

# Ehlers-Danlos syndrome type IV is associated with a novel G984R COL3A1 mutation

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**Abstract.** Ehlers-Danlos syndrome type IV is an autosomal dominant connective tissue disease. Mutations in COL3A1 have been identified to underlie this disease; however, to the best of our knowledge, no COL3A1 mutations have been reported in Ehlers-Danlos syndrome type IV patients with an ascending aortic aneurysm. In order to develop further understanding of COL3A1 mutations, an Ehlers-Danlos syndrome type IV patient diagnosed with an ascending aortic aneurysm and a familial history of sudden mortality was analyzed. Genomic DNA was isolated from the peripheral blood of the patient and his family members. All coding exons of eight aneurysm-related genes (FBN1, TGFBR1, TGFBR 2, MYH11, ACTA2, SLC2A10, NOTCH1 and COL3A1) were amplified using polymerase chain reaction (PCR). The PCR products were sequenced with the ABI 3100 Genetic Analyzer, and a mutation was predicted and identified using Polyphen-2, SIFT and Mutation Taster. The novel mutation was identified as c.2950G>A in COL3A1, which results in p.G984R. All three programs predicted this mutation to be deleterious to the protein function. The novel mutation identified in this study is potentially responsible for Ehlers-Danlos syndrome type IV in this patient, and expands the spectrum of COL3A1 mutations.

## Introduction

Ehlers-Danlos syndrome type IV (EDS IV; OMIM# 130050), also known as Ehlers-Danlos syndrome, vascular type, is an autosomal dominant disorder characterized by abnormal type III collagen, thin translucent skin, arterial/intestinal/uterine fragility, extensive bruising and a characteristic facial appearance (1). The prevalence of EDS is estimated to

range from 1/10,000 to 1/25,000 and EDS IV represents 5-10% of cases (2). As a result of spontaneous vascular/intestinal rupture, the overall life expectancy of patients with EDS IV is 48 years (3). Mutations in COL3A1, the gene that encodes type III collagen, have been associated with EDS IV. By either sequencing COL3A1 from blood samples or using biochemical tests, detection of abnormal type III collagen can identify >95% of individuals with EDS IV (4). The two methods are used to aid in the clinical diagnosis of EDS IV (4).

Vascular complications are common in patients with EDS IV, while aortic aneurysm is a rare condition. Only five mutations of COL3A1 [c.505C>T (5), c.907G>A (6), c.2356G>A (7), c.2633G>A (8) and c.1815+5G>A (9)] have been reported to result in aortic aneurysm, and no variants associated with ascending aortic aneurysm have previously been reported.

The aim of the current study was to identify the first COL3A1 mutation associated with ascending aortic aneurysm in an EDS IV patient. In addition, this study summarizes all the previously reported variants of COL3A1.

## Patients and methods

*Ethical approval and informed consent.* The current study has been approved by, and conducted according to the instructions of the Ethics Committee of Central South University (Changsha, China). The patient and the other members of his family provided written informed consent for publication.

*Patient presentation.* A 38-year-old male was admitted to the Department of Cardiothoracic Surgery, The Second Xiangya Hospital (Changsha, China) due to detection of an aortic aneurysm combined with aortic incompetence by echocardiography in medical examination. Physical examination revealed that the patient had no notable symptoms except for thin translucent skin (Fig. 1C) and a soft diastolic murmur of the aortic valve. There were no associated events in the patient's past medical history. The results of an ECG and a chest x-ray were normal. His father, grandfather and one of his four uncles suffered from sudden mortality (Fig. 2A) due to aortic rupture. No other family members, including his son, had any similar conditions or experiences.

*Imaging examination.* Computerized tomographic angiography (CTA) of the heart and thoracic aorta was performed

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in the Second Xiangya Hospital of Central South University (Changsha, China).

