• Investigation •

iTRAQ-based proteomics analysis of aqueous humor in patients with dry age-related macular degeneration

Si-Chang Qu[1], Ding Xu[1], Ting-Ting Li[1], Jing-Fa Zhang[2], Fang Liu[1]

[1]Department of Ophthalmology of Shanghai Tenth People's Hospital, Tongji Eye Institute, Tongji University School of Medicine, Shanghai 200072, China

[2]Department of Ophthalmology, Shanghai General Hospital (Shanghai First People's Hospital), Shanghai Jiao Tong University, Shanghai 200080, China

Correspondence to: Fang Liu. Department of Ophthalmology of Shanghai Tenth People's Hospital, Tongji Eye Institute, Tongji University School of Medicine, 301 Yanchang Road, Jing'an District, Shanghai 200072, China. fangliu_2004@yahoo.com; Jing-Fa Zhang. Department of Ophthalmology, Shanghai General Hospital (Shanghai First People's Hospital), Shanghai Jiao Tong University, 100 Haining Road, Hongkou District, Shanghai 200080, China. 13917311571@139.com

Received: 2019-06-29 Accepted: 2019-08-03

Abstract

• AIM: To preliminarily test proteomics in aqueous humor in patients with dry age-related macular degeneration (AMD) by using the proteomic technology.

• METHODS: Aqueous humor samples were collected from patients with or without dry AMD, who underwent cataract surgery. The aqueous samples were analyzed with isobaric tags for relative and absolute quantification (iTRAQ) combined with liquid chromatography tandem mass spectrometry (LC-MS/MS) technology. The differential expressed proteins were analyzed with gene ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein-protein interaction (PPI) network analysis. The data were partly validated by ELISA and Western blot. False discovery rate (FDR) was used for statistical analysis.

• RESULTS: A total of 244 proteins were detected, in which 38 proteins were up-regulated and 51 were down-regulated significantly in patients with dry AMD compared with that in control groups (FDR value <1.0%). Several proteins, *e.g.*, protein S100-A8 (S10A8), dystroglycan (DAG1), Ig alpha-1 chain C region (IGHA1), carbonic anhydrase 3 (CAH3) and alpha-1-acid glycoprotein (A1AG1) were increased more than 5 times of that in control group. The bioinformatics analysis showed that dry AMD is closely associated with inflammation or immune reaction, oxidative stress, blood coagulation and remodeling of extracellular matrix.

• CONCLUSION: iTRAQ-based proteomic analysis of aqueous humor demonstrate the differential expressions of proteins between dry AMD and control groups, providing the clues to understand the mechanisms and possible treatments of dry AMD.

• KEYWORDS: age-related macular degeneration; protein biomarker; isobaric tags for relative and absolute quantification; differential expression of proteins; aqueous humor

DOI:10.18240/ijo.2019.11.15

Citation: Qu SC, Xu D, Li TT, Zhang JF, Liu F. iTRAQ-based proteomics analysis of aqueous humor in patients with dry age-related macular degeneration. *Int J Ophthalmol* 2019;12(11):1758-1766

INTRODUCTION

Age-related macular degeneration (AMD) is a severe disease with the chronic progressive loss of central vision, which is caused by environmental and multi-genes interactions[1]. It is the leading cause of blindness in patients over 60 years old, and the number of patients with AMD is expected to reach 288 million by 2040[2]. Recent study showed that the morbidity caused by this globalized disease in Asian populations was similar to or even higher than Caucasians[3-6]. AMD is divided into atrophic (dry) form with drusen deposition, in which geographic atrophy (GA) was the severe form, and neovascular (wet) form with formation of choroidal neovascularization (CNV). Currently, anti-vascular endotheliall growth factor (VEGF) reagents, like ranibizumab, aflibercept and conbercept, are widely used to treat CNV secondary to wet AMD in clinical practice, showing great efficacy in regressing CNV. However, there are no effective treatments for dry AMD. It is of the importance to elucidated the mechanisms of dry AMD and thus to find effective treatments for it. Recent studies reported some differential expressed proteins, identified from aqueous humor, vitreous body, Bruch's membrane and plasma[7-11], might be used as the promising biomarkers for dry AMD, which are associated with oxidative stress, immune activation and metabolic dysfunction, *etc*.

The current methods for protein quantitative partition include two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled with stain and mass spectrometry-based detection. 2D-electrophoresis is widely used for its

feasibility and low cost, but it has some limitations, such as to effectively separate extreme high or relative low molecular weight proteins (>200 kD or <8 kD), low abundant proteins, hydrophobic proteins and basic proteins. Mass spectrometry-based detection can be divided into labeling quantitation and label-free quantitation. Labeling quantitation includes metabolic labeling and isotope-coded affinity tags (ICAT) *in vivo* as well as stable isotope labeling of amino acids in culture (SILAC) and isobaric tags for relative and absolute quantification (iTRAQ) *in vitro*. Among them, iTRAQ is an approach utilizing isotope labeling technology introduced by the American applied biological systems corporation[12]. Comparing with other methods, iTRAQ has several virtues, *e.g.*, analysis of a wide range of samples including cellular extracts and complex mixture samples, high-throughput simultaneously quantifying four to eight kinds of samples, good repeatability and high result consistency, *etc*[12].

In this study, we used iTRAQ to detect the differential expressions of aqueous proteins in patients with or without dry AMD, followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) for identification. Several differentially expressed proteins (DEPs) detected by iTRAQ could be served as potential biomarkers for dry AMD, which helped to elucidate its underlying mechanisms.

SUBJECTS AND METHODS

Ethical Approval The study received Institutional Review Board (IRB) approval from Ethics Committee of Shanghai Tenth People's Hospital, Tongji University School of Medicine, and was conducted in accordance with ethical standards of the Declaration of Helsinki regulations. All participants signed the informed consents and didn't receive any stipends.

Patients and Sampling Total 24 patients were employed, including 12 patients with cataract only and 12 with both cataract and dry AMD, who underwent phacoemulsification and intraocular lens implantation at Department of Ophthalmology of Shanghai Tenth People's Hospital, Tongji Eye Institute, Tongji University School of Medicine, Shanghai, China from February 2017 to December 2017. The clinical and demographic data of patients were detailed in Table 1.

