of the upper arm by biopsy, were incubated with 2.5% glutaraldehyde (TAAB) diluted with 0.1 M phosphate buffer for 2 h or more, and then fixed with 1% osmium acid (TAAB) for 90 minutes. The fixed tissues were dehydrated with ethanol, embedded in epoxy resin (TAAB), and observed with a JEM-1011 electron microscope (JEOL).

2.9 | Clinical evaluations

The accurate clinical characteristics of the patients with *COL3A1* variants were obtained from their medical records according to major criteria and minor criteria for the diagnosis of vEDS in the 2017 International Classification (Malfait et al., 2017).

3 | RESULTS

3.1 | Variant detection

In total, 429 unrelated patients with suspected HCTDs were analyzed. In 101 patients clinically suspected to have vEDS, heterozygous variants in *COL3A1* were found in 33 patients (32.4%), including three reported cases (Koitabashi et al., 2018; Kojima et al., 2015; Sano et al., 2019); variants in *TGFBR1*, *TGFBR2*, or *FBN1* were found in three patients whose skin fibroblasts showed normal type III collagen production (i.e., *LDS or MFS*); and variants in *COL5A1* were found in three patients (i.e., cEDS). Moreover, heterozygous variants in *COL3A1* were found in a patient (Patient 9) with a clinical diagnosis of LDS/FTAAD and in another patient (Patient 10) with a clinical diagnosis of FTAAD.

The general characteristics of 35 index patients and 16 affected relatives with vEDS are shown in Table 1. The median age at genetic diagnosis was 32 years (interquartile range 24–45) for index patients and 20 years (interquartile range 7–32) for affected relatives.

A comprehensive list of 34 *COL3A1* variants detected in 35 index patients is shown in Table 2. Of these, 18 variants (51.4%) were

TABLE 1 General characteristics of 35 index patients and 16 affected relatives with vEDS

Characteristics	*n* (%)	
	Index patients (*n* = 35)	Affected relatives (*n* = 16)
Sex		
Male	15 (42.9)	7 (43.8)
Female	20 (57.1)	9 (56.3)
Age at genetic diagnosis (years)		
0–9	1 (2.9)	3 (18.8)
10–19	5 (14.3)	3 (18.8)
20–29	9 (25.7)	3 (18.8)
30–39	9 (25.7)	2 (12.5)
40–49	5 (14.3)	1 (6.3)
50–59	5 (14.3)	1 (6.3)
60–69	1 (2.9)	0 (0.0)
Unknown	0 (0.0)	3 (18.8)
Median (interquartile range)	32 (24–45)	20 (7–32)

Abbreviation: vEDS; vascular Ehlers–Danlos syndrome.

TABLE 2 A comprehensive list of 34 variants in *COL3A1* detected in 35 index patients

Patient	Variants (NM_000090.3)	Variant types	Exon	HGMD Pro. 2020.1, Number of cases	ClinVar (ID)	2015 ACMG/ AMP guidelines & SVI recommendations	gnomAD v2.1.1	Type III collagen %
1	c.547G>A:p.Gly183Ser	Missense	7	21	P (17228)	–	–	11.5
2	c.547G>A:p.Gly183Ser	Missense	7	21	P (17228)	–	–	
3	c.556G>A:p.Gly186Ser	Missense	7	2	P (101135)	–	–	
4	c.565G>C:p.Gly189Arg	Missense	7	1	P (101205)	–	–	
5	c.583G>A:p.Gly195Arg	Missense (5′ end of exon)	8	1[a]	LP (74348)	–	–	
6	c.598C>T:p.Gln200*	Nonsense	8	–	–	LP (PVS1/PM2_Supporting/PP4/)	–	
7	c.659_664del:p.Ala220_Ile221del	In-frame deletion	9	–	–	VUS (PM2_Supporting/PM4/PP4)	–	
8	c.665G>A:p.Gly222Asp	Missense	9	7	P (101393)	–	–	
9	c.724C>T:p.Arg242*	Nonsense	10	–	P (801840)	P (PVS1/PS3/PP1)	2/282242 (MAF = 0.000007086)	22.7
10	c.754G>A:p.Gly252Ser	Missense	11	–	–	LP (PM1/PM2_Supporting/PM5/PP2/PP3)	–	
11	c.755G>A:p.Gly252Asp	Missense	11	1	P (101264)	–	–	
12	c.763G>T:p.Gly255Trp	Missense	11	–	–	LP (PM1/PM2_Supporting/PM5/PP2/PP3/PP4)	–	
13	c.848T>A:p.Leu283*	Nonsense	12	–	–	P (PVS1/PS3/PM2_Supporting/PP1/PP4)	–	29.5
14	c.897+2T>G	Splice site alteration	13[b]	–	–	LP (PVS1_Strong/PM2_Supporting/PP1/PP3/PP4)	–	
15	c.897+2T>A	Splice site alteration	13[b]	1	–	–	–	
16	c.951+5G>C	Splice site alteration	14[b]	–	–	LP (PS3/PM2_Supporting/PP1/PP3/PP4)	–	
17	c.1194+1G>A	Splice site alteration	18[b]	4	P (101293)	–	–	
18	c.1330G>A:p.Gly444Arg	Missense	20	8	P/LP (101185)	–	–	34.7
19	c.1346G>T:p.Arg449Leu	Missense (3′ end of exon)	20	–	–	VUS (PM2_Supporting/PP2/PP3/PP4)	–	78.1
20	c.[1546G>T;1556_1557delinsTT] :p.[Gly516Trp;Gly519Val]	Missense	23	–	–	LP (PM1/PM2_Supporting/PM5/PP1/PP2/PP3/PP4)	–	
21	c.1662+1G>A	Splice site alteration	24[b]	49	P (101269)	–	–	
22	c.1862G>A:p.Gly621Glu	Missense	27	2	P (101344)	–	–	13.0
23	c.1977+5G>C	Splice site alteration	29[b]	–	VUS (529311)	VUS (PM2_Supporting/PP1/PP3/PP4)	–	
24	c.2134_2160del:p.Pro712_Gly720del	In-frame deletion	32	2	–	–	–	
25	c.2283+5G>T	Splice site alteration	32[b]	2	–	–	–	18.1
26	c.2356G>A:p.Gly786Arg	Missense	35	8	P (17200)	–	–	28.9
27	c.2357G>A:p.Gly786Glu	Missense	35	–	–	LP (PM1/PM2_Supporting/PM5/PP2/PP3)	–	
28	c.2518G>A:p.Gly840Arg	Missense	37	–	–	P (PS2/PM1/PM2_Supporting/PP1/PP2/PP3/PP4)	–	
29	c.2815G>A:p.Gly939Ser	Missense	40	1	P (101472)	–	–	13.2

