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Original Article

Proteomics Clustering Research of ITRAQ Markers in Plasma of AIDS Patients with Different Chinese Medicine Syndromes

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`Abstract

Background: We aimed to distinguish the different Chinese medicine (CM) syndromes of acquired immune deficiency syndrome (AIDS) patients at the proteomics level.

Methods: We collected AIDS patients diagnosed with different CM syndromes from Weishi County, Kaifeng City, Henan Province, China, including Qi-deficiency syndrome (named QD group) and dampness-heat syndrome (named DH group). Healthy people were collected as controls from Weishi County, Kaifeng city, Henan Province, China. The plasma from three groups were labeled with ITRAQ, LC/MC was used for protein quantitative analysis. Finally, sequence search and cluster analysis were performed.

Results: Overall, 27 different proteins were found. Three proteins were up-regulated and 2 proteins down-regulated in the QD group, 11 proteins up-regulated and 13 proteins down-regulated in the DH group. Compared with DH group, there were 7 different proteins in QD group, among which 5 proteins were down-regulated and 2 proteins were up-regulated. When the target protein of DH group was up-regulated, the protein of HC group was down-regulated correspondingly.

Conclusion: The significance analysis and clustering of protein results showed that DH group was significantly different from QD group and HC group at the protein level ($P<0.05$). However, the QD group could not be effectively distinguished from the HC group. AAT, PF4, C-reactive protein and c4bp may be used as potential biomarkers in DH group. Mass spectrometry based on feature selection can be used to classify different CM syndromes.

Keywords: Acquired immune deficiency syndrome; Proteomics; Chinese medicine

Introduction

AIDS is an immune deficiency disease. Chinese Medicine (CM) has clear therapeutic effect, but lacks objective diagnostic criteria. Proteomics can explain the material basis of CM syndrome from a microscopic perspective. It has been applied to search for biomarkers of AIDS and reveal the competition mechanism between the host and HIV.

Quantitative proteomics can determine the amount of proteins in a sample and help to find differences between samples by two-dimensional gel electrophoresis (2-DE) or mass spectrometry (MS). 2-DE can separate more than 5,000 mix-

tures of proteins, but it is particularly difficult to deal with a large number of proteomes (1). Based on this, a more advanced method for automated complete protein extraction, high performance liquid chromatography (HPLC), was developed to separate, identify, and quantify the components of the sample mixture as it passes through a column containing solid adsorbents. In recent years, high performance liquid chromatography (LC/MC) based on mass spectrometry can effectively detect low abundance proteins and extreme properties of proteins, and is the most important laboratory method for quantitative proteomics. Mars-depletion plasma (2) has a minimum detection limit of 1.1ug/ml. When it is MC's turn, the use of stable isotope labeling or no labeling methods is a prerequisite for obtaining quantitative results (3). ITRAQ (isobaric Relative and absolute quantitative labeling) is an in vitro peptide labeling method developed by Applied Biosystems. It uses 4 or 8 isobaric labels to simultaneously compare relative quantitative proteins in 4 or 8 samples by labeling specific polypeptide amino acids and tandem mass spectrometry.

Before using Chinese herbs to treat AIDS patients, it is crucial to diagnose the syndromes they are experiencing. If the biomarkers of CM syndromes of AIDS are determined, the patients' syndromes can be evaluated objectively and effectively cured in clinic.

Therefore, we used proteomics technology based on ITRAQ tags to detect plasma protein levels in AIDS patients with different syndromes, expecting to find biomarkers.

Methods

Research cases collection

All research cases were from Weishi County, Kaifeng City, *Henan Province,* China and provided informed consent.

Ethics approval was obtained by Ethics Committee of the First Affiliated Hospital of Henan University of Chinese Medicine.

AIDS patients diagnosed with dampness-heat syndrome were collected as DH group. Patients diagnosed with Qi-deficiency syndrome were collected as QD group. Healthy people were enrolled as controls.

