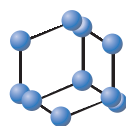
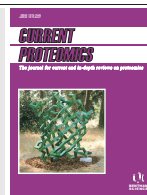


RESEARCH ARTICLE

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Proteomic Analysis of the Vitreous Body in Proliferative and Non-Proliferative Diabetic Retinopathy



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Abstract: Background: Diabetic Retinopathy (DR), one of the major microvascular complications commonly occurring in diabetic patients, can be classified into Proliferative Diabetic Retinopathy (PDR) and Non-Proliferative Diabetic Retinopathy (NPDR). Currently, available therapies are only targeted for later stages of the disease in which some pathologic changes may be irreversible. Thus, there is a need to develop new treatment options for earlier stages of DR through revealing pathological mechanisms of PDR and NPDR.

Objective: The purpose of this study was to characterize the proteomes of diabetes through quantitative analysis of PDR and NPDR.

Methods: Vitreous body was collected from three groups: control (non-diabetes mellitus), NPDR, and PDR. Vitreous proteins were digested to peptide mixtures and analyzed using LC-MS/MS. MaxQuant was used to search against the database and statistical analyses were performed using Perseus. Gene ontology analysis, related-disease identification, and protein-protein interaction were performed using the differential expressed proteins.

Results: Twenty proteins were identified as critical in PDR and NPDR. The NPDR group showed different expressions of kininogen-1, serotransferrin, ribonuclease pancreatic, osteopontin, keratin type II cytoskeletal 2 epidermal, and transthyretin. Also, prothrombin, signal transducer and activator of transcription 4, hemoglobin subunit alpha, beta, and delta were particularly up-regulated proteins for PDR group. The up-regulated proteins related to complement and coagulate cascades. Statherin was down-regulated in PDR and NPDR compared with the control group. Transthyretin was the unique protein that increased its abundance in NPDR compared with the PDR and control group.

Conclusion: This study confirmed the different expressions of some proteins in PDR and NPDR. Additionally, we revealed uniquely expressed proteins of PDR and NPDR, which would be differential biomarkers: prothrombin, alpha-2-HS-glycoprotein, hemoglobin subunit alpha, beta, and transthyretin.

Keywords: Non-proliferative diabetic retinopathy, proliferative diabetic retinopathy, vitreous body, proteomics, LC-MS/MS, gene ontology.

1. INTRODUCTION

Diabetic Retinopathy (DR), a common microvascular complication occurring in diabetic patients, is the leading cause of vision loss and blindness among working-age adults in the USA [1]. Approximately one-third of diabetic

patients have signs of DR, and about one-tenth present with vision-threatening DR, including Non-Proliferative Diabetic Retinopathy (NPDR), Proliferative Diabetic Retinopathy (PDR), and diabetic macular edema [2]. The presence of DR impacts vision and also increases the risk of systemic vascular complications [3]. Despite reasonable control of the systemic risk factors, a considerable proportion of diabetic patients progress to vision-threatening macular edema or PDR. There are currently treatment options only for PDR, such as intravitreal injections of anti-Vascular Endothelial Growth Factor (VEGF), such as bevacizumab, ranibizumab, pegaptanib, aflibercept and corticosteroids, laser photocoagulation, and vitreoretinal surgery [3, 4]. However, no proven

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method is available to prevent progression from NPDR to PDR. The development of DR can be classified into three major stages from the onset of diabetes to clinical manifestations, according to the evolution of pathophysiologic events [5]. In the first stage (several weeks to months of diabetes onset), there is no DR and vision is intact, although some metabolic insults may occur, which is due to the compensation of adaptive responses, including electrical activity reduction and biosynthetic activity reduction of some proteins and lipids [6-8]. The second stage can occur after 5-10 years with early clinical signs such as macular edema, retinal hemorrhages, vascular non-perfusion, microaneurysms, and gliosis. In this stage, NPDR develops, adaptive changes decompensate, and subtle aspects of vision are impaired (visual field and contrast sensitivity) [5, 9]. After additional time, the third stage develops with the occurrence of PDR, characterized by pathophysiologic events such as retinal neovascularization, gliosis, fibrosis, and most often irreversible loss of vision [10].

If it is possible to elucidate the pathological mechanisms underlying progression from NPDR to PDR, early intervention may be possible to treat when vision is salvageable. It may aid clinicians in recognizing high-risk patients to allow essentially personalized treatment, and also develop new and more effective treatment options [10]. Various studies have been conducted on the vitreous body to indirectly investigate the pathophysiology of neuronal and vascular changes in DR since it is in contact with and continuously exchanges substances with the retina, and the protein components in the vitreous body are impacted by the retinal pathophysiologic conditions [11, 12]. Among various approaches, proteomic analysis of vitreous body has attracted many researchers [11-16]. Mass Spectrometry (MS)-based proteomics can determine the relative abundance of different proteins in samples and provide a better insight into the pathophysiologic events and mechanisms [16-19]. Previous proteomic studies have identified several proteins that are involved in DR pathogenesis and can be potential biomarkers for PDR [4]. However, there is a long path towards reaching a comprehensive understanding of the disease regarding its complex pathophysiologic mechanisms [12]. In addition, previous studies mainly focused on the differences between PDR patients and controls, whereas there has been a limited number of studies investigating the differences between NPDR and PDR [20-24].