**Molecular genetic analysis.** Genomic DNA was prepared from the peripheral blood of the patient and his family members using a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) using the QIAcube automated DNA extraction system (Qiagen, Hilden, Germany). A total of 20  $\mu$ l QIAGEN Proteinase and 200  $\mu$ l sample (whole blood in phosphate-buffered saline; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added into a 1.5 ml microcentrifuge tube. Buffer AL (200  $\mu$ l; in DNeasy Blood & Tissue Kit) was then added and the sample was mixed by pulse vortexing (HYQ-2121A; Crystal Technology & Industries, Inc, Dallas, TX, USA) for 15 sec. The sample was then incubated at 56°C for 10 min and centrifuged at 8,000 x g for 1 min to remove drops from the inside of the lid. Subsequently, 200  $\mu$ l ethanol (96-100%; Sigma-Aldrich, St. Louis, MO, USA) was added to the sample and it was pulse vortexed again for 15 sec then centrifuged at 6,000 x g for 1 min. The sample was then added to the the QIAamp Mini spin column without wetting the rim, it was centrifuged at 6000 x g for 1 min, 500  $\mu$ l Buffer AW1 was added and it was centrifuged again at 6000 x g for 1 min. A total of 500  $\mu$ l Buffer AW2 was then added without wetting the rim, and the sample was centrifuged twice at 20,000 x g for 3 min. A total of 200  $\mu$ l Buffer AE was then added and the sample was incubated at room temperature for 1 min, and subsequently centrifuged at 6000 x g for 1 min. All buffers were from the DNeasy Blood & Tissue kit.

All coding exons of FBN1 (NM\_000138.4), TGFBR1 (NM\_004612.2), TGFBR 2 (NM\_003242.5), MYH11 (NM\_022844.2), ACTA2 (NM\_001141945.1), SLC2A10 (NM\_030777.3), NOTCH1 (NM\_017617.3) and COL3A1 (NM\_000090.3) were amplified by the polymerase chain reaction (PCR) System 9700 (Applied Biosystems Life Technologies, Foster City, CA, USA). The amplification used 25  $\mu$ l reaction mixture, which consisted of 0.3 mM deoxyribonucleotide triphosphates (BioTeke Corporation, Beijing, China), 1X PCR buffer (10 mM Tris-hydrochloric acid pH 9.0, 50 mM potassium chloride, 0.1% Triton X-100 and 0.01% w/v gelatin; BioTeke Corporation), 2.0 mM magnesium chloride (BioTeke Corporation), 0.5  $\mu$ M each primer (forward and reverse; created by Beijing Genomics Institution, Beijing, China), 1.5 U Taq polymerase and 50 ng genomic DNA. The thermal cycling conditions were as follows: Initial denaturation at 95°C for 4 min, 35 cycles of amplification consisting of denaturation at 95°C for 1 min, primer annealing at X°C for 30 sec and primer extension at 72°C for 1 min. A final extension step was performed at 72°C for 7 min. The PCR products were sequenced by the ABI 3100 Genetic Analyzer (Applied Biosystems Life Technologies). The results of sequencing were presented using Chromas software, version 2.1.1 ([http://technelysium.com.au/?page\\_id=13](http://technelysium.com.au/?page_id=13)). The overlap peaks frequently indicated variants and according to the base sequence around the variant, the location of the variant in the gene was identified. Continuous overlap peaks indicated frameshift variants and single overlap peak may have indicated nonsense, synonymous or missense variants. The identified mutation was searched in Pubmed (<http://www.ncbi.nlm.nih.gov/pubmed/>), Google scholar (<http://scholar.google.com/>), dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) and the Exome Sequencing Project (ESP; <http://evs.gs.washington.edu/>

