Identification of Vinculin as a Potential Plasma Marker for Age-Related Macular Degeneration

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PURPOSE. To identify plasma protein biomarkers for age-related macular degeneration (AMD) using a large-scale quantitative proteomic discovery procedure.

METHODS. Plasma proteomes from 20 exudative AMD patients and 20 healthy control patients were comparatively profiled by four-dimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS). Proteins existing at statistically different levels were validated by enzyme-linked immunosorbent assay (ELISA) and Western blotting in 233 case-controlled samples. Newly discovered plasma biomarkers were further confirmed using in vivo and in vitro experiments.

RESULTS. Out of 320 proteins identified, vinculin, protein S100A9, triosephosphate isomerase, protein S100A8, protein Z-dependent protease inhibitor, C-X-C motif chemokine 7, and tenascin X showed significantly differential expression in AMD patient plasma compared to control plasma. Among these, the area under the curve (AUC) for vinculin was 0.87 for discriminating between exudative AMD and controls (° n = 201) and 0.879 for discriminating between AMD and controls (° n = 233). A proteogenomic combination model using vinculin and two known risk genotypes in ARMS2 and CFH genes additionally provided excellent discrimination of AMD from controls (AUC = 0.916). The plasma level of vinculin was not associated with any confounding clinical variables, such as age, smoking, and other comorbidities. Additionally, vinculin was strongly expressed in retinal pigment epithelial cells of human eyes, and its expression was elevated when exposed to oxidative stress in vitro.

CONCLUSIONS. Vinculin was identified as a potential plasma biomarker for AMD. The early detection of AMD using novel plasma biomarkers with genetic modeling may enable timely treatment and vision preservation in the elderly.

Keywords: plasma marker, vinculin, age-related macular degeneration, 4-dimensional protein profiling, proteomics

Age-related macular degeneration (AMD) is the leading cause of blindness worldwide, with a prevalence of nearly 10% in people ages 65 years and older. The worldwide prevalence of AMD is expected to double in the next decade due to population aging.1,2 Most blindness in AMD results from the advanced form of the disease, especially exudative (neovascular or wet) AMD, characterized by the invasion of the central retina by choroidal neovascularization (CNV). Since the use of appropriate anti-vascular endothelial growth factor (VEGF) agents can stop disease progression and preserve vision, early detection and treatment of exudative AMD is essential for preventing blindness in elderly populations.

Age-related macular degeneration is a disease localized to the retina; therefore, the diagnosis of AMD relies mostly on the presentation of patients and examination by retinal specialists. In that sense, the use of blood biomarkers may theoretically enable optimal screening for AMD and can prevent referral to retinal specialists. In the last decade, genetics has been implicated as an important risk factor in the development of AMD. However, a recent study, which used the largest cohort ever studied, demonstrated that AMD predictability using genetic factors was limited, even after the inclusion of 19 single nucleotide polymorphisms (SNPs).3 Thus, the use of blood proteins as biomarkers has the potential to yield better prediction of disease
Vinculin as a Plasma Marker for AMD

**Table 1.** Characteristics of Plasma Sample Sets Used for the Experiments

<table>
<thead>
<tr>
<th>Experiments Performed</th>
<th>Proteomic Experiment</th>
<th>ELISA†</th>
<th>Western Blot 1‡</th>
<th>Western Blot 2‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. cases</td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Age, y (average)</td>
<td>70–83 (73.9)</td>
<td>70–83 (73.5)</td>
<td>70–80 (73.8)</td>
<td>70–96 (73)</td>
</tr>
<tr>
<td>Early AMD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. cases</td>
<td>-</td>
<td>19</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>Age, y (average)</td>
<td>-</td>
<td>64–80 (74.9)</td>
<td>64–80 (71.9)</td>
<td>64–79 (76)</td>
</tr>
<tr>
<td>Male:female</td>
<td>-</td>
<td>7:12</td>
<td>6:16</td>
<td>3:7</td>
</tr>
<tr>
<td>Exudative AMD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. cases</td>
<td>20</td>
<td>41</td>
<td>58</td>
<td>43</td>
</tr>
<tr>
<td>Age, y (average)</td>
<td>66–85 (73.6)</td>
<td>63–85 (72.8)</td>
<td>43–90 (70.2)</td>
<td>56–84 (71)</td>
</tr>
</tbody>
</table>

* Candidate plasma proteins: S100A8, C-X-C motif chemokine, tenascin X, triosephosphate isomerase, and protein Z-dependent protease inhibitor.
† Candidate plasma proteins: S100A9 and vinculin.
‡ Candidate plasma protein: vinculin.