The purpose of this study was to perform a comparative analysis of the composition and abundance of vitreous proteins among PDR, NPDR, and controls. A bottom-up proteomic approach was used to determine Differentially Expressed Proteins (DEPs) for each group-group comparisons (PDR *versus* control, NPDR *versus* control, and PDR *versus* NPDR). Further analyses were also carried out, including Gene Ontology (GO), protein-protein interaction, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to identify the implicated biologic pathways and essentially reveal potential pathophysiologic mechanisms underlying DR progression.

2. MATERIALS AND METHODS

2.1. Materials

Tris (2-carboxyethyl)phosphine (TCEP) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Formic acid and iodoacetamide (IAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was supplied by Promega (Madison, WI, USA). High-Performance Liquid Chromatography (HPLC)-grade water and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Sample Collection

Diabetic and non-diabetic patients were recruited at the Seoul Metropolitan Government-Seoul National University Boramae Medical Center, Seoul, Korea from 2014 to 2016. The study was approved by the Institutional Review Board (approval number 16-2017-4), adhered to the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects prior to the study. Samples were collected through a syringe connected to the ocutome from the vitreous core with the infusion line turned off during the initial stages of a 23-gauge pars plana vitrectomy. Patients were classified into three groups: control (non-diabetes mellitus, n = 4, C1-C4), NPDR (n = 4, N1-N4), and PDR (n = 5, P1-P5). Table 1 summarizes patient demographics. The control group underwent vitrectomy for rhegmatogenous retinal detachment (n=1), vitreomacular traction syndrome (n=1), and macular hole (n=2).

Table 1. Summary of patient demographics.

Group	Control	NPDR	PDR
Sample size	4	4	5
Age (years)	73 ± 11	74 ± 4	66 ± 12
Gender (male:female)	4:0	4:0	5:0

2.3. Sample Preparation

Vitreous samples were centrifuged (16,000g, 30 min, 4°C) to remove cell debris, and supernatants were collected following depletion using a 30KDa membrane. The concentration of proteins in the samples was determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein mixtures (100 µg) were loaded into a 96-well plate. Samples were then subjected to automated in-solution digestion using a liquid handling robotic system (Agilent Technologies, Santa Clara, CA, USA) and the VWorks software. Protein mixtures were reduced with TCEP (37°C, 30 min), alkylated with IAA (25°C, 30 min, in the dark), and digested using trypsin at an enzyme: protein ratio of 1:50 (w/w) (37°C, 18 h). Then, the digestion was stopped by adjusting the pH of the samples to 2-3 with 15 µL of formic acid. Sample desalting was conducted using C18 Micro SpinColumn™ (Harvard Apparatus, Holliston, MA, USA). The column was washed with 100 µL of methanol followed by 100 µL of solvent A (0.1% formic acid in water), equilibrated with 100

μL of solvent B (0.1% formic acid in acetonitrile: water (80:20, v/v)), and then washed with 100 μL of solvent A. The digested samples were loaded onto the column and washed with 50 μL of solvent A. The peptides were eluted with 100 μL of solvent B and subsequently dried in the Scan-Speed 40 centrifugal evaporator at 1,800 rpm for 3 h.

2.4. LC-MS/MS Analysis

The peptide mixtures were reconstituted in 50 μL of solvent A prior to LC-MS/MS analysis with positive nano-electrospray ionization mode. Each sample was analyzed in triplicate. The LC-MS/MS system consisted of a Dionex Ultimate 3000 HPLC coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The peptides were loaded onto an Acclaim™ PepMap™ 100 C18 nano-trap column (75 $\mu\text{m} \times 2\text{ cm}$, 3 μm particles, 100-Å pores, Thermo Fisher Scientific) at a flow rate of 2.5 $\mu\text{L}/\text{min}$ for 5 min using solvent A. The peptides were then separated in an Acclaim™ PepMap™ C18 100A RSLC nano-column (75 $\mu\text{m} \times 50\text{ cm}$, 2 μm particles, 100-Å pores, Thermo Fisher Scientific). The mobile phase solvent consisted of solvent A and solvent B with a flow rate of 300 nL/min. The gradient was set up for solvent B as follows: equilibration at 4% (0-10 min), 4-40% (10-52 min), 40-96% (52-52.1 min), holding at 96% (52.1-60 min), 96-4% (60-60.1 min), and holding at 4% (60.1-75 min) for re-equilibration of the column. The following parameters were set in MS/MS: spray voltage, 2.2 kV; capillary temperature, 320°C; isolation width, $\pm 2\text{ m/z}$; scan range, 400 to 2000 m/z ; resolution in full-MS scans, 70,000; and resolution in MS/MS scans at 400 m/z ; 17,500. The MS was operated using a data-dependent acquisition method. Top ten precursor ions with the highest intensity were isolated in the quadrupole and fragmented by the higher-energy collisional dissociation with 27% normalized collisional energy. Dynamic exclusion was set at 20 s to minimize repeated analyses of the same abundant precursor ions.

