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Original Article

Analysis of the contribution of 129 candidate genes to thoracic aortic aneurysm or dissection of a mixed cohort of sporadic and familial cases in South China

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Abstract: Thoracic aortic aneurysm or dissection (TAAD) is a group of life-threatening complex diseases after symptomatic onset with genetic heterogeneity accounting for approximately 20% of cases. Previously, we identified 40 rare variants in 11 TAAD-related core genes among 70 TAAD patients by next-generation sequencing. In this study, we further analyzed the variants in the disease-causing genes in 129 cases of sporadic TAAD and 22 familial cases by whole-exome sequencing. A total of 116 variants in 47 TAAD-related genes were identified, 64.7% (75/116) of which occurred in sporadic TAAD without syndromes, and among these genes, *FBN1* was the most common TAAD-related gene. Of the 26.7% (31/116) that were pathogenic or likely pathogenic, almost one third were from sporadic cases without syndromes involving FBN1, *SMAD3, SMAD6, MYH11, TGFBR1, MYLK, LOX and LTBP3*. Interestingly, the novel VUS (variant of uncertain significance) *879Glu in *MCTP2* occurred in two unrelated probands with sporadic acute aortic dissection without a bicuspid aortic valve. Furthermore, more than one variant was detected in 24 patients, and 70.8% (17/24) occurred in sporadic cases. Younger individuals were more likely to carry P/LP (pathogenic or likely pathogenic) variants and harbor more variants. P/LP carriers seem to have a larger aortic diameter, lower D-dimer levels, and a shorter ICU length of stay but longer hospitalization time. In conclusion, we expanded the candidate gene profile of TAAD, especially for sporadic cases without syndromic features. VUSs need further clarification.

Keywords: Thoracic aortic aneurysm or dissection, exome sequencing, *FBN1* gene, sporadic TAAD

Introduction

Thoracic aortic aneurysm or dissection (TAAD) refers to a group of rare underrecognized life-threatening cardiovascular disorders that account for more than 17000 deaths annually in the United States [1] and are the most severe diseases of acute aortic syndrome (AAS). Medial degeneration and increased aortic wall stress are frequently involved in the formation of aortic disease [2]. Common risk factors related to TAAD include hypertension, smoking, trauma, atherosclerosis and pheochromocytoma, which may be more likely to increase aortic wall stress. Furthermore, genetic background is a specific risk factor for TAAD [3]. It has been reported that hereditary connective tissue disorders are closely associated with conditions involving medial degeneration of the aorta, such as Marfan syndrome (MFS) [OMIM: 154700] caused by the FBN1 gene encoding fibrillin-1, Loeys-Dietz syndrome (LDS) [OMIM: 609192] resulting from mutations in transforming growth factor beta receptor, the vascular form of Ehlers-Danlos syndrome (v-EDS) [OMIM: 130050] due to mutation of the *COL3A1* gene, encoding type III collagen, and Shprintzen-Goldberg syndrome (SGS) [OMIM: 182212].

Genetic determinants greatly contribute to the occurrence of aortic disease, especially in TAAD with a family history [4-8]. Causative gene

mutations have also been found in sporadic TAAD. In addition, certain gene mutations, such as those in TGFBR and COL genes, could also be detected in nonsyndromic hereditary TAAD [9, 10]. To date, many clinicians have mainly studied syndromic and nonsyndromic hereditary TAAD cases, 20% of which were attributed to gene mutations, but the majority of patients with emergency hospitalization for acute aortic events have sporadic aneurysms or dissections without syndromic features. Lack of similar family history, abrupt asymptomatic onset, younger age at diagnosis, complex genetic heterogeneity and phenotypic diversity lead to the elaboration of the pathogenesis of sporadic TAAD remaining to be uncovered. Other than the common causative core genes FBN1, ACTA2, COL3A1, MYH11, SMAD3, TGFB2, TGFBR1, TGFBR2, MYLK and LOX, few studies have focused on other candidate genes [11-13]. With the application of next-generation sequencing (NGS) [11, 14], it is possible to identify the genes causing familial or sporadic TAAD for molecular diagnosis and better prevention.

Herein, we analyzed TAAD- related genes by whole-exome sequencing (WES) in a clinically mixed cohort of sporadic and familial TAAD patients from South China, especially nonsyndromic patients without a family history.

Materials and methods

Patients

Based on the approval of the Research Ethics Committee, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, we collected the clinical information of one hundred fifty-one consecutive probands with TAAD and seventy-one relatives between 2019 and 2020 in the Department of Cardiac Surgery, Guangdong Cardiovascular Institute, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangdong Province, China. The mixed study cohort included familial and sporadic cases of TAAD-related diseases. Familial TAAD was defined as cases in which more than one patient with TAAD existed in the family pedigree. Sporadic TAAD was defined as cases in which the probands was the first to suffer from aortic disease in their own family, regardless of the presence of abnormalities in other organs. Patients with suspected MFS were assessed according to the Revised Ghent Nosology [15]. Plain and enhanced spiral CT scans of the entire aorta and Doppler echocardiographic examination were performed on patients who underwent surgery for cardiovascular system diseases, and cardiac ultrasonography was performed on the rest of the patients. The diameter of the aortic root and ascending aorta were measured by echocardiography and CT. At the same time, medical records, including age of onset, sex, tobacco use, alcohol use, hypertension history, surgical history of cardiovascular system disease, and other conditions of cardiovascular diseases, were also collected. Blood samples were obtained from probands and their relatives when possible. Written specific informed consent was obtained from all participants of this study.

Exome sequencing

DNA extraction: In our cohort, genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) from 119 patients using Magbead Blood DNA Kit (CWbiotech), and the remaining DNA was extracted with the Solpure Blood DNA kit (Magen) according to the manufacturer's instructions.

Target capture and sequencing: Libraries were constructed according to the instructions of the manufacturer (Fast Library Prep Kit, iGeneTech) or prepared following the Illumina library preparation protocol. Briefly, 200 ng genomic DNA from 119 patients was sheared using Biorupter (Diagenode, Belgium) to obtain 150-200 bp fragments, followed by end repair, adaptor ligation and PCR amplification. Whole exons were captured from the sequencing libraries using the AIExome Enrichment Kit V1 (iGeneTech) and sequenced on the NovaSeq 6000 platform (Illumina) to obtain 150 base paired-end reads with at least 100X coverage. The other part of genomic DNA was fragmented by a Q800R Sonicator (Qsonica) to generate 300-500 bp insert fragments. Custom-designed NimbleGen SeqCap probes (Roche NimbleGen, Madison, Wis) were used for in-solution hybridization to enrich target sequences. Enriched DNA samples were indexed and sequenced on a NextSeq500 sequencer (Illumina, San Diego, Calif) with 100-150 cycles of single-end reads, according to the manufacturer's protocols.

Variant annotation and interpretation: Primary data were obtained in the Fastq format after

image analysis, and base calling was conducted using the Illumina Pipeline. Sequence alignment was performed using a Burrows-Wheeler algorithm, BWA-mem and variant calling were performed using Genome Analysis Tool Kit (GATK v4) best practices (https://software.broadinstitute.org/gatk/bestpractices/) from the Broad Institute. Sequencing reads were mapped to the reference human genome version hg19 (2009-02 release, http://genome.ucsc.edu/). Nucleotide changes observed in aligned reads were called and reviewed by using NextGENe software (SoftGenetics, State College, Pa). In addition to the detection of deleterious mutations and novel single nucleotide variants, a coverage- based algorithm developed in-house, eCNVscan, was used to detect large exonic deletions and duplications. The normalized coverage depth of each exon of a test sample was compared with the mean coverage of the same exon in the reference file, to detect copy number variants (CNVs).