Human plasma preparation

All groups whole human blood sample was taken by venipuncture with normal plasma tubes after an overnight fast in the morning. Sample was centrifuged at 3000 rpm (Revolutions per Minute, RPM) for 10 min. Supernatants were collected as plasmas. Agilent human 14 multiple affinity removal system spin cartridge (part number 5185-5990) was applied to deplete high abundance proteins of plasma samples.

SDS-PAGE (Sodium dodecyl sulfate- polyacrylamide gel electrophoresis)

Quantification of protein was measured with BOSTER Protein Quantification Assay Kit based Bradford method. Electrophoresis system ran 90 min with constant current of 14 mA. Then separating gel was stained by Coomassie brilliant blue G250 and eluted.

Protein Digestion and iTRAQ Labeling

We performed protein digestion and labeled resulting peptide mixture with iTRAQ Reagent-8plex Mixture Kit according to the FASP procedure and manufacturer's instructions from ABI (4).The collected peptides were centrifuged at 14000 g for 10min, then estimated and calculated with UV light spectral density at 280 nm (3) and basic frequency of tryptophan and tyrosine. Finally, each peptide mixture were added with respective iTRAQ reagent after dissolved and vacuum drying (5), the marking method is shown in Table 1.

Table 1: Sample labels of cases and controls

Group 1	*Sample*	*QD01-F*	*Con01-F*	*DH01-F*	*QD02-F*	*Con02-F*	*DH02-F*	*REF*
	Label	113	114	115	116	117	118	119
Group 2	Sample	QD03-F	Con03-F	DH03-F	QD04-F	Con04-F	DH04-F	REF
	Label	113	114	115	116	117	118	119

Peptide Fractionation and LC-MC Analysis

All labeled peptides were fractionated by Strong Cation Exchange (SCX) chromatography system according to application protocols (GE AKTA Purifier 100 system). For LC-MS analysis Q-Exactive mass spectrometer (Thermo Finnigan) was used according to the manufacturer's directions, then MS data was gotten by means of the method as Michalski A recorded (6). Using MASCOT engine the total spectra were searched. The options listed below were used for protein identification. Peptide mass tolerance=20 ppm, Missed cleavage=2, MS/MS Enzyme=Trypsin, tolerance=0.1 Da, Fixed modification: iTRAQ4/8plex (K), Carbamidomethyl (C), iTRAQ4/8plex (N-term), Variable modification : Oxidation (M), FDR≤0.01 (7).

Results

Clinical study cases

Sixty-four clinical cases were screened and divided into QD group (25 cases), DH group (23 cases) and Controls (16 cases). The clinical research file was established and the research procedure was entered. The baseline data of the three groups is shown in Table 2.

Table 2: General information of cases and controls

Group	*Gender*			*Age*	*Cd4*	*Cd8*	*Nk*
	Total	Male	Female				
Qd	25	11	14	49.1±7.5	485.25±207.52	965.69±567.01	5.35±5.36
Dh	23	9	14	51.7±5.7	466.25±173.53	777.13±461.22	8.22±2.51
Con	16	7	9	47.6±6.9	690.31±209.11	797.31±231.61	10.60±5.63
F		0.138*		1.858	2.551	1.455	1.641
P		0.933		0.165	0.061	0.233	0.186

*: x^2 value

Identification and quantification of peptide and protein

There were 2612 unique peptides and 279 protein groups were identified. Quantitative results for proteins and peptides are shown in Table 3. Quantitative ratio square distribution analysis was performed on samples of QD group, as shown in Fig. 1. Pearson correlation analysis was performed for each sample ratio in controls, as shown in Fig. 2.

Table 3: Quantitative results for proteins and peptides

Sample	*Concentration(μg/μl)*		*Volume(μl)*
	Proteins	Peptides	
QD group No.1-F	3.4	0.68	50
QD group No.2-F	3.3	0.82	50
QD group No.3-F	3.1	0.52	50
QD group No.4-F	3.6	0.63	50
DH group No.1-F	3.4	0.96	50
DH group No.2-F	2.5	1.17	50
DH group No.3-F	3.8	0.63	50
DH group No.4-F	3.4	0.71	50
Controls No.1-F	3.3	0.67	50
Controls No.2-F	2.8	0.52	50
Controls No.3-F	3.4	0.55	50
Controls No.4-F	3.4	0.61	50
Internal Standard	3.2	0.65	50