(Continues)

TABLE 2 (Continued)

Patient	Variants (NM_000090.3)	Variant types	Exon	HGMD Pro. 2020.1, Number of cases	ClinVar (ID)	2015 ACMG/ AMP guidelines & SVI recommendations	gnomAD v2.1.1	Type III collagen %
30	c.2869G>A:p.Gly957Ser	Missense	41	3	–	–	–	
31	c.2870G>T:p.Gly957Val	Missense	41	1	P (101446)	–	–	7.2
32	c.3256G>C:p.Gly1086Arg	Missense (5′ end of exon)	46	–	–	LP (PM1/PM2_Supporting/PM5/PP2/PP3/PP4)	–	
33	c.3338G>A:p.Gly1113Asp	Missense	46	–	–	P (PS3/PM1/PM2_Supporting/PP2/PP3/PP4)	–	5.3
34	c.3525+1G>A	Splice site alteration	48[b]	1	–	–	–	
35	ex.24–33 deletion	Multi-exon deletion	24–33	–	–	P (PVS1_Strong/PS3/PM2_Supporting)	–	4.5

Abbreviations: LP, likely pathogenic; P, pathogenic; VUS, variant of uncertain significance.
[a]Registered in Ehlers Danlos Syndrome Variant Database (https://eds.gene.le.ac.uk/home.php).
[b]Intron.

previously reported. The novel 16 variants were classified according to the 2015 ACMG/AMP guidelines and SVI recommendations as pathogenic in five cases (31.3%), likely pathogenic in eight cases (50.0%), and variants of uncertain significance (VUS) in three cases (18.8%; Patients 7, 19, and 23). The variant in Patient 7 was an in-frame deletion, and the variant in Patient 23 was a splice site alteration expected to cause exon skipping introducing an in-frame deletion. These variants were considered to exert a dominant-negative effect resulting in an eighth of the amount of normal type III collagen production. The pathogenicity of the variant in Patient 19 is discussed in the case report section.

The locations of all detected variants are shown in Figure 1. Twenty cases (57.1%) had missense variants (including two missense variants at the 5′ end of the exon) leading to Gly substitutions in the triple helical domain, whereas one case (2.9%) had a missense variant at the 3′ end of the exon leading to a non-Gly substitution in this domain. Eight cases (22.9%) had splice site alterations, three cases (8.6%) had nonsense variants, two cases (5.7%) had in-frame

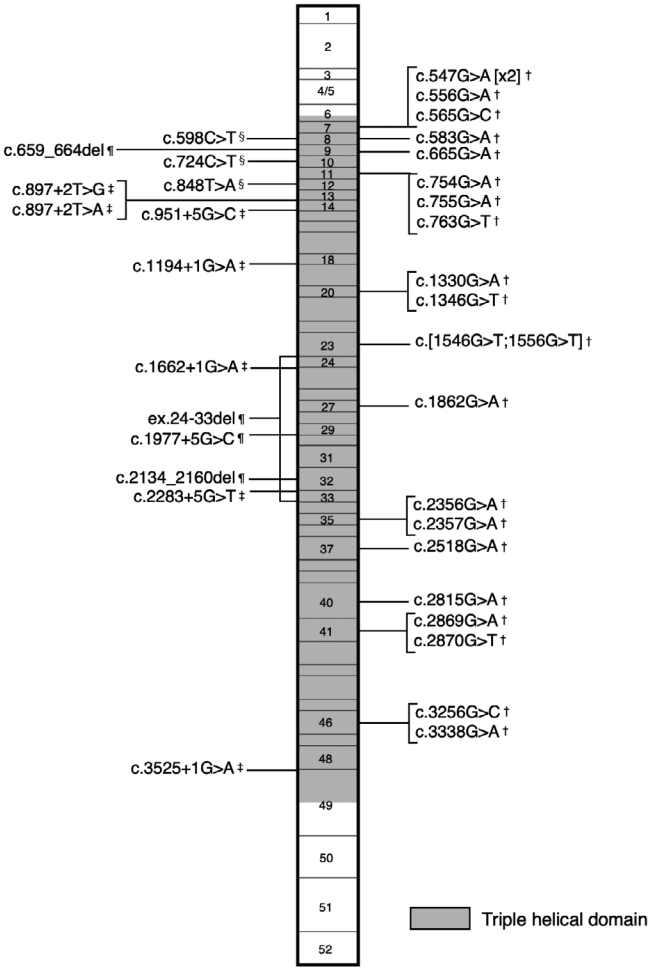

FIGURE 1 The distribution of variants in *COL3A1* detected in this study. The triple helical domain is located within exon 6 to exon 49. Exons 7–48 begin with the codon for Gly and end with the codon for the Y in the Gly-X-Y repeat. †Missense variant; ‡Splice site alteration; §Nonsense variant; ¶In-frame deletion

deletions, and one case (2.9%) had multi-exon deletion. The genotypes, phenotypes, and family histories of the 35 index patients are shown in Table 3. Twenty-two index patients had a family history relevant to vEDS, including critical events (arterial rupture, aortic dissection, and uterine rupture) and milder phenotypes (easy bruising, joint hypermobility). Twenty-seven index patients had at least one major criterion in the 2017 International Classification of the EDSs. Among eight index patients with no major criterion-related episodes, five index patients (Patients 18, 25, 26, 31, and 35) experienced spontaneous pneumothorax as a major medical concern, two younger patients (Patients 5 and 15) with skin and joint features had parental early deaths probably associated with vEDS, and the remaining patient (Patient 32) showed skin and joint features.