Sequence variants were annotated using population and literature databases including 1000 Genomes (http://www.1000genomes.org/), dbSNP, GnomAD (http://gnomad.broadinstitute.org/), Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/), HGMD and Online Mendelian Inheritance in man (OMIM, http://omim.org/). Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/www/SIFT_BLink_submit.html) and Polymorphism Phenotyping version 2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/) software were used to analyze the structure of the protein, predict the conservation and function domains and perform multiple sequence alignment. Variant interpretation was manipulated according to the American College of Medical Genetics (ACMG) guidelines [16].

We focused on 129 TAAD-related candidate genes in the literature [17-20] and database (https://hpo.jax.org/app/). The gene list is shown in Table S1.

Sanger sequencing: Sanger sequencing was performed for 71 relatives based on the consent of some patients.

Statistical analysis

Significant differences in normally distributed continuous variables were detected by one-way ANOVA, and those in nonnormally distributed variables in multiple groups were estimated by the Kruskal-Wallis test. Fisher's exact test was used for the assessment of categorical variables between different groups. Spearman correlation was used to compare the percentages of rank variables. *P* values less than 0.05 were considered statistically significant (two-sided). All calculations were performed using SPSS 24.0 software.

Results

Patient clinical data

One hundred fifty-one consecutive patients from South China were collected in our cohort, including 129 sporadic and 22 familial TAAD patients. Males composed 80.1% (121) of the cohort, with 32.2% (39) having a history of smoking and 13.2% (16) having a history of alcohol use. Eighty-eight (58.3%) patients had hypertension; of these patients, 84.1% (74) were male, and only 15.9% were female. Thoracic aortic dissection (consisting of 108 Stanford A dissections and 14 Stanford B dissections) accounted for 80.8% patients, while thoracic aortic aneurysm accounted for 15.9% (24) of patients. Of 142 TAAD patients underwent cardiovascular surgery, 9 of 30 suspected MFS did not undergo surgery because they did not meet the surgical criteria, and 5 of them did not have aortic dilation or dissection but had a distinct family history or obvious manifestations of multiple systems. In particular, one pregnant woman suffering from Stanford type A aortic dissection at the age of twenty-eight, and who underwent complex surgery for aortic repair after delivery was also included. Notably, the average age at diagnosis of sporadic TAAD was older (48.5 years, range: 2 months-76 years) than that of familial TAAD (36.0 years, range: 19-50 years). All three patients under the age of eighteen were sporadic cases. In familial cases, the mean maximum aortic dilation diameter (5.9 cm) was larger than that in cases without any family history (5.0 cm), as expected. Clinical characteristics are shown in **Table 1**.

Variants of causative genes of TAAD

In the mixed cohort, we identified 116 variants in 47 TAAD-related genes in 94 (62.3%) subjects (**Figure 1**). Of the 73.3% (85/116) that

Table 1. Patient clinical data of the cohort

Clinical characteristics of the cohort	N (%)
Number	151 (100)
Male	121 (80.1)
Female	30 (19.9)
Age, Mean	46.7
Min	2 M
Max	76 Y
<40	43 (28.5)
40-60	82 (54.3)
>60	26 (17.2)
Tobacco use	39 (25.8)
Alcohol use	16 (10.6)
Hypertension	88 (58.3)
Family history	22 (14.6)
Undergone Aortic Surgery	142 (94.0)
Thoracic AD	122 (80.8)
Stanford A	108 (88.5)
Stanford B	14 (11.5)
TAA	24 (15.9)
Suspected MFS	36 (23.8)
BAV	12 (7.9)
Aortic Root Size	
Maximum Aortic Diameter (cm), Median	4.8
Min	1.2
Max	12.3
Other CV	37 (24.5)

AD: aortic dissection, TAA: thoracic aortic aneurysm, MFS: Marfan syndrome, BAV: Bicuspid aortic valve, CV: cardiovascular, Other CV: includes coronary artery disease, atherosclerosis of thoracic aorta, abdominal aorta, arteria cervicalis, aortic regurgitation, mitral valve prolapse or congenital heart disease or arrhythmia, M: month, Y: year.

were variants of uncertain significance (VUS), 26.7% (31/116) were pathogenic or likely pathogenic (P/LP), including *FBN1, MYH11, SMAD3, TGFBR2, TGFBR1, LOX, B3GAT3, MYLK, SMAD6, and LTBP3* (**Figure 2A**; **Table 2**), 56.0% (65/116) were novel, and three were *de novo*. In contrast to previous reports, the main mutations were concentrated in the following genes: *FBN1* (32, 27.6%), *MYH11* (5, 4.3%), *MCTP2* (4, 3.4%), *SMAD3* (4, 3.4%), *NOTCH3* (3, 2.6%), *COL5A1* (3, 2.6%), *APC* (3, 2.6%), *COL1A2* (3, 2.6%) and *FBN2* (3, 2.6%) (**Figure 1**). Of note, *FBN1* was still the most common causative gene in this study, regardless of family history and syndrome (**Figure 2B**, **2C**); and the only pathogenic gene in familial cases (**Figure 2B**). The total frequency of TAAD in our cohort with disease-causing variants (P/LP) was 22.5% (34/151), which was far greater than the prevalence of deleterious mutations (3.9%, 4/102) in 102 patients with TAAD [14]. P/LP variants of sporadic TAAD were slightly more numerous than the P/LP variants of familial TAAD (17 vs 15, **Figure 2B**), and Arg1125Ter in *FBN1* was identified in both unrelated probands from the aforementioned two cohorts. More than one variant was identified in 24 patients, 17 with sporadic TAAD (Table S2) and 7 with familial TAAD (Table S3) (**Figure 3A**). Four harbored three mutations, and one harbored four mutations. Interestingly, only 37.5% (9/24) present with syndrome manifestations. (**Figure 3B**) Of those variants, most were occurred in sporadic TAAD, whereas *FBN1, COL5A1, MYH11* occurred in both two groups (**Figure 3C**).

Sporadic TAAD

A total of 92 variants were identified in sporadic TAAD, the majority of which were VUSs (81.5%). Among the 17 P/LP variants, more than half (10/17, 58.8%) were in nonsyndromic subjects without a family history. Seventy-five (81.5%) variants were found to occur in 114 nonsyndromic TAAD patients without family history, and of these, 13.3% (10/75) were P/LP, which mainly involved *FBN 1* (3, 30.0%), *SMAD3* (1, 10.0%), *SMAD6* (1, 10.0%), *MYH11* (1, 10.0%), *TGFBR1* (1, 10.0%), *MYLK* (1, 10.0%), *LOX* (1, 10.0%) and *LTBP3* (1, 10.0%) (**Table 2**). Among the VUSs, the proportion in *FBN1* was 6.2% (4), *in MCTP2* was 6.2% (4), and in each of the following genes was 4.6% (3): *SMAD3, MYH11, COL1A2 and FBN2*. Moreover, 44.6% of VUSs were predicted as "deleterious" by both SIFT and Polyphen2_HDIV. Interestingly, the novel VUS variant c.2635T>G (*879Glu) in *MCTP2* appeared in two unrelated probands with type A aortic dissection without BAV.

