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CLINICAL REPORT

Identification of two novel *COL3A1* variants in patients with vascular Ehlers-Danlos syndrome

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Abstract

Background: Vascular Ehlers-Danlos syndrome (vEDS) is an autosomal dominant disease caused by aberrations in *COL3A1*, which encodes type III collagen. Sanger sequencing has limitations for diagnosis since exon deletion/duplication and splicing alterations are not uncommon in *COL3A1*. We report 2 patients with vEDS who were not diagnosed by conventional Sanger sequencing.

Methods: We performed either targeted panel or whole-genome sequencing. Complementary DNA (cDNA) sequencing was performed using cultured skin fibroblasts. Sanger sequencing of DNA was performed for the confirmation of breakpoints in the case of exon deletion. We also evaluated the sensitivity of the splicing prediction tool, SpliceAI.

Results: An exon 27 deletion was suspected on targeted panel sequencing of 1 patient. The deletion was confirmed using cDNA sequencing (r.1870_1923del) and breakpoints were confirmed (c.1870-109_1923+10del). On targeted panel sequencing in the other patient, we found a novel intronic variant of c.1149+6T>C that leads to skipping of exon 16 (r.1051_1149del) by cDNA sequencing. SpliceAI showed 98.8% sensitivity for known splicing variants in *COL3A1*.

Conclusion: Our study highlights the necessity of a comprehensive approach to the genetic diagnosis of vEDS. In addition, cDNA sequencing was useful as an auxiliary method, especially considering the limited sensitivity of the splicing prediction tool.

KEYWORDS

COL3A1, mRNA sequencing, SpliceAI, vascular Ehlers-Danlos syndrome, whole-genome sequencing

1 | INTRODUCTION

Vascular Ehlers-Danlos syndrome (vEDS, OMIM #130050), also known as EDS type IV, is a rare autosomal dominant disorder caused by genetic variants in the *COL3A1* gene (OMIM #120180). Of the 14 subtypes of EDS classified by the 2017 international EDS classification (Malfait et al., 2017), vEDS has one of the shortest life expectancies, with a median age of 48 years (ranging from 6 to 73 years) and over 80% of patients experiencing at least one complication by age 40 (Pepin et al., 2000). There is an overlap in symptoms between vEDS and other hereditary connective tissue disorders such as Marfan syndrome, Loeys-Dietz syndrome, or familial arterial aneurysm and dissection syndromes. The *COL3A1* encodes collagen alpha 1(III) chain, which constitutes to a homotrimer to form type III collagen. It is a major fibrillar collagen for the structural stability of hollow organs like arteries, uterus, and bowel (Frank et al., 2015; Malfait, 2018), as it constitutes 5%–20% of the human body's entire collagen content. Type III collagen consists mainly of a triple-helical region containing triplet repeats of $[Gly\text{-}X\text{-}Y]_{343}$ (Dalgleish, 1998). Most vEDS cases are diagnosed due to an acute event such as arterial dissection or bowel rupture, or a positive family history (Pepin et al., 2014). Identifying pathogenic variants in *COL3A1* is important for timely management and diagnosis, including family studies for vEDS. In this study, we discovered two novel pathogenic variants in *COL3A1* that had not been detected by conventional Sanger sequencing.