The relative concentration of enzymatic peptides was assessed by their OD280 absorption value

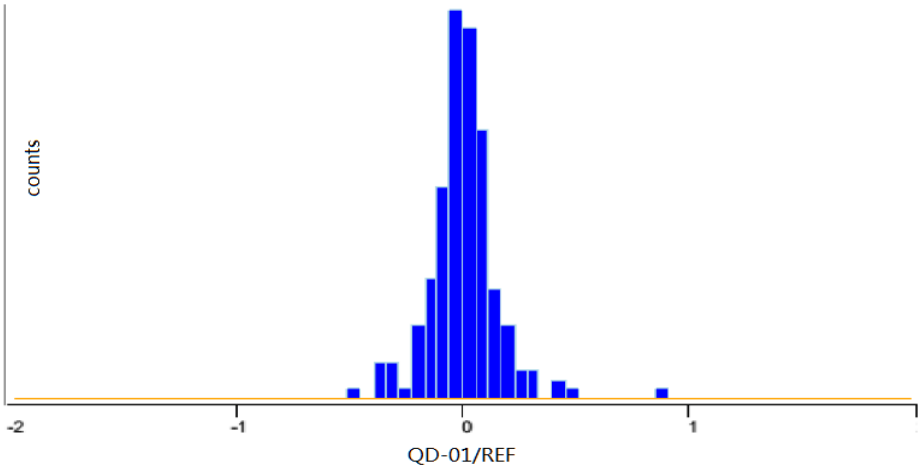

Fig. 1: Quantitative ratio square distribution analysis. The iTRAQ quantitative data were analyzed using frequency distribution histogram; the logarithm of ratio between each set of tags and reference tags was taken. Drawing by Perseus, the number of proteins is on the ordinate, the logarithm of ratio between a sample from QD group and the reference is on the abscissa

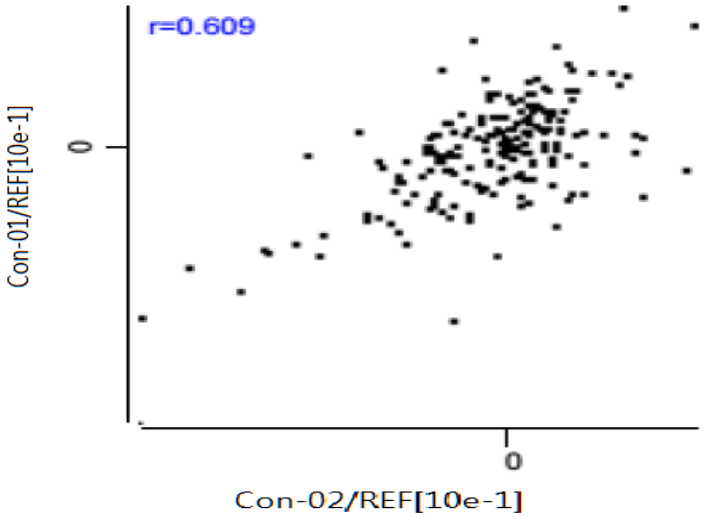

Fig. 2: Pearson correlation analysis for the ratio of the sample. The abscissa and ordinate of respectively represented ratios for iTRAQ of the sample markup tags from controls to internal reference

Significance analysis

Three groups were executed a pairwise comparison with Student's *t*-test. Then significant proteins were filtered with the condition of 1.2-fold changes and *p*-value less than or equal to 0.05. The ratio that the reference protein sample value to the marked protein sample value was less than or equal to 0.83 was regarded as downregulation, if the ratio was more than or equal to 1.2 the protein was upregulated. Finally, 27 differential proteins were found (Table 4). Compared the controls, QD group had 3 upregulated proteins and 2 downregulated proteins, although DH group had 11 upregulated proteins and 13 downregulated proteins, there were only 7 differential proteins including 5 down regulations and 2 up regulations when QD group was compared with DH group.