The aim of this study was to discover novel plasma markers for AMD by investigating the near-total set of proteins in the plasma of the peripheral blood, which has not been attempted before. We used a four-dimensional (4D) protein profiling method developed for the comprehensive proteomic analyses of peripheral blood plasma. Major protein depletion followed by higher-dimensional separation strategies is an efficient approach to identify a wide range of proteins in complex biological fluids, such as serum. Subsequent higher-dimensional separation using liquid chromatography–tandem mass spectrometry (LC-MS/MS) usually detects a substantial portion of the low-abundance plasma proteome and therefore represents the most promising strategy for discovering novel specific cancer biomarkers with high potential for achieving clinical utility. To the best of our knowledge, this is the first comprehensive proteomic analysis of the plasma of AMD patients, the results of which demonstrate that differentially expressed proteins (DEPs) are related to AMD.

**Materials and Methods**

**Patients and Controls**

The study was approved by the institutional review board of Seoul National University Bundang Hospital (SNUBH) and followed the tenets of the Declaration of Helsinki. Age-related macular degeneration patients were recruited from the retina clinic of SNUBH between January 2008 and January 2012, and informed consent was obtained from all participants. Clinical data for each patient were reviewed; and variables, including age at presentation, sex, smoking history, any accompanying diseases (e.g., diabetes and hypertension), and detailed ophthalmologic data, were recorded. Age-related macular degeneration patients were subclassified according to the Age-Related Eye Disease Study (AREDS) classification system as follows: (1) early AMD, presence of at least one large druse, numerous medium-sized drusen, and geographic atrophy that does not extend to the center of the macula; (2) late exudative AMD, CNV, and any of its potential sequelae including a fibrotic scar. For the healthy control (HC) group, subjects were recruited from individuals visiting the SNUBH healthcare center for regular medical checkups and from participants in the Korean Longitudinal Study on Health and Aging (KLoSHA; randomly sampled community-dwelling Koreans ages 65 years or older) in the same study period. Normal control subjects underwent visual acuity examination, fundus photography, and/or optical coherence tomography to ensure that no intermediate-sized drusen or RPE changes were present. For blood preparation, 16 mL blood was collected in EDTA tubes, and plasma was prepared as suggested by the HUPO Plasma Proteome Project. Plasma samples were collected and immediately frozen at –80°C until use.

**Table 2.** Clinical Variables of AMD Patients and Control Subjects and the Association With Plasma Vinculin Levels

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls, n = 100</th>
<th>Early AMD, n = 32</th>
<th>Exudative AMD, n = 101</th>
<th>P Value, AMD vs. Controls*</th>
<th>P Value, Association With Plasma Vinculin Levels‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>70.7 ± 5.4</td>
<td>72.8 ± 5.0</td>
<td>72.5 ± 5.4</td>
<td>&lt;0.001</td>
<td>0.134</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>38.0</td>
<td>28.1</td>
<td>47.5</td>
<td>0.455</td>
<td>0.442</td>
</tr>
<tr>
<td>Smoking, current or ex-smoker, %</td>
<td>32.7</td>
<td>21.9</td>
<td>45.6</td>
<td>0.037</td>
<td>0.756</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>24.0</td>
<td>29.0</td>
<td>22.0</td>
<td>0.953</td>
<td>0.651</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>54.0</td>
<td>56.3</td>
<td>61.4</td>
<td>0.347</td>
<td>0.102</td>
</tr>
<tr>
<td>Cardiovascular or cerebrovascular accident, %</td>
<td>17.0</td>
<td>12.5</td>
<td>13.9</td>
<td>0.473</td>
<td>0.550</td>
</tr>
<tr>
<td>Cancer history, %</td>
<td>3.0</td>
<td>9.7</td>
<td>2.0</td>
<td>1.000</td>
<td>0.394</td>
</tr>
</tbody>
</table>

* Independent t-test, χ² test, or Fisher’s exact test were used where appropriate.
‡ ANOVA or logistic linear regression analysis.
A total of 233 plasma samples were collected from 101 patients with exudative AMD, 32 patients with early AMD, and 100 HCs (Tables 1, 2). Fifty-nine of 101 (58.4%) patients with exudative AMD were treatment naïve.