3.2 | Reports of atypical cases

3.2.1 | Patient 9 (nonsense variant)

Patient 9, a 50-year-old man (Figure 2a, III-5), and his cousin, a 51-year-old man (Figure 2a, III-3), developed dissection of ascending aorta at age 50 and 51 years, respectively (Table 3). Both underwent valve-replacement surgery safely with no fragility-related complications. Patient 9 had skin striae (Figure 2b-I) but no other skeletal or skin features associated with MFS or vEDS, and his cousin (III-3) had atrophic scars and skin translucency (Figure 2b-II). They were suspected to have LDS or FTAAD. The current NGS-based investigation revealed a heterozygous nonsense variant in *COL3A1* (c.724C>T:p.Arg242*), which was registered in gnomAD (2/282242, MAF = 0.000007086). Skin fibroblasts cultured from Patient 9 showed a decreased level of type III collagen production (22.7% of normal, Figure 3a). They were both diagnosed with vEDS, followed by initiation of celiprolol therapy. His cousin's asymptomatic daughter and son were also diagnosed molecularly and have begun regular vascular surveillance.

3.2.2 | Patient 13 (nonsense variant)

Patient 13, a 37-year-old woman, developed an abdominal muscle rupture and was found to have an aortic aneurysm (Figure 2b-III) during her high school days, which ruptured at 32 weeks of gestation of her first pregnancy (Table 3). She underwent an aortoplasty following an emergency cesarean section, which saved her life with no sequelae but resulted in severe hypoxic ischemic encephalopathy in her daughter. During her second pregnancy, she was suspected to have vEDS because of skin translucency (Figure 2b-IV) and easy bruising in addition to the previous events. Her cultured skin fibroblasts showed a decreased amount of type III collagen production (29.5% of normal, Figure 3a), whereas mRNA-based direct sequencing of *COL3A1* showed no pathogenic variants. She underwent an elective cesarean section at 32 weeks of gestation with no consequences for her or her son. Obstetricians noticed an extreme fragility in her uterine wall during the cesarean. The current NGS-based investigation revealed a heterozygous nonsense variant in *COL3A1* (c.848T>A:p.Leu283*), which was not registered in gnomAD or 8.3KJPN. She began celiprolol therapy. Her daughter was bedridden with mild skin translucency and easy bruising, and her son had moderate skin translucency, easy bruising, and several occurrences of muscle rupture while playing football. Both were found to have the variant and her son has begun celiprolol therapy.

3.2.3 | Patient 6 (nonsense variant)

Patient 6, a 54-year-old woman, developed a right carotid-cavernous sinus fistula at age 47 years, which was treated with coil embolization (Table 3). During the procedure, a left internal carotid artery dissection occurred and was treated with stent placement. Asymptomatic bilateral external iliac artery dissection and right colic artery dissection were found on thoracoabdominal CT angiography. At age 53 years, coil embolization was performed three times for the recurrent left carotid-cavernous sinus fistula. The current NGS-based investigation revealed a heterozygous nonsense variant in *COL3A1* (c.598C>T:p.Glu200*), which was not registered in gnomAD or 8.3KJPN. She also had gingival recession, easy bruising, thin and translucent skin, and mild keratoconus.

3.2.4 | Patient 19 (non-Gly substitution)

Patient 19 developed an arterial rupture and multiple arterial aneurysms at age 55 years. She had translucent and easy bruising skin with multiple atrophic scars (Figure 2b-V, VI), finger joint hypermobility, and gingival recession (Table 3), and was clinically suspected to have vEDS. The current NGS-based investigation revealed a heterozygous missense variant (c.1346G>T:p.Arg449Leu) in *COL3A1*, leading to non-Gly substitution in the triple helical domain. The variant was classified as a VUS (Table 2) according to the 2015 ACMG/AMP guidelines and SVI recommendations, based on the criteria as follows: absent in gnomAD and 8.3KJPN (PM2_Supporting); missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease (PP2); and multiple lines of computational evidence support a deleterious effect on the gene or gene product (PP3) (i.e., 6 out of 10 in silico predictive algorithms for the pathogenicity of missense variants (SIFT, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, fathmm-MKL, MetaSVM, MetaLR, and M-CAP) supported a deleterious effect) (Table S3). The amount of type III collagen production from her cultured skin fibroblasts was mildly decreased (78.1% of the control sample) (Figure 3a), and transmission electron microscopy showed that the collagen fibers were irregular in size, the irregularity of which was found to be milder than an age- and sex-matched Gly substitution case (Patient 1) (Figure 3b). The variant was also suggested to cause a splicing alteration because of its location at the 3′ end of exon 20. dbscSNV1.1 supported a deleterious effect, and MaxEntScan