Seventeen mutations were identified in 15 patients with suspected syndromic TAAD without family history, almost half of which were novel, three were *de novo* (two were P/LP in *FBN1* and one was a VUS in *TGFBR2*). Of the patients displaying a phenotype highly suggestive of syndromic TAAD, ten were eventually confirmed as MFS, two were LDS with *TGFBR2* mutations (P and VUS, respectively), one was *B3GAT3*-related syndrome (two compound het-

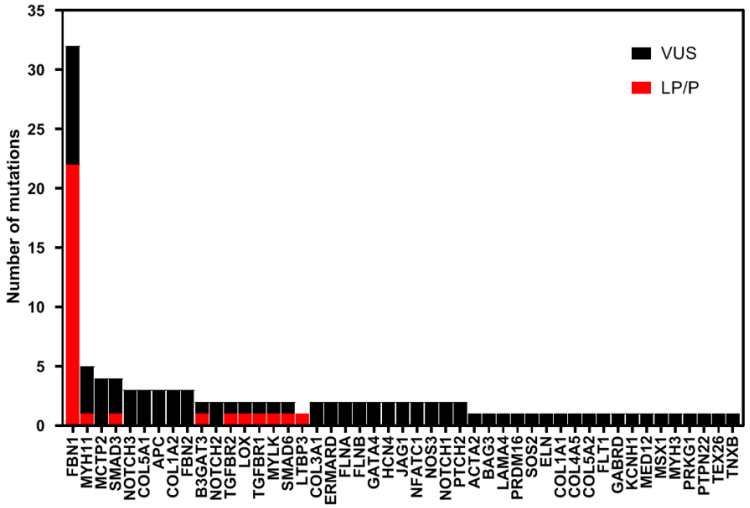

Figure 1. All mutations including pathogenic/likely pathogenic and variants of uncertain significance in the mixed cohort identified in 47 genes. P/LP: pathogenic or likely pathogenic, VUS: variant of uncertain significance.

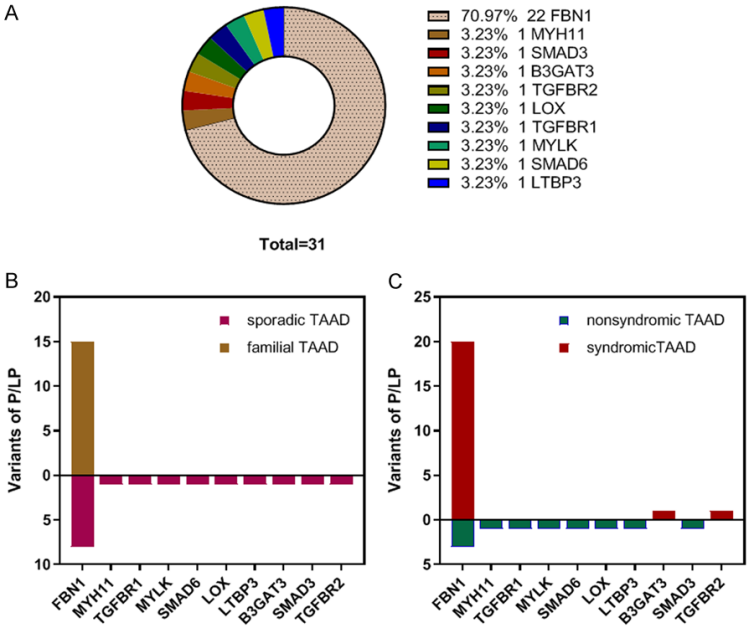

Figure 2. Pathogenic/likely pathogenic mutations identified in 10 genes. A: Gene distribution of pathogenic/likely pathogenic mutations; B: Pathogenic/likely pathogenic mutations in sporadic TAAD and familial TAAD. One mutation of *FBN1* was identified in both unrelated probands from the aforementioned two cohorts; C: Pathogenic/likely pathogenic mutations in syndromic TAAD and non-syndromic TAAD. One mutation of *FBN1* was identified in both unrelated probands from the aforementioned two cohorts. P/LP: pathogenic or likely pathogenic, VUS: variant of uncertain significance, sporadic TAAD: sporadic thoracic aortic aneurysm or dissection, familial TAAD: familial thoracic aortic aneurysm or dissection, syndromic TAAD: syndromic thoracic aortic aneurysm or dissection, nonsyndromic TAAD: nonsyndromic thoracic aortic aneurysm or dissection.

erozygous variants in *B3GAT3*), one harbored a VUS in *COL4A5*, and one patient was normal without gene mutation. The abnormal absent of α5 (type IV collagen) in the aortic media arising from the *COL4A5* gene seemed to be related to the particular genotypes of early onset aortic dissection and aneurysm with Alport syndrome [21]. In addition, more than one mutation (in both *APC* and *TEX26*) was identified in patient TAAD0046 with LDS, both of which were associated with BAV [22, 23], whereas the patient did not suffer aortic valve malformation and no previous evidence supports the correlation between each of the two genes and TAAD.

Unexpectedly, seven variants of the *FBN1* gene were identified in seven patients (six were AD, one was thoracic aortic aneurysm [TAA] with BAV) with a mean age of 45.9 (range: 28-69) who had not been previously suspected to have MFS; three variants were P/LP, and three VUSs were predicted to be "deleterious" by both SIFT and Polyphen2_HDIV (**Table 3**). Indeed, after we revisited the clinical data, none of the patients presented obvious clinical manifestations according to the MFS Ghent criteria [15]. A 28-year-old pregnant woman with acute Stanford type A dissection harbored a pathogenic mutation in *FBN1* (Arg1125Ter), and she gave birth to a child without congenital malformation at 36+ gestational weeks. Her mother died at the age of thirty because of an allergy as she described. Of the two patients who had a history of aortic surgery, patient TAAD0005 with a VUS (Glu616Lys) in *FBN1* developed Stanford type A dissection after TEVAR (thoracic endovascular aortic repair), and patient TAAD0021 with novel LP (Ser824AlafsX22) in *FBN1* underwent

Table 2. Clinical characteristics of patients with pathogenic/likely pathogenic mutations in the study

