

Proteomic analysis of calcified abdominal and thoracic aortic aneurysms

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Abstract. Aortic aneurysm is a complex multifactorial disease with genetic and environmental risk factors. It is often accompanied by aortic calcification. Here, to uncover proteins that are significantly changed in calcified abdominal aortic aneurysms (CAAs) and calcified thoracic aortic aneurysms (CTAs) compared with those in adjacent normal aorta tissues, comprehensive analysis of differentially expressed proteins in their tissues was performed by a quantitative proteomic approach with iTRAQ labeling in combination with nanoLC-MALDI-TOF/TOF-MS/MS followed by ProteinPilot analysis. The proteomic analysis revealed 138 and 134 proteins differentially expressed in CAAs and CTAs in contrast to neighboring normal aorta tissues with high confidence, respectively. Significantly increased expression (≥ 1.3 -fold) was found in 41 and 28 proteins, whereas decreased expression (< 0.77 -fold) was found in 4 and 60 proteins in CAAs and CTAs, respectively. Among them, we identified already known proteins involved in aneurysm formation and vascular calcification, such as type I and III collagen, matrix Gla protein, and α -2-HS-glycoprotein in CAAs and fibrinogen α , β and γ chains and α -2-HS-glycoprotein in CTAs with increased expression and mimecan in CAAs and fibulin-5 in CTAs with decreased expression. Based on the Panther pathway and Genesis clustering analyses, some of the proteins could be linked to corresponding biochemical pathways, such as the integrin signaling pathway with increased expression in CAAs, the blood coagulation pathway with increased expression in CTAs, and the inflammation mediated by chemokine and cytokine signaling pathway and the glycolysis pathway with decreased expression

in CTAs. Interestingly, it was found by clustering analysis that samples from CAAs of patients with both CAAs and CTAs were clustered outside the samples of patients with CAAs and were clustered with samples of patients with CTAs. Our results provide a comprehensive patient-based proteomic analysis for the identification of potential biomarkers for CAAs and CTAs.

Introduction

Aortic aneurysm is a severe cardiovascular disease with high mortality and morbidity rates. It is associated with advanced age, male gender, cigarette smoking, atherosclerosis, hypertension, family history, and genetic predisposition (1). Aortic aneurysms most frequently arise in the infrarenal abdominal aorta [so-called abdominal aortic aneurysm (AAA)] and the descending or ascending portion of the thoracic aorta [so-called thoracic aortic aneurysm (TAA)].

Previous studies have provided evidence for involvement of proteolysis and the immune system in aortic aneurysm formation and progression at the molecular level (2,3). Histologically, fragmentation and degeneration of elastin and collagen are generally observed in the media in the aneurysm wall. Numerous molecular mediators and extracellular matrix-degrading proteinases participate in the progression of AAAs. Matrix metalloproteinases (MMPs) and their antagonists, tissue inhibitors of metalloproteinases (TIMPs), are instrumental in aneurysm formation and progression. Members of the gelatinase subclass, MMP-2 and MMP-9, have been studied with regard to aneurysm formation (2). Growth and rupture of AAAs result from increased collagen turnover by increased type I collagen degradation within AAAs (4). Other causes of aortic wall weakening are apoptosis of vascular smooth muscle cells (VSMCs) and transmural infiltration of lymphocytes and macrophages within AAA aortas providing the stimulation for a chronic inflammatory response (5). An association of atherosclerotic lesions with the formation of AAAs in the intima of the aorta has also been proposed, although it is still controversial. Supporting evidence for the association of AAAs with atherosclerosis includes similar aortic regions being prone to both diseases (6). On the other hand, there are several lines of evidence suggesting that atherosclerosis is not a prerequisite for AAA formation (7).

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The prevalence of TAAs is only about one-third of that of AAAs (3). Structural heterogeneity of the aorta has been shown between the thoracic and abdominal aortic regions (8). The structural heterogeneity of thoracic and abdominal aortas may contribute to the differences observed in the pathogenesis of AAAs and TAAs. The thoracic aorta has a thinner intima, thicker media, and more medial lamellar units than those of the abdominal aorta. In addition, the thoracic aorta has significantly higher elastin and collagen contents and a lower collagen-to-elastin ratio than those of the abdominal aorta. In contrast to the abdominal region, atherosclerosis rarely presents in the thoracic region. Medial degeneration is qualitatively and quantitatively much greater in patients with TAAs than in patients with AAAs. Lesauskaite *et al* (9) demonstrated that MMP-2 and MMP-9 activities were increased at portions of medial degeneration in aortas from patients with TAAs. Furthermore, ~20% of TAAs are attributed to some genetic syndromes (10).

In many cases, aortic aneurysms are accompanied by vascular calcification. Calcified arteries lose their elasticity, resulting in exacerbation of ischemia, and increase in cardiac afterload. Vascular calcification occurs at two histological sites of arteries, the intima and the media. Intimal calcification is associated with late-stage atherosclerosis with a passive deposition of hydroxyapatite (calcium phosphate) into necrotic and apoptotic lesions of the plaques (11). In areas with tissue degeneration in the vascular wall, hydroxyapatite precipitation occurs when the physiological calcium phosphate concentration exceeds the threshold of solubility (12). On the other hand, medial calcification, such as that in Mönckeberg's medial sclerosis results from active processes with some similarities to osteogenesis (13). The vascular calcification process has been proposed (14). Inflammation triggered by elastin degradation, lipid deposition or pathogens in the vascular wall induces activation and proliferation. Activated macrophages induce proliferation and differentiation of neighboring VSMCs or stem cells with mesenchymal differentiation potential into osteogenic precursor cells. Through secreted molecules and direct cell-cell interaction, the osteogenic precursor cells differentiate to osteoblast-like cells. These osteoblast-like cells deposit bone matrix proteins that are subsequently mineralized. At that time, matrix vesicles and apoptotic bodies from VSMCs functioning as the nidus for mineral nucleation facilitate vascular calcification. In the process, the gain of calcification promoters, such as bone morphogenetic proteins (BMPs) and the loss of calcification inhibitors, such as matrix Gla protein (MGP) and fetuin-A (also known as α 2-Heremans-Schmid glycoprotein or α -2-HS glycoprotein) are involved. The factors governing bone formation overlap with those of vascular calcification (15).

The development of proteomic analyses and cDNA microarray techniques has made it possible to examine comprehensive differential expression of numerous proteins or genes between two conditions. Recently, proteomic analyses have been applied to clarify molecular alterations in AAAs (16). cDNA microarray analysis has also been applied to diverse disease processes including AAAs (17) and TAAs (18). However, the spectrum of molecular alterations that may occur in calcified aneurysms remains unknown.

The purpose of this study was to establish proteomic profiles of differentially expressed proteins in calcified

abdominal aortic aneurysms (CAAs) and calcified thoracic aortic aneurysms (CTAs) relative to adjacent normal aorta tissues. Recently, a variety of large-scale mass spectrometry (MS)-based analyses with high sensitivity for detection and quantification of differentially expressed proteins between two conditions have been developed. In order to uncover proteins that are significantly changed in CAAs and CTAs, the isobaric tag for absolute and relative quantitation (iTRAQ) technology (19) coupled with nano liquid chromatography (nanoLC)-matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF/TOF)-tandem MS (MS/MS) followed by ProteinPilot analysis (20) was used in this study as a quantitative proteomic approach. We performed a detailed characterization of CAAs and CTAs and revealed differences between normal aorta tissues and CAAs or CTAs.