Procedures for Proteomic Strategy of Plasma Marker Discovery and Validation

The flow diagram in Figure 1 summarizes our 4D protein profiling method for quantitative comparisons of plasma proteins from AMD patients and HCs (refer to Supplementary Methods for details). We analyzed results of the LC-MS/MS data and selected target proteins for further validation of their potential as biomarkers. The criteria for selection of DEPs were (1) G value > 3.841 (P < 0.05) and spectral count ratio > 2 and (2) G value > 2.71 (P < 0.1) and spectral count ratio > 5 (Table 3).

The validation process was carried out on the selected proteins with available enzyme-linked immunosorbent assay (ELISA) kits and antibodies for Western blotting. The concentrations of triosephosphate isomerase, protein S100A8, tenascin X, protein Z-dependent protease inhibitor, apolipoprotein E, and CX-C motif chemokine 7 in the plasma of AMD patients and HCs were measured using commercially available human ELISA kits (USCN Life Science, Inc., Houston, TX, USA; Abcam, Cambridge, MA, USA). The expression levels of S100A9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and vinculin (Abcam) were confirmed by Western blotting due to unreliable results of available commercial ELISA kits.

Vinculin Expression in Human Ocular Tissues and APRE-19 Cells

Expression of the plasma biomarker vinculin in human retina and choroid tissues was assessed in cadaver eyes of two AMD patients and two age-matched normal individuals using immunofluorescence. In addition, to reveal the pathogenic role of vinculin in AMD, ARPE-19, human diploid RPE cell line cells (Cat. No. CRL-2302; American Type Culture Collection, Bethesda, MD, USA) were exposed to graded oxidative stress using 0, 10, 50, 100, or 300 μM H2O2 for 24 hours. Western blotting for detection of vinculin in cell extracts and supernatants was performed to quantify the expression and secretion of vinculin (refer to Supplementary Methods for exposure of ARPE-19 cells to oxidative stress).

RESULTS

Patient Characterization

Individual plasma samples from 20 patients with exudative AMD and 20 age-matched HCs were used for comprehensive proteome profiling and subsequent semiquantitative comparison of identified plasma proteins. For further development of potential biomarker candidates, immunoblotting verification was performed on an independent set of 233 plasma samples (100 HCs, 32 patients with early AMD, and 101 patients with exudative AMD). Characteristics of enrolled patients are summarized in Tables 1 and 2.

Proteomic Analysis for Discovery of Plasma Markers Using a 4D Protein Profiling Method

The flow diagram in Figure 1 summarizes our 4D protein profiling method for quantitative comparisons of plasma from AMD patients and HCs. Using the profiling strategy, 8832 unique peptides matching 320 proteins, including proteins with reported plasma concentrations in the pg/mL to μg/mL range, were identified (Supplementary Tables S1, S2). Most of the proteins (53.9%) were overlapped by both groups (Fig. 2). There were 173 proteins showing differential expression of more than 2-fold between patients with AMD and HCs (Supplementary Table S3). Of these DEPs, 35 showed significant differences (G values > 3.841) of more than 2-fold; 26 were elevated, and 9 were decreased in patients with AMD compared to HCs. Additionally, three proteins were selected as having a G value greater than 2.71 and a spectral ratio greater than 5. Table 3 summarizes the selected 38 candidate protein biomarkers identified by this method.

To confirm our quantification results, we assayed the plasma concentration of six proteins, that is, vinculin, complement component C9, S100A8, S100A9, antithrombin-III, and thrombospondin-1, by Western blotting (Supplemen-
Immunoblotting results were highly consistent with the proteomic data (Spearman’s rank correlation, $r = 0.78$, $P < 0.01$); that is, proteins with high levels by proteomic analysis also exhibited high expression by Western blotting (e.g., vinculin). Similarly, proteins that were not differentially expressed by proteomic data showed no difference by Western blotting (e.g., complement component C9). Thus, these data demonstrated that for a subset of DEPs, proteomic discovery data and Western blotting data were broadly concordant.

