

Proteomic analysis of aqueous humor from patients with branch retinal vein occlusion-induced macular edema

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Abstract. The mechanisms responsible for macular edema with branch retinal vein occlusion (BRVO) remain to be elucidated. It is known that the expression profile of certain proteins in the aqueous humor (AH) changes in some diseases. Accordingly, determining the expression of these AH proteins may aid in the understanding of their potential role in this pathogenesis. The aim of this study was to identify the possible mechanisms involved in the development of BRVO-induced macular edema. A proteomic analysis of the AH composition in the eyes of patients with BRVO was performed and compared with that in the eyes of patients with cataract (non-BRVO; controls). AH from 6 patients with macular edema due to BRVO and 6 patients with cataract (non-BRVO) was collected. A proteomic approach which included 2-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) and bioinformatics analysis were used to identify AH proteins with altered expression in patients with macular edema due to BRVO compared with the controls. An enzyme-linked immunosorbent assay was used to validate the proteomic results. The total protein concentration in the AH of patients with BRVO-induced macular edema was significantly greater than that of the controls. In the patients with BRVO, a total of 56 protein spots were significantly altered on the 2D gels. A total of 49 protein spots were identified by MS; many of these proteins have been implicated in angiogenesis, oxidative stress and collagen synthesis. In conclusion, the protein composition in AH differed significantly between the patients with BRVO and the controls. The identified proteins may be potential biomarkers for the development of macular

edema due to BRVO and may play a role in the mechanisms responsible for it.

Introduction

Branch retinal vein occlusion (BRVO) is the second most common cause of retinal vascular abnormality after diabetic retinopathy and a frequent cause of visual loss (1). In a pooled analysis using existing data from 11 individual population-based studies, the prevalence of BRVO was found to be 4.42 per 1,000 individuals (95% CI 3.65, 5.19). The prevalence of BRVO is greater in Asians (2,3). Visual loss in BRVO, either short or long term, may be the result of the presence of macular edema, macular non-perfusion, retinal neovascularization, vitreous or intraretinal hemorrhage, tractional retinal detachment or a combination of these disorders (4). Macular edema is the most frequent cause of visual impairment in patients with BRVO (5). Thus, understanding the cellular and molecular factors that underlie the pathogenesis of macular edema with BRVO is of critical importance.

The aqueous humor (AH) is an important intraocular fluid responsible for the supply of nutrients to and the removal of metabolic wastes from the avascular tissues of the eye. It is known that protein levels in AH are altered in various eye diseases, including anterior and posterior segment disorders. In addition, a number of studies have demonstrated that some proteins whose expression is altered in AH correlate with the mechanisms or prognosis of several eye disorders (6). A number of cytokines and other factors in the AH have been suggested to be involved in the pathogenesis of macular edema due to BRVO, such as vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) (5). However, the pathogenesis of macular edema with BRVO is complex; thus, the measurement of these cytokines may not provide enough information as to the disease process. A comprehensive list of the proteins whose expression is altered in the AH of patients with macular edema due to BRVO is still lacking.

Proteomic analysis is a valuable method for elucidating the molecular nature of AH (7). High resolution 2-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) is a technique used for the analysis of several hundred proteins in tissues, fluids or cells using only a few microliters of sample and is therefore ideal for analyzing limited volumes of AH. Some researchers have used this technology to explore the patho-

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genesis of various eye diseases (6,8-12). In this study, we used proteomics as a means to identify disease-specific proteins in AH. Through comparative analyses of the proteomes in patients with cataract (controls) and those with macular edema due to BRVO, it may be possible to obtain a better understanding of the molecular events involved in the development of macular edema due to BRVO and to generate essential data required for the identification of novel biomarkers and/or treatments. The proteomic techniques used include protein separation by 2-DE and characterization by mass spectrometry (MS) of peptides, amino acid sequencing and bioinformatics analysis. Enzyme-linked immunosorbent assay (ELISA) was used to validate the results of proteomics.