2.5. Database Search for Proteins

Raw MS/MS data files were subjected to MaxQuant version 1.5.8.3 (www.coxdocs.org) for Label-Free Quantification (LFQ). Spectra were searched with a built-in Andromeda search engine against a SwissProt human protein database (<https://www.uniprot.org/>). The following parameters were set for the search: missed cleavages with trypsin, ≤ 2 ; variable modifications, methionine oxidation (+ 15.995 Da) and carbamylation of protein in N-term (+ 43.0006 Da); static carbamidomethylation of cysteine (+ 57.0215 Da); first search peptide tolerance, 20 ppm; and main search peptide tolerance, 4.5 ppm. A False Discovery Rate (FDR) cutoff of 1% was used.

2.6. Data Processing, Gene Ontology, Protein-protein Interaction, and Pathway Analysis

Data sets from MaxQuant were imported into the Perseus software platform version 1.6.5.0 (www.coxdocs.org).

Protein LFQ intensities were transformed by $\log_2(x)$ and samples with missing values for given proteins were assigned a random value using the imputation principle (downshift 1.8, width 0.3, total matrix mode). Analyses of protein distribution including Principal Component Analysis (PCA), heatmap, and boxplot were performed. Student's t-test was used to compare protein abundances between the groups: PDR *versus* control (PDR/C), NPDR *versus* control (NPDR/C), and PDR *versus* NPDR (PDR/NPDR). DEPs of each comparison were generated with a cutoff p -value < 0.1 and $\log_2\text{FC} > 2$ (fold-change). DEP-lists were further used for GO analysis (<http://geneontology.org/>). Related diseases were generated using ClueGO version 2.5.4 (Cordeliers Research Center, France)-Cytoscape version 3.7.1 (National Institute of General Medical Sciences, MD, USA). Protein-protein interaction and KEGG pathway were analyzed through the String database (<https://string-db.org/>). KEGG pathway maps were obtained from www.kegg.jp [25-27] with permission to re-use.

3. RESULTS AND DISCUSSION

3.1. Protein Distribution and Differentially Expressed Proteins

Quantitative proteomic analyses have been conducted on DR patients using serum, tear fluid, aqueous humor, and vitreous body in several studies [11, 16, 28]. This study was performed to investigate the differences in vitreous proteins among PDR, NPDR, and non-diabetic patients (the control group). The use of non-diabetic patients as controls is an advantage in revealing a comprehensive understanding of PDR and NPDR pathogenesis. After obtaining vitreous samples, an ultra-filtration method was applied to deplete high-molecular-weight proteins. The proteins were subjected to tryptic digestion and the resulting peptide mixtures were analyzed with LC-MS/MS. By utilizing filtration, we expected to obtain information about middle- and low-MW proteins. Unexpectedly, some proteins with MW $> 30\text{ kDa}$ were identified. It might be due to the insufficiency of the filtration process. Another possibility was that some proteins might suffer from fragmentation during sample handling and then could pass the filter. The analysis was conducted three times per sample. Overall, 15, 12, and 12 raw data files were obtained for PDR, NPDR, and control groups, respectively, for further data analyses. The distribution of protein abundances in each sample is shown in Fig. (S1). In general, there is no considerable difference among the groups. One sample in the PDR group (P4) and one sample in the NPDR group (N3) show a wider distribution range with a higher average protein abundance compared with the others. Correlation analysis was performed to confirm the distribution of the identified proteins. A positive correlation coefficient value indicates a positive correlation and vice versa, whereas its absolute value suggests the strength of correlative relation. It can be observed from Fig. (1A) that most of the correlation coefficient values range from 0 to 1. However, some correlation coefficient values were close to zero, suggesting the high variation among samples. In addition, within the sam-

ple, Relative Standard Deviation (RSD) values of three replicate ranged from 0.5% to 173% (Table S1). Correlations between PDR samples, except P4, are stronger than those between NPDR samples. Control samples show weaker correlations. It is reasonable since they were from non-diabetic patients with different eye-related diseases. In addition, PDR and NPDR patients might be in various stages of the disease, resulting in a large variation.

All the quantified proteins were subjected to the PCA. The PCA yielded a two-dimensional principal component model that accounted for 55.9% of the total variance. The separation of the PDR group and the control group in Fig. (1B) demonstrates their remarkable difference in protein expression. The NPDR group is close to the control groups, indicating a non-distinct difference. One patient with PDR (P4) and one patient with NPDR (N3) are far from their groups, which can be due to the patients' individual differences. Interestingly, P4 and N3 locate close to each other and also exhibit a strong correlation (Fig. 1A). The number of quantified proteins was quite low. Among 87 vitreous proteins that were quantified (Table S1), 20 proteins differed significantly in PDR/C or NPDR/C. Fig. (2) presents the relative abundances of these proteins and Fig. (1C) illustrates them on a heatmap.

