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ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · JUNE 2012

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Proteomic Analysis of the Aqueous Humor in Age-related Macular Degeneration (AMD) Patients

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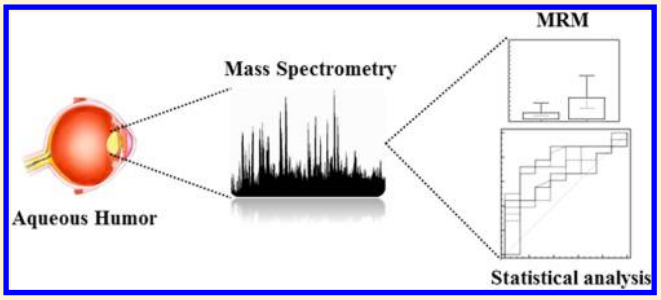
S *Supporting Information*

ABSTRACT: Age-related macular degeneration (AMD) can lead to irreversible central vision loss in the elderly. Although large number of growth factor pathways, including the vascular endothelial growth factor (VEGF), has been implicated in the pathogenesis of AMD, no study has directly assessed the whole proteomic composition in the aqueous humor (AH) among AMD patients. The AH contains proteins secreted from the anterior segment tissue, and these proteins may play an important role in the pathogenesis of AMD. Thus, comparisons between the AH proteomic profiles of AMD patients and non-AMD controls may lead to the verification of novel pathogenic proteins useful as potential clinical biomarkers. In this study, we used discovery-based proteomics and Multiple Reaction Monitoring Mass Spectrometry (MRM-MS) to analyze AH from AMD patients and AH from controls who underwent cataract surgery. A total of 154 proteins with at least two unique peptides were identified in the AH. Of these 154 proteins identified by discovery-based proteomics, 10 AH proteins were novel identifications. The protein composition in the AH was different between AMD patients and non-AMD controls. Subsequently, a systematic MRM-MS assay was performed in seven highly abundant differentially expressed proteins from these groups. Differential expression of three proteins was observed in the AH of AMD patients compared with that of cataract controls ($p < 0.0312$). Elucidation of the aqueous proteome will establish a foundation for protein function analysis and identify differentially expressed markers associated with AMD. This study demonstrates that integrated proteomic technologies can yield novel biomarkers to detect exudative AMD.



KEYWORDS: *aqueous humor, age-related macular degeneration, mass spectrometry, proteomics, multiple reaction monitoring*

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of legal blindness among the elderly in industrialized countries.[1] In particular, exudative AMD is responsible for 80% of significant visual loss related to AMD. Integrity of the Bruch's membrane is known to be compromised due to aging, oxidative stress, and inflammation, especially in people with genetic susceptibility. Early signs are characterized by the presence of soft drusen, areas of increased pigment or hyperpigmentation in the outer retina or choroid, and/or areas of depigmentation or hypopigmentation of the retinal pigment epithelium. Advanced disease manifests as either geographic atrophy or choroidal neovascularization (CNV). CNV invades through the site of rupture of the Bruch's membrane and induces sudden loss of vision.

Although a myriad of growth factor pathways have been implicated in angiogenesis, various preclinical and clinical studies suggest that the vascular endothelial growth factor (VEGF) is the central mediator in pathologic neovascularization processes of the eye.[2] Elevated levels of the VEGF mRNA and protein have been found in the ocular tissue and fluid from patients with CNV.[3−8] Several reports have demonstrated that intravitreal injection of VEGF inhibitors prevents ocular neovascularization and is effective for exudative AMD treatment.[9−12] Although previous studies have focused on investigating VEGF levels, no study has directly assessed the whole proteomic composition in the aqueous humor (AH) of AMD patients. The AH is secreted into the posterior chamber of the eye by epithelial-lined ciliary processes. Because the AH faces the vitreous, which is a reservoir of growth factors and mediators that contribute to the development of CNV, analyzing proteins from the AH would make sense. Not only are such changes in AH proteins observed in anterior segment disorders but also in posterior segment disorders.[13,14] Therefore, the AH may be a valuable tool in elucidating the accurate pathogenic mechanism of various ocular diseases.

Received: January 26, 2012
Published: June 15, 2012

dx.doi.org/10.1021/pr300080s | *J. Proteome Res.* 2012, 11, 4034−4043

Proteomics is the study of protein expression in biological fluids or tissues. More specifically, the LC−MS/MS-based proteomics technique, coupled with proper separation methods, is a powerful tool for analyzing several hundred proteins within the tissue, fluid, or cell by using only a few microliters of a sample. These methods are therefore theoretically ideal for analyzing limited volumes of the AH. Analyzing a wide range of proteins using traditional methods was previously limited due to the small sampling volume and low protein concentration in the AH. Proteomics technology has evolved to expand opportunities in discovering disease-specific proteins involved in the AH circulation, and has been used to study ocular diseases, such as primary open angle glaucoma (POAG),[15,16] primary congenital glaucoma,[17] cataract,[18−20] myopia,[21] and acute corneal rejection.[22]

In this study, we directed our attention to proteins in the AH. It is important to study the composition of these AH proteins because such information might offer new insights in elucidating the pathogenesis of AMD and identifying potential biomarkers of this disease. Furthermore, newly identified marker may also serve as therapeutic targets for AMD treatment.