Before the surgery, all patients were examined thoroughly to exclude systemic diseases and evaluate eye conditions including routine blood test, slit-lamp examination, fundus photography, fluorescence fundus angiography (FFA), indocyanine green angiography (ICGA) and optical coherence tomography (OCT), *etc*. The patients with cataract only were served as control. Total twenty-four aqueous humor samples (0.1 mL/patient) were obtained *via* paracentesis of anterior chamber by using 23G needle before phacoemulsification. The aqueous humor samples were immediately transferred to cryogenic vials and stored at -80°C until assay.

Table 1 The clinical and demographic data of the subjects

Items	AMD group	Control group
Age (mean±SD, y)	80.82±4.17	68.81±18.82
Gender (man *vs* women)	5:7	6:6
Stages of dry AMD		
Early stage	7	0
Intermediate stage	2	0
Advanced stage	3	0

AMD: Age-related macular degeneration. Early stage: Significant for the presence of multiple drusen (each drusen <125 μm in size); Intermediate stage: Confluent drusen (≥125 μm in size) and the RPE often appears atrophic, with easier visualization of the underlying choroid vascular plexus; Advanced stage: Coalescence of focal islands of atrophy and formation of large zones of atrophy.

Protein Preparation and iTRAQ Labeling Protein concentrations were determined by BCA protein assay according to the manufacturer's instruction (Merck, Darmstadt, Germany). Considering the total concentration was five times lower in aqueous humor than in plasm, the aqueous samples were not processed to remove the high abundant proteins to prevent the loss of certain binding proteins. The protein samples (30 μg per sample) were mixed separately as AMD group or control group, and digested with trypsin (the proportion of trypsin: protein=1:25). The digestion was carried out overnight at 37°C, and then followed by iTRAQ labeling.

The peptide mixture was labeled with iTRAQ reagent according to the manufacturer's instruction (AB Sciex, Foster City, CA, USA). Briefly, the aqueous samples from 12 AMD patients were pooled together, divided equally into two groups and labeled with 114 and 115 tags, respectively. And the samples from control group were processed with the same way and labeled with 116 and 117 tags respectively. The quantitative values of iTRAQ ratios expressed as the average of 114:116 and 115:116 for AMD group, and as the average of 116:116 (equal to 1) and 117:116 for control group. After reaction at room temperature for one hour, all labeled samples were collected into a tube for subsequent reaction.

Quantitative Proteomic Analyses and Data Processing Firstly, the complex mixed peptides were classified using strong cation exchanger (SCX) column according to the ICAT Cation Exchange Buffer Pack kit. Gradient elution was done in order of increasing KCL concentrations (40, 60, 80, 100, 120, 140, 160, 200, 240 and 460 mmol/L). Eluant was gathered, concentrated and desalted after SCX grading. Peptides of twice eluting were pooled and dried for liquid chromatography (LC). The complex peptides were separated into simple peptides by using LC. A binary gradient with solvent A [2% acetonitrile (ACN) and 0.1% formic acid (FA)] and solvent B (98%ACN and 0.1% FA) was employed as the mobile phase. The dried SCX fractions were dissolved in 20 μL of solvent A, followed

by centrifugation at 12 000 r for 10min. Totally 8 μL of each sample was loaded and flow rate of loading pump was controlled at 2 μL/min. The peptide solutions were desalted for 15min and then separated peptides online at 0.3 μL/min. Solvent B was ramped up from 5% to 48% in 85min and increased to 80% maintained for 5min to elute the highly retained peptide segments. After that, the concentration was changed to 5% for 10min. The peptide fractions were finally collected and entered MS analysis after ionized.

The identification of platform of MS analysis was ABSECX TripleTOF™ 4600, acquisition map model was in Data Dependent Acquisition (DDA) scanning mode, and the sprayer used New Objective. The ionization voltage maintained at 2.3 kV and the mass-to-charge ratio of MS scanning was in the range of 350-1250 (m/z), with cumulative time of 0.25s. The top 30 multiply-charged ions were selected for MS/MS analysis of each scan from an m/z 100-1500 range. The cumulative time of MS/MS analysis was 0.1s, dynamic elimination time was 25s, fluctuation state of collision energy was set as enabled, and collision voltage difference was 5.

In the LC-MS/MS analysis, a protein with an unused score below 1% false discovery rate (FDR) and at least 2 unique peptides with 95% probability should be accepted.

Bioinformatics Analysis Gene ontology (GO) annotation was performed using the Blast2GO Bioinformatics software (V3.1.3)[13]. GO enrichment, protein-protein interaction (PPI) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed with the OmicsBean multi-omics data analysis tool (http://www.omicsbean.com:88/)[14], based on gene/protein fold change. In PPI analysis, solid lines represented interactions with higher confidence scores. The confidence cutoff was defined as 400.

Enzyme-linked Immunosorbent Assay Concentration of the high-expressed protein from plasma samples were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D systems, Human alpha 1-Acid Glycoprotein Quantikine ELISA Kit). Briefly, 100 μL of assay diluent and 50 μL of standard, plasma samples or control were added to each well and incubate for 2h at room temperature. Then, the wells were sequentially aspirated, washed four times, followed by adding 200 μL of conjugate to each well and incubated at room temperature for 2h. After aspirated and washed four times, the wells were further incubated with 200 μL substrate solution at room temperature for 30min in darkness. Finally, 50 μL of stop solution was added to each well and optical density was read at 450 nm on an enzyme label colorimeter (Multiskan FC, Thermo Scientific, USA).

Western Blot Analysis Five isopyknic protein samples with the same concentration of total proteins were mixed and subjected to 10% polyacrylamide gels for electrophoresis, and were transferred onto PVDF membrane and incubated with the primary antibody (Human alpha 1 Acid Glycoprotein antibody, 1:500; R&D system, Mab3694) overnight at 4℃. The membrane was washed three times with Tris-Buffered saline Tween (TBST) and labeled with the secondary antibody (1:2000) for 1h at room temperature. After three washes with TBST, the image was captured with electrochemiluminescence (ECL; BIO-RAD, Molecular Imager).