Materials and methods

Patients and controls. Twelve AH samples were included in this study, 6 from patients with BRVO-induced macular edema (mean age, 53 ± 4.98 years; 3 males and 3 females) and 6 from age-matched patients with cataract without BRVO (mean age, 53.5 ± 2.35 years; 3 males and 3 females). The disease course was between 6 and 16 months (mean, 10 ± 3.406 months). Clinical data from the patients are summarized in Table I.

The study followed the tenets of the Declaration of Helsinki, and informed written consent was obtained from all patients and controls after we explained the nature and possible consequences of the study. The protocol for this research project was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China.

All participants went through a standard examination including best-corrected visual acuity, slit lamp biomicroscopy, optical coherence tomography (OCT), fundus photo, and fluorescein angiography (FFA). The presence of macular edema was confirmed with FFA and OCT in all patients. No patient had been treated previously for BRVO. None of the controls had any eye diseases other than cataract.

In both groups of examined patients (controls and BRVO), a certain degree of cataract was present. Patients with severe cataract determining blindness or unacceptable vision were not included in the control group.

Sample collection. AH samples were obtained from the eyes of patients with BRVO just before an intravitreal injection of bevacizumab (Avastin, treatment for macular edema due to BRVO) was administered. All sample collections were performed using a standard sterilization procedure as previously described (12). A mean volume of 100 μ l of AH was collected by anterior chamber limbal paracentesis with a 27-gauge needle attached to an insulin syringe. The intravitreal injection of bevacizumab was then administered through the pars plana. Antibiotic ointment was administered after surgery for 4 days. Immediately after collection, the AH samples were transferred to sterile plastic tubes and stored at -80°C until analysis.

AH samples from patients before cataract surgery were obtained for this study. AH samples from 6 controls (cataract patients without other eye diseases) were also collected as previously described (8). A total of 100-200 μ l of sample from each patient sample was pooled. All samples were stored at -80°C until analysis.

Table I. Data from patients with BRVO and the controls.

	No.	Age (years)	Gender ^a	Course of disease (months)	Baseline central macular thickness (μm)
Patients	1	56	F	16	496
	2	47	F	6	523
	3	60	M	10	457
	4	48	F	8	539
	5	52	M	11	431
	6	55	M	9	511
Controls	1	56	M		
	2	52	F		
	3	50	M		
	4	54	M		
	5	56	F		
	6	53	F		

^aF, female; M, male.

Sample preparation. AH samples from patients or controls were pooled to ensure there was sufficient protein in the extracts for matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS).

Excess salts followed by precipitation of proteins using the ProteoExtract™ Protein Precipitation kit (Calbiochem, San Diego, CA, USA) were removed in each of the pooled samples. The samples were processed according to the manufacturer's instructions. The protein concentrations of the AH samples were determined using the Bradford method (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA).

2-DE. 2-DE was performed as previously described (13-15). Twenty-four centimeter, pH 4-7, NL IPG strips (Amersham Bioscience, Uppsala, Sweden) were rehydrated with 80 μ g solubilized protein (for silver staining) in a rehydration buffer. After isoelectric focusing, the IPG strips were equilibrated. They were then loaded onto pre-cast 12.5% homogeneous polyacrylamide gels for electrophoresis, ran in an Ettan-Dalt II system (Amersham Biosciences, San Francisco, CA, USA) and visualized.

Image analysis. The stained gels were scanned and the resulting images were analyzed using ImageMaster™ 2D Platinum software (version 5.0, Amersham Bioscience, Swiss Institute of Bioinformatics, Geneva, Switzerland) for spot detection, quantification, comparison and analyses, as previously described (12,13). The relative intensities of the spots were used for a comparison between the BRVO and control groups. The statistical comparisons between the intensity of the control and the BRVO protein spots were conducted using the Student's t-test (ImageMaster™ 2D platinum software, with $p < 0.05$ considered to be significant). The commonly differentially expressed spots (2-fold increase or decrease) were further identified by MALDI-TOF/TOF MS.