We investigated the differential proteomes of the AH in AMD patients (patients) and in matched, non-AMD cataract patients (controls). Abnormal expressions and distributions of proteins from the AH were identified and evaluated in age-paired clinical specimens with a quantitative MRM-MS method. For the quantitative verification of selected protein marker candidates, MRM-MS, which enables detection of peptides at low attomole levels with high reproducibility and sufficient selectivity, was applied to the highly complex AH samples.[23,24]

MATERIALS AND METHODS

Patients and Controls

The AH samples were collected from 17 patients. Participants who were diagnosed with exudative AMD (n = 9) served as the patients group and those with cataract surgery served as a control group (n = 8). All patients were recruited from the retina clinic at Seoul Metropolitan Government Seoul National University Boramae Medical Center (SMG-SNU BMC) between June 2010 and January 2011 for global profiling of the AH proteome and quantitative validation of selected proteins. Other etiologies were excluded after a complete ocular examination which included slit-lamp biomicroscopy, indirect ophthalmoscopy, color fundus photography, spectral domain optical coherence tomography (OCT), and fluorescein angiography (FA). Patients who had received intravitreal injection, photodynamic therapy, and intraocular surgery, such as cataract surgery at the time of enrollment, were also excluded. This study adhered to the tenets of the Declaration of Helsinki. Approval was received from the Investigational Review Board of SMG-SNU BMC Clinical Research Institute, and written informed consent was obtained from all subjects after an explanation of the research purpose.

Clinical data of each patient were reviewed and variables, including age at presentation, sex, history of treatment, and duration of disease, were recorded. Patient demographic and clinical data are summarized in Table 1. Aqueous samples were collected in sterile Eppendorf tubes and frozen rapidly at −80 °C until assay.

Identification of the Global Profiling of AH Proteome

Individual AH samples were depleted using Agilent multiple affinity removal spin cartridges-Human 6 to remove the top six abundant proteins in AH, such as albumin, transferrin, IgG, IgA, antitrypsin, and haptoglobin, etc., following the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Protein concentration after depletion was determined by the BCA protein concentration assay (Pierce, Rockford, IL). An equivalence to 80 μg of the depleted AH samples were separated by SDS-PAGE. Coomassie-stained protein bands were divided into five sections and performed for in-gel digestion with sequencing grade modified trypsin (Promega, Madison, WI). The digested samples were lyophilized in a Speed-Vac and resuspended in 10 μL of 0.1% TFA in HPLC water, followed by desalting with ZipTip C-18 (Millipore Co., Billerica, MA). The tryptic peptides were subjected to nano-LC−ESI−MS/MS in a linear trap quadrupole mass spectrometer (LTQ-XL, Thermo Fisher, Waltham, MA) coupled online with a nanoliquid chromatography (Nano LC) system from Eksigent (Dublin, CA). The electrospray voltage was set at 2.1 kV and the threshold for switching from MS to MS/MS was 250. The normalized collision energy for MS/MS was 35% of the main RF amplitude and the duration of activation was 30 ms. All spectra were acquired in data-dependent mode. Each full MS scan was followed by five MS/MS scans for the five most abundant precursor ions in the MS survey scan. All tandem spectra were searched using SEQUEST (rev.3.3.1 sp1) search engine (Thermo Fisher, Waltham, MA) with the IPI human protein database (version 3.70, 87091 entries) for peptide identification. Two missed cleavage sites were allowed. Methionine oxidation and cysteine carbamidomethylation were assigned to variable modifications as search parameters. The peptide tolerance of 2.0 Da and an MS/MS tolerance of 1.0 Da were used. Scaffold (version Scaffold_3_00_01, Proteome Software Inc., Portland, OR) was used to validate the MS/MS based peptide and protein identifications. Peptide identifications were accepted if they were established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[25] Protein identifications were accepted if they established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[26]

Table 1. Demographic Characteristics of Enrolled Patients with Exudative AMD and Controls

	AMD (n = 9)	controls (n = 8)
Age (Years)	68.9 ± 12.8	73.0 ± 11.4
Sex (M:F)	5:4	4:4
Diabetes mellitus (No.)	3	4
Hypertension (No.)	3	4

Quantitative Analysis of Selected Candidate Proteins by MRM

Each of AH samples for quantitative validation were digested in solution with trypsin without prior affinity depletion of the top six abundant proteins in AH. All AH samples (each 50 μg) were resolved in 6 M urea and 40 mM ammonium bicarbonate in HPLC grade water. Denatured AH proteins were reduced with 10 mM DTT for 45 min, followed by 30 min of 55 mM iodoacetamide treatment in the dark for alkylation. Alkylated AH samples were digested in-solution with sequencing grade modified trypsin (Promega, Madison, WI) for 16 h at 37 °C. Subsequently, formic acid was added on sample solution to stop digestion.[27]

An MRM mode was performed on a Q-TRAP 5500 hybrid triple quadrupole/linear ion trap mass spectrometer (Applied biosystems/MDS Sciex, Carlsbad, CA) equipped with a nanospray ionization source for the quantitative analysis on

dx.doi.org/10.1021/pr300080s | J. Proteome Res. 2012, 11, 4034−4043

specific peptides of an interested protein. A given MRM Q1/Q3 ion value (precursor/fragment ion pair) was monitored to select a specifically targeted peptide corresponding to the candidate proteins. The MRM scan was performed in a positive mode with ion spray voltages in the 2200−2500 V range. The MRM mode setting was as follows: curtain gas and spray gas were 10 and 20 psi, respectively, and collision gas was set to high. The declustering potential (DP) was set to 100 V. The mass resolution was set to unit using an advanced MS parameter. For the correct MRM, monitoring of the selected peptide enhanced product ion (EPI) scan was performed with the settings as follows: threshold switching 100 counts and selection of rolling collision energy. In positive mode, a product of 30, scan range 100−1000, the number of scans was two. In advance MS tab, the Quadrupole resolution was low, the scan speed was 10000 amu/s, and a dynamic fill time was selected.