2 | METHODS

For Patient 1 (P1), we conducted targeted gene panel sequencing, which included 12 genes associated with familial thoracic aortic aneurysms and aortic dissections: *ACTA2*, *CBS*, *COL3A1*, *FBN1*, *FBN2*, *MYH11*, *MYLK*, *SLC2A10*, *SMAD3*, *TGFB2*, *TGFBR1*, and *TGFBR2*. For Patient 2 (P2), a whole-genome sequencing (WGS) library was constructed using TruSeq Nano DNA Library Prep Kit (Illumina Inc.) and WGS was performed using a NovaSeq6000 platform (Illumina Inc.) with paired-end reads of 150 bp, in accordance with the manufacturer's instructions. RNA extracted from cultured fibroblasts was analyzed using targeted cDNA sequencing for both patients and whole-transcriptome sequencing for P2, utilizing Omniscript Reverse Transcriptase (Qiagen) and TruSeq Stranded mRNA Library Prep (Illumina Inc.), in accordance with the manufacturer's instructions. The nucleotide and corresponding protein sequences were described based on reference sequences NM_000090.4 and NP_000081.2, respectively. SpliceAI (Jaganathan et al., 2019), a deep-learning-based algorithm, was used to calculate the probability of splice-altering variants, providing a Δ score between 0 and 1 with a predictive sensitivity greater than 80% at a cutoff of 0.2. The variant classification was determined in accordance with the 2015 American College of Medical Genetics and Genomics and the Association for Molecular Pathology standards and guidelines (ACMG/AMP 2015 guidelines) (Richards et al., 2015) and ClinGen recommendations for interpreting the loss of function PVS1 criterion (Abou Tayoun et al., 2018). We retrieved the three-dimensional structures of the normal type III collagen protein (UniProt P02461 (The UniProt Consortium, 2022)) from the AlphaFold Protein Structure Database (DeepMind and EMBL-EBI) (Varadi et al., 2021). The structures of the variants in our study were predicted using AlphaFold v2.3.2 (Jumper et al., 2021), and the resultant protein structure was processed with ChimeraX (Pettersen et al., 2021) to visualize the structures and delineate the regions affected by the variants. Each structure's per-residue confidence score was measured by the predicted local distance difference test (pLDDT) produced by AlphaFold. Root mean square deviation (RMSD) analysis was performed using MatchMaker in ChimeraX.

3 | RESULTS

3.1 | Clinical reports

P1 was a 21-year-old woman with a history of easy bruising who was admitted to the Division of Cardiology at Samsung Medical Center in Seoul, Korea in December 1998 due to a traumatic carotid-cavernous fistula (CCF). The CCF was successfully treated with transarterial detachable balloon embolization. Physical examination revealed thin skin with visible blood vessels, large hematomas, and multiple ecchymoses at puncture sites of angiography, but skin hyperextensibility and finger joint hypermobility were not observed. Further imaging studies identified multiple aneurysms of the left renal artery

near the renal hilum, celiac artery, and splenic artery, as well as a segmental dissection of the right iliac artery (Figure 1a). Although the clinical findings, particularly the CCF in combination with multiple aneurysms and/or dissections, were suggestive of vascular Ehlers-Danlos syndrome (vEDS), no abnormalities were identified by Sanger sequencing of *COL3A1* in 2006. Family history of vEDS or early death was not fully documented, but the proband's father died of heart failure in his 50s, and the proband's paternal grandfather died in his 40s. During follow-up outpatient visits, a CT angiography was conducted biennially, and no significant interval change of multiple aneurysms and focal dissection was noted. However, P1 passed away at the age of 39 due to a ruptured left renal artery aneurysm.

P2 was a 42-year-old man referred to our center for evaluation of aneurysmal dilatation of the celiac axis identified during a medical check-up in January 2007. He had not suffered from easy bruising, epistaxis, or abdominal angina, and his first imaging studies were done at the age of 42. On physical examination, atrophic cigarette-paper scars were seen on both knees and shins, and thin transparent vessels over the trunk were observed. Further studies revealed multiple aneurysms of both renal arteries, the left external iliac artery, and the right internal iliac artery, as well as dissecting aneurysms of the celiac artery and superior mesenteric artery (Figure 1b). In addition, he presented with numbness in his left 3rd, 4th, and 5th fingers, and upper extremities CT angiography showed left ulnar distal vessel occlusion.

Although a diagnosis of vEDS was suspected due to his clinical examination features and the presence of multiple arterial aneurysms and dissections, Sanger sequencing of *COL3A1* did not detect any pathogenic variants. Furthermore, Sanger sequencing of *TGFBR1* and *TGFBR2*, which was performed to rule out Loeys-Dietz syndrome, did not reveal any variants. During follow-up visits, a CT angiography was conducted every 2 years, and no significant change of multiple aneurysms was noted. While comprehensive segregation analysis of vEDS was not conducted, relevant familial history was noted. The proband's father died of heart failure in his 50s, and the proband's grandfather passed away in his 40s. The proband has three male siblings, none of whom were suspected of having vEDS. However, three of these brothers were found to carry the *TGFBI* p.R124H variant, which is associated with Avelino corneal dystrophy. Patient 2 had his first major complications at the age of 42 and is now 57, on regular outpatient follow-up without surgical treatment.