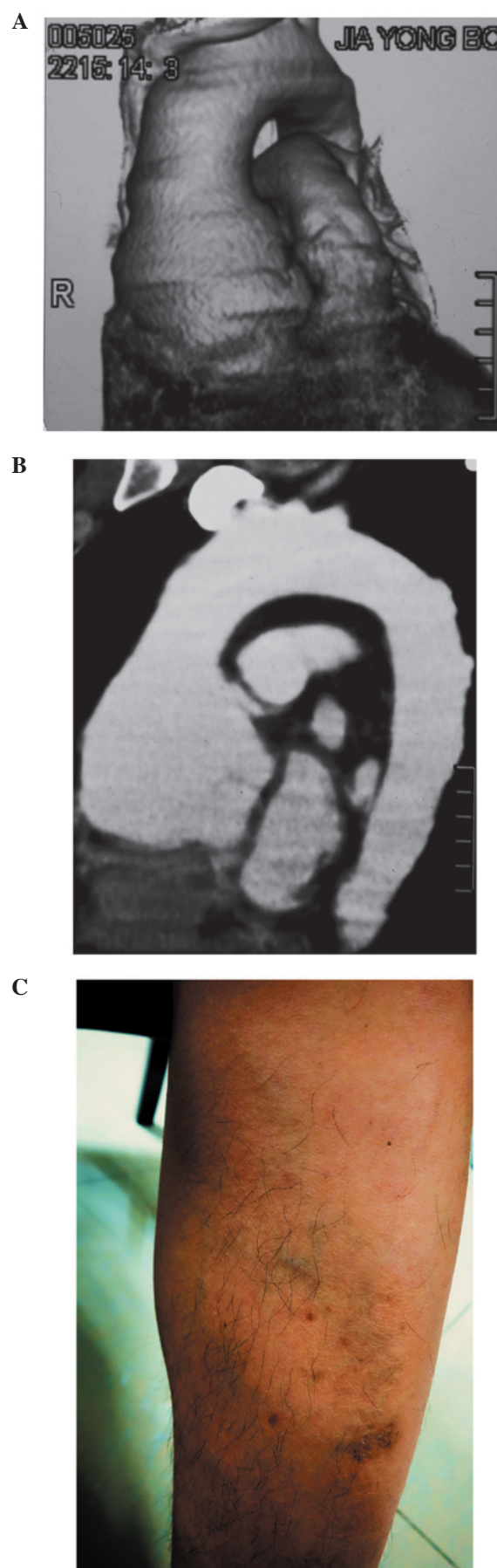


Figure 1. Two major criteria of the patient. (A) Computerised tomography (CT) reconstruction image of the ascending aorta and heart; (B) CT angiography of the ascending aorta and heart; (C) Veins visible under the thin translucent skin on the front of the left calf.

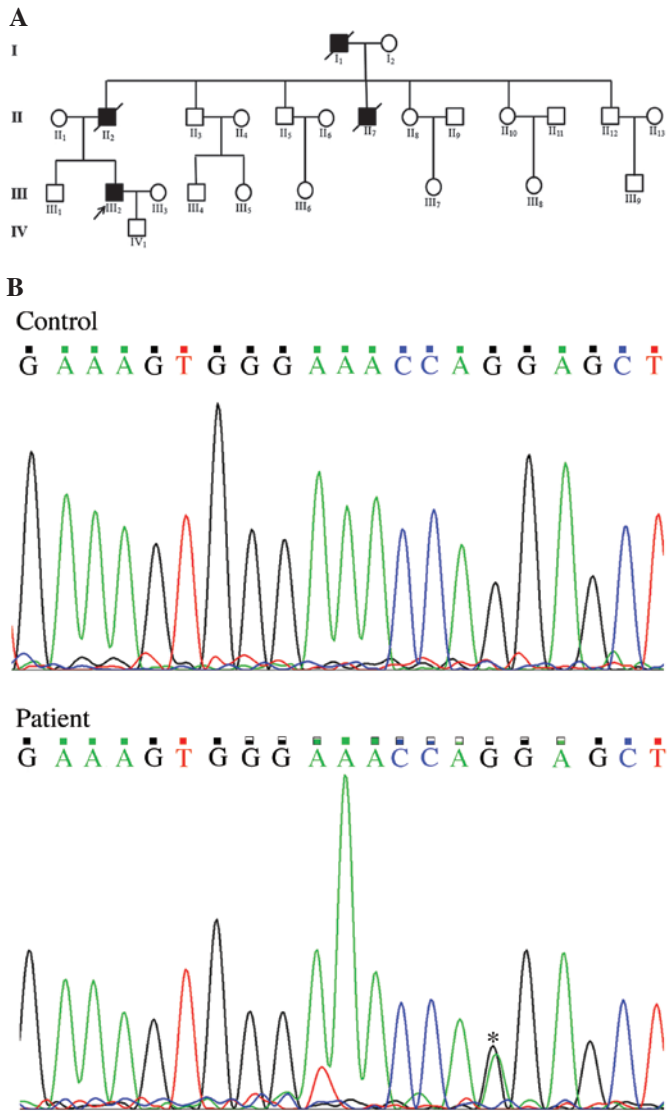


Figure 2. Patients familial history and mutation sequencing. (A) Pedigree of the family affected with EDS IV. Family members are identified by generations and numbers. Squares indicate males; circles, females; closed symbols, affected members; open symbols, unaffected members; arrow, proband; (B) Sequencing results of the COL3A1 mutation. \*Sequence chromatogram indicates a G to A transition of nucleotide 2950.