ID	Sex	Age (year)	Disease	Clinical MFS	Family history	Max-aortic diameter (cm)	Surgery	Gene	Variant	Classification	Reported
TAAD0043	F	37	TAA	Y	Y	50	B+MVP+TVP	FBN1	c.2023_2026del, p. Phe675ValfsX41	P	Y
TAAD0027	M	28	AD	Y	Y	85	B+TAR+DASI	FBN1	c.2430delA, p. Glu810AspfsX36	P	Novel
TAAD0038	M	48	AD	Y	Y	NA	TEVER	FBN1	c.3373C>T, p. Arg1125Ter	P	Y
TAAD0080	F	6	/	Y	N	18	/	FBN1	c.5788+5G>A	P	Y
TAAD0015	M	49	AD	N	N	60	B+TAR+DASI+MVP+TVP	SMAD3	c.1102C>T, p. Arg368Ter	P	Y
TAAD0046	M	52	AD	Y	N	48	B+PAR+DASI+MVP	TGFBR2	c.1483C>T, p. Arg495Ter	P	Y
TAAD0049	F	28	AD	N	N	64	B+TAR+DASI[a]	FBN1	c.3373C>T, p. Arg1125Ter	P	Y
TAAD0083	M	50	AD	N	N	50	B+TAR+DASI	FBN1	c.2860C>T, p. Arg954Cys	P	Y
TAAD0069	M	32	AD	Y	N	44	AOR+TAR+DASI	FBN1	c.6000C>A, p. Cys2000Ter	LP	Y
TAAD0074	F	23	TAA	Y	N	56	B	FBN1	c.2648G>A, p. Trp883Ter	LP	Y
TAAD0086	M	27	AD	Y	N	100	B+TAR+DASI	FBN1	C.3047delC, p. Thr1016LysfsX19	LP	Novel
TAAD0091	F	23	TAA	Y	N	43	/	FBN1	c.6253T>G, p. Cys2085Gly	LP	Novel
TAAD0097	M	2 months	AoD	Y	N	13	/	B3GAT3	C.47C>A, p. Ser16Ter	LP	Novel
TAAD0017	F	44	AD	N	N	44	AOR+TAR+DASI	MYH11	c.5838_5839del, p. Arg1946SerfsX2	LP	Y
TAAD0021	F	50	AD	N	N	45	AOR+TAR+DASI	FBN1	c.2470delA, p. Ser824AlafsX22	LP	Novel
TAAD0028	M	48	AD	N	N	63	B+TAR+DASI+CABG	TGFBR1	c.1459C>T, p. Arg487Trp	LP	Y
TAAD0031	M	47	AD	N	N	37	AOR+TAR+DASI+AVP	MYLK	c.3665_3666del, p. Val1222GlufsX22	LP	Novel
TAAD0041	M	58	TAA	N	N	43	B	SMAD6	c.220C>T, p. Gln74Ter	LP	Y
TAAD0048	M	38	AD	N	N	40	AOR+TAR+DASI+AVP+MVR	LOX	c.433C>T, p. Gln145Ter	LP	Novel
TAAD0056	M	51	AD	N	N	49	B+TAR+DASI+CABG	LTBP3	c.169_170insAGGCGGGGGCGGGGCGC, p. Leu57GlnfsX16	LP	Novel
TAAD0072	M	34	TAA	Y	Y	65	B	FBN1	c.4583-1G>A	LP	Y
TAAD0079	M	49	AD	Y	Y	NA	B	FBN1	c.7754T>C, p. Ile2585Thr	LP	Y
TAAD0081[b]	M	31	/	Y	Y	30	/	FBN1	c.7113G>A, p. Trp2371Ter	LP	Y
TAAD0082[b]	M	26	TAA	Y	Y	43	/	FBN1	c.7113G>A, p. Trp2371Ter	LP	Novel
TAAD0084	F	34	TAA	Y	Y	48	MVP+TVP	FBN1	c.6825_6837delins14, p. Gly2277AspfsX9	LP	Novel
TAAD0085	F	44	AD	Y	Y	86	B+TAR+DASI	FBN1	c.2089delC, p. Gln697SerfsX21	LP	Novel
TAAD0087[b]	F	43	AD	Y	Y	72	B+TAR+DASI+MVP+TVP	FBN1	c.6575G>T, p. Cys2192phe	LP	Novel
TAAD0088[b]	F	41	TAA	Y	Y	50	/	FBN1	c.6575G>T, p. Cys2192phe	LP	Novel
TAAD0092	M	49	AD	Y	Y	70	B+TAR+DASI	FBN1	c.316C>T, p. Gln106Ter	LP	Novel
TAAD0096	M	32	TAA	Y	Y	59	B+TAR+DASI	FBN1	c.1165_1166delTG, p. Cys389LeufsX62	LP	Novel
TAAD0051	M	30	AD	Y	Y	NA	B+TAR+DASI+MVP+TVP	FBN1	c.2886C>G, p. Tyr962Ter	LP	Novel
TAAD0047	M	27	AD	Y	Y	68	B+TAR+DASI	FBN1	c.1838-2A>G	LP	Novel
TAAD0014	M	26	AD	Y	Y	74	B+TAR+DASI	FBN1	c.2538delA, p. Ile846MetfsX25	LP	Novel
TAAD0099	M	27	TAA	Y	Y	87	B+MVP+TVP	FBN1	c.5296+1G>T	LP	Novel

F: female; M: male; TAA: thoracic aortic aneurysm; AD: aortic dissection; AoD: aortic root dilatation NA: not available; B: Bentall procedure; TAR: total arch replacement; PAR: partial arch replacement; DASI: descending aorta stent implantation; MVP: mitral valve plastic; MVR: mitral valve replacement; TVP: tricuspid valve plastic; AVP: aortic valvuloplasty; AOR: ascending aorta replacement; CABG: coronary artery bypass grafting; TEVAR: thoracic endovascular aortic repair; P: pathogenic; LP: likely pathogenic; Two unrelated patients harboring the same mutation of FBN1 was Underlined. [a]aortic surgery was performed after Caesarean section and subtotal hysterectomy at the same stage. [b]two pairs of siblings.

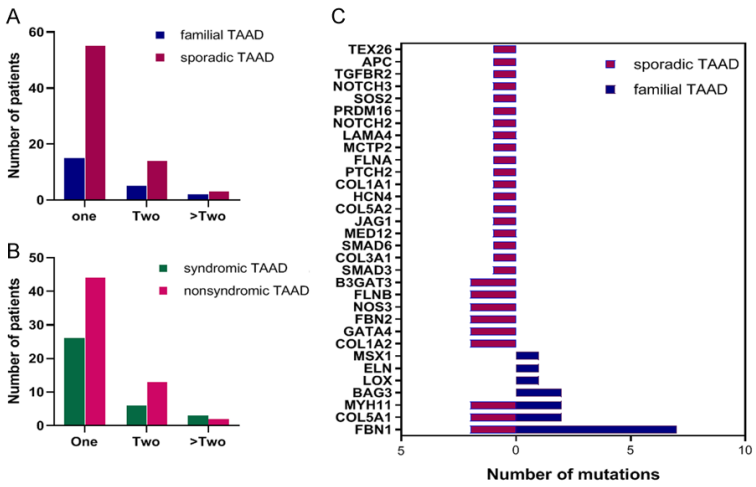

Figure 3. Multiple mutations identified in the study. A: The number of patients with family TAAD or sporadic TAAD in groups with different numbers of mutations; B: The number of patients with syndromic TAAD or nonsyndromic TAAD in groups with different numbers of mutations; C: Gene mutations in individuals with two or more mutations in sporadic TAAD and familial TAAD. familial TAAD: familial thoracic aortic aneurysm or dissection, sporadic TAAD: sporadic thoracic aortic aneurysm or dissection, syndromic TAAD: syndromic thoracic aortic aneurysm or dissection, nonsyndromic TAAD: nonsyndromic thoracic aortic aneurysm or dissection.

the Bentall procedure due to an aneurysm of the aortic sinus two years ago. Both patient TAAD0083 with the P variant Arg954Cys and patient TAAD0012 with the novel VUS Glu1449Val underwent emergency hospitalization after a sports accident. The first was tall but not thin (height of 1.83 m, weight of 85 kg) with a Z-score of 5.52, and the latter had a Z-score of 10.18 at the diagnosed age of 37. Moreover, TAAD0083 also harbored *NOTCH3* (Val237Met) in addition to *FBN1* variants. A variant (Val237Met) in NOTCH3 with a 0.002 frequency in East Asian was reported in a 70-year-old Japanese woman diagnosed with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) with phenotypes of gait disturbance and dementia [24]. The same two variants were also present in the daughter of TAAD0083 without any clinical symptoms.

