Comparative proteomic analysis of plasma proteins in patients with age–related macular degeneration

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Abstract
• AIM: To find the significant altered proteins in age–related macular degeneration (AMD) patients as potential biomarkers of AMD.
• METHODS: A comparative analysis of the protein pattern of AMD patients versus healthy controls was performed by means of proteomic analysis using two-dimensional gel electrophoresis followed by protein identification with MALDI TOF/TOF mass spectrometry.
• RESULTS: We identified 28 proteins that were significantly altered with clinical relevance in AMD patients. These proteins were involved in a wide range of biological functions including immune responses, growth cytokines, cell fate determination, wound healing, metabolism, and anti–oxidance.
• CONCLUSION: These results demonstrate the capacity of proteomic analysis of AMD patient plasma. In addition to the utility of this approach for biomarker discovery, identification of alterations in endogenous proteins in the plasma of AMD patient could improve our understanding of the disease pathogenesis.
• KEYWORDS: age-related macular degeneration; clinical patients; comparative proteomics; plasma proteins; two-dimensional electrophoresis

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INTRODUCTION
Age-related macular degeneration (AMD) is one of the most common pathologies in the retina, consisting of a chronic degenerative disorder that constitutes the leading cause of vision loss and blindness in elder individuals. AMD clinically manifests as changes in the retinal pigment epithelium (RPE), a monolayer of cells that lie between the photoreceptors and Bruch’s membrane, separating the photoreceptors from the highly vascular choroid [1]. AMD develops in two different stages: the early AMD known as dry AMD and the late stage of AMD also referred to as wet AMD by virtue of extensive neovascularization taking place in the retina choroid. Dry AMD is characterized by the appearance of lipoproteinaceous deposits between the RPE and choroid, as well as changes in RPE pigmentation. Later stages of AMD accompanied by vision loss are characterized by increased drusen accumulation, RPE atrophy, choroidal neovascularization, and loss of photoreceptors [2]. Unfortunately, current treatments for AMD are constrained by our limited understanding of the molecular mechanisms underlying AMD. Elucidation of the mechanisms responsible for AMD onset and progression is critical for developing treatments to prevent late AMD and, hence, preserving visual function in the aging population.

Preclinical studies have proposed several pathogenic mechanisms that could explain the complex etiology of AMD, including RPE cell death, oxidative damage of cellular components, mitochondrial dysfunction, and the accumulation of toxic compounds, such as lipofuscin and advanced glycation end products [3,4]. In addition, inflammation and activation of the innate immune system probably contribute to the pathogenesis of AMD[6]. However, the molecular events that mediate these pathogenic mechanisms must be more clearly defined in clinical context to facilitate the development of targeted therapies for AMD. Furthermore, the pathogenesis of AMD is associated with a number of genes and proteins. However, little is known about the role and clinical relevance of these molecules in AMD. Therefore, identifying AMD-related genes and proteins will beneficial for understanding of AMD pathogenesis and clinical manifestation, and for early prevention, diagnosis and treatment.
Proteomic technologies have been used to examine both normal physiology and disease mechanisms in various medical fields [7]. Over the past five years proteomic studies have provided important information in the fields of ophthalmology. Proteomic studies encompass the analysis of protein expression, functional products of genes, as well as protein interactions. Various aspects of individual proteins or groups of proteins have been investigated associated with ophthalmic disease but modern proteomic technologies have the advantage of simultaneous analysis and identification of large numbers of proteins [8,9]. Currently, few clinical reports are seen on AMD proteomics. In the present study, we applied comparative proteomics strategy to examine the global plasma protein expression changes in patients with wet and dry AMD and identified 28 marker proteins involved in various pathophysiological processes. These data provided direct evidence for specific changes in the expression of AMD-related proteins in clinical context.