Protein identification. Protein identification was performed as previously described (12,14,16,17). In brief, the common differ-

entially expressed protein spots were excised and the proteins within were reduced, alkylated and digested with trypsin. Digests were immediately spotted onto 600 μm anchochips (Bruker Daltonics, Bremen, Germany). The Bruker Peptide Calibration Mixture was spotted for external calibration. MALDI-TOF MS and tandem TOF/TOF MS were carried out on a time-of-flight Ultraflex II mass spectrometer (Bruker Daltonics). Using the MASCOT search engine [http://www.matrixscience.com; Database: NCBI nr 20100409 (10,820,686 sequences; 3,689,795,467 residues); Taxonomy: *Homo sapiens* (human) (231,301 sequences)] based on the Swiss-Prot protein database, peptide mass fingerprinting was performed for the identification of proteins from tryptic fragment sizes using the assumption that peptides are monoisotopic. One missed trypsin cleavage was allowed. A mass tolerance of 100 parts per million (ppm) was the window of error allowed for matching the peptide mass values.

Gene Ontology analysis. All the proteins identified in this experiment were subjected to Gene Ontology (www.geneontology.org/) for molecular function, biological process and cellular component analysis.

ELISA. Concentrations of alpha crystallin A chain (CRYAA) in the AH samples were verified and quantified using commercially available human cytokine ELISA kits from Usen Life Science Inc. (Catalog no. E9662h; Wuhan, China). The recommended protocol of the manufacturer was followed in all cases. Briefly, standards and AH samples were added to antibody-coated 96-well plates and incubated for 2 h at room temperature, followed by the addition of biotin-conjugated polyclonal antibody specific for CRYAA and incubation for an additional 1 h. The plates were then washed and incubated with avidin conjugated to horseradish peroxidase for 1 h at 37°C. Subsequently, a tetramethylbenzidine substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of sulfuric acid solution. The color change was measured by spectrophotometry at a wavelength of 450 nm. A standard curve was plotted from measurements made with the standard solution (from 0.78 to 50 ng/ml for CRYAA) and was used to determine the concentration of CRYAA in each sample. The concentration of CRYAA in the samples was determined by comparing the OD of the samples to the standard curve. All measurements were performed in duplicate. CRYAA concentrations were calculated as per nanogram of protein.

Statistical analysis. The protein spots were visualized using ImageMaster™ 2D Platinum software as described in the image analysis section. The variation in protein spot intensity within a sample map and between 2 sample maps was analyzed using the Student's t test. The ELISA results were also analyzed using the Student's t test.

Results

Protein content in AH from patients with BRVO and controls. A total of 12 AH samples were included in this study, 6 from patients with BRVO and 6 from age-matched cataract patients without BRVO. There was no statistically significant differ-



Figure 1. Two-dimensional (2D) gel images of aqueous humor (AH) samples from the controls. The relative spot intensity was calculated based on the spot volume using ImageMaster™ 2D platinum software and the labels indicate proteins whose expression was altered by a factor of >2 in the control group. Only the spots labeled with numbers were used for MALDI-TOF/TOF MS/MS analysis.