Familial TAAD

A total of 25 variants were identified among 22 familial TAAD patients; 60.0% (15) were classified as P/LP, all of which were associated only with *FBN1*. Of the 21 cases of clinically suspected MFS, 19 cases were ascertained to be MFS due to the *FBN1* gene, one was familial TAA with an *ACTA2* mutation, and one was the carrier of a VUS in *APC* mutation, which was predicted to be "deleterious" by SIFT and Polyphen2_HDIV. Notably, the nineteen-year-old subject with the APC mutation displayed aortic root aneurysm with BAV (Z-score of 13.28) and had a surgical history of aortic coarctation and ligation of PDA (patent ductus arteriosus) at the age of five and a family history of long slender limbs. The remaining patient suffered from TAA with BAV harboring a novel mutation in *ERMARD*. In addition, more than one mutation in genes other than *FBN1* was detected in 7 suspected MFS patients from 4 unrelated families, including three pairs of siblings (Table S3).

Genotype-phenotype correlation

Age under 45 years at diagnosis, female sex, family history, and syndromic manifestations involving the aortic root were found to increase the likelihood of carrying a P/LP variant (**Table 4**). The younger patients were when they developed TAAD, the more likely they were to carry P/LP variants. The median age at diagnosis of people harboring LP/P variants was 35.5 years, which was lower than 48.5 years for VUSs (P=0.003) and then 53.0 years for no variant (P=0.000001) (**Figure 4A**). After ruling out syndromic TAAD, the median age at diagnosis of the P/LP group and the VUS group increased to 49.5 years and 51.0 years, respectively (**Figure 4B**). Moreover, the pathogenicity of the variant increased with the degree of aortic regurgitation (P=0.253, P=0.002). More than one variant was identified in 25.53% (24/94) of gene-positive probands. The no variant group was significantly different from the one variant group (P=0.003). The median age decreased with the increasing number of variants in all but the group of four variants (P=-0.286; P=0.000377) (**Figure 4C**). After excluding syndromic TAAD, the median age of the three variant group greatly increased from 39.5 years to 48 years, which was slightly higher than that of

Table 3. FBN1 mutation identified in nonsyndromic TAAD without family history

ID	Sex	Age	Hypertension	Gene	Variant	Classification	SIFT	Polyphen2_HDIV	Disease	Surgery procedure	History of aortic surgery
TAAD0005	M	49	N	FBN1	c.1846G>A p. Glu616Lys	VUS	D	D	AD	AVP+AOR+TAR+DASI	Y
TAAD0012	M	37	N	FBN1	c.4346A>T p. Glu1449Val	VUS	D	D	AD	B+PAR	N
TAAD0021	F	50	N	FBN1	c.2470delA, p. Ser824AlafsX22	LP	/	/	AD	AOR+TAR+DASI	Y
TAAD0029	M	69	Y	FBN1	c.1825C>T p. Arg609Cys	VUS	D	D	AD	AVR+AOR+PAR+DASI	N
TAAD0037	M	38	N	FBN1	c.7559C>T p. Thr2520Met	VUS	T	D	TAA	AVR	N
				FBN2	c.577C>T p. Pro193Ser	VUS	T	D			
TAAD0049	F	28	Y[a]	FBN1	c.3373C>T p. Arg1125Ter	P	/	/	AD	B+TAR+DASI; cesarean section + hysterectomy[b]	N
TAAD0083	M	50	N	FBN1	c.2860C>T p. Arg954Cys	P	/	/	AD	B+TAR+DASI	N
				NOTCH3	c.709G>A p. Val237Met	VUS	/	/			

[a]transient gestational hypertension; [b]delivery before aortic repair in the same stage. VUS: variant of uncertain significance; P: pathogenic; LP: likely pathogenic; D: deleterious; T: tolerated; B: Bentall procedure; AVP: aortic valvuloplasty; AOR: ascending aorta replacement; TAR: total arch replacement; DASI: descending aorta stent implantation; PAR: partial arch replacement; AVR: aortic valve replacement.

Table 4. Risk of carrying pathogenic /likely pathogenic mutations based on diverse phenotypes

	Total	Pathogenic/Likely pathogenic (percentage)	RR (95% CI)	*P*-vale
Family history	22	17 (77.3)	5.864 (3.565-9.644)	$2.6432E^{-9}$
syndromic	36	24 (66.7)	7.667 (4.060-14.477)	$1.327E^{-11}$
Age <45	64	22 (34.4)	2.492 (1.334-4.656)	0.003
Female	30	11 (36.7)	1.929 (1.062-3.504)	0.038
Maximum aortic size>5 cm	67	19 (28.4)	1.791 (0.980-3.273)	0.077
Aortic root	110	30 (27.3)	2.795 (1.049-7.446)	0.027
D-dimmer>20000	21	3 (14.3)	0.599 (0.147-1.928)	0.410

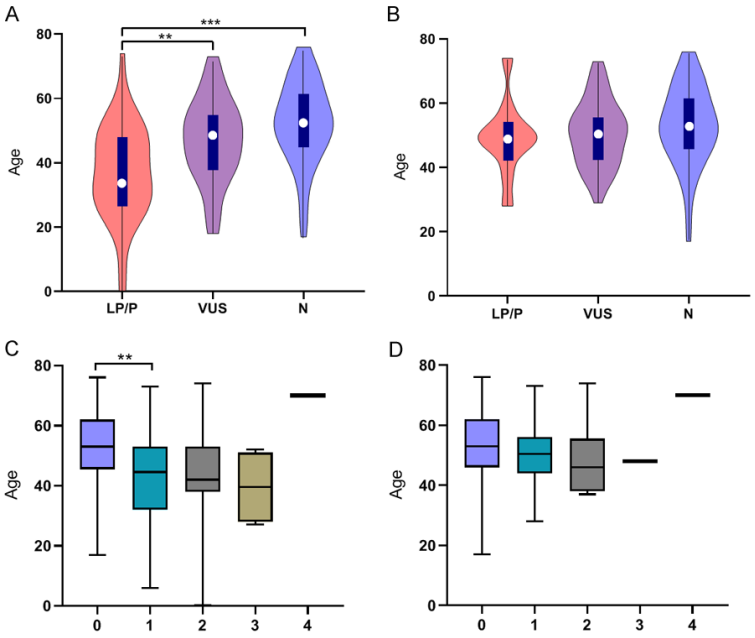

Figure 4. Correlations between clinical phenotype of the diagnosed age and genotypes. A: The diagnosed age of all patients with different mutation types; B: The diagnosed age of patients with different mutation types after eliminating clinically syndromic individuals; C: The diagnosed age of all patients with different numbers of mutations; D: The diagnosed age of patients with different numbers of mutations after eliminating clinically syndromic individuals. P/LP: pathogenic or likely pathogenic, VUS: variant of uncertain significance, N: no mutation. *P<0.05; **P<0.01; ***P<0.001.

the two variants group (46 years) (**Figure 4D**). To compare the severity of the surgery patients with variant types, we assessed the maximum aortic diameter, length of hospital stay, length of ICU (intensive care unit) stay, and D-dimer level. P/LP variant carriers were apt to have a greater median aortic diameter (P=0.038; P= 0.006) than the VUS or the N group; and a lower median D-dimer level (P=0.004) than the N group (**Figure 5A**, **5B**). Interestingly, the median ICU duration of the P/LP group was lower than that of the N group (P=0.031), although the median hospitalization time was higher than that of the N or VUS group (differences were not significant) (**Figure 5C**, **5D**). However, among the surgery subjects, the number of variants did not seem to correlate with length of hospital stay, length of ICU stay, maximum aortic diameter, or D-dimer level, all of which were significantly associated with disease severity.