SUBJECTS AND METHODS

Subjects This study involved 13 patients with wet AMD (7 males, 6 females, age 68.74 ±7.53) and 11 patients with dry AMD (5 males, 6 females, age 68.21±7.39). They were clients admitted to our hospital during August 2010 to July 2011. These AMD subjects were all a mixture of early and advanced AMD. The 6 healthy volunteers (3 males, 3 females, age 66.21±5.28) as control were from the Medical Examination Center at our hospital. Clinical Diagnostic Criteria for Age-Related Macular Degeneration established in 1986 was used as diagnostic criteria in this study [10]. Fluorescein angiography shows that there may be no fluorescein leakage in dry AMD, but this may take place concomitant with retinal neovascularization in wet AMD. The inclusion criteria included routine examination, fluorescein fundus angiography and/or optical coherence tomography with signed informed consents. The exclusion criteria included combination of other pathological changes of ground retina (i.e. Stargardt’s disease, pathologic changes of macular hole and macular preretinal membrane, retinal vein obstruction, central serous chorioretinopathy), and fundus oculi disease associated with diabetes mellitus or hypertension. The study followed the tenets of the Declaration of Helsinki, and informed written consent was obtained from all patients and controls after we explained the nature and possible consequences of the study. The study protocol was approved by the Medical Ethical Committee of Jiangsu Province Hospital of Traditional Chinese Medicine.

Methods

Sample preparation Vein blood of 2 mL was obtained from each fasting subject at morning. Anticoagulant ethylene diamine tetraacetic acid (EDTA) was added to the samples and stored at 4°C immediately followed by standing for 30min. Blood was centrifuged at 3000 r/min for 20min to separate the plasma, which then was aliquoted and stored at -80°C until further analysis. Centrifugation at 4°C, 10 000 r/min for 1h was performed prior to experiments in order to eliminate the nucleic acids, lipids, polysaccharides and some large molecular weight proteins that could interfere the subsequent two-dimensional electrophoresis. Bradford method was used to determine the protein concentration.

Two-dimensional electrophoresis Samples were analyzed by two-dimensional gel electrophoresis as described [11]. Briefly, for the first dimension, total protein (100 µg) was mixed with 350 mL in a rehydration buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 0.5% ampholytes, 50 mmol/L DTT, and 0.004% of bromophenol blue), loaded onto an 24 cm IPG strip (pH 3-10, GE Healthcare, Hong Kong, China) with rehydration solution overnight, and focused in an IPImphor 2 (GE Healthcare, Hong Kong, China) for 80 kVh. The second dimension was performed on 12.5% polyacrylamide gels in Ettan Dalt six apparatus (GE Healthcare, Hong Kong, China). Gels containing Cy5 labeled protein were scanned with the Image Scanner at a resolution of 200 mm, and photomultiplier (PMT) values were adjusted to optimize sensitivity and prevent oversaturation. An excitation/emission wavelength for Cy5 was 633/670.

Gels were stained using colloidal coomassie blue G-250. The gels were fixed in 40% methanol and 10% acetic acid in water for 60min. Gels were washed thrice in water for 10min each and then stained overnight in a staining solution containing 10% phosphoric acid, 10% ammonium sulfate, 0.12% coomassie blue G-250, and 20% methanol. Gels were then destained in water. Silver staining was done as described[15]. After protein fixation for 60min with 40% ethanol and 10% acetic acid in water, the gels were sensitized in 30% ethanol, 0.2% sodium thiosulfate, 6.8% sodium acetate in water with 0.5% glutaraldehyde for 30min. Gels were washed thrice, 15min each and the gels were stained with 0.1% silver nitrate, 0.04% formalin for 30min. After 10s wash, the gels were developed in 2.5% sodium carbonate and 0.03% formalin. Development was stopped with 1.86% EDTA in water and then the stained gels were washed twice in water for 10min each.