Gene Ontology Analysis of DEPs

To put our proteomics data into biological context, the proteomic discovery data were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). The major molecular functions of the plasma proteins associated with AMD were cellular growth and proliferation (10.3%), cell death (9.4%), cellular movement (8.1%), and cell-to-cell signaling and interactions (7.2%; Fig. 2). The 147 DEPs were mapped onto known canonical signaling pathways in order to obtain useful information on molecular interaction networks. The pathway with the most significant change was liver X receptor (LXR)/retinoid X receptor (RXR) activation ($P < 10^{-9}$). There were 10 proteins (ATP-binding cassette subfamily G, apolipoprotein C-IV, haptoglobin-related protein, lipopolysaccharide binding protein, phospholipid transfer protein, retinol binding protein 4, S100A8, serum amyloid A1, serpin peptidase inhibitor clade A, and transferrin) in this pathway that significantly changed in the plasma. Secondly, 11 proteins involved in actin cytoskeleton signaling showed increased expression in patients with AMD ($P < 10^{-6}$). In particular, proteins involved in the focal adhesion assembly pathway, including vinculin, profilin 1, actin-b, myosin 6, and thymosin $\beta$4, showed significantly increased expression in patients with AMD.

Validation of DEPs

The initial 38 candidate proteins were narrowed down with regard to their biological functions drawn from IPA and the
FIGURE 2. Comparison of proteins identified by mass spectrometry between AMD and healthy controls (HC). (A) Venn diagram for plasma proteins identified from LC-MS/MS analysis. (B) Classification of 147 differentially expressed proteins according to molecular functions. A 2-fold change cutoff was applied to the list (173 DEPs), resulting in selection of 147 proteins for generation of biological networks using Ingenuity Pathway Analysis.
Availability of commercial antibodies and ELISA kits. Finally, 26 proteins were chosen as candidate proteins for the next validation step (Table 3).

To ascertain the feasibility of candidate proteins as plasma biomarkers for AMD, ELISA was performed on 24 proteins for two sets of human plasma samples collected from 41 patients with exudative AMD, 19 patients with early AMD, and 40 HCs. Out of 24 proteins assayed using ELISAs, only five showed significantly different concentrations between patients with AMD and controls: S100A8, C-X-C motif chemokine 7 (CXCL7), tenasin X (TNX), triosephosphate isomerase (TPI), and protein Z-dependent protease inhibitor (ZPI; Supplementary Fig. S2).

Two other proteins whose ELISA kits were not commercially available (S100A9) or not optimized properly (vinculin) were tested by Western blotting. Plasma samples from 58 patients with exudative AMD, 22 patients with early AMD, and 40 HCs were used for these experiments. Plasma levels of S100A9 increased 2.7-fold in patients with exudative AMD compared to HCs (P < 0.0001; area under the curve [AUC] = 0.763; Supplementary Fig. S3). The mean level of vinculin was confirmed to increase 3.4-fold in plasma samples from patients with exudative AMD and 3.6-fold in those of patients with early AMD compared to those of HCs (P < 0.0001; Figs. 3A, 3B). The AUC value for AMD patients versus HCs was 0.895 (sensitivity, 74.1%; specificity, 92.5%; Fig. 3C).

Vinculin expression levels were reconfirmed in another set of plasma from 43 patients with exudative AMD, 10 patients with early AMD, and 60 HCs. Vinculin expression was elevated 5.3-fold in AMD patient plasma (P < 0.0001; Figs. 3D, 3E). The AUC value was 0.840 (sensitivity, 69.8%; specificity, 95%; Fig. 3F).

Table 4 summarizes the discrimination power of seven candidate plasma proteins. Vinculin showed consistently high values among candidate proteins, suggesting the most probable biomarker for AMD.

Using a logistic model, the two datasets were combined, which yielded the discrimination power of vinculin between patients with AMD and HCs (AUC = 0.879, n = 133 vs. 100), between patients with exudative AMD and HCs (AUC = 0.871, n = 101 vs. 100), and between patients with early and exudative AMD (AUC = 0.575, n = 32 vs. 101; Fig. 4A). Clinical variables, such as age, sex, smoking, diabetes, hypertension, cardiovascular or cerebrovascular incidents, and cancer history, were not associated with the plasma levels of vinculin in 233 patients and control subjects (Table 2).