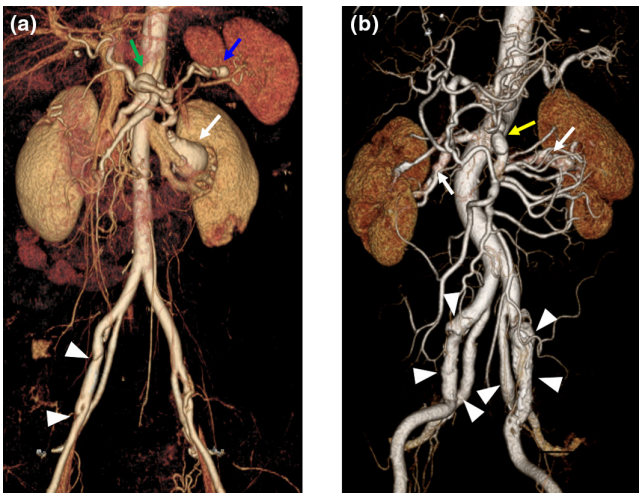


FIGURE 1 CT angiography of Patient 1 and Patient 2. CT angiography shows multiple aneurysms and/or dissections of the renal artery (white arrow), celiac artery (green arrow), splenic artery (blue arrow), superior mesenteric artery (yellow arrow), and iliac artery (arrowhead) in patient 1 (a) and patient 2 (b).

3.2 | Molecular studies

In September 2016, a targeted gene panel was performed on P1, which did not detect any pathogenic sequence variation, but it did show significantly lower coverage in exon 27 of the *COL3A1* compared to other exons, indicating a possible deletion of the exon (as shown in Figure 2a). Sanger sequencing of cDNA using cultured skin fibroblasts confirmed the exon 27 deletion, and subsequent Sanger sequencing of genomic DNA clarified the deletion margins, which were identified as c.1870-109_1923+10del, r.1870_1923del, p.(P625_G642del) (as shown in Figure 3a). As exon 27 is located within a triple-helical domain and deletion of this exon may affect the number of glycine residues, it is possible that this could impact protein function. Since the deletion was not found in the population database (gnomAD), it was classified as a likely pathogenic variant based on PVS1_strong, PM2_supporting, and PP4 criteria.

For P2, in November 2021, WGS identified a novel variant in intron 16 of the *COL3A1* (c.1149+6T>C, heterozygous), which had not been previously reported in the literature or the population database (gnomAD). The variant showed a relatively low score of 0.13 Δ on SpliceAI, which suggested a low likelihood of splicing alteration. The interpretations of deleteriousness for this variant were conflicting according to different in-silico splice-site predictors: high score on TraP-score v3 (0.905) and dbscSNV ADA (0.9946), but uncertain score on MaxEntScan (3.8389). RNAseq using cultured skin fibroblasts identified the aberrant splicing isoform, of which sequence reads were 1/3 of the normal transcript (Figure 2b). Sanger sequencing of cDNA also confirmed skipping of