MALDI TOF/TOF mass spectrometry Spots of interest were excised from the gel with a razor blade, placed in an Eppendorf tube, and destained by washing three times for 10min in 200 mL of 50% (v/v) acetonitrile, 50 mmol/L NH₄HCO₃ solution. Proteins were then reduced by 20 mmol/L dithiothreitol, 100 mmol/L NH₄HCO₃, and acetonitrile 5% for 1h at 55°C. The gel pieces were dehydrated at room temperature and covered with 10 mL of modified trypsin (Promega, Madison, WI; sequencing grade) in Tris buffer (2.5 mmol/L; pH 8.5) and were left at 37°C overnight. The spots were crushed and the peptides were extracted in an ultrasonic bath (15min) with 15 mL aqueous solution of
acetonitrile and formic acid (49/50/1 v/v/v). After extraction, the solution of the peptides was lyophilized and redissolved in water. The aqueous solutions of the lyophilized protein tryptic digests were purified by using C\textsubscript{18} solid phase extraction and were directly loaded onto the target plate (MTP 384 massive target T; Bruker Daltonics, Bremen, Germany) by mixing 1.0 mL of each solution with the same volume of a saturated matrix solution, prepared fresh every day by dissolving a-cyano-4-hydroxy cinnamic acid (CHCA) in acetonitrile/0.1% TFA (1/2, v/v). The mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics) operated in the reflector for MALDI TOF peptide mass fingerprint (PMF) or LIFT mode for PSD (post source decay) and CID (collision-induced decay) MALDI TOF/TOF with an automated mode using FlexControl 2.4 software. An accelerating voltage of 20 kV was used for PMF. The instrument uses a 337-nm pulsed nitrogen laser (Model MNL-205MC; LTBL Lasertechnik Berlin GmbH, Berlin, Germany). External calibration was performed in each case using a Bruker Peptide Calibration Standard (Bruker Daltonics). Peptide masses were acquired with a range of 800 to 5000 m/z. Each spectrum was produced by accumulating data from 500 consecutive laser shots. Singly charged monoisotopic peptide masses were searched against MSDB, Swiss-Prot, and NCBIInr databases by utilizing the MASCOT Server 2.2 search engine (www.matrixscience.com; Matrix Science Ltd., London, United Kingdom) and Bruker BioTools 3.0 software (Bruker Daltonics). Up to one missed tryptic cleavage was considered, and the mass tolerance for mono isotopic peptide masses was set to 100 ppm. For proteins not identified by MALDI TOF, we proceeded with PSD and CID MALDI TOF/TOF analysis. Bruker FlexControl 2.4 software (Bruker Daltonics) was used for control of the instrument, and Bruker FlexAnalysis 2.4 software (Bruker Daltonics) for spectra evaluation.

**Spot quantitation and statistical analysis** The significance of spot differences and statistical analysis were performed using Image Master 2D Platinum software version 7.0 (GE Healthcare, Hong Kong, China). Spots within the region of analysis were detected and then matched between each gel and the reference gel. Visual inspection was used to confirm the data points wherever needed. The quantification value used for comparison was intensity volume, which refers to the intensity volume of the protein spot taken against the total intensity volume of all proteins in the gel region selected for analysis. ANOVA test with a confidence interval at 95%. Student’s t-test was used and P value of less than 0.05 was considered to be significant by the software. The samples with three experimental replicates were used for the analysis.

**RESULTS**

The representative spectrums of two-dimensional electrophoresis analyses of plasma proteins from the control subjects and patients with wet AMD and dry AMD were depicted in Figure 1, showing that the protein spots were completely separated without apparent background interference. The spectra of healthy, dry AMD and wet AMD samples are shown in Figures 2-4, respectively. Each spectrum could identify 1460-1480 protein spots with good stability and repeatability. The matching rate for the protein spots in each group were 91.5% (control), 92.2% (dry AMD), and 92.9% (wet AMD). Analyses of gel electrophoresis with software and mass spectrum showed that the expression of
Figure 2 Two-dimensional electrophoresis analyses of plasma proteins in 6 healthy subjects. The matching rate for the protein spots was 91.5%.

Figure 3 Two-dimensional electrophoresis analyses of plasma proteins in 11 patients with dry AMD. The matching rate for the protein spots was 92.2%.