As shown in Table 2, 13 proteins were up-regulated in PDR/C, whereas 1 protein (statherin) was down-regulated.

Table 3 lists 14 up- and 1 down-regulated proteins in NPDR/C. In addition, 4 proteins were defined as up-regulated in PDR/NPDR, whereas transthyretin was the only down-regulated protein in this comparison (Table 4). From the DEP lists, it can be observed that some proteins increase their abundances in PDR and NPDR compared with the control group, such as alpha-1-antitrypsin, immunoglobulin kappa constant, immunoglobulin heavy constant gamma 1, apolipoprotein A-1, alpha-1-acid glycoprotein 1, alpha-2-HS-glycoprotein, immunoglobulin lambda constant 3, and clusterin. In addition, statherin shows a decreased abundance in PDR and NPDR compared with the control group. Thus, these proteins are critical to detect DR, but unable to distinguish the progress of the disease (PDR and NPDR). Five up-regulated proteins in NPDR/C, including kininogen-1, serotransferrin, ribonuclease pancreatic, osteopontin, and keratin type II cytoskeletal 2 epidermal, do not appear in the DEP list of PDR/C. In addition, transthyretin exhibited an increased abundance in the NPDR compared with the PDR and control group. Those proteins are probably potential biomarkers of NPDR, which can facilitate in detecting DR in the early stages. Five proteins including prothrombin, signal transducer and activator of transcription 4, hemoglobin subunit alpha, beta, and delta are up-regulated in PDR/C but do not appear in the DEP-list of NPDR/C. The increases in their abundances could be critical indicators for DR in the late stages when PDR occurs.

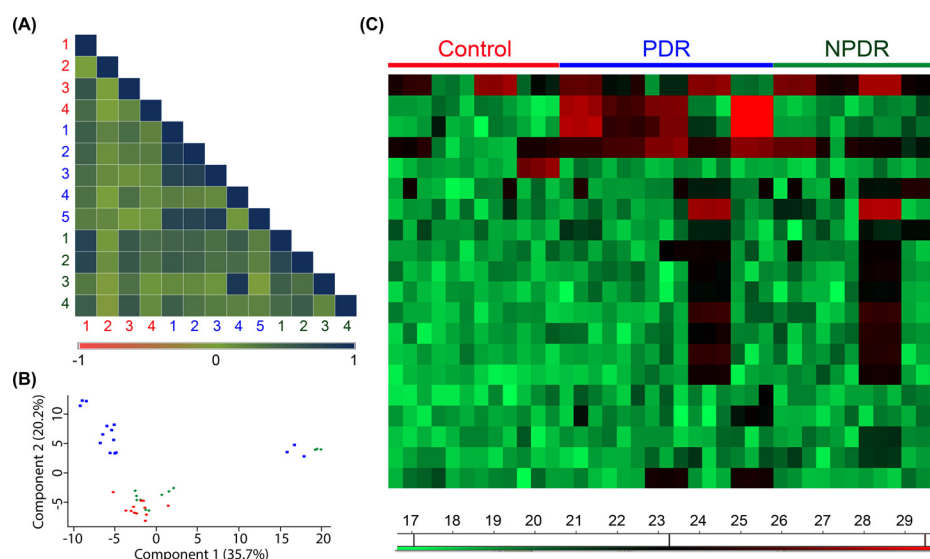


Fig. (1). (A) Correlation analysis of protein abundance between all pairs of samples: control (red), PDR (blue), and NPDR (green). (B) Principal Component Analysis (PCA) of all proteins in control (red circle), PDR (blue square), and NPDR (green diamond) groups. (C) Heatmap presenting relative abundances (log2Intensity) of 20 differentially expressed proteins in 3 groups. The proteins from bottom to top are: Prothrombin (THRB), Kininogen-1 (KNG1), Ribonuclease pancreatic (RNAS1), Hemoglobin subunit Delta (HBD), Signal Transducer and Activator of Transcription 4 (STAT4), Alpha-1-Antitrypsin (A1AT), Immunoglobulin Kappa Constant (IGKC), Alpha-1-Acid Glycoprotein 1 (A1AG1), Immunoglobulin Heavy Constant Gamma 1 (IGHG1), Apolipoprotein A-1 (APOA1), Clusterin (CLUS), Immunoglobulin Lambda Constant 3 (LAC3), Keratin, type II cytoskeletal 2 Epidermal (K22E), Serotransferrin (TRFE), Osteopontin (OSTP), Statherin (STAT), Alpha-2-HS-glycoprotein (FETUA), Hemoglobin subunit Beta (HBB), Hemoglobin subunit Alpha (HBA), Transthyretin (TTHY). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

