Identification Novel Biomarker for Acute Aortic Dissection Using 4D-Label-Free Proteomics

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Research Article

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Abstract

Purpose: we aimed to identify potential candidate biomarkers in aorta tissue from AAD patients.

Methods: We used 4D label-free quantification (4D-LFQ) mass spectrometry to screen differentially expressed proteins in aorta tissues of AAD patients. Then we performed protein annotation, unsupervised hierarchical clustering, functional classification, functional enrichment and cluster, and protein-protein interaction analyses. Parallel Reaction Monitoring (PRM) technology was used to accurately and quantitatively confirm the selected target proteins.

Results: A total of 3350 proteins were identified. Taking 1.5 times as the differential expression threshold, 139 were upregulated and 108 were downregulated as compared to the control groups. Bioinformatics analysis showed that the differential proteins were mainly distributed in extracellular matrix and cytoplasm. And their functions mainly involve cell migration and proliferation, inflammatory cell activation, cell contraction, muscle organ development and other processes. PRM technology accurately quantified the selected 20 target proteins, and found SAA1, LBP, MPO, and ENG were confirmed to be enriched in the aorta tissue of AAD patients.

Conclusions: This is the first application of a 4D-LFQ-PRM workflow to identify and validate biomarkers in AAD patients. SAA1, LBP, MPO, and ENG represent novel biomarkers for the pathogenesis of AAD and might be a therapeutic target in the future.

1. Introduction

Acute aortic dissection (AAD) is a serious life-threatening cardiovascular disease ^[1, 2]. For untreated Stanford type A of AAD, the mortality rate increased by 1–2% every 1 hour ^[3–4]. 30-day mortality in hospitalized patients with any acute aortic dissection was 37% ^[5]. Due to the rapid development of AAD and the high mortality rate, early diagnosis and progress evaluation are clinically important issues. At present, computed tomography angiography (CTA) and magnetic resonance angiography (MRA) is the main method for diagnosing AAD. However, many primary hospitals lack of the CTA or MRA equipment, which may cause delays in the diagnosis and treatment of AAD. Therefore, we urgently need to develop reliable early diagnostic biomarkers for AAD. D-dimer, C-reactive protein, soluble ST2, smooth muscle myosin heavy chain(sm-MHC) and calponin are commonly used in the diagnosis of AAD ^[6–10]. However, these biomarkers have low sensitivity and specificity for the diagnosis of AAD, which can easily cause missed or misdiagnosis and delay the disease. Therefore, it is urgent to find new biomarkers for AAD.

Studies have shown that inflammation, angiogenesis, activation of transforming growth factor- β 1 (TGF- β 1) signaling pathway and degradation of extracellular matrix, abnormal phenotypic transition of vascular smooth muscle cells (VSMCs) is mainly involved in the pathogenesis of AAD. However, the detailed molecular mechanism remains to be explored ^[11–15].

Quantitative proteomics is the most promising technology in screening biomarkers for early diagnosis of diseases ^[16–18]. It has been widely used to analyze all protein levels expressed in a wide range of body fluids and tissues obtained from patients in order to identify potential clinical biomarkers ^[19, 20]. Some previous studies used proteomics techniques to search for AAD biomarkers ^[21, 22–24]. However, due to the limitation of the scanning speed, sensitivity and resolution of mass spectrometers, the markers screened in previous studies are not of high diagnostic value for AAD, which is not conducive to clinical application. The 4D-LFQ used a highly sensitive quantitative approach, including tandem mass tag (TMT) labeling and an LC-MS/MS platform combined with

advanced bioinformatics analysis to improve in detecting flux, depth and accuracy [25, 26]. 4D-LFQ adds a fourth dimension-ion mobility on the basis of the three dimensions of the traditional 3D-LFQ, which not only greatly improves the scanning speed, detection sensitivity and quantitative accuracy, but also improves the possibility of identifying important proteins with low abundance [27].

In this study, we have applied 4D-LFQ and followed by targeted parallel reaction monitoring (PRM) to discover and validate acute aortic dissection protein changes in ascending aorta tissue samples from multiple individual patients.

2. Experimental Section

2.1 Reagents and Materials

Trypsin was purchased from Promega (Fitchburg, WI). Tetraethylammonium borohydride (TEAB), urea, dithiothreitol, iodoacetamide and trifluoroacetic acid were obtained from Sigma (St. Louis, MO). Protease Inhibitors were purchased from Calbiochem (Darmstadt, Germany). Formic acid (FA) was obtained from Fluka (Buchs, Germany). Pure water and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA). BCA kit was purchased from Beyotime (Shanghai, China).