219 proteins in the three groups was altered with statistical significance. These altered proteins were compared to each individual sample. The proteins with more than 2-fold changes in expression, or appeared or absent completely, were identified as significantly changed proteins in the samples. Consequently, we obtained 28 such protein spots and their MS characterization was shown in Table 1. These proteins are involved in a wide range of pathophysiological processes including immune responses, growth cytokines, cell fate determination, wound healing responses, metabolism, and anti-oxidance, which were discussed below in detail.
DISCUSSION

Proteins Related to Immune System

Clinical and basic studies have identified the involvement of immune responses especially complement system in the pathogenesis of...
AMD. Complement 1 and complement factor B may activate complement system; whereas complement factor I has inhibitory effects. Our current data showed that the expression of complement 1 and complement factor B was upregulated in wet AMD, but complement factor I was downregulated. Moreover, HLA-DRB as the basis of immune responses was also upregulated in wet AMD, which was in consistence with previous proteomics analysis of Bruch membrane and choroid in AMD patients showing that HLA-DRB 1-15β was upregulated accompanied by increase in many complement factors. Our data herein suggested that immune inflammatory responses could be important mechanisms underlying the pathogenesis of wet AMD.

Proteins as Growth Factors Wet AMD is characterized by formation of CNV. The present study showed that the expression of fibroblast growth factor (FGF) 6, FGF 13, hepatocyte growth factor (HGF) isoform 6, and placental growth factor (PIGF) isoform 3 was increased in wet AMD compared to that in dry AMD. However, the expression of pigment epithelium derived factor (PEDF) was decreased in wet AMD. These growth factors are all implicated in ophthalmology. FGF promotes proliferation of retinal endothelial cells and RPE cells, and induces subretinal neovascularization. HGF stimulates motility, growth and morphogenesis of retinal vascular endothelial cells beneficial for angiogenesis. PIGF has base sequences highly homologous to vascular endothelial growth factor, thereby showing strong pro-angiogenic effects. However, PEDF is an important anti-angiogenic factor and can prevent vascularization and protect nerve cells, RPE and nutrition photoreceptors. Our present data indicated a disrupted balance between pro-proliferative factors and inhibitory factors in wet AMD, leading to significant enhancement of cell proliferation.

Proteins Regulating Cell Fate We identified 5 proteins associated with cell fate determination. Ubiquitin-conjugating enzyme E2 plays an important role in transferring ubiquitin molecules to target proteins via ubiquitin-proteasome pathway, a critical quality control system of proteins controlling a wide range of physiological processes including apoptosis, major histocompatibility complex I antigen presentation, and cell cycle in eukaryotic cells. Coffin critically regulates cell motility, promotes cell migration and cytokinesis, and also plays a role in embryogenesis, organogenesis, angiogenesis, tumor metastasis, and nerve construction. Moreover, enhanced activity of eukaryotic translation initiation factor 4 results in abnormal translation of a series of proteins related to cell growth and malignant transformation, thus leading to cell survival, invasion, and angiogenesis. Furthermore, proliferating cell nuclear antigen (PCNA) modulates cell proliferation and DNA synthesis, and is involved in cellular DNA damage repair, cell cycle regulation, chromosome recombination, DNA methylation and apoptosis. Finally, Akt 2 via PI3K/Akt pathway participates in cell differentiation, growth, apoptosis and migration and some other biological processes. Our data herein demonstrated that ubiquitin-conjugating enzyme E2 was downregulated in wet AMD and coflin upregulated in dry AMD. The other three proteins were all increased in wet AMD. To date, little is known about the roles of these molecules in AMD pathogenesis. We presumed that they were associated with RPE cell apoptosis and endothelial cell proliferation.