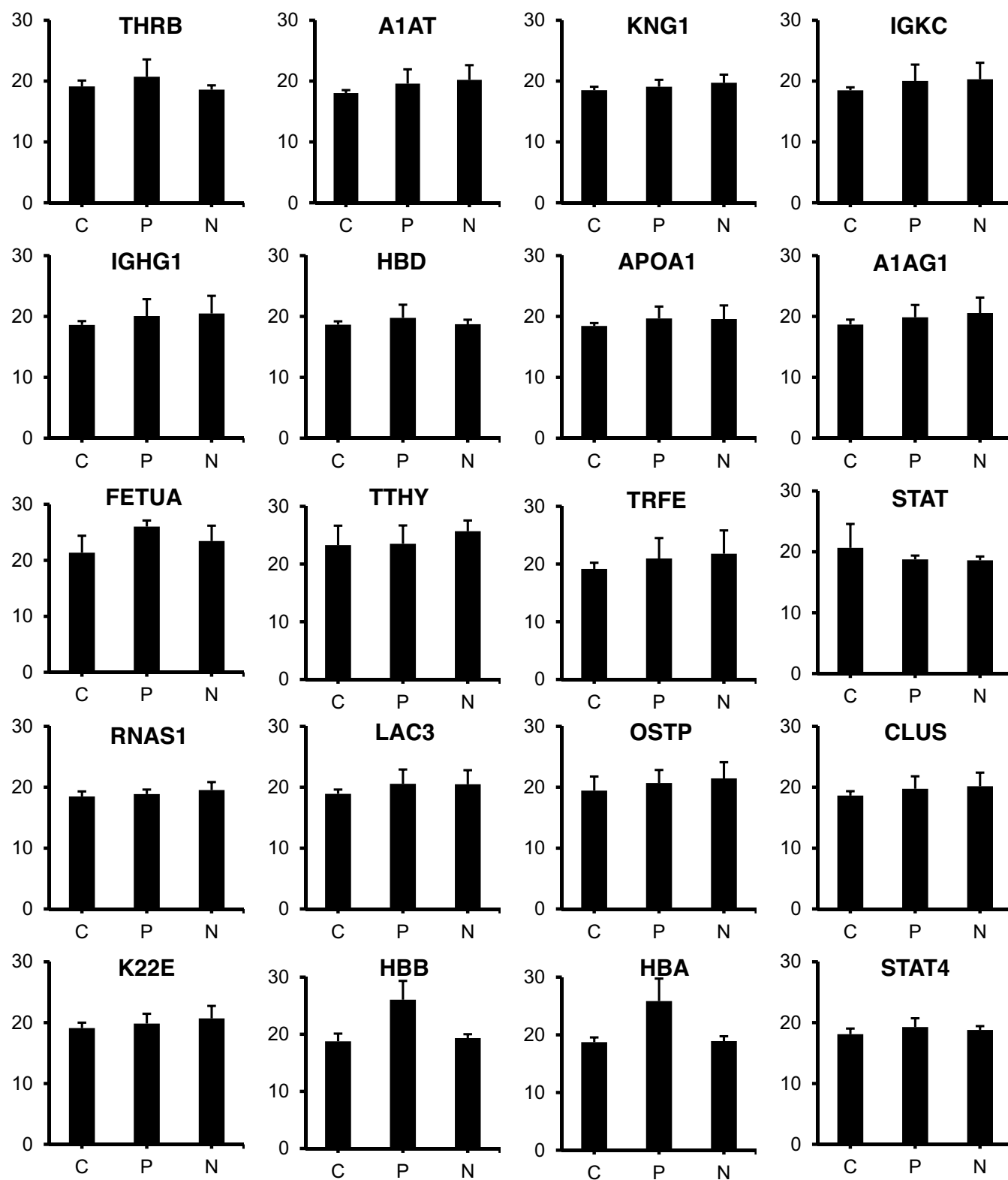


Fig. (2). Comparison of relative abundance of differentially expressed proteins (log2Intensity). Control: C, PDR: P, and NPDR: N.

Table 2. Differentially expressed proteins between PDR group and control group.

Protein ID	Protein Description	Regulation	p-value	log ₂ FC
P00734	Prothrombin (THRB)	Up	0.0774	1.5852
P01009	Alpha-1-antitrypsin (A1AT)	Up	0.0360	1.5424
P01834	Immunoglobulin kappa constant (IGKC)	Up	0.0630	1.5302
P01857	Immunoglobulin heavy constant gamma 1 (IGHG1)	Up	0.0846	1.4688
P02042	Hemoglobin subunit delta (HBD)	Up	0.0943	1.1220
P02647	Apolipoprotein A-1 (APOA1)	Up	0.0433	1.2363
P02763	Alpha-1-acid glycoprotein 1 (A1AG1)	Up	0.0697	1.1853
P02765	Alpha-2-HS-glycoprotein (FETUA)	Up	9.05E-06	4.6471
A0A075B6L0	Immunoglobulin lambda constant 3 (LAC3)	Up	0.0268	1.6570
P10909	Clusterin (CLUS)	Up	0.0848	1.1159
P68871	Hemoglobin subunit beta (HBB)	Up	1.58E-07	7.2937
P69905	Hemoglobin subunit alpha (HBA)	Up	1.82E-06	7.1071
Q14765	Signal transducer and activator of transcription 4 (STAT4)	Up	0.0210	1.1840
P02808	Statherin (STAT)	Down	0.0768	-1.8900

Table 3. Differentially expressed proteins between NPDR group and control group.