EVS/) (10), and mutations were only considered as candidate mutations if they had never been previously reported in humans, and were absent from dbSNP and ESP. Three online protein prediction software packages, Polyphen-2 (polymorphism phenotyping) (11), SIFT (Sorting Intolerant From Tolerant) (12) and Mutation Taster (13) were used to evaluate the effect of the novel mutations. In the current study, frameshift and nonsense variants were not observed and G984R in COL3A1 was the only candidate mutation. All of the three protein prediction software packages considered this variant to be damaging and it was located in a conserved region in COL3A1. This mutation also led to the replacement of one glycine in the (Gly-Xaa-Yaa) n repeat of the collagen triple helix, which was considered pathogenic in various previous studies (3,14-21). Thus, G984R in COL3A1 was identified as a pathogenic mutation in the current study. The amino acid sequence of human COL3A1 from positions 977 to 996 was compared with similar regions

Table I. Overview of six mutations associated with aortic aneurysm.

Mutation	Exon	Mutation effect	Protein change	dbSNP	ESP	Mutation Taster	SIFT	Polyphen-2	Reference	Disease
c.505C>T	6	Missense	L169F	rs111391222	-	Polymorphism (score: 0.60)	Tolerated (score 0.731)	-	22	AAA
c.907G>A	14	Missense	G303R	rs121912919	+	Disease causing (score: 3.41)	Damaging (score 0.000)	-	23	AAA
c.2356G>A	35	Missense	G786R	rs113485686	-	Disease causing (score: 3.41)	Damaging (score 0.000)	-	10	TAA/AAA
c.2633G>A	39	Missense	R878H	-	-	Disease causing (score: 0.79)	Damaging (score 0.600)	-	11	TAA
c.1815+5G>A	-	Splice site	-	rs146652498	-	-	-	-	12	TAA
c.2950G>A	42	Missense	G984R	-	-	Disease causing (score: 3.41)	Damaging (score 0.000)	Probably damaging (score 1.000)	Present study	TAA

AAA, abdominal aneurysm; TAA, thoracic aorta aneurysm; ESP, exome sequencing project; SIFT, sorting intolerant from tolerant; Polyphen-2, polymorphism phenotyping).