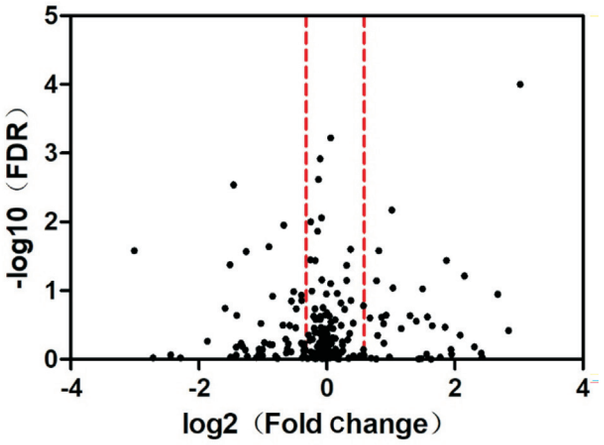

Figure 1 The Volcano pattern of DEPs The graph's horizontal axis represents log2 (fold change) and the vertical axis represents -log10 (FDR). Two dotted red lines separates the Figure into three parts. The rightmost part shows 38 proteins were up-regulated and the leftmost part shows 51 proteins were down-regulated. And the middle part remains relatively unchanged.

Statistical Analysis Protein ratio was analyzed by FDR, a built-in procedure of ProteinPilot software, which is the corrected *P* values. Peptide identifications required an FDR value <1.0%.

RESULTS

Proteomic Identification of Differentially Expressed Proteins Profiling of DEPs between dry AMD group and control group were created by quantitative proteomic analysis. To highlight the key proteins, two key criteria were used to define the specific proteins, *i.e.*, 1) protein levels in AMD group higher than at least 1.5-fold or lower than at least 0.8-fold of that in control group; 2) protein levels in control group changed between 0.8-fold and 1.2-fold. Based on this criterion, a total of 244 proteins were detected; among them, 89 proteins were identified differentially expressed, with 38 up-regulated proteins and 51 down-regulated proteins (Figure 1). The details of the DEPs were listed in Tables 2 and 3.

Gene Ontology Analysis of Differentially Expressed Proteins GO enrichment characterized the DEPs on biological process (BP), cell components (CC), and molecular function (MF). Each top 10 categories were calculated based on the protein counts and were shown in Figure 2.

Table 2 List of up-regulated proteins in AMD group compared with control group

Accession No.	Gene name	Protein name	Peptides (95%)	Fold change	FDR (%)	Stability ratio	FDR (%)
P05109	S10A8	Protein S100-A8	8	8.11285	0.0363	1.16525	0.3044
Q14118	DAG1	Dystroglycan	2	7.144	0.5497	0.9954	0.4929
P01876	IGHA1	Ig alpha-1 chain C region	30	6.36825	0.2284	0.8273	0.5461
P07451	CAH3	Carbonic anhydrase 3	3	5.37175	0.0713	1.05845	0.8471
P02763	A1AG1	Alpha-1-acid glycoprotein	44	5.3211	0.3553	1.0333	0.6994
P06727	APOA4	Apolipoprotein A-IV	47	4.9457	0.0001	1.1966	0.3151
P02788	TRFL	Lactotransferrin	25	4.43605	0.1164	0.90085	0.9119
P30838	AL3A1	Aldehyde dehydrogenase	9	4.239	0.8126	1.1902	0.3191
P01042	KNG1	Kininogen-1	41	3.8584	0.1879	1.05845	0.8961
P63261	ACTG	Actin, cytoplasmic 2	15	3.8377	0.2427	1.10115	0.2811
P19823	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	8	3.65595	0.1392	1.0741	0.9467
P02647	APOA1	Apolipoprotein A-I	78	3.59195	0.0914	1.11795	0.6642
P19652	A1AG2	Alpha-1-acid glycoprotein 2	24	3.4143	0.3822	1.1966	0.7947
P07225	PROS	Vitamin K-dependent protein S	2	3.1293	0.7689	1	0.9165
P25311	ZA2G	Zinc-alpha-2-glycoprotein	33	3.09115	0.0271	0.8365	0.2774
P43652	AFAM	Afamin	26	2.96685	0.2994	0.84275	0.1039
P80748	LV302	Ig lambda chain V-III region LOI	6	2.94375	0.7735	0.88635	0.339
P00338	LDHA	L-lactate dehydrogenase A chain	6	2.818	0.6028	0.8556	0.956
P01024	CO3	Complement C3	139	2.7808	0.0006	1.02355	0.8534
P02750	A2GL	Leucine-rich alpha-2-glycoprotein	6	2.7016	0.0263	1.10115	0.7073
P02760	AMBP	Protein AMBP	13	2.63815	0.0112	0.8758	0.1804
P02675	FIBB	Fibrinogen beta chain	13	2.46095	0.023	0.8556	0.3175
P02774	VTDB	Vitamin D-binding protein	58	2.23515	0.2346	1.0093	0.37
P04196	HRG	Histidine-rich glycoprotein	27	2.048	0.9573	0.9315	0.5461
P00734	THRB	Prothrombin	20	2.02405	0.7763	0.91975	0.8888
P00747	PLMN	Plasminogen	32	1.9724	0.582	1.1067	0.3485
P05546	HEP2	Heparin cofactor 2	12	1.96475	0.8765	1.09015	0.5298
Q9HCQ7	RFRP	FMRFamide-related peptides	2	1.90125	0.2499	0.956	0.9658
P61626	LYSC	Lysozyme C	11	1.8574	0.3403	1.15915	0.5153
P04406	G3P	Glyceraldehyde-3-phosphate dehydrogenase	9	1.8478	0.9072	0.9731	0.1866
P01775	HV314	Ig heavy chain V-III region LAY	2	1.81	0.5162	1.0636	0.8005
P00751	CFAB	Complement factor B	36	1.75395	0.4939	1.0333	0.3176
P10451	OSTP	Osteopontin	31	1.73075	0.5831	0.90085	0.6573
P01023	A2MG	Alpha-2-macroglobulin	67	1.70955	0.2094	1.00465	0.6274
P35749	MYH11	Myosin-11	2	1.7088	0.4505	1.10115	0.6608
P31025	LCN1	Lipocalin-1	7	1.61465	0.2414	1.14115	0.8803
P01861	IGHG4	Ig gamma-4 chain C region	157	1.59305	0.8834	0.9688	0.9524
P01772	HV311	Ig heavy chain V-III region KOL	5	1.5013	0.2508	1.0956	0.5357

Fold change: The average of 114:116 and 115:116, represents the differences between dry AMD and control group. Stability ratio: The average of 117:116 and 116:116, represents the stability of experimental.