RESULT AND DISCUSSION

Patient Characterization

A total of 17 AH samples were analyzed in this study for AH proteome identification and quantitative verification. Individual AH samples from 3 AMD patients and 3 matched controls that underwent routine cataract surgery were used for comprehensive proteome profiling and subsequent semiquantitative comparison of identified AH proteins. For further development of potential biomarker candidates, MRM verifications were performed on an independent set of 11 AH samples (6 AH from AMD and 5 AH from cataract controls). Demographic characteristics of enrolled patients are summarized in Table 1.

Proteomic Analysis

The proteomic analysis of human biological fluids is especially complicated due to the presence of few highly abundant proteins that make the identification of low abundant proteins difficult. Since AH also contains highly abundant serum proteins typical of other human biological fluids, such as albumin and IgG, it is necessary to remove the abundant proteins in order to detect enough numbers of proteins and ultimately identify potential biomarkers of AMD. To detect low abundant proteins from the AH samples, we removed the six highly abundant proteins, including albumin, transferrin, antitrypsin, haptoglobin, IgG, and IgA with MARS column.

The protein concentrations of AH samples ranged from 2.4 to 3.7 mg/mL as determined by BCA. Depletion of the six most abundant proteins with MARS column resulted in a concentration range between 0.2 and 0.5 mg/mL. The depleted human AH samples equivalent to 80 μg were loaded onto a 4−20% SDS-PAGE gel and separated. As expected, the SDS-PAGE analysis of the AH from the MARS affinity chromatography showed successful removal of the highly abundant proteins from AH (data not shown). The separated gel bands were cut into 5 parts followed by in-gel trypsin digestion. The resulting peptides were subjected to an LC−ESI−MS/MS for protein identification of the AH.

In the LC−ESI−MS/MS analysis, a total of 154 AH proteins were identified with high confidence using Scaffold from the AH samples of AMD patients (n = 3) and cataract surgery controls (n = 3) in duplicate (Figure 1, Table 2). Scaffold was applied to the three criteria for cutoffs: 95% of semitryptic peptide identification probability, 99% of protein identification probability, and at least two unique peptides. A total of 132 proteins were identified in the AMD and 102 from the control group (Figure 1, Supplementary Table 1, Supporting Information). The detailed information for unique peptides identified by the LC−ESI−MS/MS for these 154 proteins is listed in Supplementary Table 2 (Supporting Information).

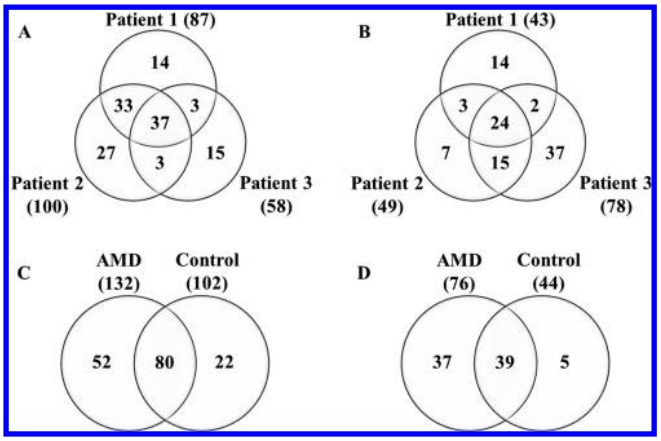


Figure 1. Venn diagram of identified proteins from both groups: (A) and (B) show identified AH proteins from individual AMD patients (A) and identified AH proteins from individual cataract surgery controls (B). (C) and (D) represent Venn diagrams of total AH proteins (C) and AH proteins identified in at least 2 patients (D).

Among the identified AH proteins, a total of 81 proteins identified in at least 2 subjects were analyzed for further validation and marker development. Our list of identified proteins was also compared to previous human AH studies and human plasma protein lists. Among the proteins identified in at least 2 patients, 10 proteins were newly identified in the AH compared to the previous reports on proteome of the AH, 30 proteins were previously identified in the AH of cataract patients,[18] 35 proteins were reported in the AH of 16 patients,[28] 68 proteins were identified in the AH obtained during elective cataract surgery,[19] and 40 proteins were previously identified in the plasma proteome (Table 3A).[29] The newly identified AH proteins are listed in Table 3B.

GO and Pathway Analysis of Identified Proteins from AH

The 81 proteins that were detected in at least two subjects were classified according to the related Gene Ontology (GO) terms using DAVID (http://david.abcc.ncifcrf.gov). Major biological processes of these proteins were biological regulations, including a homeostasis process, metabolic process, immune responses including acute inflammatory responses and defense response, response to stimulus, and response to wounding that reflect enriched biological process terms of the AH (Figure 2A). Major molecular functions of the AH proteins enriched among AMD patients were assigned as enzyme inhibitor activity and binding, including protein biding and lipid binding, and transporter activity (Figure 2B). For a given functional class, most of the AH proteins were categorized as extracellular proteins according to cellular component terms of the GO (Figure 2C). Interestingly, the AH proteins from the AMD patients contained vesicle proteins unlike those of control AH.