TABLE 3 Genotypes, phenotypes, and family histories of 35 index patients

					Major criteria							Minor criteria												
Patient	Variants (NM_000090.3)	Suspected disease	Age at genetic diagnosis (years)	Sex	Aortic dissection	Aortic rupture	Arterial dissection	Arterial rupture	Uterine rupture	Sigmoid colon perforation	Carotid-cavernous sinus fistula	Easy bruising	Thin, translucent skin	Characteristic facial features	Spontaneous pneumothorax	Acrogeria	Talipes equinovarus	Congenital hip dislocation	Hypermobility of small joints	Tendon and muscle rupture	Gingival recession/fragility	Keratoconus	Early onset varicose veins	Family history
1[a]	c.547G>A:p.Gly183Ser	vEDS	59	F	−	−	−	+		+	+	+	+	−	−	−	−	−	−	−	−		−	−
2	c.547G>A:p.Gly183Ser	vEDS	29	F	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−		−	Mother, died, carotid-cavernous sinus fistula
3	c.556G>A:p.Gly186Ser	vEDS	d.15	M	+	+	−	−		−	−	−	−	−	−	−	−	−	−	−	−		−	Mother, died at 30s, subarachnoid hemorrhage
4	c.565G>C:p.Gly189Arg	vEDS	45	F	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−		−	−
5	c.583G>A:p.Gly195Arg	vEDS	17	F	−	−	−	−		−	−	+	+	+	−	−	−	−	+	−	−		−	Mother, died at 32 years, aortic dissection; grandfather, died at 30s, aortic dissection
6	c.598C>T:p.Gln200*	vEDS	54	F	−	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	+	+	−	−
7	c.659_664del: p.Ala220_Ile221del	vEDS	21	F	−	−	−	−		−	+	+	−	−	−	−	+	−	+	−	−		−	Positive family history (details unknown)
8	c.665G>A:p.Gly222Asp	vEDS	34	F	+	−	+	−	−	−	−	+	+	−	−	+	−	−	−	−	+		−	−
9	c.724C>T:p.Arg242*	LDS/FTAAD	50	M	+	−	−	−		−	−	−	−	−	−	−	−	−	−	−	−		−	Father, died at 62 years, aortic dissection; sister (46 years), variant positive; cousin (51 years), aortic dissection, variant positive; cousin's daughter (21 years) and son (19 years), variant positive
10	c.754G>A:p.Gly252Ser	FTAAD	27	M	+	−	−	−		−	−				+									Mother died, aortic dissection
11	c.755G>A:p.Gly252Asp	vEDS	38	F	−	−	−	+	−	−	−	+	+	−	+	−	−	−	+	−	−		+	Father, died, sudden death after chest trauma
12	c.763G>T:p.Gly255Trp	vEDS	d.15	M	−	−	−	+		−	−	−	−	−	+	−	−	−	−	−	−		−	Father, died at 30 years, arterial rupture
13	c.848T>A:p.Leu283*	vEDS	37	F	−	+	−	−	+	−	−	+	+	+	−		−	−	−	+	+			Daughter, thin skin, variant positive; son (10 years), thin skin, muscle rupture, variant positive
14	c.897+2T>G	vEDS	28	M	−	+	−	+		+	−	+	+	+	+		−	−	+	−	+		+	Son (6 years), easy bruising, spontaneous pneumothorax, thin skin, joint hypermobility, variant positive; daughter (6 years), thin skin, joint hypermobility, variant positive
15	c.897+2T>A	vEDS	8	M	−	−	−	−		−	−	+	+	+	−	−	−	−	+	−	−		−	Father, died at 25 years, aortic dissection
16[b]	c.951+5G>C	vEDS	25	F	−	−	+	+	−	−	−	+	+	+	+	−	−	−	−	−	−		−	Mother, no symptoms, variant negative (germline mosaicism suspected); maternal half-brother, easy bruising, hematoma, vascular fragility, variant positive
17	c.1194+1G>A	vEDS	39	M	−	−	−	+		−	−	+	+	+	+	+	−	−	+	−	+			Daughter (6 years), easy bruising, variant positive
18	c.1330G>A:p.Gly444Arg	vEDS	32	M	−	−	−	−		−	−	+	+	+	+	+	+	−	+	−	−		−	−
19	c.1346G>T:p.Arg449Leu	vEDS	55	F	−	−	−	+		−	−	+	+	+	−	−	−	−	+	−	+			−

TABLE 3 (Continued)

Patient	Variants (NM_000090.3)	Suspected disease	Age at genetic diagnosis (years)	Sex	Major criteria							Minor criteria											Family history
					Aortic dissection	Aortic rupture	Arterial dissection	Arterial rupture	Uterine rupture	Sigmoid colon perforation	Carotid-cavernous sinus fistula	Easy bruising	Thin, translucent skin	Characteristic facial features	Spontaneous pneumothorax	Acrogeria	Talipes equinovarus	Congenital hip dislocation	Hypermobility of small joints	Tendon and muscle rupture	Gingival recession/fragility	Keratoconus	Early onset varicose veins	
20[c]	c.[1546G>T;1556G>T]:p.[Gly516Trp;Gly519Val]	vEDS	38	F	+	−	+	+	−	−	−	+	+	−	−	+	−	−	−	−	−		−	Mother, died at 33 years, arterial rupture, variant positive
21	c.1662+1G>A	vEDS	42	F	−	−	−	−	−	−[d]	+	+	−	+	−	−	−	−	+	−	−		−	−
22	c.1862G>A:p.Gly621Glu	vEDS	30	F	−	−	+	−		−	−	+	+	+	−	+	−	−	+	−	−		−	Mother, easy bruising, pulmonary valve stenosis, meniscus injury, variant positive
23	c.1977+5G>C	vEDS	65	F	+	−	+	−	−	−	−	+	+		−				+				+	Daughter (37 years), easy bruising, joint hypermobility, tendon and muscle rupture, variant positive
24	c.2134_2160del: p.Pro712_Gly720del	vEDS	48	F	+	−	−	+		−	−	+	+	+	+		−	−	+	−	+			Sister, uterine rupture
25	c.2283+5G>T	vEDS	16	F	−	−	−	−		−	−	−	+	+	+	−	−	−	+	−	−		−	−
26	c.2356G>A:p.Gly786Arg	vEDS	31	F	−	−	−	−		−	−	+	+	+	+	−	−	−	+	−	−		−	Father, easy bruising
27	c.2357G>A:p.Gly786Glu	vEDS	33	M	−	−	+	−		−	−	−	−	−	−	−	−	−	−	−	−		−	Father, died at 54 years, aortic dissection; brother (29 years), subclavian artery aneurysm
28	c.2518G>A:p.Gly840Arg	vEDS	43	M	−	−	−	+		−	−	+	−	+	+	+	+	−	+	−	−		−	Son (14 years), easy bruising, tendon and muscle rupture, variant positive
29	c.2815G>A:p.Gly939Ser	vEDS	25	M	−	−	+	−		−	−	+	−	−	−	−	−	−	+	−	−		−	−
30	c.2869G>A:p.Gly957Ser	vEDS	d.26	M	−	−	−	+		−	−	+		−	−					−				−
31	c.2870G>T:p.Gly957Val	vEDS	24	M	−	−	−	−		−	−	−	+	−	+	−	−	−	+	−	−		−	Father, died at 30 years, aortic dissection
32	c.3256G>C:p.Gly1086Arg	vEDS	49	F	−	−	−	−		−	−	+	+	+	−	+	−	+	+	+	+			−
33	c.3338G>A:p.Gly1113Asp	vEDS	17	M	−	−	−	+		−	−	−	−	−	+	−	−	−	−	+	−		−	−
34	c.3525+1G>A	vEDS	53	F	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	+	+		+	Daughter (29 years) and son (24 years), variant positive
35	ex. 24–33 deletion	vEDS	24	M	−	−	−	−		−	−	+	+	+	+	−	+	−	+	−	−		−	−