Materials and methods

Patients and samples. The participants gave informed consent, and the studies were performed with the approval of the Ethics Committee of Shimane University School of Medicine, Japan. Calcified aortic aneurysm tissues were obtained at surgery from subjects with CAAs (n=6) and from subjects with CTAs (n=6) who underwent elective aneurysm repair. Reference samples were obtained from normal aorta tissues adjacent to CAA or CTA tissues. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until protein extraction.

Sample preparation. Sample preparation was performed according to the manual supplied by AB Sciex (Foster, CA, USA). Briefly, ~20 mg of the CAA or CTA samples and neighboring normal aorta tissues as controls were subjected to protein extraction. After adding urea lysis buffer containing 7 M urea, 0.1% Nonidet P-40 (NP-40) and 500 mM triethylammonium bicarbonate (TEAB) (Sigma, Tokyo, Japan), the sample was sonicated 5 times for 10 sec each time on ice using a Sonifier 450 sonicating probe (Branson Japan, Atsugi, Japan), incubated at 4°C for 1 h with constant shaking, and centrifuged at 10,000 x g for 10 min, and then the supernatant was collected. The supernatant was desalted and its buffer was exchanged with 50 mM TEAB using spin concentrators (Corning, Tokyo, Japan). The protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA).

Denaturation, reduction, trypsin digestion, and iTRAQ labeling. Labeling with iTRAQ was performed using iTRAQ™ Reagent from AB Sciex as described in the manufacturer's instructions. Briefly, as described previously (20), each of 50 μ g of CAA or CTA samples and neighboring normal aorta samples was denatured by sodium dodecyl sulfate (SDS) and reduced by [tris-(2-carboxyethyl)phosphine (TCEP)] as described previously. Then cysteine alkylation was performed using methylmethanethiosulfonate (MMTS). Subsequently, each sample was cleaved by trypsin. Digests were labeled with an iTRAQ Reagent Multiplex kit (AB Sciex). iTRAQ label 114 or 115 was used for labeling the control sample, and iTRAQ label 116 or 117 was used for labeling the CAA or CTA sample. Then the labeled control and the calcified aneurysmal samples were combined.

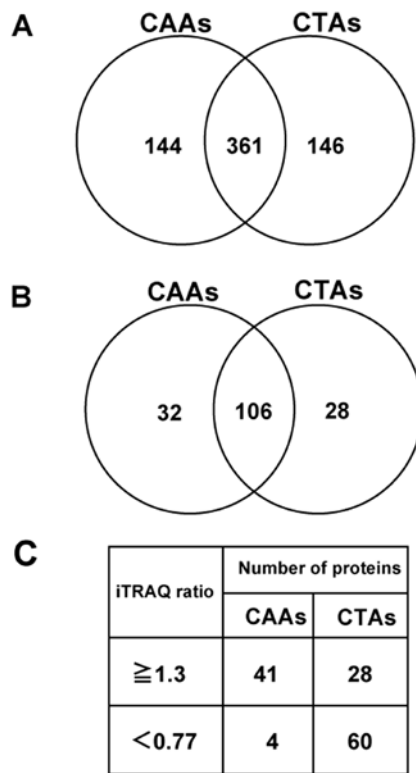


Figure 1. Venn diagrams presenting the numbers of differentially expressed proteins of CAAs, CTAs, and both calcified aneurysms compared with adjacent normal aortic tissues. (A) Number of proteins that were identified in more than one sample among 6 samples with iTRAQ ratios. (B) Number of proteins that were identified in at least 5 samples among 6 samples with iTRAQ ratios. (C) Numbers of proteins with significantly increased (≥ 1.3 -fold) and decreased expression ratios (< 0.77 -fold) that were identified in at least 5 samples among 6 samples in CAAs and CTAs.

Strong cation exchange (SCX) chromatography and nanoLC. SCX chromatography was performed with the AB Sciex cation exchange system according to the manufacturer's instructions. Subsequently, 6 fractions were collected and desalted by a Sep-Pac C_{18} cartridge according to the instructions of Waters Corporation (Milford, MA, USA). Fractionation with a NanoLC system was performed according to the instructions of the manufacturer (KYA Technologies, Tokyo, Japan). A total of 171 fractions per one fraction of SCX chromatography were collected onto an Opti-TOF LC/MALDI 384 target plate (AB Sciex) using a DiNa Map fraction collector (KYA Technologies). The methods for this step have been described in detail in our previous paper (20).

MS analysis and protein identification and relative quantification. The Mass Spectrometer 5800 MALDI-TOF/TOF Analyzer (AB Sciex) with TOF/TOF Series software (version 4.0) was used for MS data acquisition in a positive ion mode. Monoisotopic precursor selection for MS/MS was performed by automatic precursor selection with an interpretation method using the DynamicExit Algorithm (AB Sciex). The peptide data obtained by 5800 MALDI TOF/TOF MS/MS were analyzed with ProteinPilot™ 3.0 software using the Paragon protein database search algorithm (AB Sciex) (21). Each MS/MS spectrum was searched against the database constructed by AB Sciex (version 20081216; 20,489 entries).

Bioinformatic analysis. The Panther system (version 7) (<http://www.pantherdb.org/>) was used for classification and pathway analyses of proteins. The annotations of proteins were obtained from the Uniprot database (<http://www.uniprot.org/>) and appropriate literature. Clustering analysis of differentially expressed proteins was performed by the Genesis software provided by the Genesis team of Institute for Genomics and Bioinformatics, Graz University of Technology (Graz, Austria) (<http://genome.tugraz.at/>) (22).

Western blot analysis. Twenty micrograms of cell lysates extracted with urea lysis buffer as described above was electrophoresed through 10% SDS-polyacrylamide gel (PAGE), and proteins were subsequently electroblotted onto Hybond ECL nitrocellulose membranes (GE Healthcare Japan, Hino, Japan). The membranes were blocked at room temperature for 1 h with 5% nonfat dry milk in TBST [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20] and then incubated with rabbit polyclonal anti- α -2-HS-glycoprotein antibody (0.5 μ g/ml in TBST with 1% milk) (Calbiochem, Darmstadt, Germany), rabbit polyclonal anti-ceruloplasmin antibody (Epitomics, Burlingame, CA, USA), mouse monoclonal anti-periostin antibody (Sigma-Aldrich, St. Louis, MO, USA), or rabbit polyclonal anti-TGM2 antibody (Epitomics) for 1 h at room temperature. Membranes were washed three times with TBST and incubated with anti-rabbit or anti-mouse Alexa Fluor 680-conjugated or IRDye 800-conjugated immunoglobulin (IgG), followed by visualization using the infrared imaging system Odyssey (LI-COR, Inc., Lincoln, NE, USA). Data from duplicate experiments were analyzed for statistical significance by the unpaired t-test. Statistical significance was set at the 95% level ($P < 0.05$).