For the analysis of BP, majority of obtained proteins were involved in single-multicellular organism process or multicellular organismal process. Beyond that, response to stress and external stimulus accounted for a large proportion with pathological significance. In the CC analysis, the most of proteins located in extracellular regions and membrane-bounded vesicles or organelles. In terms of MF, the results indicated that protein binding is one of the important functions. GO analysis of DEPs consistent with the known pathogenic mechanism of AMD. Besides, vesicle mediated transport in BP, extracellular vesicle and exosome in CC suggested that some specific proteins were related with exosome.

Kyoto Encyclopedia of Genes and Genomes Pathway Analysis of Differentially Expressed Proteins KEGG enrichment highlighted 15 significantly accumulated pathways involving the DEPs (Figure 3).

Table 3 List of down-regulated proteins in AMD group compared with control group

Accession No.	Gene name	Protein name	Peptides (95%)	Fold change	FDR (%)	Stability ratio	FDR (%)
P49788	TIG1	Retinoic acid receptor responder protein 1	4	0.79175	0.7269	0.9477	0.3415
O95967	FBLN4	EGF-containing fibulin-like extracellular matrix protein 2	2	0.78015	0.7237	0.8365	0.456
Q9BRK5	CAB45	45 kDa calcium-binding protein	2	0.77985	0.4855	0.9518	0.4635
P13591	NCAM1	Neural cell adhesion molecule 1	2	0.7792	0.1763	0.9775	0.5542
P03950	ANGI	Angiogenin	3	0.7774	0.7888	1.04825	0.8322
P23142	FBLN1	Fibulin-1	15	0.7672	0.4822	0.9819	0.9004
P01779	HV318	Ig heavy chain V-III region TUR	5	0.7603	0.9268	0.98635	0.9713
P11021	GRP78	78 kDa glucose-regulated protein	3	0.75905	0.3494	0.9315	0.3553
P06309	KV205	Ig kappa chain V-II region GM607 (fragment)	8	0.7586	0.7575	1.0533	0.9666
O00391	QSOX1	Sulfhydryl oxidase 1	4	0.7185	0.3163	0.87235	0.3321
Q92563	TICN2	Testican-2	4	0.7145	0.3223	0.8828	0.6172
P61812	TGFB2	Transforming growth factor beta-2	2	0.69935	0.7418	0.90455	0.7235
Q16270	IBP7	Insulin-like growth factor-binding protein 7	16	0.6812	0.0721	1.10115	0.7936
P00738	HPT	Haptoglobin	21	0.67985	0.9968	1.1471	0.7978
P28799	GRN	Granulins	2	0.66815	0.8388	1.14115	0.9648
Q96KN2	CNDP1	Beta-Ala-His dipeptidase	9	0.658	0.5946	0.8793	0.5747
P30041	PRDX6	Peroxiredoxin-6	2	0.65225	0.5452	0.8126	0.8256
Q15113	PCOC1	Procollagen C-endopeptidase enhancer 1	13	0.6438	0.227	1.1123	0.8747
P51693	APLP1	Amyloid-like protein 1	5	0.63985	0.9855	1.1353	0.6148
P02452	CO1A1	Collagen alpha-1(I) chain	5	0.62645	0.5824	0.83035	0.3598
Q06481	APLP2	Amyloid-like protein 2	19	0.62055	0.3582	1.1237	0.7933
Q16568	CART	Cocaine-and amphetamine-regulated transcript protein	2	0.5856	0.947	0.8097	0.4122
O15031	PLXB2	Plexin-B2	3	0.5732	0.6615	1.18385	0.419
P35555	FBN1	Fibrillin-1	30	0.5557	0.1014	0.9236	0.9677
Q8WXD2	SCG3	Secretogranin-3	6	0.5546	0.1091	1.0432	0.4877
P02766	TTHY	Transthyretin	21	0.53735	0.0087	0.9645	0.7666
P22352	GPX3	Glutathione peroxidase 3	23	0.53365	0.0067	1.11795	0.4594
Q02809	PLOD1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	2	0.5083	0.3454	1.0093	0.9497
P06865	HEXA	Beta-hexosaminidase subunit alpha	2	0.4977	0.1839	0.90085	0.376
Q99435	NELL2	Protein kinase C-binding protein NELL2	2	0.49765	0.2352	0.9315	0.4598
P01034	CYTC	Cystatin-C	30	0.48975	0.0024	0.9477	0.6458
P05154	IPSP	Plasma serine protease inhibitor	8	0.47985	0.6772	0.99085	0.4351
Q13510	ASAH1	Acid ceramidase	3	0.47905	0.3238	1.1471	0.5318
Q12907	LMAN2	Vesicular integral-membrane protein VIP36	2	0.4665	0.346	0.90085	0.681
P06396	GELS	Gelsolin	57	0.4229	0.0012	0.956	0.6997
P13645	K1C10	Keratin, type I cytoskeletal 10	14	0.418	0.2382	0.9602	0.8642
P07339	CATD	Cathepsin D	21	0.4131	0.0792	1.01875	0.5453
P16870	CBPE	Carboxypeptidase E	11	0.4007	0.01	0.9236	0.2487
Q9HCB6	SPON1	Spondin-1	10	0.3956	0.6449	0.8334	0.9428
Q92520	FAM3C	Protein FAM3C	5	0.3774	0.5027	0.9775	0.5558
P07477	TRY1	Trypsin-1	29	0.37515	0.0695	0.9688	0.6649
Q12805	FBLN3	EGF-containing fibulin-like extracellular matrix protein 1	36	0.37485	0.0363	0.9436	0.9729
Q13822	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	35	0.36485	0	1.0093	0.7491
P39060	COIA1	Collagen alpha-1(XVIII) chain	8	0.35235	0.0136	0.9477	0.8467
P04264	K2C1	Keratin, type II cytoskeletal 1	25	0.3503	0.025	1.0741	0.5077
P10745	RET3	Retinol-binding protein 3	49	0.3321	0.0429	1.0636	0.3677
P41222	PTGDS	Prostaglandin-H2 D-isomerase	90	0.27485	0.0029	0.8184	0.3224
Q9BU40	CRDL1	Chordin-like protein 1	6	0.20485	0.1811	0.8155	0.2359
O15537	XLRS1	Retinoschisin	6	0.18485	0.0263	0.8013	0.7073
P16035	TIMP2	Metalloproteinase inhibitor 2	3	0.15235	0.1206	0.86555	0.5001
Q8N475	FSTL5	Follistatin-related protein 5	8	0.12455	0.0357	0.9159	0.9342