To reveal canonical pathways that are potentially involved in the pathology of AMD, we used the Ingenuity Pathway Analysis v7.5 (Ingenuity Systems, Redwood City, CA) to annotate the 81 AH proteins that were identified in AMD. Table 4 lists the pathways that are associated with AH proteins from AMD patients and the cataract surgery controls. The primary pathways from AMD, including an acute phase response signaling, complement system, and coagulation system, demonstrated a significant association with the AH proteins.

dx.doi.org/10.1021/pr300080s | J. Proteome Res. 2012, 11, 4034−4043

Table 2. List of AH Proteins Identified by LC−ESI−MS/MSs

IPI00029863	55 kDa protein
IPI00027780	72 kDa type IV collagenase
IPI00008603	Actin, aortic smooth muscle
IPI00019943	Afamin
IPI00216773	ALB protein
IPI00296183	Aldehyde dehydrogenase, dimeric NADP-preferring
IPI00022429	Alpha-1-acid glycoprotein 1
IPI00020091	Alpha-1-acid glycoprotein 2
IPI00022895	Alpha-1B-glycoprotein
IPI00166729	alpha-2-glycoprotein 1, zinc
IPI00478003	Alpha-2-macroglobulin
IPI00021062	Alpha-Crystallin A chain
IPI00032220	Angiotensinogen
IPI00032179	Antithrombin-III
IPI00021841	Apolipoprotein A-I
IPI00304273	Apolipoprotein A-IV
IPI00006662	Apolipoprotein D
IPI00021842	Apolipoprotein E
IPI00024284	Basement membrane-specific heparan sulfate proteoglycan core protein
IPI00298828	Beta-2-glycoprotein 1
IPI00004656	Beta-2-microglobulin
IPI00003269	Beta-actin-like protein 2
IPI00064667	Beta-Ala-His dipeptidase
IPI00216092	Beta-Crystallin B1
IPI00218748	Beta-Crystallin B2
IPI00554640	Beta-Crystallin S
IPI00218413	Biotinidase
IPI00021536	Calmodulin-like protein 5
IPI00215983	Carbonic anhydrase 1
IPI00011229	Cathepsin D
IPI00002745	Cathepsin Z
IPI00550991	cDNA FLJ35730 fis, clone TESTI2003131, highly similar to ALPHA-1-ANTICHYMOTRYPSIN
IPI00296165	cDNA FJ54471, highly similar to Complement C1r
IPI00022431	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein
IPI00019591	cDNA FLJ55673, highly similar to Complement factor B
IPI00017601	Ceruloplasmin
IPI00002147	Chitinase-3-like protein 1
IPI00291262	Clusterin
IPI00019581	Coagulation factor XII
IPI00844090	Collagen alpha-1(V) chain
IPI00019088	Collagen alpha-2(IX) chain
IPI00022394	Complement C1q subcomponent subunit C
IPI00017696	Complement C1s subcomponent
IPI00783987	Complement C3
IPI00032258	Complement C4-A
IPI00032291	Complement C5
IPI00418163	Complement component 4B
IPI00879709	Complement component 6
IPI00296608	Complement component C7
IPI00022395	Complement component C9
IPI00165972	Complement factor D
IPI00291867	Complement factor I
IPI00027482	Corticosteroid-binding globulin
IPI00002540	Cryptochrome-1
IPI00032325	Cystatin-A
IPI00032293	Cystatin-C
IPI00027547	Dermcidin
IPI00242956	IgGFc-binding protein
IPI00016915	Insulin-like growth factor-binding protein 7
IPI00292530	Interalpha-trypsin inhibitor heavy chain H1
IPI00305461	Interalpha-trypsin inhibitor heavy chain H2
IPI00013890	Isoform 1 of 14-3-3 protein sigma
IPI00299608	Isoform 1 of 26S proteasome non-ATPase regulatory subunit 1
IPI00031030	Isoform 1 of Amyloid-like protein 2
IPI00451624	Isoform 1 of Cartilage acidic protein 1
IPI00160340	Isoform 1 of Cas scaffolding protein family member 4
IPI00044678	Isoform 1 of Cell division protein kinase 15
IPI00156171	Isoform 1 of Ectonucleotide pyrophosphatase/phosphodiesterase member 2
IPI00022418	Isoform 1 of Fibronectin
IPI00026314	Isoform 1 of Gelsolin
IPI00003865	Isoform 1 of Heat shock cognate 71 kDa protein
IPI00333913	Isoform 1 of Neuroblastoma-amplified sequence
IPI00333776	Isoform 1 of Neuronal cell adhesion molecule
IPI00025426	Isoform 1 of Pregnancy zone protein
IPI00555812	Isoform 1 of Vitamin D-binding protein
IPI00216099	Isoform 1A of Desmocollin-1
IPI00291807	Isoform 2 of C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8
IPI00007257	Isoform 2 of Calsyntenin-1
IPI00428691	Isoform 2 of Desmoglein-4
IPI00071509	Isoform 2 of Plakophilin-1
IPI00023283	Isoform 2 of Titin
IPI00021000	Isoform A of Osteopontin
IPI00015614	Isoform A of Trypsin-3
IPI00465248	Isoform alpha-enolase of Alpha-enolase
IPI00013933	Isoform DPI of Desmoplakin
IPI00032328	Isoform HMW of Kininogen-1
IPI00215894	Isoform LMW of Kininogen-1
IPI00011836	Isoform UNPEL of Ubiquitin carboxyl-terminal hydrolase 4
IPI00554711	Junction plakoglobin
IPI00328609	Kallistatin
IPI00022417	Leucine-rich alpha-2-glycoprotein
IPI00152871	Leucine-rich repeat-containing protein 15
IPI00009650	Lipocalin-1
IPI00020986	Lumican
IPI00019038	Lysozyme C
IPI00029260	Monocyte differentiation antigen CD14
IPI00007856	Myosin-2
IPI00001753	Myosin-4
IPI00012102	*N*-acetylglucosamine-6-sulfatase
IPI00009997	*N*-acetyllactosaminide beta-1,3-*N*-acetylglucosaminyltransferase
IPI00016150	Neuroserpin
IPI00299547	Neutrophil gelatinase-associated lipocalin
IPI00002678	Opticin
IPI00163563	Phosphatidylethanolamine-binding protein 4
IPI00006114	Pigment epithelium-derived factor
IPI00291866	Plasma protease C1 inhibitor
IPI00007221	Plasma serine protease inhibitor
IPI00019580	Plasminogen
IPI00007118	Plasminogen activator inhibitor 1
IPI00299738	Procollagen C-endopeptidase enhancer 1
IPI00013179	Prostaglandin-H2 D-isomerase
IPI00022426	Protein AMBP
IPI00334282	Protein FAM3C
IPI00219806	Protein S100-A7