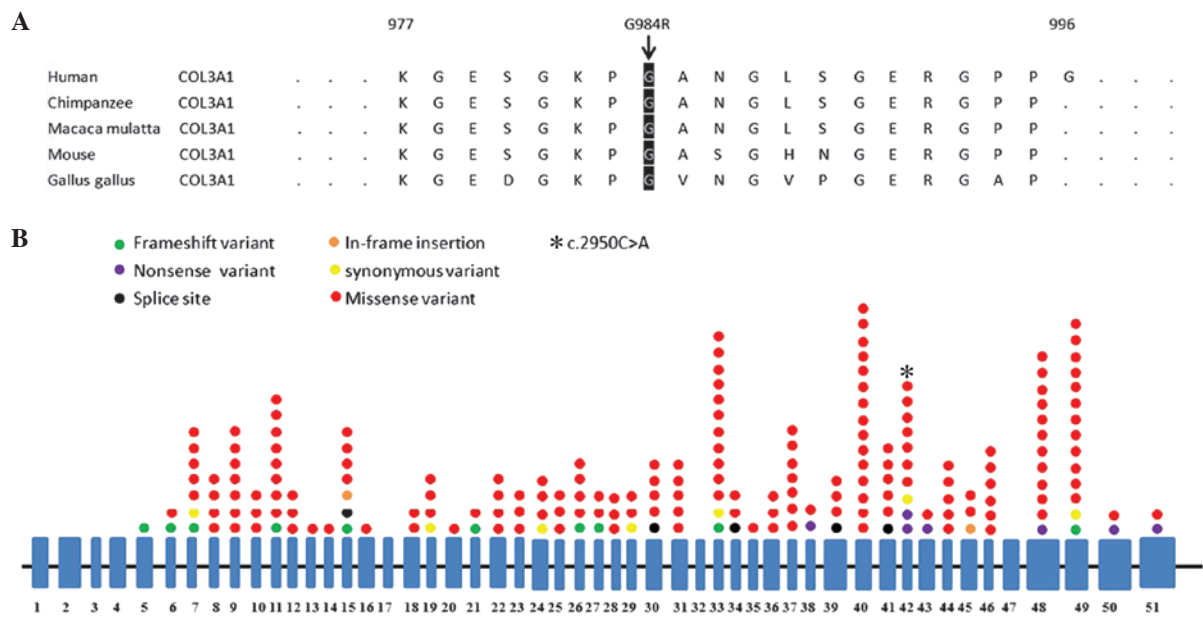


Figure 3. COLA1 protein and mutations in the COLA1 gene. (A) Alignment of multiple COL3A1 protein sequences across species. The G984 affected amino acid is located in the conserved amino acid region in different mammals (from Ensembl). The black column shows the G984 site. (B) Distribution of the 204 reported variants and the novel mutation in the exons of COL3A1. Circles with different colors represent variants with a matched mutation effect. \*Novel mutation c.2950C>A identified in this article. Blue rectangles represent the exons of COL3A1 and the numbers under them denote the exon order.

in the chimpanzee, *Macaca mulatta*, mouse and *Gallus gallus* using Ensembl (<http://asia.ensembl.org/index.html>).

**Variants summary.** The variants previously reported in COL3A1 were searched in Pubmed in order to identify mutations associated with aortic aneurysm. All of the variants were matched in dbSNP and ESP to identify variants previously associated with disease in healthy populations. Mutations leading to aortic aneurysm were evaluated by Polyphen-2, SIFT and Mutation Taster.

Results

**Result of computerized tomographic angiography (CTA).** The images from the CTA revealed that the root of the ascending aorta was dilated and had formed a sacculated aortic aneurysm (Fig. 1A and B) with a diameter of >7 cm.

**Novel mutation and functional prediction.** Sequencing the PCR products of the eight aortic aneurysm-related genes, mentioned in Patients and methods, identified the nucleotide A instead of G at position 2950 of the coding sequence (c.2950G>A, Fig. 2B) in COL3A1, leading to an amino acid change from glycine to arginine at position 984 of the protein (p.G984R). This c.2950G>A mutation was only present in the patient and not in his family members or our control cohorts as well as dbSNP and ESP. A search of Pubmed and Google Scholar revealed that this mutation has not been previously reported. The prediction results of Polyphen-2, SIFT and Mutation Taster were probably damaging, damaging and disease causing, respectively (Table I). Alignment of multiple COL3A1 protein sequences across species showed the G984 affected amino acid is located in the conserved amino acid region in different mammals (Fig. 3A).

**Variants review.** A literature review revealed that 268 variants, including 66 variants in the introns and 204 variants in the exons

Table II. Diagnostic criteria for EDS IV caused by abnormal type III collagen.

Major criteria	Minor criteria
Thin translucent skin	Acrogeria
Arterial/intestinal/uterine fragility or rupture	Hypermobility of small joints
Extensive bruising	Tendon and muscle rupture
Characteristic facial appearance	Clubfoot
	Early-onset varicose veins
	Pneumothorax
	Gingival recession
	Positive family history/sudden death
	Arteriovenous, carotid-cavernous sinus fistula

(Fig. 3B), were identified in COL3A1. Of the 268 mutations, 257 variants were previously reported to be pathogenic. In total, 1.9% (5/257) of the mutations were found in the ESP. Of the exon variants, 159 of 204 variants lead to the replacement of one glycine in the (Gly-Xaa-Yaa)<sub>n</sub> repeat of the collagen triple helix. All of the 159 missense variants have been previously reported as pathogenic and were not found in the ESP database. In total, five mutations [c.505C>T (5), c.907G>A (6), c.2356G>A (7), c.2633G>A (8) and c.1815+5G>A (9)] were reported as causes of aortic aneurysm. The results of functional prediction by Polyphen-2, SIFT and Mutation Taster are listed in Table I.