2.2 Clinical Samples

All patients met the following inclusion criteria: i) AAD group: Diagnosis of Stanford type A AAD through CTA of the aorta. ii) CABG group: Patients with severe and complex atherosclerotic heart disease require to undergo Coronary Artery Bypass Grafting (CABG). Patients with the following diseases were excluded: Patients with other macrovascular disease, liver and kidney diseases, chronic wasting disease, autoimmune diseases, infection, and mental illness. The study was approved by the scientific ethics committee of the First Affiliated Hospital of Xinjiang Medical University and conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki.

A total of 10 AAD patients who were diagnosed and treated by surgery in the First Affiliated Hospital of Xinjiang Medical University from December 2018 to August 2019 were selected as the case group. At the same period, 10 patients with coronary atherosclerotic heart disease who were required to undergo CABG were selected as the control group. The whole ascending aorta tissue samples were obtained during the operation. Specimens of AAD patients were taken from a 2.0×2.0 cm² round aortic tissue at the rupture of ascending aortic dissection. The tissue of CABG patients were obtained from the anastomosis of the ascending aorta and bridge vessels, about 1.5×1.5 cm² of ascending aorta tissue. The selected aortic tissue specimens strictly follow the collection and storage standards of tissue specimens. After the tissue was removed during the operation, it was immediately put into the cryopreserved tube, and then transferred to liquid nitrogen for rapid freezing and storage in the refrigerator at -80°C until the experiment.

2.3 4D-Label-Free Quantitation Mass Spectrometry and Data Analysis

2.3.1 Protein extraction

The sample was grinded by liquid nitrogen into cell powder and then transferred to a 5-mL centrifuge tube. After that, four volumes of lysis buffer (8 M urea, 1% Protease Inhibitor) was added to the cell powder, followed by sonication three times on ice using a high intensity ultrasonic processor (Scientz). The remaining debris was removed by

centrifugation at 12000 g at 4°C for 10 min. Finally, the supernatant was collected and the protein concentration was determined with BCA kit according to the manufacturer's instructions.

2.3.2 Trypsin digestion

The protein solution was reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

2.3.3 LC-MS/MS Analysis

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15-cm length, 75 μ m i.d.). The gradient was comprised of an increase from 6–23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23–35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC^[28]. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.

2.3.4 Database Search

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8)^[29]. Tandem mass spectra were searched against human uniprot database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 4 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in First search and 5 ppm in Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and acetylation modification and oxidation on Met were specified as variable modifications. FDR was adjusted to < 1% and minimum score for modified peptides was set > 40.

2.3.5 Bioinformatics Methods

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>). Firstly, Converting identified protein ID to UniProt ID and then mapping to GO IDs by protein ID. If some identified proteins were not annotated by UniProt-GOA database, the InterProScan soft would be used to annotated protein's GO functional based on protein sequence alignment method. Then proteins were classified by Gene Ontology annotation based on three categories: biological process, cellular component and molecular function.

GO enrichment analysis classified the proteins by GO annotation into three categories: biological process, cellular compartment and molecular function. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. The GO with a corrected p-value < 0.05 is considered significant. Encyclopedia of Genes and Genomes (KEGG) database was used to identify enriched pathways by a two-tailed Fisher's exact test to test the enrichment of the differentially expressed protein against all identified proteins. The pathway with a corrected p-value < 0.05 was considered significant. These pathways were classified into hierarchical categories according to the KEGG website. For each category proteins, InterPro (a

resource that provides functional analysis of protein sequences by classifying them into families and predicting the presence of domains and important sites) database was researched and a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. Protein domains with a corrected p-value < 0.05 were considered significant.

For further hierarchical clustering based on differentially expressed protein functional classification. We first collated all the categories obtained after enrichment along with their P values, and then filtered for those categories which were at least enriched in one of the clusters with P value < 0.05. This filtered P value matrix was transformed by the function $x = -\log_{10}(P \text{ value})$. Finally these x values were z-transformed for each functional category. These z scores were then clustered by one-way hierarchical clustering (Euclidean distance, average linkage clustering) in Genesis. Cluster membership were visualized by a heat map using the "heatmap.2" function from the "gplots" R-package.