Discussion

The etiology and pathogenesis of aortic aneurysm/dissection is extremely complex and genetically heterogeneous. Causative genes could be responsible for the severe morbidity of 4.9%-18.6% TAAD [9, 10, 25-27]. Herein, we primarily focused on 129 sporadic TAAD patients in addition to 22 familial TAAD patients, which were categorized into syndromic and nonsyndromic groups, and spared no effort to explore the possible reason using the power of gene sequencing.

A total of 116 variants in 47 genes were identified by WES, 64.7% of which existed in sporadic TAAD without syndromes. Of the 26.7% that were pathogenic or likely pathogenic, almost one third were from sporadic patients without syndromes and involved *FBN1, SMAD3, SMAD6, MYH11, TGFBR1, MYLK, LOX and LTBP3*. The frequency of pathogenetic/likely pathogenetic variants was 22.5% in our cohort, which

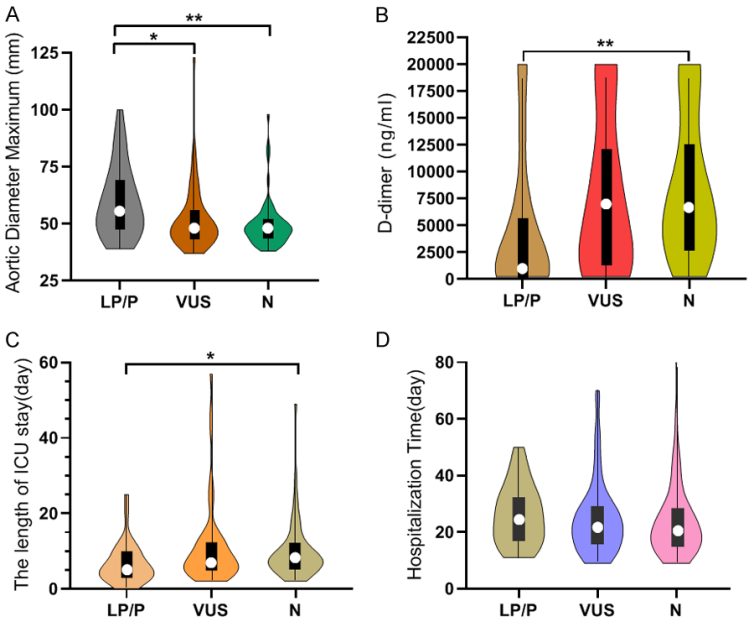

Figure 5. Correlations between clinical phenotypes and genotype of different mutation types in surgery subjects. A: Aortic diameter maximum of surgery patients in different mutation type groups; B: D-dimer of surgery patients in different mutation type groups; C: The length of ICU stay of surgery patients in different mutation type groups; D: The hospitalization time of surgery patients in different mutation type groups. P/LP: pathogenic or likely pathogenic, VUS: variant of uncertain significance, N: no mutation. *P<0.05; **P<0.01; ***P<0.001.

was higher than that in our previous reports [27]. The majority of variants were in *FBN1* as expected, accounting for 27.6%, and 53.1% were in sporadic cases, which was in line with the suggestion of a common pathogenesis of aortic events in MFS and sporadic TAAD [28]. In contrast to other reports [27, 29], the *ACTA2* gene was not second to *FBN1* as the most common mutated gene, as it accounted for only 0.9% of cases in this study. However, in other countries, *ACTA2* mutations have been reported to be the most common cause of familial TAAD and causative of first-time sporadic and young-onset TAAD [30, 31]. In agreement with a previous study, *MYH11* and *SMAD3* mutations were responsible for TAAD with frequencies of 4.3% and 3.4%, respectively [12]. Both altered smooth muscle cell (SMC) contraction and impaired TGF-β signaling proteins have adverse effects on the development of the aortic wall.

There are few reports about the *LTBP3* gene (latent-transforming growth factor beta-binding protein 3) in correlation with thoracic aneurysm or dissection. Recently, three rare variants from two unrelated families were found to be causative of heritable TAAD, and heterozygous mutations in *LTBP3* might increase the risk for later-onset thoracic aortic disease [7]. Moreover, the novel LP variant Leu57GlnfsX16 in *LTBP3* was identified in a 51-year-old male who suffered acute Stanford type A aortic dissection without either family or syndromic history in our cohort. In fact, LTBP-3 mRNA is expressed at high levels in some tissues, including the human heart and skeletal muscle, which participate in the formation of a large latent complex (LLX) with LAP (latency-associated peptide) and TGFβ and the regulation of TGFβ activity [32-34]. Interestingly, LTBP3 has a similar structure of 8-Cys domains specific to FBN1; they colocalize in tissues, and loss of fibrillin 1 in the ECM prevents matrix incorporation of LTBP-3 [33, 35]. Zilberberg et al proved that improper localization of LLX composed of LTBP-3 and TGFβ contributes to aortic disease progression in MFS [36].

To the best of our knowledge, four variants of the *MCTP2* (multiple C_2 domain and transmembrane region protein) gene were first reported to be responsible for nonsyndromic TAAD without a family history, accounting for 3.4% of TAAD cases (**Figure 6A**, **6B**). The VUS *879Glu was present in two unrelated TAAD probands without a history of familial or syndromic TAAD. Both were Stanford Type A aortic dissections without BAV, which contrasts with what has been reported in a study identifying the *MCTP2* gene in BAV patients [20, 23]. However, the patient with Arg542* underwent surgery for aortic valve replacement due to the BAV phenotype. The *MCTP2* gene located at chromosomal 15q26.2 is composed of three significant C_2 domains, two transmembrane domains, a variable N-terminal region and a C-terminal sequence, which are involved in the biological processes of calcium-mediated signaling and multicellular organism development. In con-