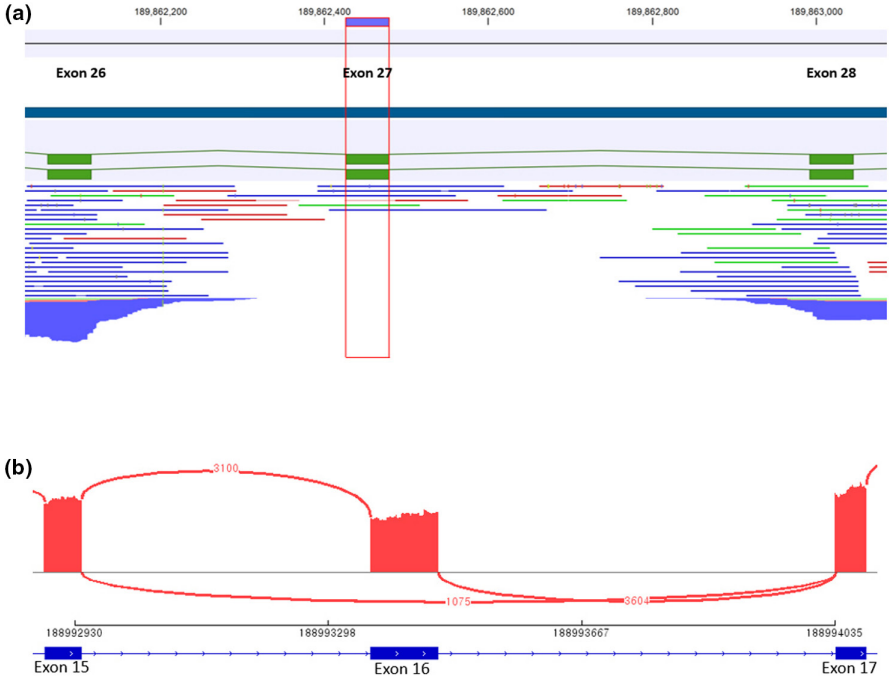
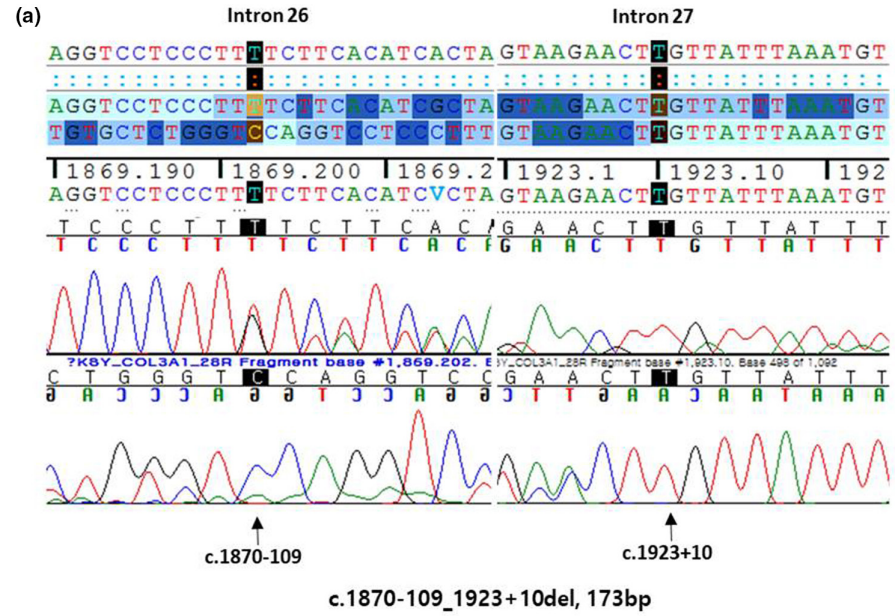


FIGURE 2 Molecular study of the patients. (a) Lower coverage depth of exon 27 in NGS study of Patient 1. (b) Quantitative visualization (Sashimi plot) for *COL3A1* inclusion/exclusion of exon 16 in Patient 2.



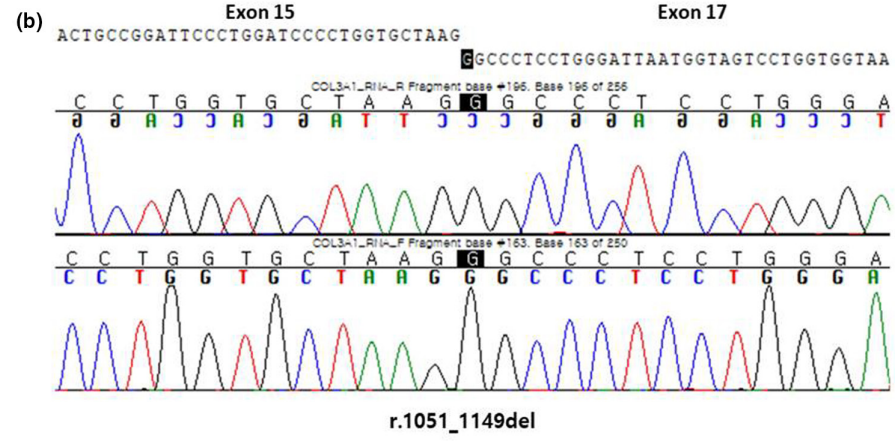


FIGURE 3 Chromatogram and sequence (a) Demarcation of deletion in genomic DNA (NM_000090.4:c.1870-109_1923+10del, 173 bp) in Patient 1. (b) Chromatogram and sequence of mRNA showed an exon 16 deletion (r.1051_1149del) in Patient 2.

exon 16 (r.1051_1149del), which resulted in a deletion of 33 amino acids (p.(E352_G384del)) as predicted by the RNA change (as shown in Figure 3b). As with P1, exon 16 deletion may also affect protein function. Based on these findings, the variant was classified as a likely pathogenic variant according to PVS1_strong, PM2_supporting, and PP4 criteria.