All differentially expressed protein database accession or sequence were searched against the STRING database version 10.1 for protein-protein interactions. Only interactions between the proteins belonging to the searched data set were selected, thereby excluding external candidates. STRING defines a metric called "confidence score" to define interaction confidence; we fetched all interactions that had a confidence score ≥ 0.7 (high confidence). Interaction network from STRING was visualized in R package "networkD3".

2.4 Parallel Reaction Monitoring (PRM) and Data Analysis

2.4.1 PRM analysis

The remain peptides of 4D-LFQ were used for PRM analyses^[30] to verify 20 proteins that are closely related to AAD. The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column. The gradient was comprised of an increase from 6–25% solvent B (0.1% formic acid in 98% acetonitrile) over 40 min, 25–35% in 12 min and climbing to 80% in 4 min then holding at 80% for the last 4 min, all at a constant flow rate of 500 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.1 kV. The m/z scan range was 455 to 1085 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70000. Peptides were then selected for MS/MS using NCE setting as 27 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-independent procedure that alternated between one MS scan followed by 20 MS/MS scans. Automatic gain control (AGC) was set at 3E6 for full MS and 1E5 for MS/MS. The maximum IT was set at 50 ms for full MS and 185 ms for MS/MS. The isolation window for MS/MS was set at 1.6 m/z.

2.4.2 Data Analysis

Skyline software(v.3.6)^[31] was used for analysis of PRM data. Peptide settings: enzyme was set as Trypsin [KR/P], Max missed cleavage set as 0. The peptide length was set as 7–25, fixed modification was set as cysteine alkylation. Transition settings: precursor charges were set as 2, 3, ion charges were set as 1, ion types were set as b, y. The product ions were set as from ion 3 to last ion, the ion match tolerance was set as 0.02 Da.

3. Results

3.1 Clinical Characteristics of Patients

The clinical characteristics of patients with acute artery dissection and the CABG group are shown in Table 1. As known risk factors to AAD, white blood cell (WBC), D-dimer and C-reactive protein (CRP) showed significant differences between the AAD and control groups ($P < 0.05$). No significant difference appears based on age or gender distribution between the AAD and CABG groups.

Table 1
Clinical Characteristics of total subjects

	AAD group (n = 10)	CABG group (n = 10)	p value
Age (year)	47.80 ± 7.35	54.50 ± 7.38	0.057
Male (%)	9(90)	9(90)	1.000
Weight (kg)	88.00 ± 15.42	78.10 ± 15.94	0.175
HR(beats/min)	77.70 ± 9.19	83.00 ± 16.04	0.376
Smoking (%)	5(50)	2(20)	0.160
Drinking (%)	3(30)	11(10)	0.264
Hypertension (%)	8(80)	5(50)	0.160
Diabetes mellitus (%)	0(0)	4(40)	0.025
WBC(×10 ⁹ /L)	14.12 ± 2.16	6.66 ± 1.28	<0.001
TG (mmol/L)	2.16 ± 1.61	1.39 ± 0.34	0.172
TC (mmol/L)	3.80 ± 1.73	3.46 ± 1.12	0.607
LDL-C (mmol/L)	2.35 ± 1.02	2.27 ± 1.12	0.869
D-dimer (mmol/L)	2461.39 ± 2334.10	162.50 ± 106.24	0.027
CRP (mmol/L)	55.02 ± 57.47	9.09 ± 22.53	0.037
<i>Note:</i> HR: heart beta; WBC: white blood cell; TG: triglycerides; TC: total cholesterol; LDL-C: low-density lipoprotein-cholesterol; CRP:C-reactive protein.			

3.2 Identification of Differentially Expressed Proteins by 4D-LFQ

We have detected a total of 3985 proteins by 4D-LFQ quantitative analysis, of which 3350 can be quantified. Compared with the CABG group, the protein quantitative AAD group exceeded 1.5 as a significant up-regulation threshold value, and less than 1/1.5 as down-regulation. 139 proteins were upregulated, and 108 proteins were downregulated (Fig. 1).

We performed subcellular localization of these differentially expressed proteins and the results showed that upregulated proteins were mainly distributed in the cytoplasm (33.09%), extracellular matrix (31.65%), nucleus (12.95%) and other parts of the cell (Fig. 2A). The majority of the downregulated proteins are mainly distributed in the extracellular matrix (25%), mitochondria (23.15%), cytoplasm (20.37%) and other parts of the cell (Fig. 2B). The percentage of upregulated proteins located in the cytoplasm, extracellular, plasma membrane was higher than that of downregulated proteins. However, a higher percentage of downregulated proteins were mitochondria and nucleus located. In addition to the above, there are 8 and 3 upregulated proteins were found to be localized in the mitochondria and peroxisome respectively.