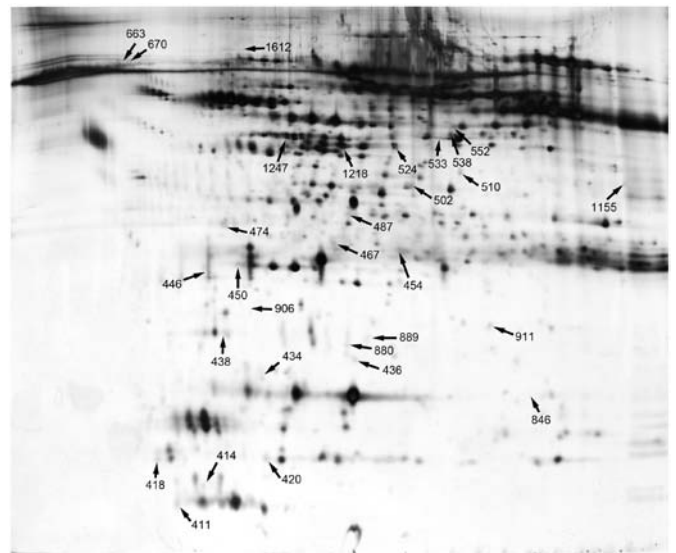


Figure 2. Two-dimensional (2D) gel images of aqueous humor (AH) samples from patients with BRVO. The relative spot intensity was calculated based on the spot volume using ImageMaster™ 2D platinum software and the labels indicate proteins whose expression was altered by a factor of >2 in the branch retinal vein occlusion (BRVO) group. Only the spots labeled with numbers were used for MALDI-TOF/TOF MS/MS analysis.

ence between the 2 groups as regards age ($p=0.074$). Clinical data from the patients are summarized in Table I.

The mean total protein level in AH from patients with BRVO was 1.124 mg/ml, while that from the controls was 0.545 mg/ml. Total protein levels in the patients with BRVO were significantly greater than those of the controls.

2-DE patterns. Figs. 1 and 2 depict the 2D gel images from patients and the controls. Gel images from patients with BRVO displayed more spots and more intensely silver stained spots than