Note: blank, data not available.

Abbreviations: d., died; F, female; FTAAD, familial thoracic aortic aneurysm and dissection; M, male; LDS, Loeys-Dietz syndrome; vEDS, vascular Ehlers–Danlos syndrome.

[a]Kojima et al. (2015).

[b]Koitabashi et al. (2018).

[c]Sano et al. (2019).

[d]Transverse or descending colon perforation.

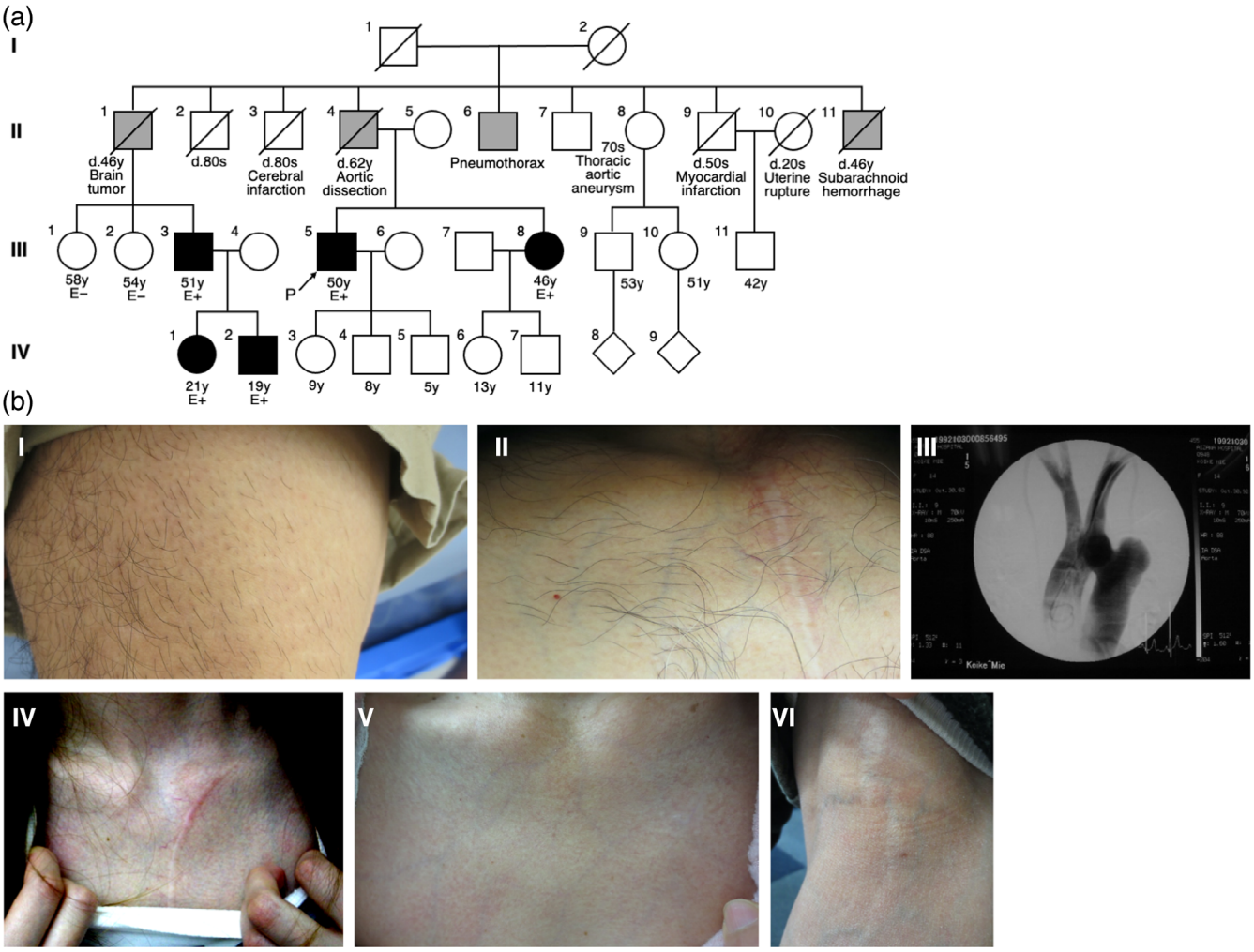

FIGURE 2 (a) Family tree of Patient 9 with the heterozygous nonsense variant c.724C>T:p.Arg242* in *COL3A1*. E(+) means that this variant was detected and E(−) means that this variant was not detected. (b) Clinical photographs of patients. (I) Skin striae in Patient 9. (II) Translucent skin in a cousin of Patient 9. (III) Aortic aneurysm in Patient 13. (IV) Significantly translucent skin in Patient 13. (V) Skin translucency in Patient 19. (VI) Skin striae in Patient 19.

supported a tolerated effect (data not shown). cDNA-based sequencing using her cultured skin fibroblasts showed a normal splicing pattern (Figure S1a).