Results

Quantitative analysis of differentially expressed proteins between CAA or CTA and adjacent normal aorta tissues. A total of 6 calcified abdominal aortic aneurysmal tissues and adjacent normal aorta tissues were collected from 6 CAA patients. Similarly, 6 calcified thoracic aortic aneurysmal tissues and adjacent normal aorta tissues were collected from 6 CTA patients. Protein expression was compared between each calcified aneurysmal tissue and adjacent normal artery using iTRAQ labeling coupled to nanoLC-MALDI-TOF/TOF-MS/MS followed by ProteinPilot analysis. A total of 505 differentially expressed proteins in 6 CAA samples and 507 proteins in 6 CTA samples with 361 proteins in both calcified aneurysmal tissues that were identified in more than one sample were shown (Fig. 1A). Among them, 138 differentially expressed proteins in 6 CAA samples and 134 proteins in 6 CTA samples with 106 proteins in both calcified aneurysmal tissues that were identified in at least 5 samples were shown using highly stringent criteria for protein identification (unused ProtScore > 2 ; confidence $> 99\%$) (Fig. 1B). The relative quantitation by ProteinPilot analysis was based on statistical analysis. However, since most biochemical methods are apt to include technical variation, we considered an additional cut-off value at 1.3-fold change (≥ 1.3 - or < 0.77 -fold) for iTRAQ ratios to reduce false positive rates for the selection of differentially expressed proteins. Finally, among them 41 and

Table I. The proteins with increased expression in CAAs compared to those in adjacent normal aortic tissues.

Unused ^a	% Cov ^b	Peptides ^c	Reproducibility ^d	UniProt number	Gene symbol	Protein name	iTRAQ ratio (Average ± SE)	Molecular function
10.0	45.6	10	5	P08493	MGP	Matrix Gla protein	2.72±1.22	Extracellular matrix
22.0	48.0	16	6	P02743	APCS	Serum amyloid P-component	2.71±0.62	Immune response
9.1	48.7	6	5	P08519	LPA	Apolipoprotein(a)	2.38±1.10	Signaling molecule
22.8	40.3	15	6	P02765	AHSG	α -2-HS-glycoprotein	2.21±0.61	Extracellular matrix
4.0	44.4	4	6	P02656	APOC3	Apolipoprotein C-III	2.01±0.32	Transporter
33.7	49.2	25	6	P10909	CLU	Clusterin	1.94±0.45	Extracellular chaperone
4.0	13.7	2	5	Q961Y4	CPB2	Carboxypeptidase B2	1.85±0.37	Protease
35.7	45.8	19	6	P00734	F2	Prothrombin	1.82±0.37	Blood coagulation
45.8	41.6	34	6	P04004	VTN	Vitronectin	1.77±0.23	Extracellular matrix
9.5	38.5	4	6	P61626	LYZ	Lysozyme C	1.77±0.44	Hydrolase
19.2	26.1	11	6	P02748	C9	Complement component C9	1.74±0.21	Complement
162.7	73.1	187	6	P02452	COL1A1	Collagen α -1(I) chain	1.71±0.57	Extracellular matrix
18.1	19.9	9	5	P02730	SLC4A1	Band 3 anion transport protein	1.67±0.60	Transporter
48.0	74.8	42	6	P02649	APOE	Apolipoprotein E	1.66±0.59	Transporter
50.7	80.2	32	6	P02647	APOA1	Apolipoprotein A-I	1.65±0.33	Transporter
3.7	14.2	2	5	Q96PD5	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	1.65±0.37	Hydrolase
9.8	47.7	5	5	P35542	SAA4	Serum amyloid A-4 protein	1.64±0.31	Acute phase reactant
13.3	59.2	7	6	P62805	HIST1H4A	Histone H4	1.61±0.67	Nucleic acid binding
8.6	68.0	4	5	P02652	APOA2	Apolipoprotein A-II	1.58±0.31	Transporter
44.9	66.6	35	6	P21810	BGN	Biglycan	1.57±0.44	Enzyme modulator
10.1	16.3	5	6	P01042	KNG1	Kininogen-1	1.52±0.30	Blood coagulation
10.6	26.5	6	6	P06727	APOA4	Apolipoprotein A-IV	1.50±0.23	Transporter
100.3	57.0	101	5	P02461	COL3A1	Collagen α -1(III) chain	1.48±0.61	Extracellular matrix
14.1	42.8	9	6	Q07507	DPT	Dermatopontin	1.47±0.36	Extracellular matrix
128.1	72.6	138	6	P08123	COL1A2	Collagen α -2(I) chain	1.47±0.48	Extracellular matrix
17.0	20.5	9	6	P19823	ITIH2	Inter- α -trypsin inhibitor heavy chain H2	1.44±0.27	Hyaluronan binding
24.0	37.6	14	5	P07339	CTSD	Cathepsin D	1.43±0.46	Protease
4.0	14.1	2	5	P00740	F9	Coagulation factor IX	1.43±0.28	Protease
14.6	33.0	9	6	P02749	APOH	β -2-glycoprotein 1	1.41±0.32	Blood coagulation
70.6	99.3	110	6	P68871	HBB	Hemoglobin subunit β	1.39±0.33	Oxygen transport

^aA score of protein confidence (ProScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already 'used' by higher scoring proteins in the experiments. ^bThe percentage of matching amino acids from identified peptides. ^cThe number of distinct peptides with at least 95% confidence in the experiments. ^dReproducibility in six experiments.

Table II. The proteins with decreased expression in CAAs compared to those in adjacent normal aortic tissues.

Unused ^a	% Cov ^b	Peptides ^c	Reproducibility ^d	UniProt number	Gene symbol	Protein name	iTRAQ ratio (Average ± SE)	Molecular function
34.0	25.2	18	5	Q05707	COL14A1	Collagen α-1(XIV) chain	0.70±0.11	Extracellular matrix
86.5	47.2	45	5	P35580	MYH10	Myosin-10	0.70±0.20	Enzyme modulator
14.6	36.9	9	6	P20774	OGN	Mimecan	0.71±0.10	Extracellular matrix
4.1	17.2	3	5	Q6NZI2	PTRF	Polymerase I and transcript release factor	0.75±0.20	Transcription factor

^aA score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already 'used' by higher scoring proteins in the experiments. ^bThe percentage of matching amino acids from identified peptides. ^cThe number of distinct peptides with at least 95% confidence in the experiments. ^dReproducibility in six experiments.

28 proteins with significantly increased expression (≥ 1.3 -fold) and 4 and 60 proteins with significantly decreased expression (< 0.77 -fold) were identified in CAAs and CTAs, respectively (Fig. 1C).

In Tables I-IV, increased and decreased proteins in CAAs and CTAs compared with those in adjacent normal aorta tissues are listed in the order of iTRAQ ratios. Among them, proteins such as type I and type III collagens (16,23) in increased proteins in CAAs, type I collagen, fibrinogen α , β , γ chains and prothrombin (24) in increased proteins in CTAs, and fibulin-5 (DANCE) (25) in decreased proteins in CTAs that are known to be involved in aortic aneurysmal development and progression were identified. Proteins such as matrix Gla protein, α -2-HS-glycoprotein (fetuin-A) (15) and biglycan in increased proteins in CAAs, mimecan (osteo-glycin) in decreased proteins in CAAs, α -2-HS-glycoprotein, and decorin and biglycan (26) in increased proteins in CTAs that are known to participate in vascular calcification were also identified.