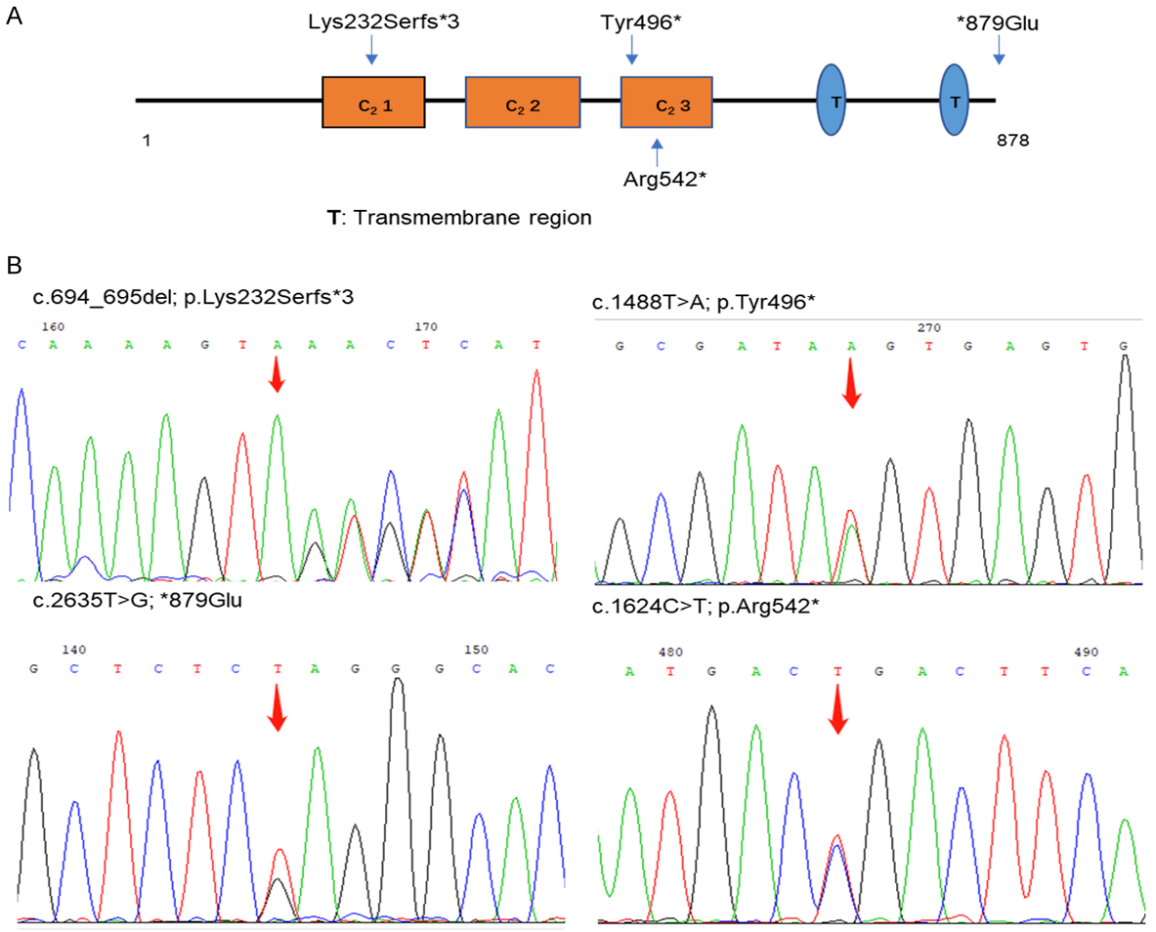

Figure 6. Four novel mutations identified in the *MCTP2* gene. A. The structure of *MCTP2* gene. B. Sequencing chromatographs of four mutations in *MCTP2* gene.

trast to the other three families of trafficking proteins containing multiple C_2 domains and one single transmembrane region, such as synaptotagmins, ferlins and E-Syts, the *MCTP2* protein binds Ca^{2+} via the C_2 domains in the absence of phospholipids with a high apparent affinity [37]. It was proven that Y235C located in the first C_2 domain was attributed to the altered calcium-binding affinity of *MCTP2* protein [38]. In this study, Lys232Serfs*3, Arg542* and Tyr496* were also located in the significant C_2 domain (the first and the third, respectively), which includes perfect Ca^{2+}-binding modules and was highly conserved.

It has been proposed that rare MCTP2 mutations might be attributed to cardiac malformations in the failure of endocardial cells to undergo EMT (epithelial-mesenchymal transformation) [38]. There is emerging evidence that the *MCTP2* gene is primarily distributed in rat heart muscle except the testis, and functional knockdown could result in abnormal cardiac development such as coarctation of the aorta (CoA) [37, 38]. EMT of endocardial cells and the morphogenesis of the endocardial cushion are fundamental to the remodeling of the outflow tract from the aorta, which is absent in *MCTP2* morphants. Further mechanistic studies are needed to explain the inconsistent phenotypes of CoA and aortic dilation/dissection with the *MCTP2* mutant.

Causative mutations tend to occur in young patients under the age of 45 years at diagnosis. Likewise, it seems that *de novo* variants were easily found in severely syndromic TAAD patients with young age at diagnosis. Furthermore, female sex, family history, and syndromic manifestations, involving the aortic root were found to increase the likelihood of carrying a P/LP variant. P/LP carriers seem to have low

D-dimer levels, short ICU stays but increased maximum aortic diameter, and long hospitalization times, which could be because most young patients with early apparent symptoms were hospitalized in a state of nonstress and a non-emergency state before surgery. Consistent with a previous study, people with one or more mutations appeared to be younger [39]. However, the number of mutations did not correlate with these aforementioned indicators of disease severity.

Exome sequencing made it easier to obtain a definitive diagnosis despite complicated overlapping phenotypes between multiple syndromes or low gene penetrance among mutation carriers. In sporadic cases, we diagnosed a two-month-old boy, under the suspicion of MFS due to arachnodactyly and mild aortic root dilation as linkeropathies owing to novel rare *B3GAT3* variants, which is an extremely rare hereditary connective tissue disorder affected by various glycosyltransferases in the biosynthesis of proteoglycans [40]. In addition, seven individuals with a mean age of 45.9 years who did not fulfill the revised Ghent criteria (prior to genetic testing), were found to harbor FBN1 variants, three of which were P/LP. Unexpectedly, none of the first-degree-relatives presented any suspected clinical features of MFS. As expert consensus suggests, screening immediate relatives, especially asymptomatic relatives, is particularly important to avoid the risk of sudden rupture of aortic aneurysms or dissection [41]. However, even among siblings with TAAD in each family, the identified variants were not entirely identical (Table S3). The *FBN1* gene might be responsible for the remarkable phenotypes, but we cannot ignore the potential mutual effect for similar phenotypes between multiple genes. Instead of Sanger sequencing, performing WES among siblings when available will be more beneficial for disease diagnosis and discovery of new candidate genes.

One of the limitations of our study is that we did not eliminate other risk factors, such as hypertension, which was responsible for a considerable proportion of aortic dissection. Second, it would be better to identify the association between genotype and phenotype if TAA and dissection were grouped separately. Third, our study had a relatively limited sample size, so more data are needed to verify and confirm the results. We focused on exome sequencing of 129 TAAD-related genes, which could account for most of the phenotypes of TAAD, but might miss other potential regions or new genes. Whole-genome sequencing could make all the difference with the development of cost-effective gene analysis.

In summary, we found that the frequency of gene positivity (including VUS and LP/P) was 62.3% in the mixed cohort of sporadic and familial TAAD, and 80.6% of cases were non-syndromic TAAD without a family history. Moreover, 26.7% of mutations were pathogenic or likely pathogenic, and almost one third of these were from sporadic cases without syndromes. In addition to focusing on core pathogenic genes involved in hereditary TAAD, paying more attention to *de novo* variants in sporadic cases and candidate genes in nonsyndromic cases without family history is more conducive to further mechanistic research about silent but life-threatening aortic events.

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Disclosure of conflict of interest

None.