3.3 | 3D modeling through AlphaFold2

The three-dimensional structures were obtained and pLDDT scores calculated for all three products: a normal alpha 1 chain of type III collagen and two different structures resulting from variants in this study. The pLDDT showed a high confidence score in the noncollagenous (NC1) domain, which consists of about 230 residues at the C-terminal, containing mostly helixes and beta-strands (Figure 4a–c; blue color indicates high pLDDT). On the other hand, a low confidence score (as indicated by orange or yellow color) was seen for the triple-helical region that occupies the majority of alpha 1(III) chain. From normal structure, P1 and P2 had 0.6 Å RMSD (45 pruned atom pairs) and 1.2 Å RMSD (16 pruned atom pairs), respectively.

4 | DISCUSSION

We have identified two novel likely pathogenic variants in *COL3A1*; an exon 27 deletion (c.1870-109_1923+10del) and an intronic variant (c.1149+6T>C), which were not detected by Sanger sequencing. Both variants led to in-frame deletions within the collagen type III triple-helix. In P1, the exon 27 deletion was identified through targeted gene panel sequencing using copy-number variation detection tools (Moreno-Cabrera et al., 2020), and RNA-level confirmation and gap PCR with direct sequencing were used to determine the deletion margins, as commercialized multiplex ligation-dependent probe amplification probes (SALSA MLPA Probemix P155-E1 EDS, MRC Holland) do not cover all exons of *COL3A1*. RNA studies are recommended in cultured skin fibroblasts (1932 transcripts per million (TPM)) since *COL3A1* RNA expression is low in whole blood (0.4798 TPM) (Aguet et al., 2020). In P2, although the SpliceAI Δ score of 0.13 for the c.1149+6T>C variant represented a lower probability of splice-altering compared to an adjacent variant, c.1149+5G>A (Pepin et al., 2014), is 0.54. RNA studies and whole-transcriptome sequencing confirmed that both variants had the same RNA sequence, indicating exon 16 skipping (r.1051_1149del, p.(E352_G384del)) (Figure 2b).