Fold change: The average of 114:116 and 115:116, represents the differences between dry AMD and control group. Stability ratio: The average of 117:116 and 116:116, represents the stability of experimental.

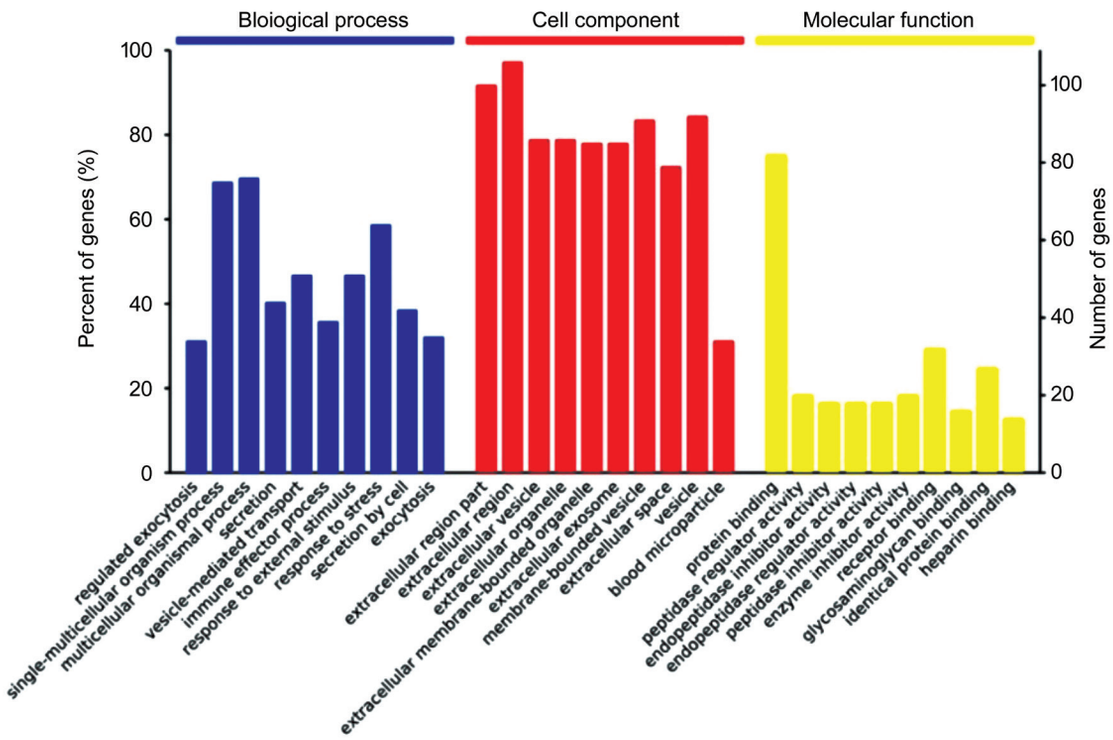

Figure 2 GO enrichment analysis of DEPs GO enrichment characterized the DEPs on BP, CC, and MF. Each top 10 categories were calculated based on the protein counts. The left and right y-axes represent percent of genes and number of genes, respectively.

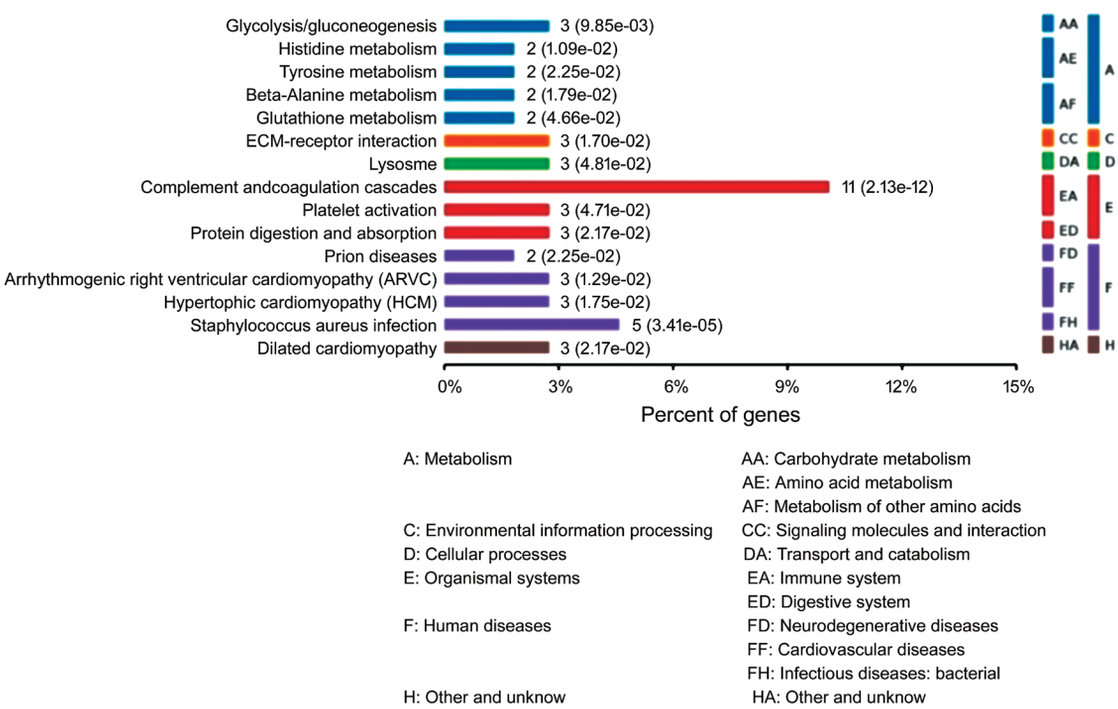

Figure 3 KEGG pathway analysis of DEPs Eleven DEPs which account for 10.09% were accumulated in the complement and coagulation cascades pathway. The cross columns refer to different pathways and the longitudinal column refer to classification of pathways.