**Prediction Model Using a Combination of Plasma Protein Markers and Risk Genotypes**

The final proteogenomic prediction model for exudative AMD was constructed through combination of the plasma protein marker vinculin with two known risk SNPs, rs10490924 (ARMS2) and rs800292 (CFH), which were recently reported as the most significant SNPs in Korean exudative AMD (Park KH, et al. IOVS 2012;53;ARVO E-Abstract 3304). The proteogenomic combination model showed excellent discrimination power between patients with AMD (n = 106) and HCs (n = 92; AUC = 0.916; Fig. 4B). Plasma vinculin levels were not associated with the two SNPs after adjusting for the effect of AMD.

**Vinculin Expression in Retinas From Patients With or Without AMD and in RPE Cells Exposed to Oxidative Stress**

We compared vinculin protein expression by immunofluorescence in cadaveric human retina and choroid tissue from two patients with AMD and two age-matched normal individuals. Immunofluorescence experiments revealed that vinculin (red) expression was strongest in RPE cells and weak in the choroid of normal control eyes (Fig. 5A). In eyes of patients with exudative AMD, vinculin was colocalized within the CNV endothelium (Fig. 5B). However, in eyes of patients with early AMD, drusen showed no expression of vinculin (Fig. 5C).

After exposure to oxidative stress, ARPE-19 cells showed a dose-dependent significant increase in vinculin production inside and outside the cells, as demonstrated by Western blotting of vinculin in cell extracts and supernatants compared to controls (P < 0.05 by t-test; Fig. 5D, 5E).

**Discussion**

Our proteomic strategy and validation process was successful in elucidating plasma biomarkers for AMD. Compared to prior studies investigating candidate plasma markers, a considerable number of plasma proteins were covered and highly probable plasma proteins, including vinculin, were discovered in our study. In addition, by analyzing the list of plasma proteins showing discrepancies between patients with AMD and HCs, we were able to describe altered systemic pathways of AMD that may contribute to AMD pathogenesis. We also showed that by combining known risk gene variants of AMD (ARMS2 and CFH), the discrimination between AMD patients and HCs could be more accurate. Receiver operating characteristic (ROC) curves suggested that vinculin alone could discriminate between AMD and HCs with approximately 88% accuracy and, in combination with genomic markers, provided up to approximately 92% discrimination accuracy (Fig. 4). We believe that a blood-based diagnostic system for AMD, combining plasma proteins and genotype analysis, may revolutionize diagnoses and treatment of patients with AMD.

**Identified Plasma Markers Associated With AMD**

While AMD is not a classic inflammatory disease, inflammation has been found to play an important role in the pathogenesis and progression of AMD. Genetic susceptibility (several SNPs including complement pathways) and local factors (macrophages) are thought to induce inflammatory conditions in the RPE and choroid, thereby contributing to AMD. Among our plasma biomarker candidates, S100A8, S100A9, and CXCL7 are known to be involved in inflammation. C-X-C chemokine 7 is a small CXC family chemokine that promotes angiogenesis. The protein levels of S100A8 and S100A9 in human serum have been found to be elevated in various inflammatory diseases, and S100A8 and S100A9 exist as either homodimers, heterodimers, or heterotetramer type S100A8/A9. Interestingly, the expression of both proteins in the plasma was increased in patients with exudative AMD; therefore, the S100A8/A9 complex and the individual proteins may be involved in AMD pathogenesis. S100A9 suppresses VEGF-independent angiogenesis through interaction with tasquinimod in myeloid cells. Thus, the elevation of S100A9 in the plasma of patients with exudative AMD indicated changes in angiogenesis pathways and resulting vulnerability to CNV development in patients with AMD; however, further research is necessary to support this hypothesis.