Table 4: Differentially expressed proteins identified by iTRAQ analysis

Accession no.	*Protein name*	*MW [kDa]*	*calc.pl*	*Coverage*	*Proteins*	*Unique Peptides*	*QD vs. Con*		*QD vs. DH*		*DH vs. Con*	
							Fold	*P*	Fold	Fold	*P*	Fold
B4E334	Phosphate-regulating neutral endopeptidase	53.03	9.32	1.33	5	1	1.10	0.31	1.24	0.03	0.88	0.11
Q13747	a-1 antitrypsin	22.81	6.55	12.69	8	2	0.98	0.50	0.64	0.02	1.54	0.02
B4E1D8	cDNA FLJ51597	60.37	6.65	15.3	5	7	0.98	0.78	0.78	0.01	1.26	0.01
Q9NZP8	Complement C1r subcomponent-like	53.46	7.20	12.94	5	4	0.88	0.06	1.10	0.10	0.80	0.01
B4E1Z4	Complement factor B	140.85	7.18	41.07	17	30	0.95	0.33	1.15	0.05	0.82	0.01
P01023	a-2-macroglobulin	163.19	6.46	39.28	11	42	1.43	0.12	0.71	0.15	2.02	0.01
O00187	Mannan-binding lectin serine prote-	75.65	5.63	2.19	1	1	1.10	0.06	0.92	0.05	1.21	0.01
Q59HB3	Apolipoprotein B variant	183.46	6.80	50.28	12	1	0.92	0.15	1.11	0.04	0.83	0.01
Q68BL8	Olfactomedin-like protein 2B	83.95	5.20	0.8	3	1	1.12	0.38	1.48	0.02	0.76	0.03
P02776	Platelet factor 4	10.84	8.62	19.8	2	2	1.02	0.74	0.81	0.02	1.25	0.01
P40197	Platelet gly copro-tein V	60.92	9.63	4.64	1	2	0.92	0.06	0.82	0.02	1.12	0.13
Q5VVP7	C-reactive	11.62	8.46	20.59	3	2	0.96	0.54	0.76	0.01	1.27	0.01
P02652	Apolipoprotein A-II	11.17	6.62	62	1	6	1.34	0.01	0.80	0.09	1.67	0.01
B2R812	cDNA-FLJ93914, highly similar to HRG	59.47	7.44	32.76	3	17	1.29	0.05	1.26	0.09	0.02	0.80
B2R6W1	cDNA-FLJ93143	93.49	6.48	43.53	5	1	1.25	0.01	1.03	0.60	1.21	0.01
Q96L50	Leucine-rich repeat protein 1	46.69	9.09	1.45	1	1	0.80	0.03	1.18	0.21	0.68	0.02
Q15849	Urea transporter 2	101.14	6.95	0.65	1	1	0.73	0.04	1.13	0.48	0.65	0.01
B4DI70	cDNA FLJ53509	46.39	5.26	7.26	6	2	1.12	0.01	0.83	0.07	1.35	0.02
Q86TT1	Full-length cDNA clone CS0DD006-YL02 of euroblas-toma	41.25	6.79	6.13	3	2	1.17	0.15	0.75	0.09	1.56	0.03
A8K2T4	cDNA FLJ78207	93.35	6.51	44.72	6	2	1.33	0.08	1.06	0.63	1.25	0.01
E7EQ48	Proteoglycan 4	102.45	9.64	4.39	7	3	1.06	0.05	0.87	0.01	1.22	0.01
B7Z832	cDNA FLJ51409, high similar to Thrombospondin-4	95.89	4.64	3.22	3	1	1.10	0.69	1.71	0.13	0.64	0.01
F5H1A8	Gelsolin	81.43	5.85	32.48	15	19	0.91	0.03	1.11	0.08	0.82	0.01
P0C7M7	Acyl-coenzyme A synthetase ACSM4, mitochondrial	65.66	8.59	1.03	1	1	0.88	0.19	1.11	0.26	0.79	0.02
C9J9F8	Coiled-coil domain-containing protein 173	19.65	9.22	3.77	2	1	0.92	0.19	1.19	0.07	0.77	0.02
Q8WY91	THAP domain-containing protein 4	62.85	9.28	1.04	1	1	0.86	0.04	1.05	0.54	0.82	0.01
B4E1B3	cDNA FLJ53950, high similar to An-giotensinogen	51.03	6.16	32.83	12	7	0.89	0.14	1.17	0.09	0.76	0.01

Feature Selection

Characteristic difference proteins were filtrated based on WEKA5 software (4) which used the algorithms of information gain attribute evaluator and correlation-based feature selection (CFS). At last, we got seven characteristic proteins, four in Table 5, three in Table 4 (E7EQ48, B4DI70, and P02652).