Molecular function classification of differentially expressed proteins. For the functional distribution, analysis of the identified proteins with significant differential expression (≥ 1.3 - or < 0.77 -fold) in CAAs and CTAs was performed using the Panther classification system, which sorts the proteins into respective classes based on their biological process (Fig. 2). We classified the identified variable proteins with increased expression in the following groups with more than 4 proteins: transporter [7 proteins (17.1%)], signaling molecule [6 proteins (14.6%)], protease [5 proteins (12.2%)] and transfer/carrier protein [4 proteins (9.8%)] among 41 proteins of CAAs, and signaling molecule [8 proteins (28.6%)] and enzyme modulator [4 proteins (14.3%)] among 28 proteins of CTAs. On the other hand, the groups with decreased expression were cytoskeletal protein [13 proteins (21.7%)], signaling molecule [8 proteins (13.3%)], transfer/carrier protein [5 proteins (8.3%)], isomerase [4 proteins (6.7%)], oxidoreductase [4 proteins (6.7%)], and structural proteins [4 proteins (6.7%)] among 60 proteins of CTAs. There were no classified groups for decreased proteins in CAAs. The transporter group included apolipoprotein C-III (apoC-III), band 3 anion transport protein, apolipoprotein E (apoE), apolipoprotein A-I (apoA-I), serum amyloid A-4 protein, apolipoprotein A-II (apoA-II), and apolipoprotein A-IV (apoA-IV) with increased expression in CAAs. The signaling molecule group included fibrinogen γ , β , α chains, periostin, thrombospondin-1, complement C1q subcomponent subunit C, complement C3, and type I collagen α -1 with increased expression in CTAs. The cytoskeletal protein group included transgelin, myosin regulatory light polypeptide 9, tropomyosin β chain, cysteine and glycine-rich protein 1, calponin-1, caldesmon, lipoma-preferred partner, myosin light polypeptide 6, talin-1, moesin, vinculin, PDZ and LIM domain protein 7, and gelsolin with decreased expression in CTAs.

Pathway analysis of differentially expressed proteins. The Panther software was also used for pathway analyses of proteins that were shown to have significant differential expression (≥ 1.3 - or < 0.77 -fold) in our study. It was notable that 6 proteins with increased expression in both CAAs and CTAs correspond to proteins found in the blood coagulation

Table III. The proteins with increased expression in CTAs compared to those in adjacent normal aortic tissues.

Unused ^a	% Cov ^b	Peptides ^c	Reproducibility ^d	UniProt number	Gene symbol	Protein name	iTRAQ ratio (Average ± SE)	Molecular function
54.7	64.9	37	6	P02679	FGG	Fibrinogen γ chain	3.80±1.74	Blood coagulation
77.7	75.2	69	6	P02675	FGB	Fibrinogen β chain	3.61±1.65	Blood coagulation
76.0	50.5	44	6	P02671	FGA	Fibrinogen α chain	3.15±1.22	Blood coagulation
14.4	31.1	8	6	P02765	AHSG	α -2-HS-glycoprotein	2.91±0.83	Extracellular matrix
35.4	43.1	17	6	P00734	F2	Prothrombin	2.85±0.80	Blood coagulation
41.2	74.5	30	6	P02649	APOE	Apolipoprotein E	2.33±0.63	Transporter
10.0	10.3	5	6	P01042	KNIG1	Kininogen-1	2.32±0.45	Blood coagulation
30.9	35.7	15	6	Q15063	POSTN	Periostin	2.21±0.68	Extracellular matrix
42.9	41.6	31	6	P04004	VTN	Vitronectin	2.18±0.46	Extracellular matrix
26.0	27.8	13	5	P07996	THBS1	Thrombospondin-1	2.14±0.68	Extracellular matrix
21.1	39.0	11	5	P07585	DCN	Decorin	1.98±0.51	Extracellular matrix
44.5	74.6	23	6	P00738	HP	Haptoglobin	1.95±0.33	Hemoglobin binding
27.1	53.3	18	6	P51884	LUM	Lumican	1.89±0.35	Extracellular matrix
6.0	37.6	5	6	P02747	C1QC	Complement C1q subcomponent subunit C	1.81±0.44	Complement
2.0	16.2	1	5	P02656	APOC3	Apolipoprotein C-III	1.80±0.20	Transporter
10.0	13.7	5	5	P19827	ITIH1	Inter- α -trypsin inhibitor heavy chain H1	1.63±0.39	Protease inhibitor
53.4	73.1	37	6	P21810	BGN	Biglycan	1.53±0.25	Extracellular matrix
44.0	74.2	25	6	P02647	APOA1	Apolipoprotein A-I	1.50±0.27	Transporter
57.2	28.8	29	6	P01024	C3	Complement C3	1.48±0.30	Complement
27.9	56.1	23	6	P02743	APCS	Serum amyloid P-component	1.46±0.20	Immune response
10.7	10.9	5	5	P19823	ITIH2	Inter- α -trypsin inhibitor heavy chain H2	1.43±0.15	Hyaluronan binding
36.2	53.8	20	6	P01009	SERPINA1	α -1-antitrypsin	1.41±0.21	Protease inhibitor
122.0	71.1	99	6	P08123	COL1A2	Collagen α -2(I) chain	1.40±0.31	Extracellular matrix
15.0	43.3	9	6	P02763	ORM1	α -1-acid glycoprotein 1	1.38±0.21	Immune response
152.5	71.5	163	6	P02452	COL1A1	Collagen α -1(I) chain	1.38±0.32	Extracellular matrix
56.6	98.0	86	6	P68871	HBB	Hemoglobin subunit β	1.33±0.40	Oxygen transport
15.7	44.8	12	6	Q07507	DPT	Dermatopontin	1.31±0.19	Extracellular matrix
18.0	7.2	9	6	P04114	APOB	Apolipoprotein B-100	1.30±0.23	Transporter

^aA score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already 'used' by higher scoring proteins in the experiments. ^bThe percentage of matching amino acids from identified peptides. ^cThe number of distinct peptides with at least 95% confidence in the experiments. ^dReproducibility in six experiments.

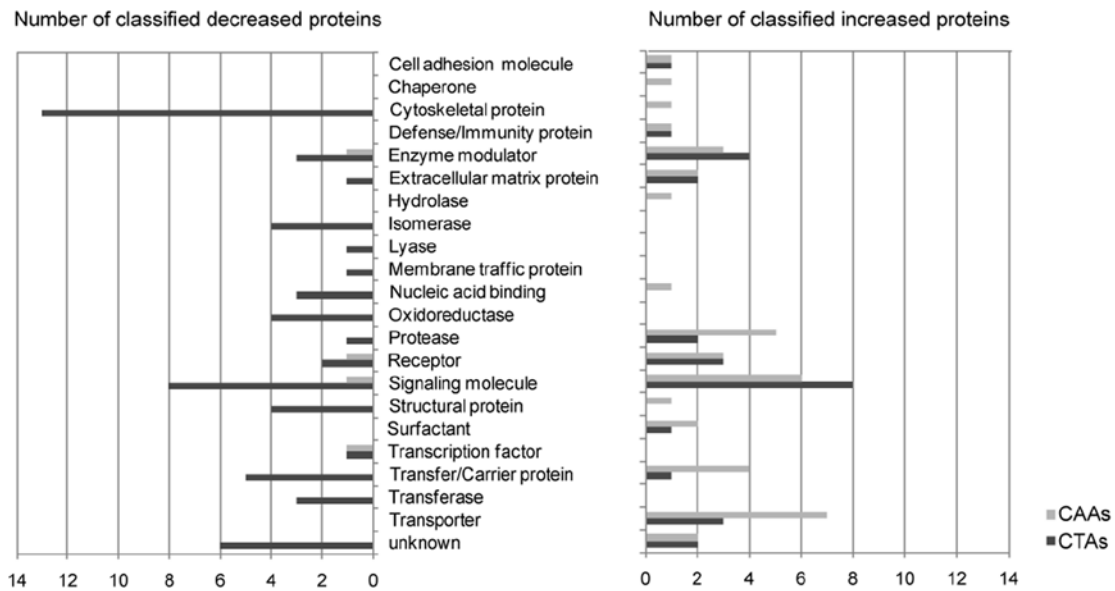


Figure 2. Classification analysis of proteins that show significantly increased (≥ 1.3 -fold) and decreased expression (<0.77 -fold) in CAAs and CTAs by the Panther software. Number of increased or decreased proteins are shown in each group.