Protein ID	Protein Description	Regulation	p-value	log ₂ FC
P01009	Alpha-1-antitrypsin (A1AT)	Up	0.0057	2.1753
P01042	Kininogen-1 (KNG1)	Up	0.0074	1.2327
P01834	Immunoglobulin kappa constant (IGKC)	Up	0.0327	1.8135
P01857	Immunoglobulin heavy constant gamma 1 (IGHG1)	Up	0.0375	1.8921
P02647	Apolipoprotein A-1 (APOA1)	Up	0.1000	1.1375
P02763	Alpha-1-acid glycoprotein 1 (A1AG1)	Up	0.0231	1.8841
P02765	Alpha-2-HS-glycoprotein (FETUA)	Up	0.0927	2.0712
P02766	Transthyretin (TTHY)	Up	0.0422	2.3921
P02787	Serotransferrin (TRFE)	Up	0.0397	2.6429
P07998	Ribonuclease pancreatic (RNAS1)	Up	0.0244	1.0774
A0A075B6L0	Immunoglobulin lambda constant 3 (LAC3)	Up	0.0356	1.5713
P10451	Osteopontin (OSTP)	Up	0.0594	2.0176
P10909	Clusterin (CLUS)	Up	0.0362	1.5220
P35908	Keratin, type II cytoskeletal 2 epidermal (K22E)	Up	0.0217	1.5948
P02808	Statherin (STAT)	Down	0.0886	-2.0433

Table 4. Differentially expressed proteins between PDR group and NPDR group.

Protein ID	Protein Description	Regulation	p-value	log ₂ FC
P00734	Prothrombin (THRB)	Up	0.0187	2.1240
P02765	Alpha-2-HS-glycoprotein (FETUA)	Up	0.0028	2.5759
P68871	Hemoglobin subunit beta (HBB)	Up	2.77E-07	6.7446
P69905	Hemoglobin subunit alpha (HBA)	Up	2.74E-06	6.9268
P02766	Transthyretin (TTHY)	Down	0.0471	-2.1783

Previously, several proteins were identified as critical in PDR and NPDR as compared with the control patients. In the vitreous body of PDR patients, up-regulated proteins were angiopoietin-related protein 6, estrogen receptor-alpha [29], enolase, catalase [30], fibrinogen-gamma, alpha-1-B-glycoprotein, complement component C3, vitamin D-binding protein [31], clusterin [28], apolipoprotein A-1 [32], hemo-

globin subunit beta [33], and alpha-2-HS-glycoprotein [34]. In addition, a previous study on aqueous body of DR patients revealed the increases in apolipoprotein A-1 and serotransferrin abundance [13]. Those results are similar to our findings, which suggest some critical proteins as potential biomarkers. Our study contributes to the up-regulated proteins in the DEP list of PDR/C by finding out prothrombin,