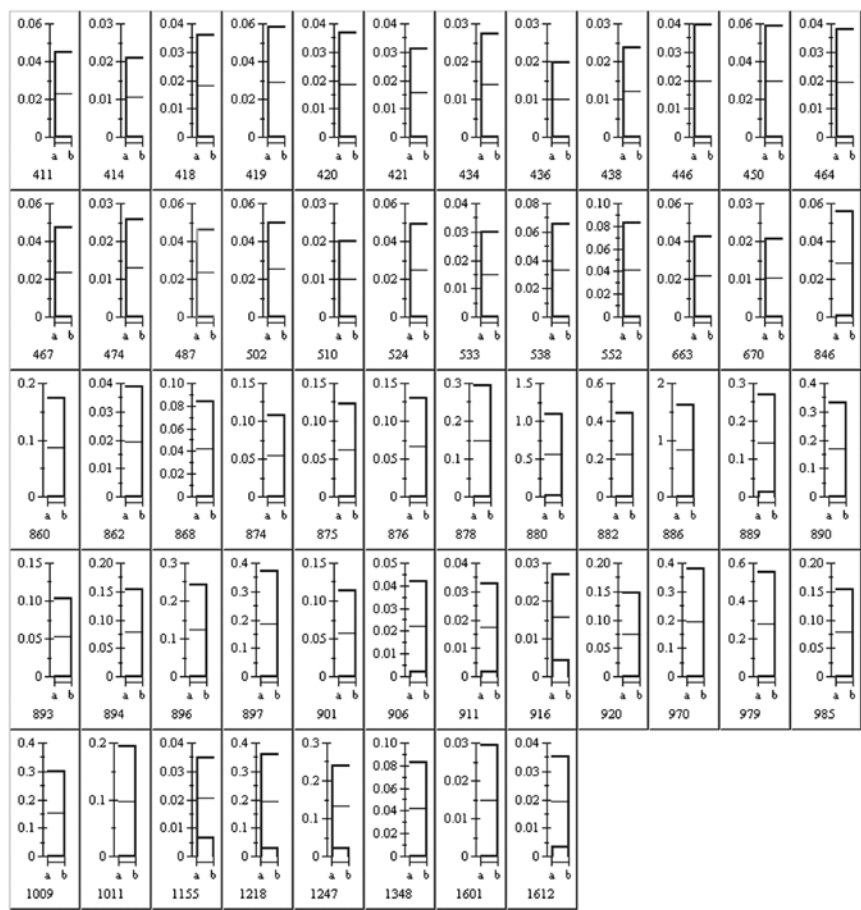


Figure 3. Comparing the relative intensities of spots between the branch retinal vein occlusion (BRVO) and the control groups. The stained gels were scanned, and resulting images were analyzed using the ImageMaster™ 2D Platinum software for spot detection, quantification, comparison and analyses. The relative intensities of spots were used for comparison between the BRVO and control groups. A total of 56 spots were altered by >2-fold in 2-dimensional (2D) gels from patients with BRVO-induced macular edema.

the gel images from the controls. There were significant differences in relative spot volumes (% volume) in the gel patterns; patients with BRVO showed greater volumes than the controls. The stained gels were scanned and the resulting images were analyzed using ImageMaster™ 2D Platinum software for spot detection, quantification, comparison and analyses. A total of 56 protein spots were altered by >2-fold in the 2D gels from patients with BRVO-induced macular edema (Fig. 3).

Identification of proteins. Based on the results presented above, 56 protein spots were isolated for further analysis. Each spot was acquired from the gel and digested extensively with trypsin. The resulting peptides were applied to a MALDI TOF/TOF MS for measurements. A total of 49 protein spots were identified by MS, including fibroblast growth factor-4 (FGF-4), hepatoma-derived growth factor (HDGF) and crystallins. Many of these proteins have been implicated in angiogenesis, oxidative stress and collagen synthesis. The identified proteins [name, function, molecular weight (MW), isoelectric point (PI) and sequence coverage] are listed in Table II.

Gene Ontology analysis. All the proteins identified in this study were subjected to Gene Ontology (www.geneontology.org/) for molecular function, biological process and cellular component analysis. The results are listed in Table II.

Aqueous levels of CRYAA in BRVO patients and controls. Aqueous levels of CRYAA in the AH of BRVO patients or controls were below the minimum detectable concentration (data not shown).

Discussion

BRVO is a common cause of retinal vascular abnormality and a frequent cause of visual loss. Macular edema is the most frequent cause of visual impairment in patients with BRVO. Therefore, understanding the cellular and molecular factors that underlie the pathogenesis of macular edema with BRVO is of particular importance. However, the majority of studies on BRVO have focused on the treatment and only a few studies have emphasized the pathogenesis of macular edema due to BRVO (5,18-22,26).

To assess the severity of macular edema with BRVO by obtaining a sample of the AH or vitreous fluid at surgery is of critical importance. Several cytokines and other factors in the ocular fluid have been suggested to be involved in the pathogenesis of macular edema due to BRVO, such as VEGF and