We performed a functional enrichment analysis of Gene Ontology (GO), KEGG pathways and protein domains to further understand the differentially expressed proteins in the ascending aorta tissue of patients with AAD.

The enrichment of differentially expressed proteins in this classification or function was positively correlated with the length of the bar chart. The GO enrichment analysis demonstrated that upregulated proteins were significantly enriched in cell components and biological processes, but at a lower level of molecular function (< 4.0). The terms involved in upregulated proteins in the above three parts mainly include extracellular space, secretory granule, calcium ion binding, transmembrane signaling receptor activity, secretion by cell, leukocyte and granulocyte activation. The down-regulated proteins are significantly enriched in cellular components and biological processes, not significantly enriched in molecular functions (< 4.0), being associated with terms including extracellular matrix, myofibril, ADP-forming activity, telethonin and actin binding, aerobic respiration, actin-myosin filament sliding (Fig. 3).

KEGG is an information network that connects known molecular interactions. The enrichment analysis of KEGG pathway showed that the differentially expressed proteins were mainly enriched in 23 pathways. Among them, ECM receptor interaction, renin secretion, TGF-beta signaling pathway, platelet activation, complement and coagulation cascades and energy-producing pathways (tricarboxylic acid cycle and propanoate metabolism) are perhaps the most relevant pathways associated with AAD. The other pathways include valine, leucine and isoleucine metabolism, protein digestion and absorption, cortisol synthesis and secretion etc (Fig. 4).

The domain enrichment analysis indicated that many of the upregulated proteins contained the following domains: EGF-like calcium-binding domain; growth factor receptor cysteine-rich domain; integrin domain; complement C1r-like EGF domain (Fig. 5A). By contrast, the down regulated proteins contained leucine-rich repeat N-terminal domain; leucine-rich repeat domain, L domain-like; von Willebrand factor, type A; amine oxidase (Fig. 5B).

In order to find the nature of the differentially expressed proteins in AAD patients, we carried out GO enrichment-based clustering, KEGG pathway enrichment-based clustering, and protein domain enrichment-based clustering analysis. We divided the differentially expressed proteins into four quantitative categories according to their different expression folds: Q1 (Ratio < 0.667 , 108 proteins), Q2 ($0.667 < \text{Ratio} < 0.769$, 35 proteins), Q3 ($1.3 < \text{Ratio} < 1.5$, 153 proteins) and Q4 (Ratio > 1.5 , 139 proteins). Cluster analysis based on GO enrichment showed that in the biological process category, the upregulated proteins were mainly associated with secretion by cell, leukocyte activation and neutrophil mediated immunity, granulocyte activation. The downregulated proteins were mainly associated with the cellular respiration, aerobic respiration, actin-myosin filament sliding and energy metabolism process (Fig. 6A). In the molecular function, the upregulated proteins were mainly enriched in calcium ion binding, transmembrane signaling receptor activity, drug binding, ion binding and glycoprotein binding. However, most of the downregulated proteins were associated with the succinate-CoA ligase (ADP-forming) activity, telethonin binding, actin binding (Fig. 6B). The enrichment analysis of the cellular component category showed that the upregulated proteins were highly enriched in extracellular space, secretory granule and vesicle. For the downregulated proteins, we found that were associated with extracellular matrix, myofibril, contractile fiber (Fig. 6C).

The KEGG-based enrichment analysis indicated that the upregulated proteins are mainly enriched in the phagosome, hematopoietic cell lineage, dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM) pathways. Conversely, ECM-receptor interaction, amino acid metabolism and energy metabolism pathways enriched in the downregulated proteins (Fig. 7A). For the differentially expressed proteins in the domain enrichment-based clustering analysis, the upregulated proteins were clustered with EGF-like domain, growth factor receptor cysteine-rich domain, integrin α and β related domain. The downregulated proteins were associated with Leucine-rich repeat

N-terminal and L domain; von Willebrand factor, type A and type D domain; dimeric alpha-beta barrel; fibronectin type III (Fig. 7B).