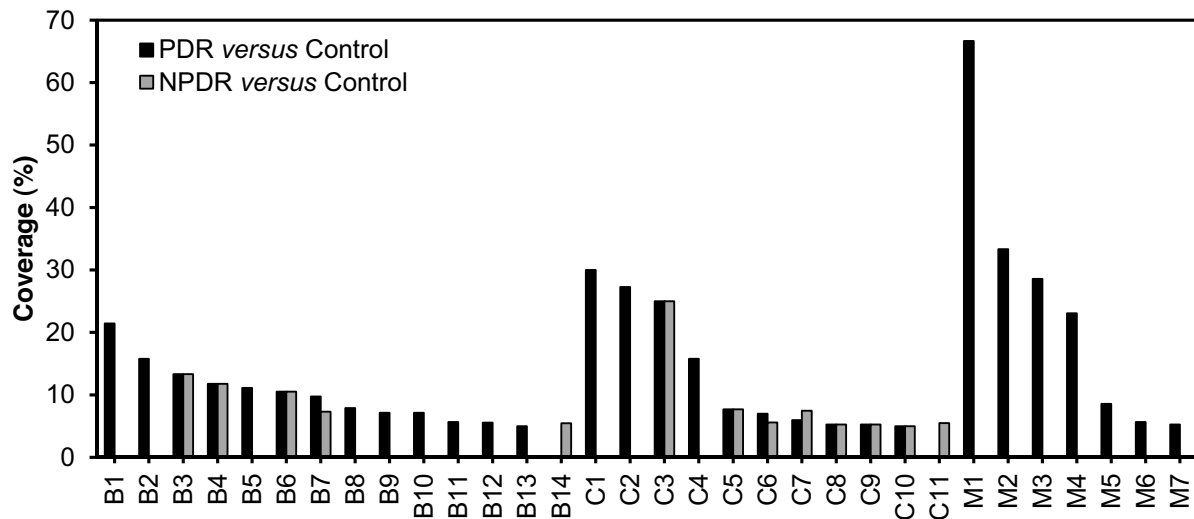


Fig. (3). Gene Ontology (GO) classified into biological processes (B1-B14), cellular components (C1-C11), and molecular functions (M1-M7) with >5% coverage from up-regulated proteins of two DEP lists: PDR versus control and NPDR versus control. The GO terms are: oxygen transport-B1, gas transport-B2, chaperone-mediated autophagy-B3, reverse cholesterol transport-B4, hydrogen peroxide catabolic process-B5, negative regulation of bone mineralization-B6, acute-phase response-B7, hydrogen peroxide metabolic process-B8, negative regulation of biomineralization-B9, negative regulation of biomineral tissue development-B10, antibiotic catabolic process-B11, acute inflammatory response-B12, cofactor catabolic process-B13, platelet degranulation-B14; haptoglobin-hemoglobin complex-C1, hemoglobin complex-C2, spherical high-density lipoprotein particle-C3, endocytic vesicle lumen-C4, high-density lipoprotein particle-C5, blood microparticle-C6, platelet alpha granule lumen-C7, lipoprotein particle-C8, plasma lipoprotein particle-C9, protein-lipid complex-C10, platelet alpha granule-C11; hemoglobin alpha binding-M1, haptoglobin binding-M2, hemoglobin binding-M3, oxygen carrier activity-M4, oxygen binding-M5, peroxidase activity-M6, oxidoreductase activity-M7.

immunoglobulin kappa constant, immunoglobulin heavy constant gamma 1, immunoglobulin lambda constant 3, alpha-1-acid glycoprotein 1, hemoglobin subunit alpha, beta, and delta. Some proteins were found to be up-regulated in the vitreous body of NPDR patients, including glyceraldehyde-3-phosphate dehydrogenase hepatocyte growth, factor activator, kallistatin, gamma-glutamyl hydrolase [24], beta-2 microglobulin, and thyroxine-binding globulin [35]. In our study, several new proteins were found to up-regulate in NPDR/C, including, alpha-1-antitrypsin, kininogen-1, apolipoprotein A-1, alpha-1-acid glycoprotein 1, alpha-2-HS-glycoprotein, transthyretin, ribonuclease pancreatic, osteopontin, immunoglobulin kappa constant, immunoglobulin heavy constant gamma 1, immunoglobulin lambda constant 3, and keratin type II cytoskeletal 2 epidermal. In addition, one proteomic study was previously conducted to compare PDR and NPDR patients, which identified some up-regulated proteins in PDR/NPDR such as peroxiredoxin-2, peroxiredoxin-6, reactive species modulator 1, catechol 1,2-dioxygenase, hypoxia-upregulated protein-1, and nitric oxide synthase [11]. Our study contributes several proteins to the list of up-regulated proteins in PDR/NPDR, including prothrombin, alpha-2-HS-glycoprotein, hemoglobin subunit alpha, and beta. Particularly, transthyretin was detected for the first time as a down-regulated protein in PDR/NPDR.

3.2. Gene Ontology and Related Disease Analysis

GO analysis was performed using up-regulated proteins in DEP lists of PDR/C and NPDR/C. GO terms were classi-

fied into three databases: biological processes, cellular components, and molecular functions. In PDR/C, a total of 68 biological processes, 32 cellular components, and 10 molecular functions were identified (Table S2). Top 5 of GO terms according to coverage percentage are presented in Fig. (S2). In NPDR/C, 28 biological processes, 33 cellular components, and 1 molecular function were identified (Table S3). Fig. (S3) exhibits the top 5 of GO annotations. To compare GO terms related to up-regulated proteins in PDR/C and NPDR/C, those with coverage $\geq 5\%$ in lists are presented in Fig. (3). Some GO terms are unique for PDR/C, such as oxygen transport, gas transport, hydrogen peroxide metabolic process, haptoglobin-hemoglobin complex, haptoglobin binding, hemoglobin binding, oxygen binding, and peroxidase activity, whereas platelet degranulation and platelet alpha granule only appear in NPDR/C. Related disease classification was conducted using up-regulated proteins in PDR/C and NPDR/C (Fig. (S4), Table S4, S5). In addition, the distribution of classified diseases is depicted in Fig. (S5). It is noticeable that in PDR/C, the top 2 disease categories are alpha thalassemia and prothrombin deficiency.