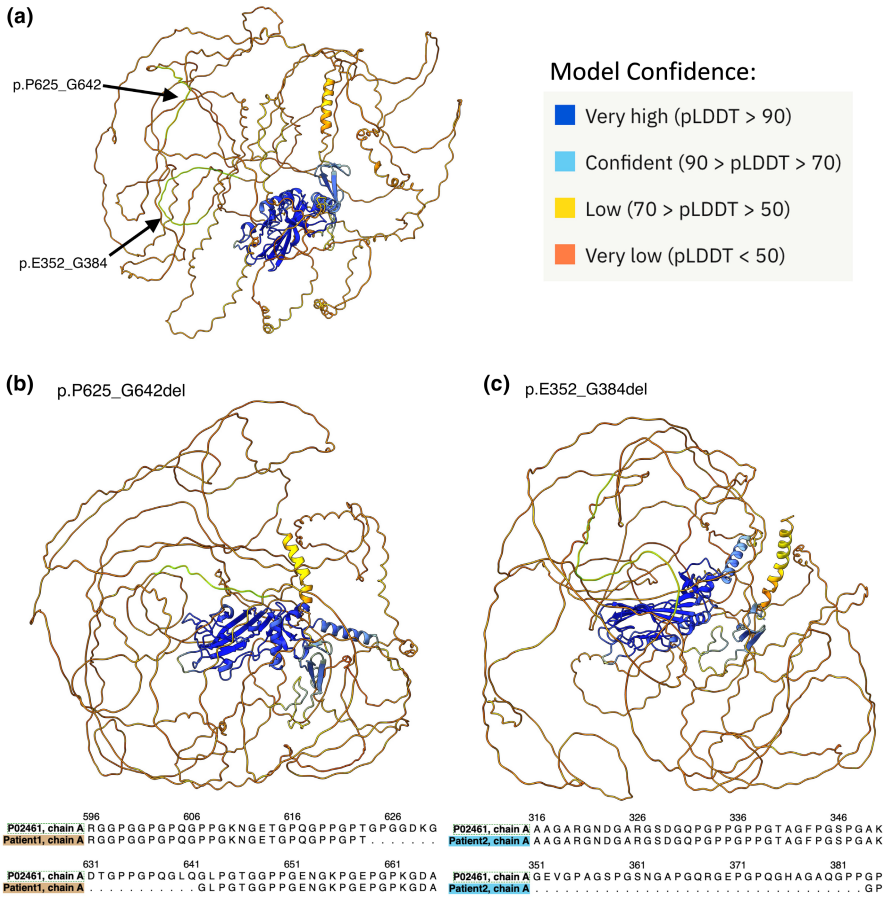


FIGURE 4 Three-dimensional modeling from AlphaFold2 with per-residue confidence score (pLDDT) (a) Normal type III collagen protein (UniProt P02461) from the AlphaFold Protein Structure Database; the variant region of Patient 1 and Patient 2 were filled with green color. (b, c) Predicted structures and comparative sequence of normal from Patient 1 (b) and Patient 2 (c).

We assessed the efficacy of SpliceAI in predicting splicing in *COL3A1*, given that splice-site variants are crucial genetic alterations in this gene. We analyzed 721 variants from the Human Gene Mutation Database 2022.1 (HGMD) (Stenson et al., 2020), with 76% (547/721) being located in exons and the remaining 24% (174/721) being splicing variants. While most of the splicing mutations were in the canonical splice site (122/174), 30% of the splicing mutations were noncanonical intronic variants (52/174).

We categorized the 721 variants into three groups based on their location and analyzed the Δ scores: (i) Canonical splice-site group, (ii) Noncanonical intron group, and (iii) Exon group (Figure 5a). As expected, the distribution of Δ scores was highest for the Canonical group, followed by the Intron group and Exon group. We observed a Δ score <0.2 for one canonical site variant (c.1815+2T>C) (Haer-Wigman et al., 2019) and three noncanonical intronic variants (c.583-3T>C (Stranneheim et al., 2021), c.1348-3C>T (Chen et al., 2021), and c.1815+5G>A (Wai et al., 2020)), the latter variant of which had a normal splicing result through blood RNA analysis. We evaluated performances of SpliceAI in the intronic region. Of 160 variants classified as disease-causing mutations by HGMD, 158 variants had Δ scores ≥0.2 (98.8% of sensitivity).

In the Exon group, nine variants had a Δ score ≥0.2, and only two had a Δ score ≥0.9 (c.2195G>T, p.G732V, 0.96; c.2222G>T, p.G741V, 0.90), but there was no proven splicing effect confirmed by RNA studies (Berezowska et al., 2018; Yang et al., 2007). We also analyzed 337,827 variants from Illumina BaseSpace (SpliceAI v1.3) (Figure 5b), and found 1511 noncanonical site variants with a high Δ score (≥0.8), which could indicate a potential splicing variant. Most potential splicing variants were located within 10 bp from exon–intron boundaries, but extension of intronic sequence analysis up to 100 bp could increase the diagnostic yield. One variant (c.3256-43T>G) was confirmed to affect splicing by the acceptor gaining mechanism through RNA study of the affected vEDS patient (p.P1085_G1086insVCVYMTSIQNMFLK) (Pepin et al., 2014). Since the sensitivity of splicing prediction tools is not 100%, RNA study of cultured fibroblasts is recommended if there is a strong clinical suspicion of vEDS with potential splice variants, even if in-silico prediction tools do not strongly suggest a potential splicing effect.