Table II. Identified proteins associated with BRVO-induced macular edema.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
411	NP_085915	Lens intrinsic membrane protein 2, 19 kDa isoform 1	LIM2	Structural constituent of eye lens	Cell-cell junction assembly	Cell junction/integral to membrane	161	24,514	10.04	30%	11	Bs only
414	AAH57766	Ninjurin-2	NINJ2	Protein binding	Nervous system development/neuron cell-cell adhesion/tissue regeneration	Integral to plasma membrane	172	17,461	9.52	53%	15	Bs only
418	NP_034601	Neuron-specific calcium-binding protein hppocalcin	Hpca	Actin binding/calcium ion binding		ND	200	22,527	4.87	72%	14	Bs only
420	NP_037370	DnaI homolog subfamily C member 15	DNAJC15	Heat shock protein binding	ND	Cytoplasm/focal adhesion/integral to membrane/nucleus	443	16,373	10.08	100%	27	Bs only
434	BAC81643	PMS2-C terminal-like	PMS2CL	ATP binding/mismatched DNA binding	Mismatch repair	ND	182	21,851	5.85	58%	14	Bs only
436	AAF37290	p37 TRAP/SMCC/PC2 subunit	Mediator of RNA polymerase II transcription subunit 27 (MED27)	Protein binding/co-activator activity/transcription regulator activity	Regulation of transcription from RNA polymerase II promoter/transcription initiation from RNA polymerase II promoter	Cytoplasm/nucleolus/transcription factor complex	202	31,540	9.45	52%	17	Bs only
438	AAH45686	ANAPC5 protein	ANAPC5	Binding	ND	ND	123	36,007	7.18	40%	10	Bs only
446	BAG62828	cDNA FLJ58860, highly similar to <i>Homo sapiens</i> trichoplein, mRNA		ND	ND	ND	300	27,353	9.69	60%	33	Bs only
450	NP_001138549	LRRC10-like protein		Protein binding	ND	ND	201	32,864	6.88	63%	17	Bs only
454	AAH20843	HAVCR2 protein	Hepatitis A virus cellular receptor 2	ND	ND	Integral to membrane	119	16,594	5.15	27%	9	Bs only
467	AAK07550	PNAS-139		ND	Regulation of actin filament polymerization	Cytoskeleton	170	22,952	5.83	50%	13	Bs only
474	EAW87342	hCG15971, isoform CRA_b		ND	ND	ND	228	13,907	5.24	78%	16	Bs only
487	Q6NV95	Putative protein PABPC1-like		ND	ND	ND	146	30,256	8.88	43%	16	Bs only
502	AAP49001	Programmed death ligand 2 type III isoform	PDCD1LG2	Receptor activity	Immune response	Endomembrane system/extracellular region/integral to membrane/plasma membrane	96	2,1017	6.94	25%	8	Bs only
510	AAF64257	BM-001 (cyclin-L1)	CCNL1	ND	Regulation of transcription/transcription	Nuclear speck	121	37,420	11.18	29%	13	Bs only

Table II. Continued.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
524	NP_001092	Actin, cytoplasmic 1 (β actin)	ACTB	ATP binding/ kinesin binding/ nitric-oxide synthase binding/ nucleotide binding/ protein binding/ protein kinase binding/ structural constituent of cytoskeleton	Axogenesis/cellular component movement	MLL5-L complex/ NuA4 histone acetyltransferase complex/axon/ cortical cytoskeleton/ cytoplasm/ cytoskeleton/cytosol/ protein complex/ ribonucleoprotein complex/soluble fraction	109	42,052	5.29	31%	11	Bs only
533	BAG58981	cDNA FLJ55029, highly similar to creatine kinase, ubiquitous mitochondrial (EC 2.7.3.2)		Kinase activity	ND	ND	61	16,823	7.82	21%	5	Bs only
538	NP_000468	Isoform 1 of serum albumin precursor	ALB	DNA binding/ antioxidant activity/ chaperone binding/ copper ion binding/ drug binding/fatty acid binding/metal ion binding/oxygen binding/protein binding/ pyridoxal phosphate binding/toxin binding	Cellular response to starvation/hemolysis by symbiont of host erythrocytes/maintenance of mitochondrion location/negative regulation of apoptosis/negative regulation of programmed cell death/transport	Extracellular region/ extracellular space/ platelet α granule lumen/protein complex	62	71,317	5.92	11%	9	Bs only
552	NP_878907	Prolyl 4-hydroxylase, alpha III subunit precursor	P4HA3	L-ascorbic acid binding/ iron ion binding/ metal ion binding/ oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen/oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen/ procollagen-proline 4-dioxygenase activity	Oxidation reduction	Endoplasmic reticulum/ endoplasmic reticulum lumen	64	61,430	6.05	16%	8	Bs only
663	EAW48192	Minichromosome maintenance deficient domain containing 1, isoform CRA_b			ND	ND	183	63,435	5.89	34%	22	Bs only