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Table S1. TAAD-related Gene list

TAAD-related Genes									
ACTA2	GATA5	PTPN22	TGFBR1	ARFGEF2	COL1A1	ERMARD	HGD	MED12	POR
FBN1	LOX	SLC2A10	TGFBR2	B3GAT3	COL5A1	FBLN5	HLA-B	MYH3	PRDM16
COL3A1	MFAP5	SMAD3	BGN	BCR	COL5A2	FBN2	IFIH1	MYPN	PTEN
ELN	MYH11	SMAD4	PLOD1	C12orf57	CYP11B1	FLNA	IL12B	NEDD4L	RERE
ENG	MYLK	SMAD6	ADAR	CHD7	CYP17A1	FLNB	KANSL1	NOD2	RIN2
PRKG1	NKX2-5	TGFB2	ALDH18A1	CHRNG	DNMT3A	FMR1	KCNH1	NSMCE2	RNASEH2A
FOX3	NOTCH1	TGFB3	ARF1	CHST3	EFEMP2	GABRD	LMNA	PIGN	RNASEH2B
RNASEH2C	TAB2	SMAD2	HCN4	TNXB	SLC25A24	ZMPSTE24	EMILIN1	CBS	MAT2A
ROBO4	TMTC3	TPM3	SEMA3E	ADAMTS-2	TIMP3	ARIH1	LTBP1	NOTCH2	MSTN
SAMHD1	TPM2	TREX1	SKI	COL4A5	TIMP1	COL1A2	LTBP3	NOTCH3	*
BAV-related Genes									
JAG1	COLLAGEN3	AXIN1	MSX1	GLI1	ELASTIN	PDIA2	NOS1	VEGFC	WNT4
SNAI3	MCTP2	SOX9	TBX5	SLC35B2	NFATC1	VEGFB	TEX26	APC	AXIN2
ZNF236	GATA4	PAX6	FLT1	PIGF	NOS3	KCNJ2	PTCH2	PPP3CA	EGFR

*Genetic analysis of both LAMA4 and SOS2 genes were performed only in patient TAAD0077.

Table S2. Multiple gene mutations identified in Sporadic TAAD

ID	Sex	Age	Disease	Hypertension	Gene	variant	Type	classification	Syndrome
TAAD0001	M	37	AD	N	COL1A2	c.3338A>T, p. Asp1113Val	Missense	VUS	N
					SMAD3	c.245G>A, p. Gly82Glu	Missense	VUS	
TAAD0004	M	41	AD	Y	GATA4	c.487C>T, p. Pro163Ser	Missense	VUS	N
					COL5A1	c.1970C>T, p. Pro657Leu	Missense	VUS	
TAAD0006	M	37	AD	Y	FBN2	c.6665C>T, p. Pro2222Leu	Missense	VUS	N
					NOS3	c.2984+4A>G	Splicing	VUS	
TAAD0009	M	46	AD	Y	COL3A1	c.3490G>T, p. Gly1164Trp	Missense	VUS	N
					FLNB	c.2183C>T, p. Pro728Leu	Missense	VUS	
TAAD0010	M	53	AD	Y	SMAD6	c.919A>C, p. Asn307His	Missense	VUS	N
					MED12	c.2665C>G, p. Leu889Val	Missense	VUS	
TAAD0017	F	74	AD	N	MYH11	c.5838_5839del, p. Arg1946SerfsX2	Frameshift	LP	N
					JAG1	c.3286C>T, p. Arg1096Trp	Missense	VUS	
TAAD0030	M	56	AD	Y	COL5A2	c.3884C>A, p. Thr1295Lys	Missense	VUS	N
					HCN4	c.995G>A, p. Arg332Gln	Missense	VUS	
TAAD0035	M	43	TAA+BAV	N	COL1A1	c.1072C>G, p. Gln358Glu	Missense	VUS	N
					GATA4	c.221C>A, p. Ala74Asp	Missense	VUS	

TAAD0036	M	48	AD	Y	COL5A1	c.5045T>G, p. Phe1682Cys	Missense	VUS	N
					MYH11	c.4786A>G, p. Arg1596Gly	Missense	VUS	
					PTCH2	c.2483G>C, p. Gly828Ala	Missense	VUS	
TAAD0037	M	38	TAA+BAV	N	FBN2	c.577C>T, p. Pro193Ser	Missense	VUS	N
					FBN1	c.7559C>T, p. Thr2520Met	Missense	VUS	
TAAD0044	M	55	AD	Y	FLNA	c.1484T>C, p. Val495Ala	Missense	VUS	N
					MCTP2	c.694_695del, p. Lys232SerfsX3	Frameshift	VUS	
TAAD0048	M	38	AD	Y	LOX	c.433C>T, p. Gln145Ter	Nonsense	LP	N
					NOS3	c.1399T>A, p. Tyr467Asn	Missense	VUS	
TAAD0050	M	58	AD	N	FLNB	c.6028C>T, p. Arg2010Cys	Missense	VUS	N
					COL1A2	c.1976A>G, p. Glu659Gly	Missense	VUS	
TAAD0077	M	70	AD	Y	LAMA4	c.307C>G, p. Arg103Gly	Missense	VUS	N
					NOTCH2	c.4666G>A, p. Glu1556Lys	Missense	VUS	
					PRDM16	c.3623T>C, p. Val1208Ala	Missense	VUS	
					SOS2	c.3027C>G, p. Asn1009Lys	Missense	VUS	
TAAD0083	M	50	AD	N	NOTCH3	c.709G>A, p.Val237Met	Missense	VUS	N
					FBN1	c.2860C>T, p.Arg954Cys	Missense	P	
TAAD0097	M	2 months	TAA	N	B3GAT3	c.47C>A, p.Ser16Ter	Nonsense	LP	Y
					B3GAT3	c.752T>C, p.Val251Ala	Missense	VUS	
TAAD0046	M	52	AD	N	TGFBR2	c.1483C>T, p. Arg495Ter	Nonsense	P	Y
					APC	c.1865A>G, p. Tyr622Cys	Missense	VUS	
					TEX26	c.31C>T, p. Gln11Ter	Nonsense	VUS	

F: female; M: male; AD: aortic dissection; TAA: thoracic aortic aneurysm; BAV: bicuspid aortic valve; VUS: variant of uncertain significance; P: pathogenic; LP: likely pathogenic.

Table S3. Multiple mutations of different genes in Familial TAAD

ID	Sex	Age	Disease	Hypertension	Gene	Variant	Type	Classification	Syndrome
TAAD0047	M	27	AD	Y	FBN1	c.1838-2A>G	Splicing	LP	Y
					MSX1	c.458C>A, p. Pro153Gln	Missense	VUS	
TAAD0075	M	40	AD	N	FBN1	c.5797G>A, p. Glu1933Lys	Missense	VUS	Y
					BAG3	c.317G>A, p. Arg106Gln	Missense	VUS	
TAAD0076	F	42	AD	Y	FBN1	c.5797G>A, p. Glu1933Lys	Missense	VUS	Y
					BAG3	c.317G>A, p. Arg106Gln	Missense	VUS	
TAAD0081	M	31	/	N	FBN1	c.7113G>A, p. Trp2371Ter	Nonsense	LP	Y
					COL5A1	c.526A>C, p. Asn176His	Missense	VUS	
					MYH11	c.33G>T, p. Glu11Asp	Missense	VUS	
TAAD0082	M	27	TAA	N	FBN1	c.7113G>A, p. Trp2371Ter	Nonsense	LP	Y
					COL5A1	c.526A>C, p. Asn176His	Missense	VUS	
					MYH11	c.33G>T, p. Glu11Asp	Missense	VUS	
TAAD0087	F	43	AD	N	FBN1	c.6575G>T, p. Cys2192phe	Missense	LP	Y
					LOX	c.218G>A, p. Gly73Asp	Missense	VUS	
TAAD0088	F	41	TAA	N	FBN1	c.6575G>T, p. Cys2192Phe	Missense	LP	Y
					ELN	c.1876G>A, p. Ala626Thr	Missense	VUS	

M: male; F: female; AD: aortic dissection; TAA: thoracic aortic aneurysm; LP: likely pathogenic; VUS: variant of uncertain significance. Three pairs of siblings from three unrelated families were underlined.