Table 5: Four differentially expressed proteins from feature selection

Accession no.	*Protein name*	*MW [kDa]*	*calc.pI*	*Coverage*	*proteins*	*Unique Peptides*	*QD vs. Con*		*QD vs. DH*		*DH vs. Con*	
							Fold	*P*	Fold	Fold	*P*	Fold
A8K8Z4	cDNA FLJ78071, highly similar to Human MHC class III complement component C6 mRNA OS=Homo sapiens PE=2 SV=1 - [A8K8Z4_HUMAN]	104.65	6.62	28.37	3	22	0.94	0.02	1.08	0.02	0.87	0.00
Q14520	Hyaluronan-binding protein 2 OS=Homo sapiens GN=HABP2 PE=1 SV=1 - [HABP2_HUMAN]	62.63	6.54	17.5	2	9	1.10	0.10	0.96	0.30	1.15	0.03
Q6MZL2	Putative uncharacterized protein DKFZp686M0562 (Fragment) OS=Homo sapiens GN=DKFZp686M0562 PE=2 SV=1 - [Q6MZL2_HUMAN]	35.12	5.97	3.12	1	1	0.85	0.22	1.15	0.01	0.74	0.09
B2R9F2	cDNA, FLJ94361, highly similar to Homo sapiens serine (or cysteine) proteinase inhibitor, clade A(alpha-1 antiproteinase, antitrypsin), member 6 (SERPINA6), mRNA OS=Homo sapiens PE=2 SV=1 - [B2R9F2_HUMAN]	45.07	6.04	26.67	4	9	1.11	0.05	1.07	0.33	1.04	0.64

Clustering

For testing rationality and accuracy of selected target proteins in each group, class hierarchy clustering was executed. Statistics indicated proteins set were highly accurate for sample classification (Fig. 3). Target proteins could reflect not only the changes of protein expression levels between different samples, but also help us to select the key targets to determine differences among different samples.