3.2.5 | Patient 35 (multi-exon deletion)

Patient 35 had talipes equinovarus, easy bruising, translucent skin, bilateral pneumothorax, pulmonary hemorrhage, habitual dislocation of the shoulder, and cryptorchidism (Table 3), and was clinically suspected to have vEDS. The CNV visualization method for Ion AmpliSeq data (Nishio et al., 2018) revealed a heterozygous multi-exon deletion in exons 24–33 (Figure 3c), which was confirmed by MLPA (Figure 3d). The amount of type III collagen production from his skin fibroblasts was measured as 4.5% of the control sample (Table 2).

3.2.6 | Patients 3 and 12 (analyses using FFPE samples)

Patients 3 and 12 had died in their teens from a rupture of a dissecting thoracic aortic aneurysm and a rupture of the right internal thoracic artery, respectively. NGS analyses were performed on DNA extracted from their FFPE samples. The current NGS-based investigation revealed heterozygous Gly substitutions in *COL3A1* (Patient 3, c.556G>A:p.Gly186Ser; Patient 12, c.763G>T:p.Gly255Trp). In Patient 12, no variant was detected using UDG-untreated DNA because regions with ≥20× depth of coverage reached only 14.7% in *COL3A1* or 29.1% in total (i.e., only 14.7% in *COL3A1* or 29.1% in total was sequenced 20 or more times, which is necessary to identify the genotype), but the variant was detected using UDG-treated DNA because regions with ≥20× depth of coverage markedly increased to 91.2% in *COL3A1* or 91.9% in total (Figure S1b).

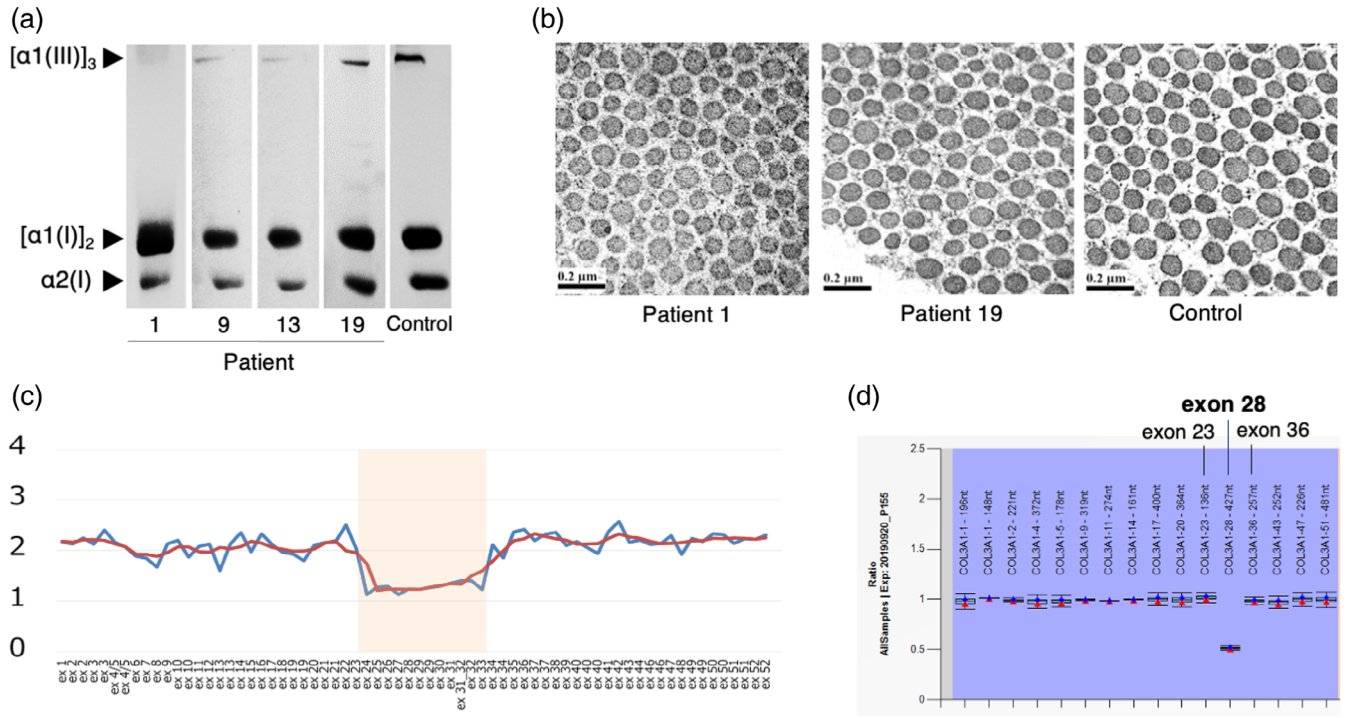

FIGURE 3 Protein analysis, electron microscopy, and copy number variation (CNV) analysis and multiplex ligation-dependent probe amplification (MLPA) validation. (a) The amount of type III collagen production from skin fibroblasts in Patient 1 with the heterozygous missense variant leading to glycine (Gly) substitution c.547G>A:p.Gly183Ser, Patient 9 with the heterozygous nonsense variant c.724C>T:p.Arg242*, Patient 13 with the heterozygous nonsense variant c.848T>A:p.Leu283*, and Patient 19 with the heterozygous missense variant leading to non-Gly substitution c.1346G>T:p.Arg449Leu. Control is a healthy individual. (b) Electron microscopy of the collagen fibers in Patient 1, a 59-year-old woman with the heterozygous missense variant leading to Gly substitution c.547G>A:p.Gly183Ser, Patient 19, a 55-year-old woman with the heterozygous missense variant leading to non-Gly substitution c.1346G>T:p.Arg449Leu, and a control, a 59-year-old woman. (c) A multi-exon deletion in Patient 35 detected by the CNV visualization method for ion AmpliSeq data. (d) Validation of multi-exon deletion in Patient 35 by MLPA. Between exons 24 and 33, the SALSA MLPA Kit P155-D2 for the *COL3A1* gene contained only an exon 28 probe.