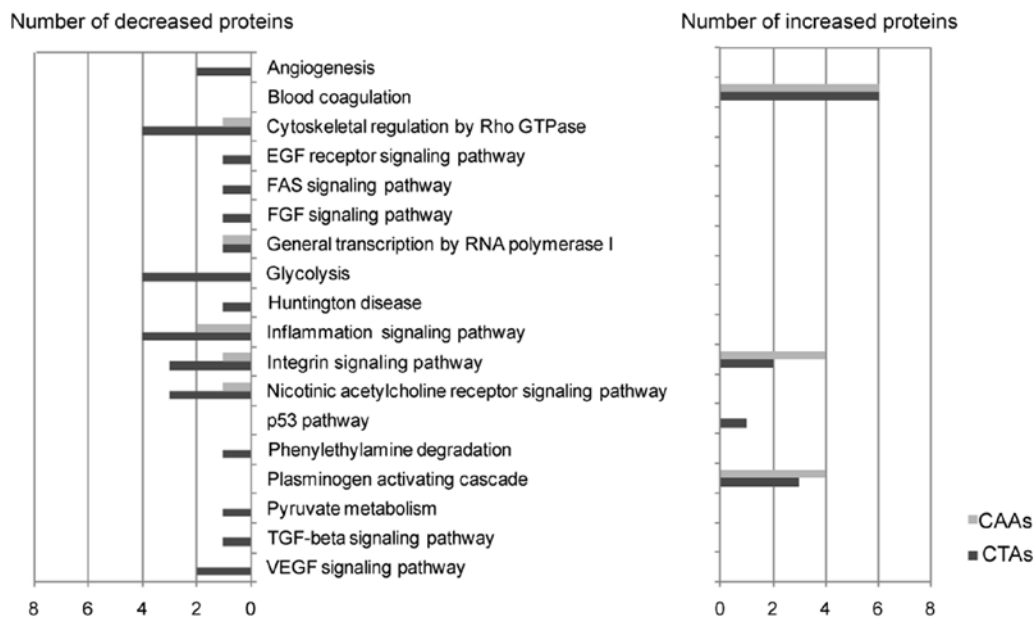


Figure 3. Pathway analysis of proteins that show significantly increased (≥ 1.3 -fold) and decreased expression (<0.77 -fold) in CAAs and CTAs by the Panther software. Number of increased or decreased proteins are shown in each pathway.

pathway illustrated in Fig. 3. In addition, two pathways each consisting of 4 proteins with increased expression in CAAs corresponded to proteins in the plasminogen activating cascade and the integrin signaling pathway. On the other hand, three pathways consisting of each of 4 proteins with decreased expression in CTAs corresponded to proteins in the cytoskeletal regulation by Rho GTPase pathway, the glycolysis pathway, and the inflammation mediated by chemokine and cytokine signaling pathway.

Hierarchical clustering analysis of differentially expressed proteins in CAAs and CTAs. Cluster analysis with the Genesis software was performed for our identified proteins to evaluate

the relation between differentially expressed proteins that were identified in at least 5 samples among 6 CAA samples or 6 CTA samples and to identify interesting protein expression clusters (Fig. 4A). We examined 138 differentially expressed proteins in CAAs and the 134 differentially expressed proteins in CTAs. Interestingly, in CAAs, three proteins with increased expression (type I collagen α -1 and α -2, and type III collagen α -1) included in the integrin signaling pathway (Fig. 3) were clustered (Fig. 4A, left panel). On the other hand, in CTAs, three proteins with increased expression (fibrinogen α , β , γ chains) included in the blood coagulation pathway (Fig. 3), three proteins with decrease expression (myosin-10, -11 and type XIV collagen α -1) included in the