Proteins Involved in Tissue Wounding We also identified 5 significantly altered proteins involved in injury responses, namely heat shock protein B1 (HSPb1), α l-antitrypsin (α l-AT), α l-antichymotrypsin (α l-ACT), kininogen 1 and vitamin D binding protein. The primary biological function of HSPb1 is to protect tissue cells against various stress factors of injury, and is also involved in the signal transduction controlling cell proliferation, differentiation and apoptosis. α l-AT is the most important protease inhibitor in human plasma, protecting normal cells and organs from injury caused by proteases and inhibiting infection and inflammation so as to maintain the internal homeostasis. Serine protease inhibitor α l-ACT can inhibit neutrophil chemotaxis, superoxide production, and tumor necrosis factor-α-induced synthesis of platelet activating factor. Therefore, α l-ACT plays an important role in inhibition of inflammatory process. Kininogen 1 is related to chronic inflammatory injury, and was found in the serum and vitreous of patients with proliferative vitreoretinopathy. Kininogen 1 is also involved in the formation of CNV. Our current results demonstrated that the expression of HSPb1, α l-AT and α l-ACT was low in wet AMD, but kininogen 1 was increased in wet AMD, suggesting that inflammatory responses and injury could play important roles in the development of wet AMD. Vitamin D binding protein is a multifunction plasma protein and cooperates with gelsoin to form actin removal system so as to attenuate the damage of actin. The present study showed that vitamin D binding protein was decreased in dry AMD. However, the biological relevance of this alteration awaits further investigation.

Proteins Associated with Metabolism Guanylyl cyclase activation protein plays a key role in dark adaptation recovery after light stimulation of rod cells. Our present data showed that this protein was decreased in wet AMD. Retinol-binding protein (RBP) is a specific carrier protein transporting retinol from the liver to target tissues to maintain intracellular retinol metabolism. Thus it plays a key physiological role in storage and metabolism of retinol. Our data showed that RBP was upregulated in wet AMD. NADH dehydrogenase is an
important player in the citric acid cycle. Decrease in NADH dehydrogenase content may lead to inhibition of respiratory chain and blockade of adenosine triphosphate synthesis \[30\]. This protein was downregulated in wet AMD shown in our present study. Apolipoprotein (Apo) A1, a major component of high density lipoprotein, is a key factor regulating reverse cholesterol transport, and has anti-oxidant, anti-inflammatory, and anti-endotoxin effects \[10\]. Apo A4 can promote the transferring of cholesterol from extrahepatic tissues and cells to the liver, thereby accelerating the metabolism of cholesterol. Recent evidence also indicated that Apo A4 has antioxidant properties due to its binding to low density lipoprotein (LDL) resulting in inhibition of LDL oxidation \[26\]. Apo H participates in triglyceride metabolism and its phenotype affects the levels of triglyceride. Apo H and its antibody have been demonstrated to be associated with thrombus formation, atherosclerosis, renal diseases, cerebrovascular diseases and some autoimmune diseases \[30\].

Our present data demonstrated that Apo A1 was downregulated but Apo A4 was upregulated in dry AMD. However, Apo H was increased in wet AMD but decreased in dry AMD. These results indicated a close association between AMD pathogenesis and lipid metabolism, which could also be confirmed by recent epidemiological evidence \[34\]. Furthermore, oxidative damage is considered to be another important mechanism underlying AMD pathogenesis. Superoxide dismutase and peroxide reductase are primary antioxidases \textit{in vivo} performing in concert to scavenge the active free radicals. We here found that these two antioxidant enzymes were all decreased in both wet and dry AMD.

In summary, we herein used comparative proteomics approaches to establish the expression spectrum of plasma proteins in patients with dry and wet AMD, and identified 28 proteins that were significantly altered with clinical significance. These proteins are associated with activation of complement system, release of cytokines or inflammatory factors, cell proliferation and apoptosis, and lipid accumulation caused by hypoxia, metabolic disorders and oxidative injury. Investigation into these proteins and their biological relevance may contribute to further understanding the molecular mechanisms of the pathogenesis of AMD and providing basis for discovery of markers for diagnosis and treatments of AMD.

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