4 | DISCUSSION

This is the largest case series of Asian patients with vEDS, and the first case series applying an amplification-based NGS with custom HCTD panels, including a CNV analysis. The variants detected were as follows: missense variants leading to Gly substitutions (57.1%), a missense variant leading to non-Gly substitution (2.9%), splice site alterations (22.9%), nonsense variants (8.6%), in-frame deletions (5.7%), and a multi-exon deletion (2.9%). The frequency of variant types in the current study was compatible with those reported in the previous literature. At present, 1272 cases with heterozygous variants in *COL3A1* are registered in HGMD Professional 2020.1. Of these, 745 cases (58.5%) had missense variants leading to Gly substitutions of the Gly-X-Y repeats in the triple helical domain, 20 cases (1.6%) had missense variants leading to non-Gly substitutions in the triple helical domain, nine cases (0.7%) had missense variants outside the triple helical domain, 327 cases (25.7%) had splice site alterations, 108 cases (8.5%) had deletions or/and insertions, 48 cases (3.8%) had nonsense variants, two cases (0.2%) had stop-loss variants, and 13 cases (1.0%) had multi-exon deletions. These findings represent an appropriate throughput of this multiplex PCR-based comprehensive NGS screening.

The current panel-based NGS sequencing system is useful for the management of vEDS through appropriate differential diagnosis with other clinically overlapping HCTDs, such as LDS, MFS, and FTAAD. Heterozygous pathogenic/likely pathogenic variants in *COL3A1* were found in a patient with a clinical diagnosis of LDS/FTAAD (Patient 9) and in another patient with a clinical diagnosis of FTAAD (Patient 10). Among patients with a clinical diagnosis of vEDS in the current cohort, a 42-year-old woman with skin hyperextensibility and hypermobility of small joints who developed multiple arterial aneurysms was found to have a VUS in *TGFBR1*, another patient with no available clinical information was found to have a VUS in *TGFBR2*, and a third patient with thin skin and hypermobility of finger joints who underwent surgery for an aortic aneurysm was found to have a reported pathogenic variant in *FBN1*. Gly substitutions in *COL5A1* were found in three patients: one with joint hypermobility and skin features (easy bruising, translucent, hyperextensible, fragile with atrophic scars, and acrogeria) who developed multiple arterial lesions (carotid artery dissection and dissection/aneurysm of splenic and superior mesenteric artery); another with no specific skin features who developed arterial lesions (vertebral artery dissection, bilateral internal carotid artery dissection, and arterial tortuosity); and one with no available clinical information. No pathogenic/likely pathogenic variants were found in *COL3A1* in

these six patients. Arterial lesions (dissection, aneurysm, or rupture) are the clinical hallmark of vEDS, and are rarely found in patients with cEDS (Bowen et al., 2017). Thus far, six relevant variants (four Gly substitutions and two nonsense variants) in *COL5A1* have been reported in patients with EDS and vascular fragility: a family clinically suspected to have vEDS (Monroe et al., 2015), and five independent patients clinically suspected to have cEDS, but who developed vascular events (Borck et al., 2010; de Leeuw et al., 2012; Karaa and Stoler, 2013; Mehta et al., 2012; Yasuda et al., 2013).

HGMD Professional 2020.1 contains 1272 cases with heterozygous variants in *COL3A1*, and 101 of them (7.9%) are registered as having variants that may cause haploinsufficiency of *COL3A1*, including nonsense variants, frameshift alterations, and splice site alterations predicted or confirmed to cause exon skipping introducing a premature termination codon. Leistritz et al. (2011) reported that the lifespan in patients with variants leading to haploinsufficiency was extended and the age at the first complication was delayed by almost 15 years. They also reported that about half of the index patients and affected relatives had no minor diagnostic criteria for vEDS according to the previous Villefranche classification (Beighton et al., 1998). Pepin et al. (2014) reported that the survival of patients with variants leading to haploinsufficiency was significantly longer than those with missense variants leading to Gly substitutions in the triple helical domain. Frank et al. (2015) reported that the median age at diagnosis of patients with in-frame alterations was 25 years, the median age of patients with Gly substitutions in the triple helical domain was 34 years, and the median age of patients with variants leading to haploinsufficiency was 46 years. Thus, patients with variants leading to haploinsufficiency are expected to survive longer than those with variants leading to a dominant-negative effect, and may not be diagnosed with vEDS at younger ages because of the delayed onset of the first complication or a milder phenotype. A pLoF (predicted loss-of-function) search in gnomAD reveals the carrier status of pathogenic variants in the general population: a variant (c.724C>T:p.Arg242*) in Patient 9, clinically suspected as LDS/FTAAD, is also included (Table S2). Two other patients with variants leading to haploinsufficiency are included in the current study: Patient 13 (c.848T>A: p.Leu283*) with translucent and easy bruising skin developed an aortic aneurysm rupture during pregnancy in her 20s, and Patient 6 (c.598C>T:p.Glu200*) with translucent and easy bruising skin had recurrent cavernous sinus fistula as well as asymptomatic bilateral external iliac artery dissection and right colic artery dissection. Considering the limited information regarding patients with variants in *COL3A1* leading to haploinsufficiency, it would be reasonable to judge the severity not only based on the genotype but also the relevant histories of the patient and the affected family members. Shalhub et al. (2014) also reported that patients with variants leading to haploinsufficiency had milder arterial lesions, but a significantly higher prevalence of aortic disease than patients with variants leading to a dominant-negative effect.