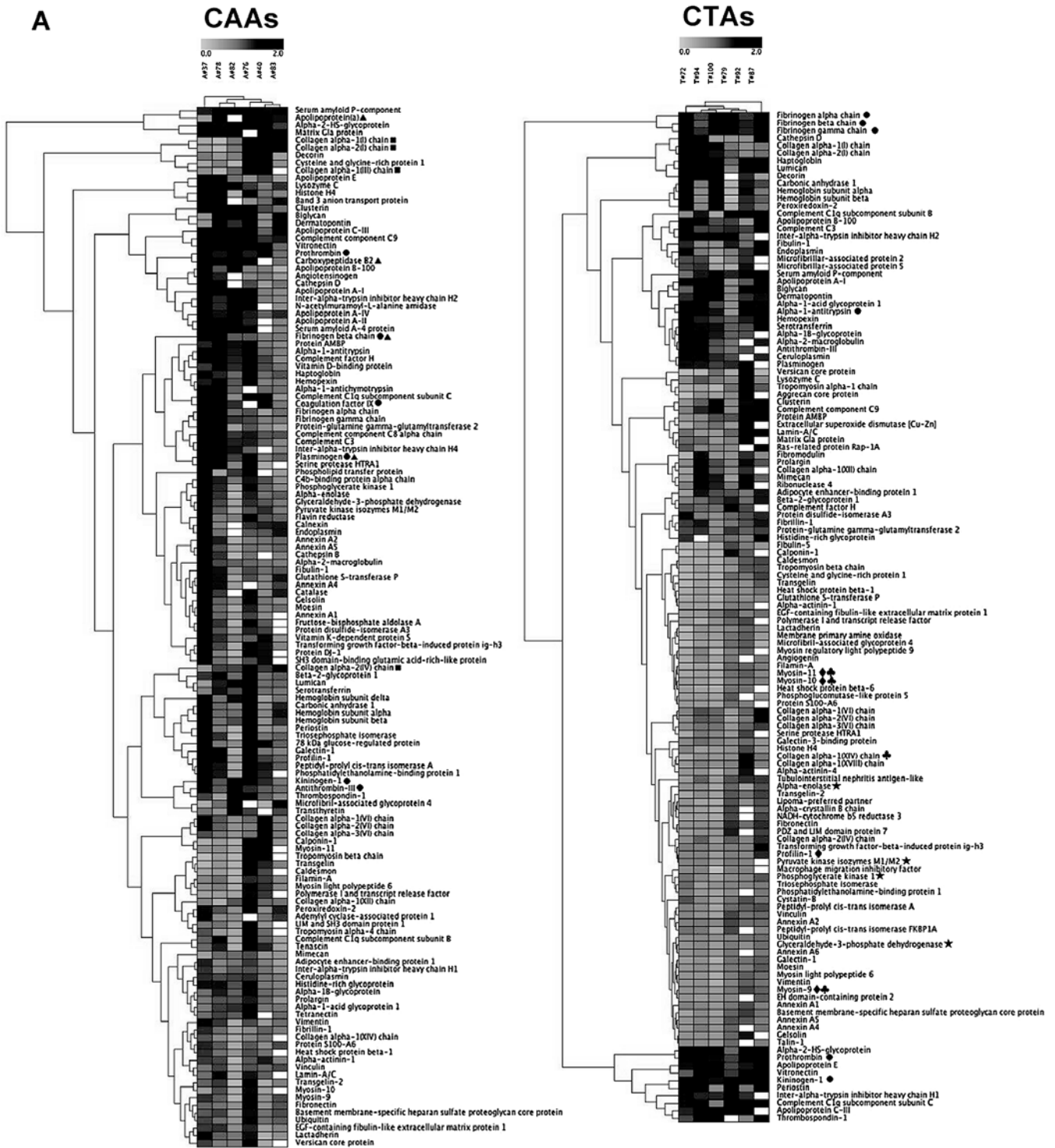


Figure 4A. Hierarchical clustering analysis of differentially expressed proteins in CAAs and CTAs by Genesis software. (A) The dendrogram displays two-way hierarchical clustering analyses of 138 differentially expressed proteins that were commonly identified in at least 5 samples among 6 CAA samples (A#37, A#40, A#76, A#78, A#82 and A#83) (left panel) and 134 differentially expressed proteins that were identified in at least 5 samples among 6 CTA samples (T#72, T#79, T#87, T#92, T#94 and T#100) (right panel). Differences in iTRAQ ratios are color-coded. Darker black spectra indicate increased expression, whereas lighter grey spectra indicate decreased expression compared with those in adjacent normal aorta tissues. White squares show no detection of indicated proteins in corresponding samples. Type I collagen α -1 and α -2 chains, type III collagen α -1 chain and type IV collagen α -2 chain included in the integrin signaling pathway with increased expression in CAAs (Fig. 3) are indicated with black squares in the left panel. Prothrombin, fibrinogen β chain, coagulation factor IX, plasminogen, kininogen-1 and antithrombin-III included in the blood coagulation pathway with increased expression in CAAs (Fig. 3) are indicated with black circles in the left panel. Apolipoprotein(a), carboxypeptidase B2, fibrinogen β chain and plasminogen included in the plasminogen activated cascade with increased expression in CAAs (Fig. 3) are indicated with black triangles in the left panel. Fibrinogen α , β and γ chains, α -1-antitrypsin, prothrombin and kininogen-1 included in the blood coagulation pathway with increased expression in CTAs (Fig. 3) are indicated with black circles in the right panel. Myosin-11, myosin-10, profilin-1 and myosin-9 included in the cytoskeletal regulation by Rho GTPase pathway with decreased expression in CTAs (Fig. 3) are indicated with black diamonds in the right panel. Myosin-11, myosin-10, type XIV collagen α -1 and myosin-9 included in the inflammation mediated by chemokine and cytokine signaling pathway with decreased expression in CTAs (Fig. 3) are indicated with black clover-shapes in the right panel. α -enolase, pyruvate kinase isozymes M1/M2, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase 1 included in the glycolysis pathway with decreased expression in CTAs (Fig. 3) are indicated with black stars in the right panel.

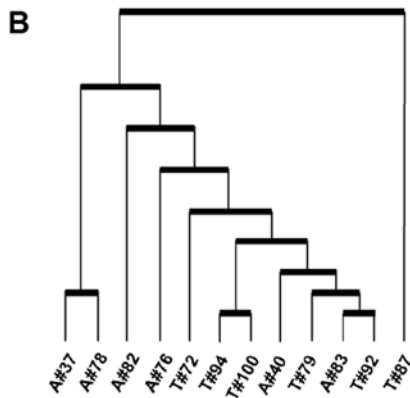


Figure 4B. Hierarchical clustering analysis of differentially expressed proteins in CAAs and CTAs by Genesis software. (B) Hierarchical clustering of 12 calcified aortic aneurysmal samples with 6 CAAs and 6 CTAs based on differentially expressed proteins compared with those in adjacent normal aorta tissues. One hundred forty differentially expressed proteins that were commonly identified in at least 9 samples among 12 samples (A#37, A#40, A#76, A#78, A#82, A#83, T#72, T#79, T#87, T#92, T#94 and T#100) were analyzed.

inflammation mediated by chemokine and cytokine signaling pathway (Fig. 3), and three proteins with decreased expression (pyruvate kinase isozymes M1/M2, phosphoglycerate kinase 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) included in the glycolysis pathway (Fig. 3) were clustered (Fig. 4A, right panel).

Subsequently, we performed cluster analysis of 12 calcified aortic aneurysmal samples from 6 CAA and 6 CTA patients based on the expression patterns of differentially expressed proteins of each sample (Fig. 4B). Samples of CAAs (patient nos. A#37, A#76, A#78 and A#82) and samples of CTAs (T#72, T#79, T#87, T#92, T#94 and T#100) were properly clustered. Interestingly, CAA samples from patients with both CAAs and CTAs (A#40 and A#83) were clustered outside the samples of patients with CAAs and were rather clustered with samples of patients with CTAs.

Validation of iTRAQ ratio by western blot analysis. To validate the accuracy of the quantitative results by iTRAQ ratios of differentially expressed proteins, some proteins with an iTRAQ quantitative ratio were quantified again by western blot analysis. Ceruloplasmin (iTRAQ ratio, 1.00) in CAAs and protein-glutamine γ -glutamyltransferase (referred to as TGM2) (iTRAQ ratio, 0.99) in CTAs were used as proteins expressed equivalently in a calcified aortic aneurysm and normal adjacent blood vessel. On the other hand, α -2-HS-glycoprotein (referred to as AHSG) (iTRAQ ratio, 2.21) in CAAs and periostin (iTRAQ ratio, 2.21) in CTAs were selected as representative proteins, since the iTRAQ ratios of these proteins were relatively large. We performed western blot analysis with anti-AHSG antibody and anti-ceruloplasmin antibody against CAA and adjacent normal aortic samples from patient A#40 (Fig. 5Aa). The ratio of increased expression of AHSG in the CAA normalized with ceruloplasmin expression was determined by band intensity and it was 2.22-fold (Fig. 5Ab). This value is consistent with the quantitative result determined by the iTRAQ ratio (2.21). Similarly, western blot analysis was performed with anti-periostin antibody and anti-TGM2 antibody against CTA and adjacent normal aortic samples from