We performed protein interaction network analysis on all groups of differential proteins, and then in order to clearly show the interaction relationship between proteins, we screened out the top 50 proteins with the closest interaction relationship and mapped the protein interaction network. This network interaction picture includes differential proteins involved in inflammation, amino acid and energy metabolism, extracellular matrix degradation, and TGF-β signaling pathways (Figure S1, Supporting Information).

3.3 Validation of Differentially Expressed Proteins Using Parallel Reaction Monitoring

Based on the proteomics and bioinformatics results, twenty differential proteins were selected as candidate biomarkers and validated in total samples. We used targeted PRM to provide high sensitivity relative peptide quantification. Validation was performed using Proteomics remaining samples from ten AAD and ten CABG patients.

Of the twenty proteins identified as differentially expression in the proteomics, significant differential expression of four of these candidate proteins was confirmed by PRM for serum amyloid A-1 protein (SAA1), lipopolysaccharide-binding protein (LBP), Myeloperoxidase (MPO) and endoglin (ENG) (Fig. 8). A comparison of the significantly differentially expressed proteins between the 4D-LFQ and PRM patient cohorts demonstrated good agreement between the two techniques (Table 2). In order to evaluate the sensitivity and specificity of each protein for the diagnosis of AAD, we performed ROC analysis, and the results support the conclusions of the differential expression analysis (Fig. 9). These results demonstrate that measuring expression of these proteins in a larger patient cohort may produce clinically applicable biomarkers of advanced ovarian disease.

Table 2
Comparison of significantly differentially enriched protein data between the LFQ and PRM.

Protein Coding	Gene Name	Protein name and accession	Peptides used for PRM quantitation	LFQ		PRM	
				Log ₂ Fold change	p value	Log ₂ Fold change	p value
P0DJ18	SAA1	serum amyloid A-1 protein	FFGHGAEDSLADQAANEWGR SFFSFLGEAFDGAR	6.40	0.011	18.71	0.004
P18428	LBP	lipopolysaccharide-binding protein	LAEGFPLPLLK ITLPDFTGDLR	3.48	0.001	3.58	0.002
P05164	MPO	Myeloperoxidase	IICDNTGITTVSK	11.33	0.001	12.14	0.007
P17813	ENG	endoglin	GPITSAAELNDPQSILLR	2.00	0.001	2.03	0.032

4. Discussion

Early and rapid diagnosis is essential to improve the survival rate of AAD [21]. Biomarkers of AAD could be potentially used to screen patients with compatible symptoms and identify patients at higher risk of AD, which can increase the diagnostic pretest probability for the AAD patients [32]. Moreover, many primary hospitals or remote areas which

have no imaging facilities to diagnose AAD currently, biomarkers can help them make early diagnosis without delaying treatment. To our knowledge, this is the first research who use 4D-LFQ-PRM workflow for AAD biomarker discovery, and establishes the methodology required for subsequent studies to evaluate specific disease biomarkers of AAD.

In this study, we used 4D-LFQ technology to screen out proteins with significantly different expression in AAD patients. We used the 1.5-fold change of LFQ-intensity between the AAD and CABG groups was set as the cut off value in differential protein screening, and obtained 139 up-regulated proteins and 108 down-regulated proteins. Among the proteins enriched in the ascending aorta tissue of AAD patients, we selected twenty proteins that may be related to the pathogenesis of AAD for PRM verification. Among them, four differential proteins are significantly expressed that may serve as novel biomarkers. Systemic inflammatory response, abnormal phenotypic transformation and ECM degradation of VSMCs mediated by TGF- β /Smad signaling pathway are considered to be the important pathological basis of AAD [33–36]. SAA1 is a major acute phase protein that is highly expressed in response to inflammation and tissue injury [37, 38]. The elevated levels of SAA1 in the tissues of AAD patients may be related to systemic inflammation. In vitro studies have shown that SAA can induce vascular smooth muscle cells (VSMCs) to produce a variety of matrix metalloproteinases, leading to extracellular matrix (ECM) degradation [39, 40]. Therefore, the increase of SAA1 level is of great significance for evaluating the development of AAD. ENG is increased during vascular injury and is an essential endothelial co-receptor in the TGF- family. ENG can promote the signal transduction of the TGF- β /Smad pathway to play an important role in the regulation of angiogenesis and the migration of VSMCs [41]. Thus, we speculate ENG plays an important role in the development of AAD by promoting TGF- β /Smad signaling to regulate angiogenesis and vascular endothelial cell migration [42]. Up-regulation of MPO expression is a marker of neutrophil activation and reflects the development of inflammation in the body to some extent. Our study and a previous analysis identified the increased abundance of MPO in AAD patients, strongly supporting its potential as a biomarker candidate for further investigation [43]. LBP rises rapidly during acute inflammation. During the pathogenesis of AAD, LBP increased and activated monocytes and neutrophils to release inflammatory factors, further damaging vascular endothelial cells and aggravating inflammatory response.