dx.doi.org/10.1021/pr300080s | *J. Proteome Res.* 2012, 11, 4034−4043

Table 2. continued

IPI00025753	Desmoglein-1
IPI00296141	Dipeptidyl peptidase 2
IPI00014424	Elongation factor 1-alpha 2
IPI00027827	Extracellular superoxide dismutase [Cu−Zn]
IPI00397801	Filaggrin-2
IPI00023673	Galectin-3-binding protein
IPI00016801	Glutamate dehydrogenase 1, mitochondrial
IPI00026199	Glutathione peroxidase 3
IPI00219018	Glyceraldehyde-3-phosphate dehydrogenase
IPI00218130	Glycogen phosphorylase, muscle form
IPI00410714	Hemoglobin subunit alpha
IPI00654755	Hemoglobin subunit beta
IPI00473011	Hemoglobin subunit delta
IPI00022488	Hemopexin
IPI00022371	Histidine-rich glycoprotein
IPI00003935	Histone H2B type 2-E
IPI00171611	Histone H3.2
IPI00453473	Histone H4
IPI00398625	Hornerin
IPI00431645	HP protein
IPI00550731	Putative uncharacterized protein
IPI00022337	Retinol-binding protein 3
IPI00025257	Semaphorin-7A
IPI00022463	Serotransferrin
IPI00019399	Serum amyloid A-4 protein
IPI00022391	Serum amyloid P-component
IPI00218732	Serum paraoxonase/arylesterase 1
IPI00152881	shroom family member 3
IPI00739237	similar to complement component 3
IPI00082931	Small proline-rich protein 3
IPI00014572	SPARC
IPI00218733	Superoxide dismutase [Cu−Zn]
IPI00292946	Thyroxine-binding globulin
IPI00018219	Transforming growth factor-beta-induced protein ig-h3
IPI00022432	Transthyretin
IPI00465028	triosephosphate isomerase 1 isoform 2
IPI00007750	Tubulin alpha-4A chain
IPI00179330	ubiquitin and ribosomal protein S27a precursor
IPI00552548	Zinc transporter 9
IPI00060800	Zymogen granule protein 16 homologue B

Table 3A. Comparison of Published Proteins in AH and Plasma

	Jeong, S.K. et al. *Korean Plasma Proteins* (185)	Escoffier et al. *J. Proteome Res.* 2009 (71)	Richardson et al. *Mol. Vis.* 2009 (54)	Richardson et al. *Mol. Vis.* 2010 (72)	Chowdhury et al. *Invest. Ophtalmol. Vis. Sci.* 2010 (355)	Bouhenni et al. *Exp. Eye. Res.* 2011 (137)	AH proteins In this paper (81)
Jeong, S.K. et al.	185	37	32	27	77	57	40
Escoffier et al.		71	35	31	53	43	35
Richardson et al.			54	33	45	41	30
Richardson et al.				72	40	37	30
Chowdhury et al.					355	88	68
Bouhenni et al.						137	48
In this paper							81

Table 3B. List of AH Proteins Identified Only in This Study

IPI00029863	55 kDa protein
IPI00008603	Actin, aortic smooth muscle
IPI00025753	Desmoglein-1
IPI00027827	Extracellular superoxide dismutase [Cu−Zn]
IPI00397801	Filaggrin-2
IPI00299608	Isoform 1 of 26S proteasome non-ATPase regulatory subunit 1
IPI00007221	Plasma serine protease inhibitor
IPI00739237	similar to complement component 3
IPI00071509	Isoform 2 of Plakophilin-1
IPI00152871	Leucine-rich repeat-containing protein 15

Selection of Potential Biomarker Candidates

For semiquantitative analysis, a normalized spectra count was calculated by dividing the number of spectra of a specific protein by the total number of spectra found in each group. These values provided the ratio between the two groups and the degree of differential expressions. A total of 52 proteins that were higher than 2-fold were chosen for potential biomarker protein candidates based on their fold changes in the protein amounts represented by spectral counts and pathological relevance reviewed by ophthalmologists. Several tryptic peptides for a subset of candidate proteins obtained by database search were monitored to optimize MRM transitions.

To develop potential biomarker proteins for AMD, we selected 7 proteins for further verification with the quantitative MRM-MS based on their biological functions, detectability in serum, and medical implications reviewed by ophthalmologists, as summarized in Table 5.

Among the potential biomarker candidates, 6 proteins, except for transforming growth factor-beta-induced protein ig-h3 (TGFBI), were previously identified in human plasma[29] and 6 proteins, except for cathepsin D, were previously identified in the AH samples from various diseases.[17] In particular, TGFBI was reported to be involved in angiogenesis and defects in TGFBI are a known cause for corneal dystrophy. It has also been demonstrated that a fibrotic scar can lead to blindness in the advanced stage of AMD. Numerous reports describe transforming growth factor (TGF)-β as a potent regulator of the extracellular matrix. TGF-β-induced extracellular matrix production and fibrosis might be implicated in the vision loss among advanced AMD patients.