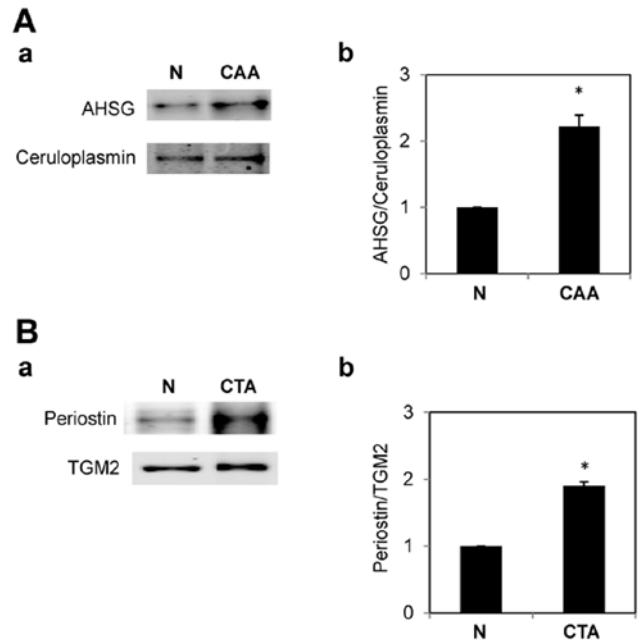


Figure 5. Validation of iTRAQ ratio by western blotting. (Aa) Western blot analysis with anti- α -2-HS-glycoprotein (referred to as AHSG) antibody and anti-ceruloplasmin antibody against CAA and adjacent normal aorta (N) samples. (Ab) Densitometric analysis in duplicated experiments for AHSG. The ratio of increased expression (2.22-fold) of AHSG normalized with ceruloplasmin expression in CAA compared with those of adjacent normal aorta samples was determined by band intensity. (Ba) Western blot analysis with anti-periostin antibody and anti-protein-glutamine γ -glutamyltransferase (TGM2) antibody against CTA and adjacent normal aorta (N) samples. (Bb) Densitometric analysis in duplicated experiments for periostin. The ratio of increased expression (1.90-fold) of periostin normalized with TGM2 expression in CTA compared with those of adjacent normal aorta samples was determined by band intensity. * $P < 0.05$.

patient T#94 (Fig. 5Ba). The ratio of increased expression of periostin in the CTA normalized with TGM2 expression was 1.90-fold (Fig. 5Bb). This value is nearly consistent with the quantitative result determined by the iTRAQ ratio (2.21). Overall, there was a good correlation between the iTRAQ ratio and band intensity of western blot analysis.

Discussion

Calcified aortic aneurysm is a cardiovascular disease. It is a complex disease with both genetic and various risk factors, including aging, gender, and inflammation factors, contributing to the development and progression of the disease. The molecular pathogenesis of calcified aortic aneurysm is poorly understood. In this study, we carried out a global proteomic study on calcified aneurysmal tissues from abdominal and thoracic aortas and adjacent normal aorta tissues. Quantitative proteomics using iTRAQ labeling followed by nanoLC-MALDI-TOF/TOF-MS/MS analysis, which is currently one of the most widely used approaches for high-throughput protein quantitation, has been proven to be a useful technique for examination of molecular mechanisms in diseases. We used the iTRAQ proteomic approach to identify proteins with differential expression between CAAs or CTAs and their adjacent normal aorta tissues. As a result, 41 and 28 proteins with increased expression (≥ 1.3 -fold) and 4 and 60 proteins with decreased

Table IV. The proteins with decreased expression in CTAs compared to those in adjacent normal aortic tissues.

Unused ^a	% Cov ^b	Peptides ^c	Reproducibility ^d	UniProt number	Gene symbol	Protein name	iTRAQ ratio (Average ± SE)	Molecular function
31.3	57.3	21	6	P55083	MFAP4	Microfibril-associated glycoprotein 4	0.53±0.12	Signaling molecule
63.9	49.3	38	5	P12814	ACTN1	α-actinin-1	0.53±0.08	Cytoskeletal protein
61.4	96.0	46	6	Q01995	TAGLN	Transgelin	0.53±0.11	Cytoskeletal protein
28.5	43.5	17	6	Q9UBX5	FBLN5	Fibulin-5	0.53±0.12	Extracellular matrix
11.2	31.4	6	6	P09211	GSTP1	Glutathione S-transferase P	0.54±0.06	Transferase
19.7	46.8	16	6	P04792	HSPB1	Heat shock protein β-1	0.55±0.08	Structural protein
23.4	34.4	10	5	Q15124	PGM5	Phosphoglucomutase-like protein 5	0.57±0.10	Isomerase
46.2	65.1	26	6	Q08431	MFGE8	Lactadherin	0.57±0.09	Signaling molecule
27.9	26.2	14	6	Q16853	AOC3	Membrane primary amine oxidase	0.57±0.10	Oxidoreductase
18.0	61.1	10	5	P24844	MYL9	Myosin regulatory light polypeptide 9	0.58±0.12	Cytoskeletal protein
6.0	34.0	3	5	P03950	ANG	Angiogenin	0.58±0.08	Endoribonuclease
4.0	23.8	2	5	O14558	HSPB6	Heat shock protein β-6	0.59±0.10	Structural protein
75.8	78.5	46	6	P07951	TPM2	Tropomyosin β chain	0.59±0.13	Cytoskeletal protein
7.3	44.4	4	5	P06703	S100A6	Protein S100-A6	0.59±0.08	Signaling molecule
180.7	55.9	108	6	P35580	MYH10	Myosin-10	0.59±0.08	Enzyme modulator
4.0	17.4	3	5	P14174	MIF	Macrophage migration inhibitory factor	0.59±0.10	Signaling molecule
159.2	57.3	104	6	P35749	MYH11	Myosin-11	0.60±0.10	Enzyme modulator
221.7	59.0	166	6	P21333	FLNA	Filamin-A	0.60±0.11	Actin-binding protein
17.9	70.5	9	6	P21291	CSRPI	Cysteine and glycine-rich protein 1	0.60±0.14	Cytoskeletal protein
36.5	58.9	22	5	P51911	CNN1	Calponin-1	0.62±0.22	Cytoskeletal protein
43.6	47.8	20	6	Q05682	CALD1	Caldesmon	0.62±0.13	Cytoskeletal protein
6.0	22.5	3	5	P30086	PEBP1	Phosphatidylethanolamine-binding protein 1	0.62±0.07	Transfer/carrier protein
10.2	15.3	5	5	Q9NZN4	EHD2	EH domain-containing protein 2	0.63±0.08	Membrane traffic protein
88.9	84.6	83	6	P08670	VIM	Vimentin	0.63±0.09	Structural protein
17.0	71.8	8	6	P62805	HIST1H4A	Histone H4	0.64±0.09	Nucleic acid binding
67.4	12.8	56	6	P13611	VCAN	Versican core protein	0.64±0.29	Extracellular matrix
16.8	65.1	9	5	P02511	CRYAB	α-crystallin B chain	0.64±0.14	Structural protein
12.0	32.3	8	6	P06733	ENO1	α-enolase	0.65±0.10	Lyase
12.3	26.4	6	5	Q6NZI2	PTRF	Polymerase I and transcript release factor	0.65±0.13	Transcription factor
18.1	29.6	12	6	Q93052	LPP	Lipoma-preferred partner	0.65±0.12	Cytoskeletal protein

^aA score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already 'used' by higher scoring proteins in the experiments. ^bThe percentage of matching amino acids from identified peptides. ^cThe number of distinct peptides with at least 95% confidence in the experiments. ^dReproducibility in six experiments.

expression (<0.77-fold) were shown to be proteins with significant differential expression in CAAs and CTAs versus adjacent normal aorta tissues, respectively.