In conclusion, we identified two novel likely pathogenic variants in *COL3A1* in two patients with clinical symptoms consistent with vEDS. Our study highlights the necessity of a comprehensive approach such as targeted NGS panel, WGS, and cDNA sequencing. cDNA sequencing in the present study was useful for the confirmation of exon deletion due to the limited availability of MLPA probes as well as the pathogenicity of a intronic variant with uncertain significance. Although obtaining RNA from patients may be difficult since it requires invasive procedures like skin biopsy due to the scarcity of mRNA expression of *COL3A1* in blood, cDNA sequencing is

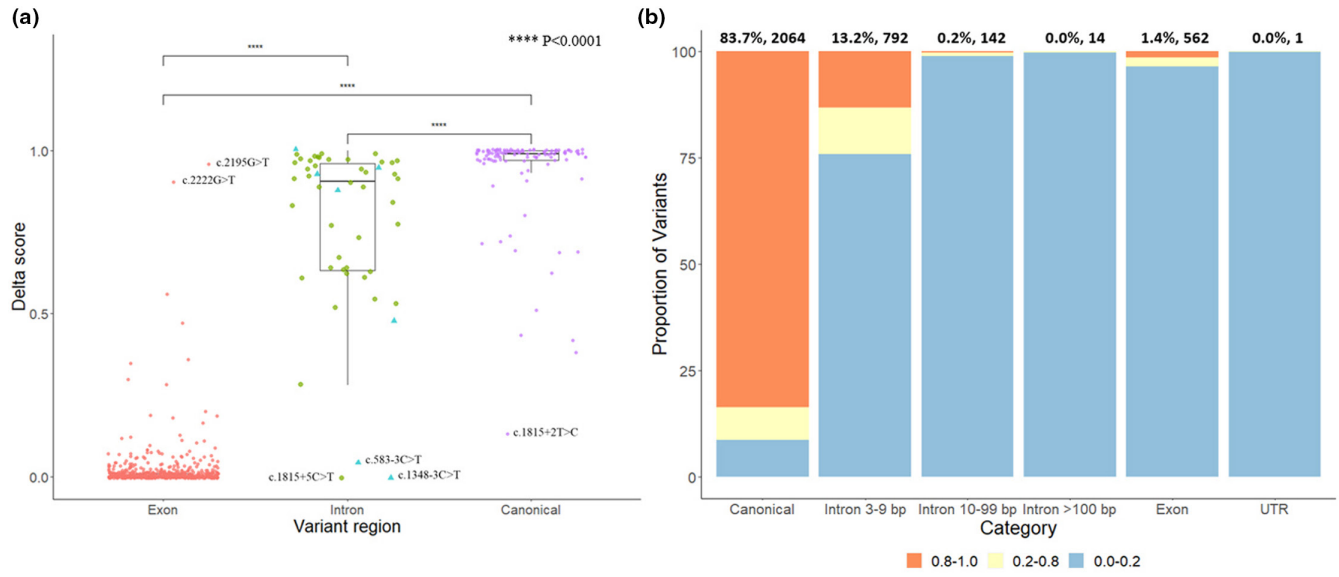


FIGURE 5 Visualization of Δ scores of SpliceAI in *COL3A1* (a) Distribution of Δ scores of SpliceAI in 721 disease-causing variants of *COL3A1* in the Human Gene Mutation Database 2021.4 (HGMD). The number of variants and median Δ scores based on location; Exon: $N = 547$, 0.00 (IQR: 0.00–0.01); Intron: $N = 52$, 0.91 (0.63–0.96); Canonical splice site: $N = 122$, 0.99 (0.97–1.00). The intronic variants were classified into an acceptor site (blue triangle) and a donor site (green circle). (b) Distribution of precalculated Δ scores of all potential *COL3A1* variants ($N = 337{,}827$) from Illumina BaseSpace (v1.3). The proportion and number of variants with Δ score of 0.8 or higher are shown above the stacked bar plot.

helpful for identifying genetically undiagnosed vEDS, especially considering the limited sensitivity of the splicing prediction tool.

AUTHOR CONTRIBUTIONS

Ja-Hyun Jang and Duk-Kyung Kim contributed to the conception and design of the study; Won Young Heo and Ja-Hyun Jang drafted the manuscript; Shin Yi Jang, Taek Kyu Park and Duk-Kyung Kim were involved in clinical evaluation; Won Young Heo, Chang-Seok Ki, Jong-Won Kim, and Ja-Hyun Jang interpreted the genomic results; Won Young Heo performed the statistical analysis; and Ja-Hyun Jang and Duk-Kyung Kim supervised the study. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the Institutional Review Board of Samsung Medical Center (IRB File No. 2016-11-039). Written informed consent was obtained from each patient prior to genetic testing.

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