Our data suggested that the canonical pathway with the most significant change in patients with AMD was LXR/RXR activation. Retinoic X receptors are nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid–mediated gene activation. Our findings correlated well with the results of Yuan et al., who recently investigated protein expression profiles in Bruch’s membrane/choroid complex from patients with AMD and found that four retinoid procession proteins were elevated only in early/
FIGURE 3. Vinculin levels in the plasma of patients with AMD and healthy controls, as measured by Western blotting. (A) Western blotting for detection of vinculin was performed with plasma samples from 40 healthy controls, 22 patients with early AMD, and 58 patients with exudative AMD. After SDS-PAGE, upper parts of the gel above 90 kDa (vinculin, 117 kDa) were excised from all the gels, arranged row by row, and electroblotted onto a single membrane. The image was rearranged for visualization. Equal loading for each sample was confirmed by Coomassie staining on the remaining portions of SDS-PAGE gels. (B) Western blotting images were scanned, and the intensities of protein bands were determined by densitometry. Data are presented as box plots. (C) The relationship between the specificity and sensitivity of vinculin measurement.
for the detection of exudative AMD is represented by an ROC curve. (D) Western blotting for vinculin was performed with another blind set of plasma samples from 60 healthy controls, 10 patients with early AMD, and 43 patients with exudative AMD. Densitometric analysis (E) and ROC curve results (F) of Western blotting images in (D) are shown. Note: Taken together, we used a total of 100 healthy controls, 32 patients with early AMD, and 101 patients with exudative AMD for Western blotting analysis of vinculin.

midstage AMD, supporting the role of retinoids in the initial development of AMD. Elevated retinoid processing proteins may reflect increased RPE synthesis of these proteins to compensate for increased oxidative damage to retinoids. Retinoids are highly susceptible to oxidation and can be toxic to cells. Additionally, their by-products accumulate in RPE lipofuscin granules with age and in AMD and can activate complement. 24, 25 The newly identified major molecular functions of the plasma proteins in patients with AMD (cellular growth and proliferation, cell death, cellular movement, and cell-to-cell signaling and interaction) also correlated with the known pathogenic mechanisms of AMD and CNV. 26

Vinculin in AMD

Vinculin is a 117-kDa membrane-associated protein that functions as a multiprotein linker connecting cell–matrix adhesions and cell–cell adhesions to the actin-based cytoskeleton. 27, 28 We showed for the first time that the plasma level of vinculin was significantly higher in patients with AMD than in HCs (Figs. 3, 4). However, results (Fig. 4) indicated that the diagnostic value of plasma vinculin was in the early detection of AMD, not in the discrimination of disease severity; further clinical studies are necessary to reveal the accurate diagnostic value. Currently, age, smoking, and cardiovascular diseases are known risk factors for AMD, 29 and the lack of association between vinculin levels and clinical variables of subjects in Table 2 confirmed that the association of AMD with the plasma level of vinculin was not biased by any potential confounding factors. Several possibilities may explain this significant diagnostic value in AMD. First, vinculin concentrations may indicate damage, that is, RPE degeneration or breakdown of the outer blood–retinal barrier. Our in vitro data showed that vinculin was expressed in and secreted from RPEs, consistent with immunofluorescence analysis on cadaver eyes (Fig. 5). The association of vinculin secretion from RPEs under conditions that mimic oxidative stress, another factor associated with the pathogenesis of AMD, may enhance upregulation of the angiogenic growth factor VEGF. Indeed, several studies have suggested a role of vinculin in angiogenesis and tumor cell invasion. 30, 31 Second, based on the cellular localization and function of vinculin, it could be hypothesized that circulating vinculin is a result of tissue leakage in ocular tissues. In recent years, emerging data have supported that endothelial dysfunc-

**Implications of Proteomic Studies for AMD**

Disregulation of alternative complement activation has been reported to be involved in AMD pathogenesis. Several groups have carried out proteomic studies of AMD using ocular tissues, such as drusen, RPE/Bruch’s membrane/choroid, and aqueous humor. 4–7 Most of the identified proteins in the posterior part of the eye are involved in immune responses and host defense, including many complement proteins and damage-associated molecular pattern proteins, such as α-defensins, protein S100s, crystallins, histones, and galectin-3. Moreover, analysis of the aqueous humor revealed significantly elevated complement components and acute-phase response signaling-related proteins in AMD. 6 We identified more than 20 complement components in the plasma by LC-MS/MS; however, there were no significant differences in protein spectral counts between patients with AMD and controls. Therefore, our data suggested that complement activation occurred only locally in the eyeballs of patients with AMD and that an attempt to identify serologic biomarkers of AMD from systemic complements may not be feasible. Although the exact association of our plasma proteins and AMD pathogenesis is unknown and some newly identified biomarkers, such as vinculin, are thought to be the consequence of AMD, it is still possible that some of the candidate proteins may be involved in the pathogenesis of AMD either directly or indirectly. Further research is necessary to investigate the potential association.