Eleven DEPs (10.09%) were accumulated in the complement and coagulation cascades pathway, and 5 DEPs (4.59%) were enriched in staphylococcus aureus infection pathway. Moreover, the enrichment of extracellular matrix (ECM)-receptor interaction pathway (3 DEPs, 2.75%) was observed, which is consistent with the GO enrichment analysis.

Protein-Protein Interaction Network Analysis PPI analysis displayed the signaling network and interactions among the DEPs (Figure 4). The result showed that up-regulated proteins were related to complement and coagulation cascades and glycolysis/gluconeogenesis, while the down-regulated proteins were involved in the pathways in protein digestion and absorption.

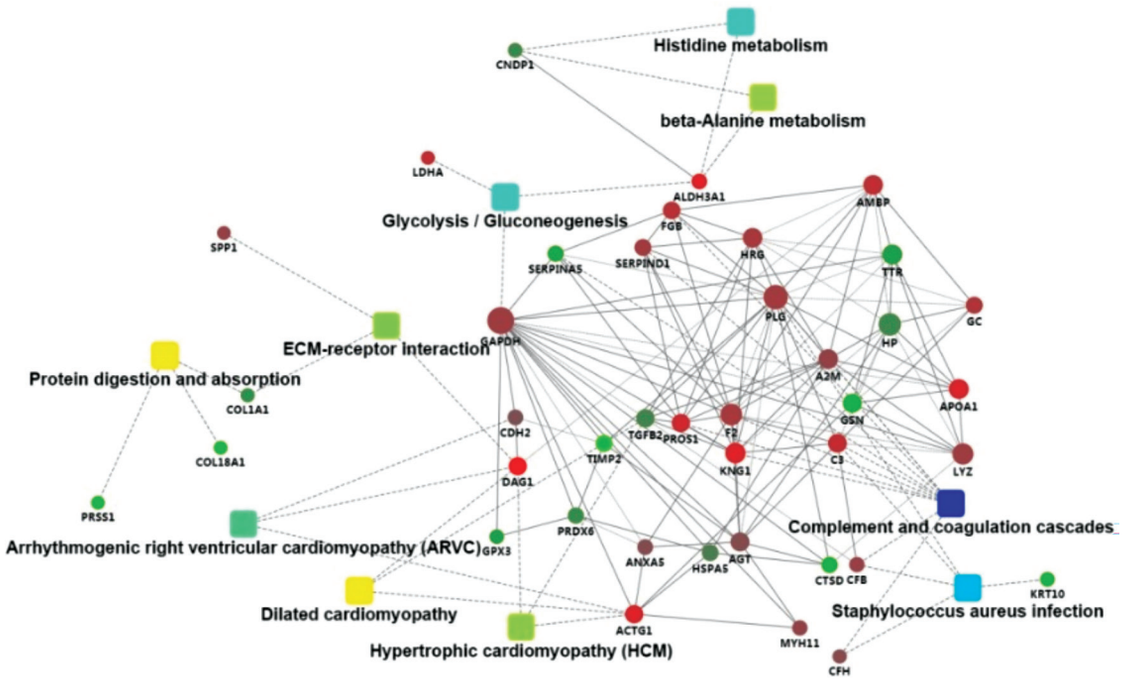

Figure 4 PPI network PPI analysis displayed the signaling network between the DEPs. Red dots represent up-regulated proteins and green dots represent down-regulated proteins. Squares link to proteins represent the main functions of relevant proteins. Solid lines represent direct relationships and imaginary lines represent predictive relationships.

Glyceraldehyde-3-phosphate dehydrogenase, plasminogen, kininogen-1, lysozyme C, and prothrombin tended to be key regulators in the network of PPI, which deserved further investigation.

Up-regulation of Immuno-inflammatory Protein in Serum

To validate the changes of DEPs detected by iTRAQ, serum alpha-1-acid glycoprotein 1 (A1AG1, aliases for ORM1 gene) was selected and verified with both Western blot (Figure 5A) and ELISA (Figure 5B), since this protein was an acute phase protein with inflammatory and immunomodulating properties[15], and also some unique drug-binding properties that differ from those of albumin[16]. As shown in Figure 5, serum A1AG1 level in dry AMD group was increased significantly than that in control group (about 5.32-fold of the control). The result suggests that immune disorders might play an important role in the pathogenesis of dry AMD.

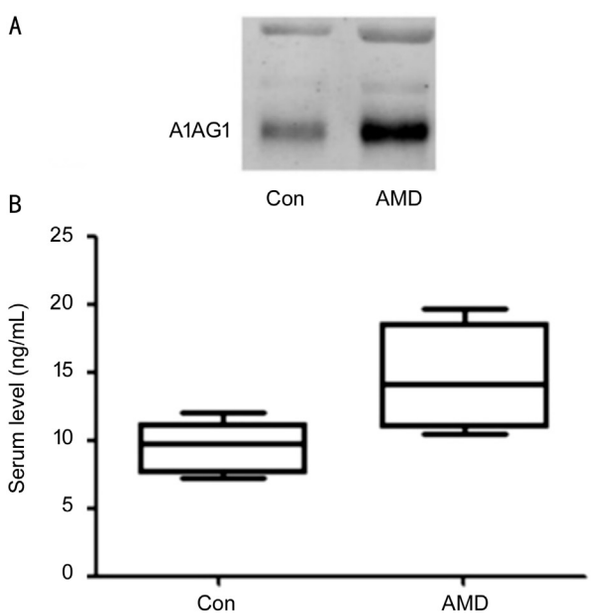

Figure 5 Serum A1AG1 protein detection with Western blot (A) and ELISA (B) Each of five protein samples from dry AMD group or control group were isovolumetric mixed and detected with Western blot and ELISA. Con: Serum from control group; AMD: Serum from patients with dry AMD.