Clusterin is associated with the disease severity of Alzheimer's disease, one of the major neurodegenerative disorders. Increased plasma concentration of clusterin was predictive of greater fibrillar amyloid-beta burden in the brain.[30] Interestingly, clusterin was found in drusen within AMD.[31] When taken together, these suggest an important role of clusterin in the pathogenesis of the neurodegenerative disorders, such as AMD or Alzheimer's disease. Additionally, it has been demonstrated that angiogenesis was inhibited by the antisense oligonucleotides to clusterin using a capillary cell viability assay.[32]

In our study, pigment epithelium-derived factor, which is known to have an antiangiogenic and neurotrophic activities that regulate vascular development within the eye, was

dx.doi.org/10.1021/pr300080s | J. Proteome Res. 2012, 11, 4034−4043

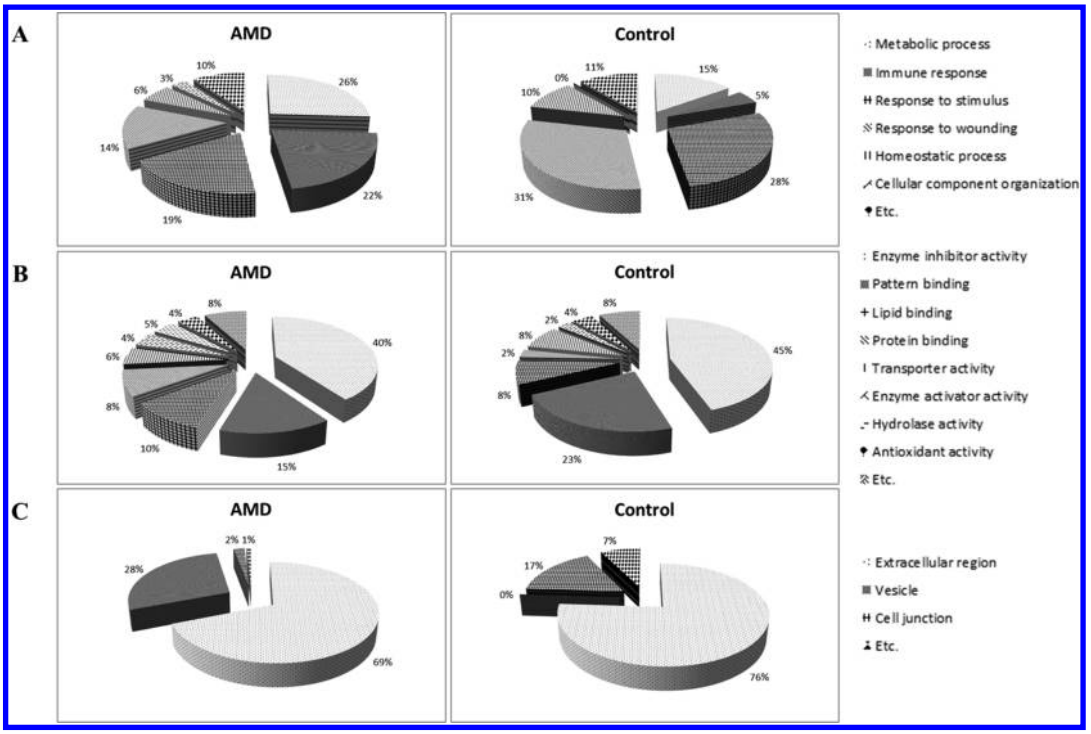


Figure 2. Gene Ontology (GO) analysis of AH proteins as determined by the DAVID. We compared identified AH proteins from both groups. (A) Biological process, (B) molecular function, and (C) cellular component.

Table 4. Pathway Analysis of AH Proteins using IPA Pathway Analysis Tools

canonical pathway	AMD	control
Acute phase Response Signaling	22	17
Complement System	8	5
Coagulation System	4	2
Germ Cell-Sertoli Cell Junction Signaling	4	2
Hepatic Fibrosis/Hepatic Stellate Cell Activation	3	2
LXR/RXR Activation	3	2
FXR/RXR Activation	2	2
Arachidonic Acid Metabolism	2	2
Neuroprotective Role of THOP1 in Alzheimer's Disease	2	2
NRF2-mediated Oxidative Stress Response	3	0
Regulation of Actin-based Motility by Rho	2	0
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2	0
LPS/IL-1 Mediated Inhibition of RXR Function	0	3

Table 5. List of Selected Potential Biomarker Candidates

		normalized spectra count		
IPI	protein name	AMD	control	fold change
IPI00017601	Ceruloplasmin	0.173	0.067	2.6
IPI00006114	Pigment epithelium-derived factor	0.143	0.058	2.5
IPI00291866	Plasma protease C1 inhibitor	0.045	0.021	2.2
IPI00018219	TGFBI	0.016	0	
IPI00291262	Clusterin	0.012	0	
IPI00011229	Cathepsin D	0.008	0	
IPI00032293	Cystatin C	0.003	0	

increased in AMD patients compared to controls. While we do not have a complete explanation for this finding, we speculate that regional abnormalities in vascularization occur in AMD as a result of an imbalance between an antiangiogenic factor, PEDF, and VEGF. The AH maintains a normal homeostatic environment. The development of CNV may cause an imbalance between the angiogenic and antiangiogenic molecules that are important in maintaining healthy posterior segment tissues. Further studies are needed to clarify this discrepancy.