3.3. Protein-Protein Interaction

Protein-protein interaction was carried out using up-regulated proteins in PDR/C, NPDR/C, and PDR/NPDR. Interactions between proteins can be known interactions (curated databases and experimental determination of literature), predicted interactions (gene neighborhood, gene fusions, and gene co-occurrence), and others (text mining, co-expression,

and protein homology). The detail information was listed in Tables S6 and S7. Most of the protein couples have a combined score >0.9, indicating the strength of protein-protein interaction. As shown in Fig. (S6A), interactions among up-regulated proteins in PDR/C are mainly from curated databases, co-expression, and text mining. Fig. (S7A) shows interactions among up-regulated proteins in NPDR/C, which belong to curated databases, experimental determination of literature, co-expression, and text mining. Fig. (S6B-D), and Fig. (S7B-D) show protein-protein interactions with the localization of proteins into different GO terms. Besides the above-mentioned interactions, it is obvious that the proteins also co-exist in various GO terms.

3.4. Regulation of Some Proteins in KEGG Pathways

Analysis of up-regulated proteins in PDR/C and NPDR/C with String database reveals that only one KEGG pathway (complement and coagulation cascades) involved in protein expression, which was in agreement with a previous study [33]. This pathway refers to four proteins, including prothrombin (F2), Alpha-1-Antitrypsin (A1AT), Kininogen-1 (KNG), and clusterin (Fig. (S8)). In addition, statherin was down-regulated in PDR/C and NPDR/C. This protein belongs to one KEGG pathway, the salivary secretion pathway (Fig. (S9)). As mentioned above, transthyretin is a unique protein that increased its abundance in the NPDR compared with the PDR and control group. It involves in thyroid hormone synthesis pathway (Fig. (S10)).

Proteomic studies on DR patients have been greatly supported by the development of analytical techniques and database search programs. The identification of DEPs is a critical key to reveal specific pathological processes. Generally, a bottom-up proteomic profiling study is able to identify a large number of proteins in an analysis [36]. However, some specific key proteins such as VEGF, platelet-derived growth factor, and placental growth factor are rarely detected in discovery proteomics [36], which can be due to the complexity of samples as well as ion suppression during MS/MS analysis. The use of some enrichment methods and fractionation techniques, as well as the improvement of MS/MS sensitivity, will contribute to better results. Proteins of particular interest that belong to the DEP lists of PDR/C, NPDR/C, and PDR/NPDR can be potential biomarkers of DR at different stages. One of the limitations in this study was the lack of the verification of DEPs. They need to be validated with orthogonal analyses, including western blot, Enzyme-Linked Immunosorbent Assays (ELISA), radioimmunoassay, and immunohistochemistry [33, 37, 38]. In addition, the number of samples used in this study is limited in providing a meaningful evaluation. Further studies should be performed with a large number of samples.

CONCLUSION

In this study, a comparative proteomic analysis was performed on PDR, NPDR, and control patients using vitreous body samples. Twenty proteins were defined as critical proteins in PDR and NPDR. GO analysis and protein-protein in-

teraction were conducted using up-regulated proteins in the DEP list of PDR/C, NPDR/C, and PDR/NPDR. These up-regulated proteins related to complement and coagulation cascades, whereas statherin was down-regulated in PDR/C and NPDR/C and belongs to the salivary secretion pathway. The unique protein that increased its abundance in the NPDR compared with the PDR and control group, transthyretin, involves in the thyroid hormone synthesis pathway. This study confirmed the differential expression of a number of proteins in PDR and NPDR that were previously reported. In addition, some new proteins identified in this study could be potential biomarkers for DR at different stages. However, some limitations in this study include the use of patients with not only macular hole as the control group, the small number of samples, the small number of quantified proteins, and the lack of DEP verification. Further studies should be conducted with a larger number of samples to validate the expression of those proteins by other analyses for future applications.

LIST OF ABBREVIATIONS

DEPs	= Differentially Expressed Proteins
DR	= Diabetic Retinopathy
GO	= Gene Ontology
HPLC	= High-Performance Liquid Chromatography
IAA	= iodoacetamide
KEGG	= Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	= Liquid Chromatography-Tandem Mass Spectrometry
LFQ	= Label-Free Quantification
MS	= Mass Spectrometry
NPDR	= Non-Proliferative Diabetic Retinopathy
PCA	= Principal Component Analysis
PDR	= Proliferative Diabetic Retinopathy
TCEP	= Tris (2-carboxyethyl)phosphine
VEGF	= Vascular Endothelial Growth Factor

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Review Board, Seoul Metropolitan Government, Seoul National University Boramae Medical Center, Seoul, Korea (approval number 16-2017-4).

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. The reported experiments in accordance with the ethical standards of the committee responsible for human experimentation (institutional

and national), and with the Helsinki Declaration of 1975, as revised in 2008 (<http://www.wma.net/en/20activities/10ethics/10helsinki/>).

CONSENT FOR PUBLICATION

Written informed consent was taken from all participants.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the ProteomeXchange Consortium via the PRIDE [39] partner repository with the dataset identifier PXD017116.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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