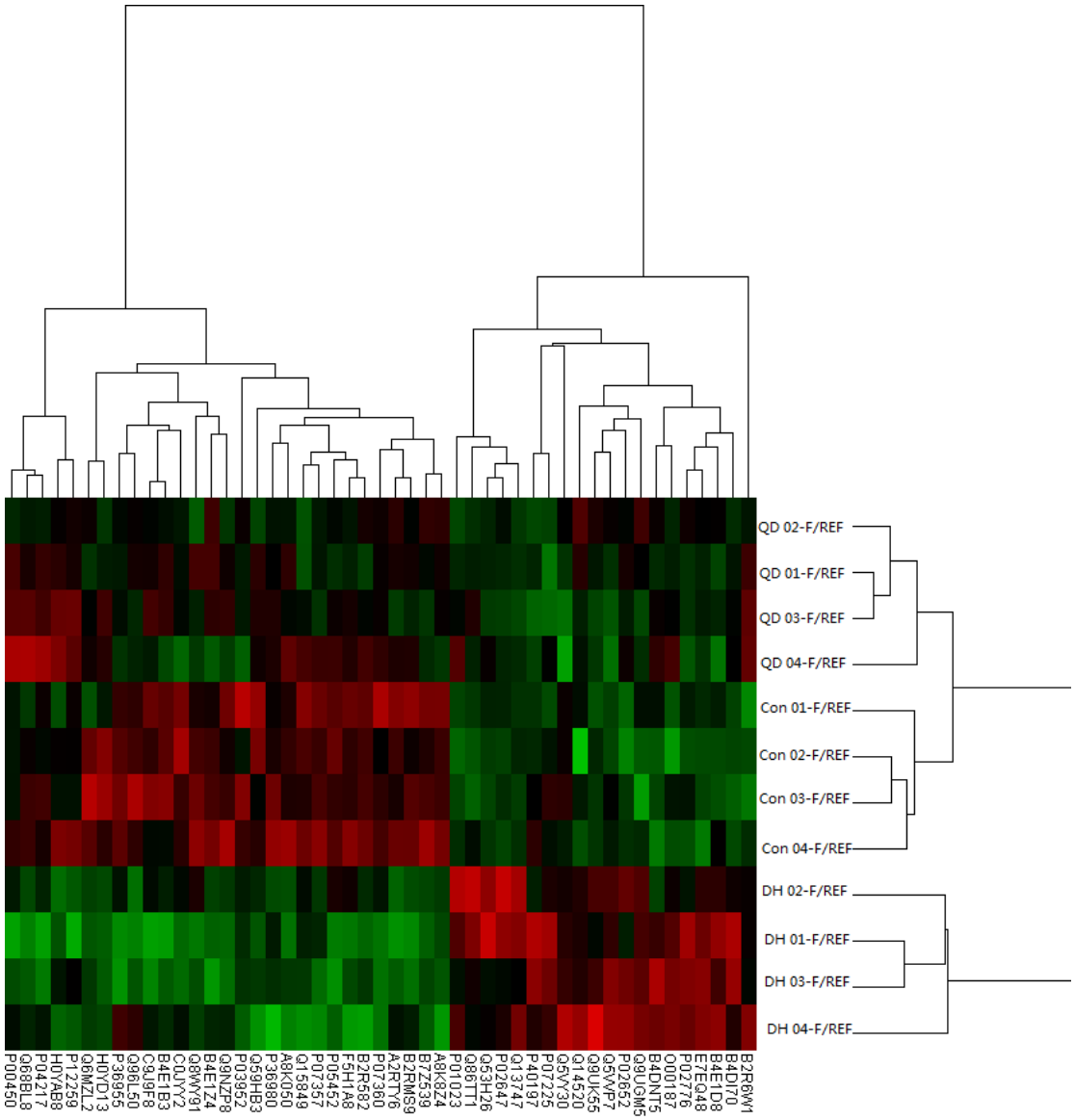

Fig. 3: Class hierarchy clustering. In this figure red blocks represented up-regulation and green blocks represented down-regulation. The x-axis stood for samples and y-axis stood for accession numbers of proteins. Controls and DH group of AIDS could be distinguished clearly in a protein level, when the target proteins upregulated in DH group of AIDS, the proteins in the controls downregulated correspondingly. The correspondence was confined when QD group compared with the controls or DH group

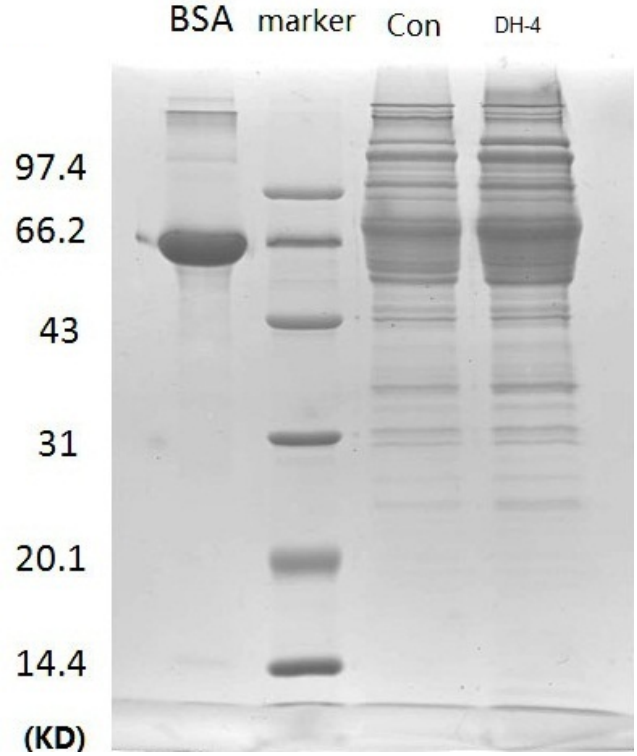

Fig. 4: Separation and enrichment of low abundance proteins. The leftmost column displayed electrophoresis spectrums of bovine plasma albumin (BSA), the second column next was protein molecular weight markers and the other columns were the research samples from controls and DH group. Compared BSA, sample bands displayed more intensive at a molecular weight of 60KD after high abundance proteins were removed, other bands displayed clearly