In addition, an increase in AH ceruloplasmin levels was observed among AMD patients compared to controls. Oxidative stress, which refers to cellular damage caused by reactive oxygen species (ROS), is implicated in AMD because the retina is particularly susceptible to oxidative stress.[33] There is increased oxidative damage in the retina of AMD patients, in which ceruloplasmin was shown to increase iron efflux from the retinal pigment epithelial cells. Interestingly, some reports have suggested that ceruloplasmin can act as an acute inflammatory biomarker in psoriasis or cancer.[34,35] Additionally, there is ample evidence that inflammation plays an important role in the pathogenesis of AMD. As an acute-phase reactant, this suggests that ceruloplasmin can serve as a potential indicator for screening the development of AMD.

Since blood sampling is a far less invasive procedure than obtaining AH, finding a serum biomarker for AMD is the ultimate goal. AMD is a well-known multifactorial disease with genetic and environmental risk factors. Numerous gene studies have repeatedly identified gene complement factor H (CFH) as the genetic risk factor in Caucasian AMD patients.[36−38] Recent studies have also shown that C-reactive protein (CRP) and acute phase protein as indicatives of systemic inflammation are elevated in AMD.[39,40] These findings commonly implicate inflammation as the major pathogenic mechanism of AMD and also imply that AMD is a systemic diseases. Hence, the goal, when investigating serum biomarkers for AMD, is to identify certain proteins most likely associated with the inflammatory process that can differentiate AMD patients from normal

dx.doi.org/10.1021/pr300080s | J. Proteome Res. 2012, 11, 4034−4043

Table 6. List of SRM Transitions

index	IPI	protein name	peptide sequence	target ion	mass info
1	IPI00017601	Ceruloplasmin	ALYLQYTDETFR	2y8	760.4/1059.5
			GAYPLSIEPIGVR	2y8	686.4/870.5
2	IPI00291866	Plasma protease C1 inhibitor	TNLESILSYPK	2y7	632.8/807.5
			FQPTLLTLPR	2y8	593.3/910.6
3	IPI00032293	Cystatin C	ALDFAVGEYNK	2y7	613.8/780.4
4	IPI00018219	TGFBI	LTLLAPLNSVFK	2y7	658.4/804.5
5	IPI00006114	Pigment epithelium-derived factor	LAAAVSNFGYDLYR	2y9	780.4/1134.5
			DTDTGALLFIGK	2y8	625.8/818.5
6	IPI00291262	Clusterin	ASSIIDELFQDR	2y7	697.3/922.4
			ELDESLQVAER	2y7	644.8/802.4
7	IPI00011229	Cathepsin D	VGFAEAAR	2y5	410.7/517.3
	IS	Beta Galactosidase	VDEDQPFPAVPK	2y5	671.3/511.3

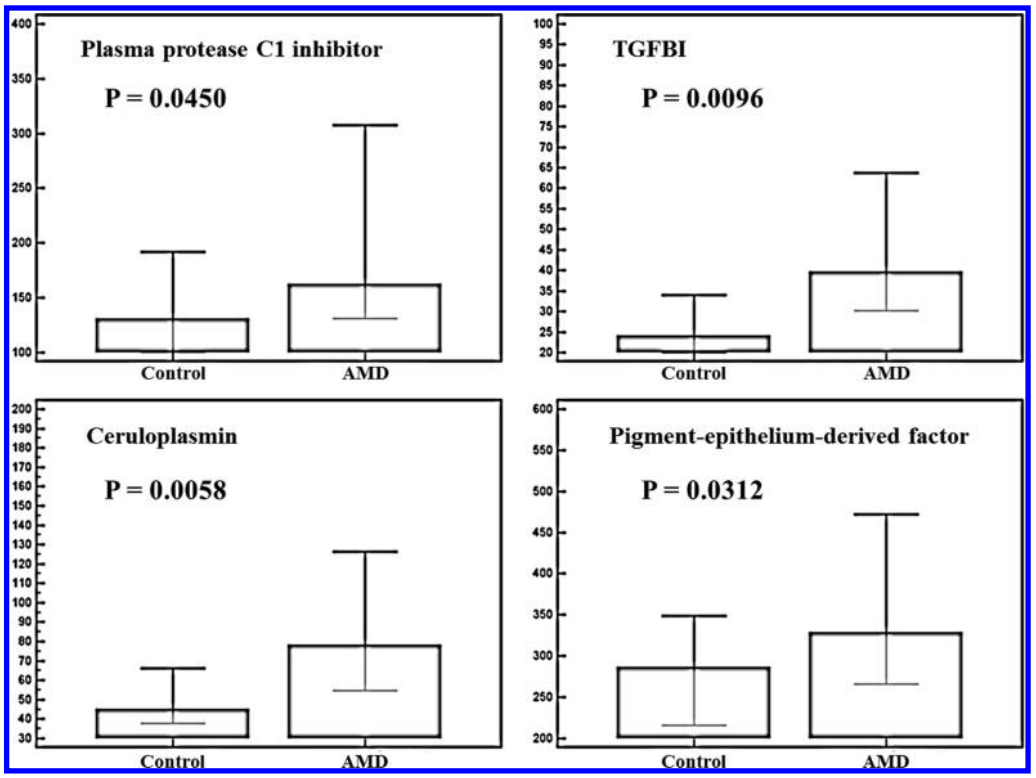


Figure 3. Box plots of results from MRM analysis in an independent sample set of 6 AH samples of AMD and 5 AH samples of cataract control. The relative abundance of plasma protease C1 inhibitor, TGFBI, ceruloplasmin, and pigment epithelium-derived factor are elevated in the AH samples of AMD compared to cataract controls as determined by *t* test analysis.

controls, and not simply find proteins which we assume to be leaked into the blood from the eye.