Discussion

Due to vascular/intestinal rupture, EDS IV is the most severe type among the different types of EDS. According to the

Villefranche nosology (1) (Table II), the combination of two major criteria is highly specific to this disease. The patient described in this study had an aortic aneurysm combined with thin translucent skin. Hence, his clinical diagnosis was EDS IV. The molecular genetic analysis revealed a mutation in COL3A1, which confirmed the diagnosis. In order to avoid aneurysmal rupture, the ascending aorta of the patient was replaced with an artificial aorta and three weeks later, following recovery, the patient was discharged from hospital.

Since Richards *et al* (24) initially described a variant of COL3A1 in EDS IV patients, 268 variants, including 66 intron variants and 204 exon variants (Fig. 3B), were identified in COL3A1. Of the exon variants, 159/204 variants led to the replacement of one glycine in the (Gly-Xaa-Yaa)<sub>n</sub> repeat of the collagen triple helix. All of the 159 missense variants have been previously reported as pathogenic and were not present in the ESP database. In total, five mutations [c.505C>T (5), c.907G>A (6), c.2356G>A (7), c.2633G>A (8) and c.1815+5G>A (9)] were reported as causes of aortic aneurysm. The results of functional prediction by Polyphen-2, SIFT and Mutation Taster are listed in Table I. However, they were all found in the ESP database. Whole exome data from the National Heart, Lung, and Blood Institute Grand Opportunity (NHLBI GO) ESP provided sequencing results of all protein-coding regions in 6,503 individuals without heart disease (12). Any reported pathogenic mutation identified in the ESP database may be false positive. This indicates that these five EDS IV-associated mutations may be SNPs or non-pathogenic.

To the best of our knowledge, the c.2950G>A mutation identified in this study has not been previously reported, and is not present in the ESP database and control cohorts. This mutation led to an amino acid change from glycine to arginine at position 984 of the protein (p.G984R), which may damage the formation of the triple helix. The prediction results of the Polyphen-2, SIFT and Mutation Taster programs were that the mutation may be damage or disease causing. A review of the literature revealed that all the missense variants leading to the replacement of one glycine in the (Gly-Xaa-Yaa)<sub>n</sub> repeat of the collagen triple helix were previously reported to be pathogenic. Hence, c.2950G>A is considered to be the cause of EDS IV in this patient, which expands the spectrum of COL3A1 mutations and may make a contribution to the genetic counseling of EDS IV in the future.

COL3A1 encodes a procollagen molecule, pro $\alpha$ 1 (III); basic collagen synthesis requires three polypeptide procollagen chains, referred to as  $\alpha$  chains, to be folded tightly into a triple helix (3). Every third amino acid in the protein triple helix is a glycine, and substitution of a glycine slows down formation of the triple helix (25). This may lead to collagen III breakdown or over modification. Collagen III is a major component of the extracellular matrix in skin, blood vessels and a variety of internal organs. Abnormal collagen III may result in thin translucent skin and thin artery walls, which leads to a number of vascular complications, including aneurysm, artery dissection and so on. Vascular complications are commonly observed in EDS IV, while ascending aortic aneurysm is extremely rare. Hetzer *et al* (26) reported an isolated giant ascending aortic aneurysm in an infant with EDS IV. The infant underwent replacement of the ascending aorta and proximal aortic arch.

Genetic analysis was not performed. To the best of our knowledge, this is the only other report of an EDS IV patient with ascending aortic aneurysm, which means the novel mutation identified in our study is the first COL3A1 mutation reported in association with ascending aortic aneurysm.

To date, including the novel mutation identified in this study, only six mutations in COL3A1 have been found to be associated with aortic aneurysms (5-9). With the exception of c.907G>A identified in ESP, the mutations are distributed in different regions of COL3A1, and the correlation between aortic aneurysm and these specific mutations remains unknown. Thus, these associations require further investigation.

In conclusion, the results of this study revealed a novel COL3A1 mutation (p.G984R) in an EDS IV patient with ascending aortic aneurysm, which expands the spectrum of COL3A1 mutations and may aid with the genetic counseling and diagnosis of EDS IV in the future.

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