Among the proteins with significant differential expression in CAAs, type I collagen α -1 and type III collagen α -1 chains were increased, whereas type XIV collagen α -1 was decreased. Collagen plays an important role in arterial wall integrity (27). Appropriate amounts of these collagens are essential for arterial integrity to endure the forces exerted by blood pressure. Either increased or decreased collagen in aortas may weaken the integrity of the aortic wall, resulting in predisposition of aortic aneurysms. Interestingly, increased amounts of collagens would lead to fibrosis, which may result in arterial stiffness and increased susceptibility to aneurysmal diseases. Similarly, not only collagens, but also other extracellular matrix proteins such as fibulin-5 (also known as DANCE) with regulated expression is essential for the functional integrity of vessel walls. Fibulin-5 showed significantly decreased expression in CTAs in this study. Fibulin-5 is essential for elastogenesis with an elastogenic organizer activity (28).

Vascular calcification is frequently detected in advanced atherosclerosis and aneurysms. Vascular calcification is a highly regulated process with inducers and inhibitors responsible for calcium deposition. An imbalance of these regulators results in the initiation of calcification. Under normal conditions, inhibitor proteins such as matrix Gla protein, BMP-7, α -2-HS glycoprotein (fetuin-A), osteoprotegerin and osteopontin prevent vascular calcification processes (29). Among these representative inhibitors, matrix Gla protein and α -2-HS glycoprotein were increased in CAAs and CTAs in this study. These findings are consistent with previous observations by Spronk *et al* (30). The upregulation of these inhibitors in calcified aneurysms might be explained by assuming a feedback mechanism of upregulated production of these inhibitors, leading to the prevention of further calcium phosphate deposition.

The abdominal and thoracic aortas share similarities in their physical structures and cellular components. In this study, 16 proteins including serum amyloid P-component, α -2-HS glycoprotein, apolipoprotein C-III, prothrombin, and vitronectin were significantly increased in both CAAs and CTAs (≥ 1.3 -fold) and 3 proteins including type XIV collagen α -1 chain, myosin-10, and polymerase I and transcript release factor were significantly decreased in both CAAs and CTAs (<0.77-fold). These proteins might provide the basis for the similar pathological phenotypes between CAAs and CTAs. However, heterogeneity also exists between abdominal and thoracic aortas. This difference contributes to vessel mechanics, proteolytic activity, signaling pathways, and atherosclerotic plaque deposition, leading to disparities in the pathologic mechanisms involved in CAAs and CTAs. In the present study, 3 proteins (histone H4, serine protease HTRA1, and cysteine and glycine-rich protein 1) that were significantly increased in CAAs but significantly decreased in CTAs were identified. On the other hand, 10 proteins (including matrix Gla protein, clusterin, lysozyme C, complement component C9, and cathepsin D) that were significantly increased only in CAAs, one protein (mimecan) that was significantly decreased only in CAAs, 12 proteins (including fibrinogen γ and α chains, periostin, thrombospondin-1, and haptoglobin)

that were significantly increased only in CTAs, 35 proteins (including microfibril-associated glycoprotein 4, α -actinin-1, transgelin, glutathione S-transferase P, and heat-shock protein β -1) that were significantly decreased only in CTAs were identified. These proteins with distinct expression patterns might be involved in the different pathogenesis associated with these two types of calcified aneurysms.

Although it is still controversial as to whether atherosclerotic lesions are a prerequisite for the formation of AAAs, it was found with the Panther classification system that several kinds of apolipoproteins such as apoC-III, apoE, apoA-I, apoA-II, and apoA-IV exhibited increased expression in CAAs compared with their expression in control tissues in this study. Apolipoproteins are deeply involved in lipid metabolism. The development of atherosclerosis is associated with disorders of lipoprotein metabolism (31). Golledge *et al* (32) demonstrated that a low high-density lipoprotein level is the most sensitive predictor of AAA among lipid markers. However, several lines of evidence have suggested potent anti-atherogenic roles of apoE (33), apoA-I (34), apoA-II (35), and apoA-IV (36) in contrast to the atherogenic role of apoC-III (37). In addition, Sadek *et al* (38) demonstrated the upregulation of ApoE mRNA synthesis in AAAs by microarray analysis. The upregulation of apolipoproteins with anti-atherogenic function in this study might be explained by assuming a feedback mechanism of increased anti-atherogenic apolipoproteins, possibly to prevent further atherosclerosis required for calcified aortic aneurysms.

The presence of a chronic intraluminal thrombus (ILT) is often found in aortic aneurysms. An ILT participates in aneurysmal dilatation. An ILT is a highly biologically active site in which fibrin formation following platelet and thrombin activation occurs. The ILT is a laminated structure with several layers of fibrin clots and an underlying hematic luminal layer with undegraded crosslinked fibrin (39). Consistent with these observations, the pathway analysis using Panther software in this study revealed that 6 proteins with increased expression in both CAAs and CTAs are involved in the blood coagulation pathway. Interestingly, Stec *et al* (40) showed strong linear relationships between plasma fibrinogen and cardiovascular diseases.

Several studies have suggested that oxidative stress is increased in aortic aneurysms and that modulation of reactive oxygen species (ROS) influences the formation of aortic aneurysms (41). In aortic aneurysms, ROS are generated by many infiltrating leukocytes, and generation of ROS leads to increased production of MMPs (42). Thus, it is important for protection of aortic aneurysms to reduce the activity of ROS-generating systems and/or to increase the levels of cellular antioxidants and antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione S-transferase (GST). It has been reported that SOD activity was reduced in AAA tissue samples (43). In addition, it is known that GST is involved in the removal of products of potential atherogenic substances, such as lipid oxides (44). In line with this evidence, it was found in the present study that SOD (iTRAQ ratio, 0.77) and GST (iTRAQ ratio, 0.54) were significantly decreased in CTAs compared with those in normal tissues.

Increasing evidence suggests that oxidative stress has effects on gene expression, protein stability, and enzymatic

activity. It has been reported that GAPDH is one of the targets for oxidative stress. GAPDH plays an important role in energy metabolism in the glycolytic pathway. Sukhanov *et al* (45) showed that proatherogenic molecule oxidized low-density lipoprotein (OxLDL) downregulates GAPDH by decreasing protein stability, resulting in a reduction in ATP levels. In the present study, it was found that GAPDH levels decreased in CTAs compared with that in controls. Although we have not shown whether oxidative stress is actually increased in aortic aneurysms in the samples used in this study, it is possible that the decreased level of GAPDH is due to the increase in oxidative stress in CTAs. Furthermore, other decreased glycolytic enzymes such as α -enolase, pyruvate kinase isozymes M1/M2, and phosphoglycerate kinase 1 in the present study may also be targets for oxidative stress.

Interestingly, as shown in Fig. 4B, it was found that the expression patterns of differentially expressed proteins in CAAs from patients with both CAAs and CTAs (A#40 and A#83) show more resemblance to those in CTAs from patients with only CTAs than those in CAAs from patients with only CAAs. We speculated that the factors that influence the protein expression in CAAs may be diffusely secreted from CTAs of patients A#40 and A#83 and, in consequence, the protein expression patterns in CAAs from these patients might be more closely related to those in CTAs than those in CAAs.

In conclusion, this is the first comprehensive study on the mechanisms of development and progression of calcified abdominal aortic aneurysms (CAAs) and thoracic aortic aneurysms (CTAs) using an iTRAQ proteomics approach. This study revealed many novel proteins that had not been known to be associated with the abdominal and thoracic aortic aneurysm formation and vascular calcification.

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