However, there are the following limitations in our study. First of all, our sample size is too small, and a larger sample size is needed to verify the research results in the future. Second, the samples we used for 4D-LFQ analysis and PRM verification were from the same population, which may limit the extrapolation of the research results. Finally, due to the difficulty in obtaining aortic tissue from normal people, we choose the aortic tissue of CABG patients as a control, which may have an impact on some indicators in the study results.

5. Conclusions

In conclusion, our 4D-LFQ-PRM workflow provides a comprehensive proteomic signature for AAD patients. Many proteins identified in this study have never been associated with AAD, and further investigations are needed to analyze and verify these changes and determine their significance in monitor therapeutic response and disease recurrence. Therefore, the present study will provide meaningful data and new ideas for the differential diagnosis of AAD.

Declarations

Authors' contributions

Wang MM and Wang BZ conceived and designed the experiments, and wrote the draft of the manuscript; MM, Shao MH, Lu CF and Zhong J collected data and undertook the statistical analyses; Wang MM, Wang BZ, Shao MH and Lu CF performed laboratory experiments; DL and Chen BD gave critical comments on the draft and contributed to the manuscript writing; Ma X and Ma YT reviewed clinical assessments in this study and supervised this study. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that they have no competing interests.

Availability of data and materials

The data will not be shared, since part of the data is being reused by another study.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Ethical Review Board of The First Affiliated Hospital of Xinjiang Medical University. Written informed consent was obtained from all enrolled patients.

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Figures

Figure 1

Differentially expressed proteins between AAD group and CABG group. Red represents up-regulated proteins, blue represents down-regulated proteins. A) Bar graph. B) Volcano graph.

Figure 2

The WoLF PSORT software was used to predict the subcellular localization of significantly differentially expressed proteins and perform classification statistics. A) upregulated proteins (% of total). B) downregulated proteins (% of total).

Figure 3

The Gene Ontology (GO) enrichment analysis of differentially abundant proteins. The vertical axis is the classification description of GO, and the horizontal axis is the enrichment test of Fisher Exact Test P value after logarithmic transformation. The longer the bar chart, the more significant the enrichment degree of the differentially expressed proteins in this classification or function. A) The red bars represent upregulated proteins. B) The green bars represent upregulated proteins.

Figure 4

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for differential abundance proteins. The vertical axis is the description of KEGG pathway, and the horizontal axis is the enrichment test of Fisher Exact Test P value after logarithmic transformation. The more significant the enrichment of differentially expressed proteins in the KEGG signaling pathway, the longer the bar graph was.

Figure 5

Protein domain enrichment analysis of differential abundance proteins. The vertical axis is the classification description of protein domain, and the horizontal axis is the enrichment test of Fisher Exact Test P value after logarithmic transformation. The longer the bar chart, the more significant the enrichment degree of the differentially expressed proteins in this classification or function. A) The red bars represent upregulated proteins. B) The green bars represent upregulated proteins.

Figure 6

GO enrichment-based clustering analysis of the quantified proteins. The color blocks corresponding to the enrichment function of differentially expressed proteins in different comparison groups indicate the degree of enrichment. Red represents strong enrichment, blue represents weak enrichment. A) Biological Process analysis. B) Molecular Function. C) Cellular Component.

Figure 7

Functional enrichment-based clustering analysis of the quantified proteins. The color blocks corresponding to the enrichment function of differentially expressed proteins in different comparison groups indicate the degree of enrichment. Red represents strong enrichment, blue represents weak enrichment. A) KEGG pathway. B) Protein domain.

Figure 8

Four differential proteins found in AAD and CABG patients validated by PRM. Box plot showing proteins relative Abundance from four proteins targeted by PRM. Four proteins were significantly increased ($p \leq 0.05$) AAD patients compared to CABG patients.

Figure 9

ROC analysis of four differential proteins between AAD patients and CABG patients verified by PRM.

Supplementary Files

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- [FigureS1Proteinproteininteractionnetwork.pdf](#)