Among 774 heterozygous missense variants reported in HGMD professional 2020.1, 745 are Gly substitutions in the triple helical domain, whereas only 20 are non-Gly substitutions in the triple helical domain (Frank et al., 2015; Ghali et al., 2019; Weerakkody et al., 2016) (Table S3). Weerakkody et al. (2016) reported "c.2417C>T:p.Pro806Leu" as a pathogenic variant in a patient with clinically diagnosed vEDS. Though the variant is registered in gnomAD (1/31398, MAF = 0.00003185), biochemical abnormalities were mild, and light microscopy and electron microscopy were abnormal (Weerakkody et al., 2016). Ghali et al. (2019) reported a comprehensive study of seven index pas and 11 affected relatives with missense variants leading to non-Gly (glutamic acid to lysine [Glu>Lys]) substitutions in the triple helical domain. The current cohort included one patient with a probably relevant non-Gly substitution variant (c.1346G>T:p.Arg449Leu) in *COL3A1*. Although the variant was classified as a VUS according to the 2015 ACMG/AMP guidelines and SVI recommendations, phenotypically she showed arterial rupture/aneurysms in her 50s and relevant physical signs (translucent and easy bruising skin and gingival fragility); biochemically, a mild reduction in the amount of type III collagen production was shown (Figure 3a); and pathologically, a mild irregularity in the collagen fibers was shown (Figure 3b). Based on these findings, a diagnosis of vEDS was thought to be reasonable, though the clinical and pathophysiological mildness could be mentioned. The clinical significance of missense variants leading to non-Gly substitution is difficult to judge, and careful discussion based on comprehensive analyses including clinical, molecular, biochemical, and pathological approaches will be required.

Among 1272 cases with heterozygous variants in *COL3A1* registered in HGMD Professional 2020.1, 13 cases (1.0%) are multi-exon CNVs. They were detected by mRNA analysis using cultured skin fibroblasts. In the current cohort, we used a recently proposed CNV visualization method for Ion AmpliSeq data we call "the Nishio Method," which is simple and cost-effective without any additional equipment or software and has been applied to genetic analysis for congenital hearing loss (Nishio et al., 2018; Sugiyama et al., 2019). The result of the Nishio Method was comparable to that of MLPA in the current study (a deletion of exons 24–33 in Patient 35) (Figure 3c, d). Further evaluation, including larger numbers of patients and genes, will be required for the Nishio Method to be established as a confirmative procedure for CNV analysis.

In the current study, pathogenic variants in *COL3A1* were successfully identified from FFPE samples of two patients who had died in their teens because of a rupture of a dissecting thoracic aortic aneurysm or a rupture of the right internal thoracic artery. Index patients with vEDS could be found in the context of autopsy examinations because of the potentially lethal complications and the limited medical awareness at younger ages (easy bruising could be the only phenotype). Thus, it would be helpful to adopt a genetic analysis procedure corresponding to the analysis using FFPE samples. Ion Torrent NGS technology is a widely and routinely applied method to the detection of somatic variants from FFPE samples of cancer tissues (Hovelson et al., 2015). Do and Dobrovic (2012) reported that deamination of cytosine to uracil of DNA from FFPE samples led to sequence artifacts, and these can be minimized without affecting true variants by UDG treatment prior to PCR amplification. In this study, regions with ≥20× depth of coverage were markedly increased

through UDG treatment prior to PCR amplification. UDG treatment is expected to be essential because the DNA polymerase used in Ion Torrent NGS technology may be one of the enzymes that specifically recognize the presence of uracil in a template DNA and stall DNA synthesis before misincorporation of adenine (Greagg et al., 1999). Detection of pathogenic variants in deceased index patients would be meaningful not only for acceptance of critical episodes by the families but also for the application of early genetic diagnosis in at-risk relatives.

The current study has several limitations. First, there are theoretical limitations regarding the amplification-based NGS system we used. Variants in deep intronic regions or regulatory regions, as well as intragenic rearrangements, are undetectable. Additionally, the presence of SNVs in the primer-binding regions could affect the PCR amplification. If these regions include SNVs and are not covered by other amplicons, the SNVs are undetectable. Second, the current cohort was not a strictly systematical one, but a research-based one to collect large numbers of patients with HCTD-related symptoms, and the phenotypic evaluation depended on the experience of the physicians. The low detection rate (32.4% of patients suspected as vEDS) might be related to this recruitment strategy. However, this wider inclusion contributed to the detection of a variant leading to haploinsufficiency in a patient clinically suspected to have LDS/FTAAD. Considering the usefulness of a gene-based surveillance and treatment strategy (e.g., indication for surgery, and application of pharmacological intervention including celiprolol for vEDS and β-blockers and angiotensin receptor blockers for MFS and LDS), it would be reasonable to perform NGS-based genetic testing for vEDS in the context of screening for such major HCTDs, just as in the current study.

In conclusion, we have presented the details and clinical usefulness of comprehensive genetic screening through an amplification-based NGS panel analysis, with the ability for CNV analysis and FFPE sample analysis. The system effectively detected a wide spectrum of variants, including nonsense variants in patients with milder features, a non-Gly substitution in an atypical case, a multi-exon deletion, and variants from FFPE samples in deceased patients. The usefulness of comprehensive clinical, molecular, biochemical, and pathological approaches for atypical cases (e.g., non-Gly substitutions) was also noted. Clinical application of this system, and its improvement based on larger numbers of cases, would be required.

AUTHOR CONTRIBUTIONS

Tomomi Yamaguchi performed all molecular experiments, interpreted the data, and wrote the first draft of the manuscript. Tomoki Kosho conceived the work, organized the data collection, and interpreted the data. Shujiro Hayashi provided clinical data and conducted type III collagen production analysis and electron microscopic investigation. Shin-Ya Nishio assisted CNV analysis. Daisuke Hayashi, Takeshi Matsuyama, Norimichi Koitabashi, Kenichi Ogiwara, Masaaki Noda, Chiai Nakada, Shinya Fujiki, Akira Furutachi, Yasuhiko Tanabe, Michiko Yamanaka, Aki Ishikawa, Miyako Mizukami, Asako Mizuguchi, Kazumitsu Sugiura, Makoto Sumi, Hirokuni Yamazawa, Atsushi Izawa, and Yuko Wada provided clinical data. Tomomi Fujikawa, Yuri Takiguchi, and Kyoko Takano helped to perform molecular analysis. Keiko Wakui established cultured skin fibroblasts. All authors participated in revision and approval of the manuscript.

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CONFLICT OF INTEREST

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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.

ORCID

Kyoko Takano https://orcid.org/0000-0002-7283-1503

Tomoki Kosho https://orcid.org/0000-0002-8344-7507

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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