**Table 4. Plasma Protein Biomarkers Validated to be Significantly Different in Plasma Concentrations Between AMD Patients and Controls**

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein</th>
<th>Normalized Ratio of MS/MS Spectra, AMD/HC</th>
<th>G Value</th>
<th>Method</th>
<th>First Dataset</th>
<th>Second Dataset</th>
</tr>
</thead>
<tbody>
<tr>
<td>P18206</td>
<td>Vinculin; metavinuculin</td>
<td>9.07</td>
<td>3.72</td>
<td>Western blot</td>
<td>40:58</td>
<td>0.895</td>
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<td>P06702</td>
<td>Protein S100-A9; calgranulin-B</td>
<td>2.15</td>
<td>4.84</td>
<td>Western blot</td>
<td>40:58</td>
<td>0.763</td>
</tr>
<tr>
<td>P05109</td>
<td>Protein S100-A8; calgranulin-A</td>
<td>19.15</td>
<td>9.99</td>
<td>ELISA</td>
<td>20:20</td>
<td>0.835</td>
</tr>
<tr>
<td>P02775</td>
<td>CX-C motif chemokine 7</td>
<td>2.15</td>
<td>4.93</td>
<td>ELISA</td>
<td>20:20</td>
<td>0.702</td>
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<tr>
<td>P22105</td>
<td>Tenascin X</td>
<td>0.08</td>
<td>6.1</td>
<td>ELISA</td>
<td>20:20</td>
<td>0.77</td>
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<tr>
<td>D5DU99</td>
<td>Triosephosphate isomerase</td>
<td>9.07</td>
<td>3.72</td>
<td>ELISA</td>
<td>20:20</td>
<td>0.803</td>
</tr>
<tr>
<td>Q9UK55</td>
<td>Protein Z-dependent protease inhibitor</td>
<td>4.23</td>
<td>5.37</td>
<td>ELISA</td>
<td>20:20</td>
<td>0.847</td>
</tr>
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</table>
FIGURE 4. Receiver operating characteristic curves. (A) Differentiation between patients with exudative AMD ($n = 101$), patients with early AMD ($n = 32$), and healthy controls ($n = 100$) using plasma vinculin levels assayed by Western blotting. (B) A combination model using vinculin and two SNPs, that is, rs10490924 ($ARMS2$) and rs800292 ($CFH$), was used to verify the results in 106 AMD cases and 92 healthy controls.
There were several limitations to our study. First, although we believe that our discovery strategy using LC-MS/MS was valid and enabled us to identify many candidate plasma protein markers for AMD, molecules with extremely low concentrations (i.e., ≤pg/mL) could not be detected with our strategy. Additionally, the use of more technical replicates would have increased the coverage and improved the overlap of the identified proteins from two groups. Furthermore, we could not detect posttranslational modifications of plasma proteins with our methodology alone. Further research using different profiling methods may reveal additional novel plasma markers for AMD. Second, the validation process mostly depended on commercial ELISA kits, which show limited accuracy and repeatability for proteins that are rarely assayed in clinical practice. Therefore, to complete the discovery of plasma biomarkers for AMD, well-designed, accurate ELISA kits are necessary for validation and eventual clinical application. Third, for predicting AMD development using blood samples, blood obtained before AMD development should be analyzed. However, our prediction model was made from a case-control design including patients with confirmed AMD before blood sampling. To make a prediction model for AMD development, a prospective cohort study is mandatory.

In conclusion, we discovered plasma protein biomarkers for AMD that can be used adjunctively with risk genotype data using in-depth analysis of the plasma proteomes of AMD patients and normal age-matched controls. Our results may enable the detection of AMD using blood samples, thereby permitting early diagnosis and treatment and ultimately leading to the prevention of blindness in the elderly.
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