DISCUSSION

AMD is one of the most serious eye diseases, still lack of effective treatments, especially for its dry form. It is necessary to find the potential biomarkers for dry AMD to understand the pathogenesis, predict its progression and prognosis, and find potential targets, thus to provide effective treatments. The proteomics for biomarkers in aqueous humor is a valuable method. Because there is evidence that pathological concentrations of several proteins present in the aqueous fluid are closely associated with fundus diseases[17].

In this study, total of 244 proteins were identified, in which 38 proteins were increased significantly in dry AMD patients. By using bioinformatics analysis (GO enrichment analysis, KEGG pathway, PPI), we found that immune and inflammation seems to play a major role in the pathogenesis of AMD. Most of the up-regulated proteins are serum proteins and primarily involved in physiological and pathological processes including inflammation and immune reaction, oxidative stress,

coagulation process and formation of the extracellular matrix. From single protein function to pathways they involved in, and signaling networks constructed from proteins, these up-regulated proteins provided the clues for the novel targets for dry AMD treatment.

Immune and Inflammation Related Proteins The KEGG pathway showed 11 DEPs were enriched in the complement and coagulation cascades accounting for the largest part. Most of these proteins were reported to have pro-inflammatory function and some of them were also found to be up-regulated in AMD patients, *e.g.*, Complement C3 (CO3)[18] and Ig gamma-1 chain C region (IGHG1)[19].

A1AG1, an immune and inflammation related protein which was found more than five times higher in our study, was proven to have the immune modulatory function, to decrease the pro-inflammatory cytokines and reduce the synthesis of cytokines of lymphocytes through changing its surface properties, thus exert the immune-suppression[15]. As an immune regulator and inflammation inhibitor, A1AG1 might play a protective role in the pathogenesis of dry AMD. In addition, A1AG1 can be induced by the acute phase inflammatory reaction[20]. The presence of acute phrase proteins suggested a local temporary inflammation in the eye of dry AMD patients and persistent inflammation will insult the retina, resulting in AMD.

To explore the potential biomarkers for dry AMD, the serum is easier to be obtained than aqueous humor, which was further considered to be a more practical approach for clinical diagnosis. Therefore, the blood samples were collected from all patients and up-regulated A1AG1 level was detected, indicating serum A1AG1 might be a potential biomarker for dry AMD.

Oxidative Stress Related Proteins Oxidative stress is involved in the pathological process of AMD, like lipofuscin in retinal pigment epithelium (RPE) cells was proved to be from oxidatively damaged photoreceptor outer segments[21]. A series of up-regulated proteins was found in our study like L-lactate dehydrogenase A chain (LDHA). It was also reported to be increased in RPE exosomes caused by oxidative stress[22]. Protein S100-A8 with highest expression level in dry AMD group, its extracellular function involves oxidant-scavenging and has a protective role in preventing exaggerated tissue damage by scavenging oxidants. Besides, carbonic anhydrase 3 (CAH3) was found, for the first time, to be five times higher in AMD group than that in control group. It is regarded as a scavenger of oxygen free radicals in many studies and has a protective effect on cells in oxidative stress reaction[23]. Roy *et al*[24] found that H_2O_2-induced apoptosis in fibroblast can be restored when CAH3 expression is forcibly increased in cells. These facts indicated the causal role of oxidative stress in the pathogenesis of dry AMD.

Metabolism Related Proteins Macular region is one of the most active metabolic areas of human body. Maintenance of the homeostasis plays a prominent role in keeping normal functions. Inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2) with three-fold increase in AMD group acts as a carrier of hyaluronan in serum or as a binding protein between hyaluronan and other matrix protein. Hyaluronan is the main component of ECM, which associated with ITIH2 might be involved in the pathological process of AMD. Up-regulation of hyaluronan means the activation of cells and tissues remodeling of physiological or pathological processes[25], and the remodeling of ECM plays an important role in the pathological process of AMD patients. The development of drusen, changes in the Bruch membrane and the infiltration of immune cells are all related to the remodeling of dense or loose extracellular structures[7]. It can be speculated that the up-regulation of ITIH2 destabilize the ECM environment by affecting the production of hyaluronan, thus promoting the development of AMD. Beyond this, proteins of actin, cytoplasmic 2 (ACTG) and zinc-alpha-2-glycoprotein (ZA2G)[26], which were proved to participate in retinal homeostasis, were found to be up-regulated in our study.

AMD is caused by multiple factors, utilizing the proteomics technology to look for biomarkers can help us to elucidate the pathogenesis of this disease and screen the novel targets. By comparing the protein abundance spectra of the same tissue under physiological and pathological conditions, we can identify DEPs, facilitating early diagnosis and potential targets screening. The result of confirmatory discovery in serum bring us a reflect on whether AMD is a systemic disease or a local lesion, but the answer is still debated. Multiple factors may interact each other in the development of dry AMD. By applying different treatments for potential targets, the progress of the disease could be regulated, meanwhile the therapeutic effect and prognosis can be evaluated. Although the limitations exist, such as small sample size caused by difficulty in obtaining samples, the present study reported the differential expressions of proteins in the aqueous humor, which could provide a clue for the elucidating the pathogenesis of dry AMD as well as the potential therapy to targeting these biomarkers.

ACKNOWLEDGEMENTS

Thanks to all the contributions of authors. Qu SC performed the experiments, analyzed the data, interpreted results and wrote the manuscript. Xu D collected the sample. Li TT participated in data analyses. Liu F designed the experiment, analyzed and discussed the data, played an important role in interpreting the results and approved the final version. Zhang JF designed the experiment, analyzed and discussed the data, and revised the manuscript.