Additionally, to assess whether intravitreal anti-VEGF injection can reverse the proteomic composition, AH samples were compared at baseline and after an intravitreal anti-VEGF injection. There was no change in the proteomic composition before and after an intravitreal anti-VEGF injection (data not shown). This implies that VEGF modulates the final stages of CNV development and blockage of the VEGF pathway cannot effectively change the proteomic profile. Furthermore, comprehensive investigations are warranted to delineate the full AH proteome spectrum in a larger number of AMD patients.

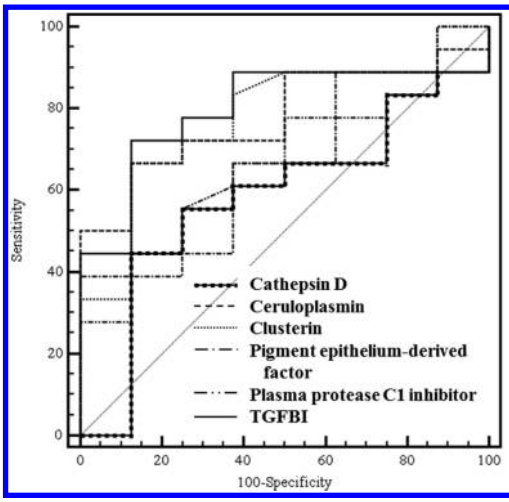
Verification of Selected Potential Biomarker Candidates

Due to the possible quantification bias in the depleted AH samples associated with sampling handling processes, we conducted an MS-based quantitative analysis, MRM analysis, for quantification instead of a label-free based method for verification of selected proteins. Relative quantification and pairwise comparisons were conducted by the MRM analysis of tryptic digests of the AH samples, in which the abundant proteins were not depleted to avoid potential errors associated with the depletion process.

All 7 candidate proteins, identified by both AH and serum, are related to the pathology of AMD, were subjected to the MRM assay for further verification. As it is critical for a successful MRM assay to select proteotypic peptides (PTPs) which give good MS responses and uniquely represent the target proteins,[9,10] we used MRMPilot software (AB SCIEX, Foster City, CA) to select multiple PTPs for a single target protein. Consequently, the MRM transitions were optimized for 11 peptides of 7 proteins, as shown in Table 6.

The selected PTPs were examined by the MRM experiments by using a Q-TRAP 5500 hybrid triple quadrupole/linear ion

dx.doi.org/10.1021/pr300080s | J. Proteome Res. 2012, 11, 4034−4043



	AUC	SE [a]	95% CI [b]
Cathepsin D	0.583	0.126	0.375 to 0.772
Ceruloplasmin	0.778	0.0924	0.573 to 0.916
Clusterin	0.781	0.0986	0.577 to 0.918
Pigment epithelium-derived factor	0.667	0.114	0.456 to 0.838
Plasma protease C1 inhibitor	0.656	0.111	0.446 to 0.830
TGFBI	0.799	0.0928	0.596 to 0.929

Figure 4. ROC curves of selected candidate proteins. The relative abundance of the candidate proteins from 6 AMD patients and 5 control samples were determined by MRM. The area under the curve (AUC) was shown for AMD at 95% confidence level.

trap mass spectrometer to select appropriate target peptides that have the strongest MS signal for a further validation of the marker proteins. We successfully established a subset of PTPs for the MRM analysis (Table 6) and applied to the AH samples from the control groups (n = 5) and the AMD patient (n = 6) as shown in Figure 3. The mean abundance of the candidate proteins was compared. The relative abundance of the plasma protease C1 inhibitor, TGFBI, ceruloplasmin, and pigment epithelium-derived factor were significantly increased between the AMD and cataract control groups, as determined by a t test analysis. Differences for clusterin, cathepsin D and cystatin C are not significant due to their low abundance or high matrix effect. Thus, these proteins were not evaluated.

To prove the statistical significance between the AMD group and the control group, we performed receiver operating characteristic (ROC) analysis (Figure 4). The MRM analysis showed high sensitivity and specificity to discriminate AMD from the control groups as shown in ROC curves (AUC, 0.7). In the case of AMD, the area under curve (AUC) is 0.799 for TGFBI, 0.778 for ceruloplasmin, 0.781 for clusterin, and 0.667 for pigment epithelium-derived factor (Figure 4).

CONCLUSION

The results of the present study reveal that the proteomic compositions of AH in AMD are different from that of cataract controls. To the best of our knowledge, this is the first proteomic analysis of AH in patients with AMD indicating that the differentially expressed proteins are related to AMD. The findings from this study suggest that alterations in proteins can contribute to pathologic changes and complications of AMD. The proteins identified may serve as potential biomarkers for the screening of AMD. In addition, our description of 3 novel AH markers and their potential use as biomarkers in detecting exudative AMD will open new opportunities for better treatment strategies in AMD patients.

ASSOCIATED CONTENT

Supporting Information

Supplementary Table 1. The list of identified unique proteins from each patient with AMD and control.

Supplementary Table 2. The detailed information for identified AH proteins and peptides from AMD group and control group.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported by grants from the National Project for Personalized Genomic Medicine (A111218-11-CP02) from the Korean Ministry of Health and Welfare, WCU program (Project No. R33-10128) from the Korean Ministry of Education, Science and Technology, and the SNUH research fund (04-2010-0410).

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