Discussions

There were rich protein and peptide (8) which was one of the best sources of samples of potential disease markers in the Human plasma fluid. Separation and enrichment of low abundance proteins played a key role in proteomics research. From the Fig. 4 we could see that Agilent human 14 multiple affinity removal system spin cartridge could remove some high abundance proteins from plasmas effectively.

In QD group, all the upregulated proteins involved in the immune system process, the down-regulated Leucine-rich repeat protein could suppress the activation of NF-KB when it overexpressed (9). In DH group, The four up-regulated proteins which consist of AAT, PF4, C-reactive protein and c4bp all involved response to stimulus in biological process.

Alpha-1-antitrypsin (AAT) is a human neutrophil serine protease inhibitor, leading to anti-inflammatory and anti-apoptotic effect (10), has been shown to counteract Ischemia/reperfusion injury (11). Some academic papers have declared the upregulation of AAT increased risk for liver diseases (12). Clinical intravenous administration of A1AT for one week can reduce serum IL-6 concentration in patients with SARS-CoV-2 (13). PF4 plays a biological role by regulating angiogenesis and different types of immune cells (14). The chemotaxis of PF4 can promote neutrophil degranulation, stimulate monocytes to produce cytokines and cause inflammation (15). PF4 has been reported to be involved in the physiological and pathological processes of many diseases, such as chronic obstructive pulmonary disease (COPD)(16), COVID-19 (17), polycystic ovary syndrome (PCOS) (18), etc.

C-reactive protein results in activation of pro-inflammatory cytokines (19, 20), when it rises can lead to inflammation and activating the complement system (21). Secretion of other pro-inflammatory cytokines also increases CRP concentrations, exacerbating the inflammatory process (22). C4BP is a glycoprotein in human serum and inhibits complement activity in classical and mannose-binding lectin pathways, preventing excessive inflammation and tissue damage (23).Pathogens can absorb C4BP on the cell surface to escape complement attack (24).The altered expression level of C4BP protein reflects the neuroinflammatory state of the host and can be used as a potential biomarker of MDD (25).
Apolipoprotein A-II was evaluated as a likely candidate gene for familial Type II diabetes for its gene variation in insulin secretion and post-challenge glucose (26). The expression of apolipoprotein A-II is low in COVID-19 patients (27). a-2M is a large homotetrameric protein and multifunctional protease inhibitor in Human plasma involved in inhibiting endopeptidase and trapping mechanism (28-33). It is considered to be involved in the pathogenesis of diabetes and Alzheimer's disease (33, 34). PRG4, which has been identified as articular superficial zone protein and megakaryocyte stimulating factor (35), was regarded as a probable biomarker for confirming COPD severity (36), PRG4 was detected to be upregulated in Osteoarthritis (37). Gelsolin was pointed out that closely related with the occurrence of some cancers, whose high expression could promote liver cancer cell proliferation and invasive (38). Apolipoprotein B levels could indicate the risk of coronary heart disease (39, 40), chronic kidney disease (41), and its high-expression may accelerate disease progression.

Conclusion

We made quantitative proteomics research for different CMS of AIDS which based on ITRAQ Labeling. Significance analysis and clustering for proteins results both reflect that DH group could be distinguished clearly with QD group and controls in the proteomics level. However, QD group could not be distinguished effectively with controls. AAT, PF4, C-reactive protein and c4bp may be considered as probable biomarkers for DH group of AIDS. Mass spectrometry based on feature selection can be used to classify different CM syndromes.

Journalism Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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