Foundations: Supported by National Natural Science Foundation of China (No.81570852); the Shanghai Municipal Health and Planning Commission Foundation (No.201540046).
Conflicts of Interest: Qu SC, None; **Xu D,** None; **Li TT,** None; **Zhang JF,** None; **Liu F,** None.

REFERENCES

1 Lim LS, Mitchell P, Seddon JM, Holz FG, Wong TY. Age-related macular degeneration. *Lancet* 2012;379(9827):1728-1738.

2 Wong WL, Su XY, Li X, Cheung CM, Klein R, Cheng CY, Wong TY. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health* 2014;2(2):e106-e116.

3 Cho BJ, Heo JW, Kim TW, Ahn J, Chung H. Prevalence and risk factors of age-related macular degeneration in Korea: the Korea National Health and Nutrition Examination Survey 2010-2011. *Invest Ophthalmol Vis Sci* 2014;55(2):1101-1108.

4 Jin G, Ding X, Xiao W, *et al*. Prevalence of age-related macular degeneration in rural southern China: the Yangxi Eye Study. *Br J Ophthalmol* 2018;102(5):625-630.

5 Song P, Du Y, Chan KY, Theodoratou E, Rudan I. The national and subnational prevalence and burden of age-related macular degeneration in China. *J Glob Health* 2017;7(2):020703.

6 Wong CW, Yanagi Y, Lee WK, Ogura Y, Yeo I, Wong TY, Cheung CMG. Age-related macular degeneration and polypoidal choroidal vasculopathy in Asians. *Prog Retin Eye Res* 2016;53:107-139.

7 Kersten E, Paun CC, Schellevis RL, *et al*. Systemic and ocular fluid compounds as potential biomarkers in age-related macular degeneration. *Surv Ophthalmol* 2018;63(1):9-39.

8 Kim HJ, Ahn SJ, Woo SJ, *et al*. Proteomics-based identification and validation of novel plasma biomarkers phospholipid transfer protein and mannan-binding lectin serine protease-1 in age-related macular degeneration. *Sci Rep* 2016;6:32548.

9 Kim TW, Kang JW, Ahn J, Lee EK, Cho KC, Han BN, Hong NY, Park J, Kim KP. Proteomic analysis of the aqueous humor in age-related macular degeneration (AMD) patients. *J Proteome Res* 2012;11(8):4034-4043.

10 Lambert NG, ElShelmani H, Singh MK, Mansergh FC, Wride MA, Padilla M, Keegan D, Hogg RE, Ambati BK. Risk factors and biomarkers of age-related macular degeneration. *Prog Retin Eye Res* 2016;54:64-102.

11 Yuan X, Gu X, Crabb JS, Yue X, Shadrach K, Hollyfield JG, Crabb JW. Quantitative proteomics: comparison of the macular Bruch membrane/choroid complex from age-related macular degeneration and normal eyes. *Mol Cell Proteomics* 2010;9(6):1031-1046.

12 Ross PL, Huang YN, Marchese JN, *et al*. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 2004;3(12):1154-1169.

13 Ashburner M, Ball CA, Blake JA, *et al*. Gene ontology: tool for the unification of biology. the gene ontology consortium. *Nat Genet* 2000;25(1):25-29.

14 Sun N, Sun W, Li S, Yang J, Yang L, Quan G, Gao X, Wang Z, Cheng X, Li Z, Peng Q, Liu N. Proteomics analysis of cellular proteins co-immunoprecipitated with nucleoprotein of influenza A virus (H7N9). *Int J Mol Sci* 2015;16(11):25982-25998.

15 Hochepied T, Berger FG, Baumann H, *et al*. α1-Acidglycoprotein: an acute phase protein with inflammatory and immunomodulating properties. *Cytokine Growth Factor Rev* 2003;14(1):25-34.

16 Huang Z, Ung T. Effect of alpha-1-acid glycoprotein binding on pharmacokinetics and pharmacodynamics. *Curr Drug Metab* 2013;14(2):226-238.

17 Pollreisz A, Funk M, Breitwieser FP, *et al*. Quantitative proteomics of aqueous and vitreous fluid from patients with idiopathic epiretinal membranes. *Exp Eye Res* 2013;108:48-58.

18 Yates JR, Sepp T, Matharu BK, *et al*. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357(6):553-561.

19 Lederman M, Weiss A, Chowers I. Association of neovascular age-related macular degeneration with specific gene expression patterns in peripheral white blood cells. *Invest Ophthalmol Vis Sci* 2010;51(1):53-58.

20 Luo Z, Lei H, Sun Y, Liu X, Su DF. Orosomucoid, an acute response protein with multiple modulating activities. *J Physiol Biochem* 2015;71(2):329-340.

21 Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 2000;45(2):115-134.

22 Biasutto L, Chiechi A, Couch R, Liotta LA, Espina V. Retinal pigment epithelium (RPE) exosomes contain signaling phosphoproteins affected by oxidative stress. *Exp Cell Res* 2013;319(13):2113-2123.

23 Räisänen SR, Lehenkari P, Tasanen M, Rahkila P, Härkönen PL, Väänänen HK. Carbonic anhydrase III protects cells from hydrogen peroxide-induced apoptosis. *FASEB J* 1999;13(3):513-522.

24 Roy P, Reavey E, Rayne M, Roy S, Abed El Baky M, Ishii Y, Bartholomew C. Enhanced sensitivity to hydrogen peroxide-induced apoptosis in Evi1 transformed Rat1 fibroblasts due to repression of carbonic anhydrase III. *FEBS J* 2010;277(2):441-452.

25 Petrey AC, de la Motte CA. Hyaluronan, a crucial regulator of inflammation. *Front Immunol* 2014;5:101.

26 Pieragostino D, Agnifili L, Fasanella V, D'Aguanno S, Mastropasqua R, Di Ilio C, Sacchetta P, Urbani A, Del Boccio P. Shotgun proteomics reveals specific modulated protein patterns in tears of patients with primary open angle glaucoma naïve to therapy. *Mol Biosyst* 2013;9(6):1108-1116.