Table II. Continued.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
670	NP_786923	Oligodendrocyte transcription factor 3	OLIG3	DNA binding/ transcription regulator activity	Regulation of transcription/ transcription	Cytoplasm/ nucleus/ plasma membrane	340	29,567	9.54	56%	36	Bs only
846	AAU34193	AKNA transcript F2	AKNA	ND	ND	ND	130	45,166	10.66	29%	10	-92.434
860	NP_000385	Alpha crystallin A chain	CRYAA	Protein binding/ structural constituent of eye lens/ unfolded protein binding	M phase specific microtubule process/ actin filament organization/ anti-apoptosis/ camera-type eye development/ lens fiber cell morphogenesis/ mitochondrion organization/ negative regulation of intracellular transport/protein homooligomerization/ protein refolding/ response to heat/ response to stimulus/ visual perception	Cytoplasm	72	20,011	5.77	45%	7	Cs only
862	Q14917	SPTAN1 protein (fragment)	SPTAN1	Actin binding/ protein binding/ calcium ion binding/ calmodulin binding/ structural constituent of cytoskeleton	Actin filament capping	Cell cortex/cytoplasm/ cytoskeleton/cytosol/ membrane fraction/ spectrin	63	54,468	5.72	24%	8	Cs only
868	A6NMY6	Putative Annexin A2-like protein	ANXA2P2	Calcium ion binding/ calcium-dependent phospholipid binding/ phospholipase inhibitor activity	ND	Basement membrane/ melanosome	65	20,146	6.76	39%	13	Cs only
874	NP_001876	E3 ubiquitin-protein ligase TTC3/DCRR1	TTC3	Protein binding/ ubiquitin-protein ligase activity/ zinc ion binding	Protein K48-linked ubiquitination/ ubiquitin-dependent protein catabolic process	Nucleus	83	222,852	8.38	8%	18	Cs only
875	Q5HYJ3	Protein FAM76B	FAM76B	ND	ND	ND	157	39,753	9.35	42%	19	Cs only

Table II. Continued.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
880	NP_000385	Alpha crystallin A chain	CRYAA	Protein binding/ structural constituent of eye lens/ unfolded protein binding	M phase specific microtubule process/ actin filament organization/ anti-apoptosis/ camera-type eye development/lens fiber cell morphogenesis/ mitochondrion organization/negative regulation of intracellular transport/ protein homooligomerization/ protein refolding/ response to heat/ response to stimulus/ visual perception	Cytoplasm		20,011	5.77	45%	9	-69.84
882	NP_001876	Alpha crystallin B chain	CRYAB	Cytoskeletal protein binding/ microtubule binding/ protein binding/ protein homodimerization activity/ structural constituent of eye lens/unfolded protein binding	Aging/anti-apoptosis/ camera-type eye development/ glucose metabolic process/ microtubule polymerization or depolymerization/ muscle contraction/ muscle organ development/ negative regulation of cell growth/ negative regulation of intracellular transport/ oxygen and reactive oxygen species metabolic process/ protein folding/ protein homooligomerization/ response to estradiol stimulus/ response to heat/ response to hydrogen peroxide/ stress-activated MAPK cascade	Golgi apparatus/ Z disc/ actin filament bundle/ cell surface/ contractile fiber/ cytoplasm/ insoluble fraction/ microtubule cytoskeleton/ plasma membrane/ soluble fraction	83	20,146	6.76	52%	10	Cs only

Table II. Continued.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
886	NP_000385	Alpha crystallin A chain	CRYAA	Protein binding/ structural constituent of eye lens/unfolded protein binding	M phase-specific microtubule process/ actin filament organization/ anti-apoptosis/ camera-type eye development/lens fiber cell morphogenesis/ mitochondrion organization/negative regulation of intracellular transport/protein homooligomerization/ protein refolding/ response to heat/ response to stimulus/ visual perception	Cytoplasm	93	20,011	5.77	45%	9	Cs only
889	BAH11709	cDNA FLJ55428, highly similar to regulator of G-protein signaling 7		Signal transducer activity	G-protein coupled receptor protein signaling pathway	Heterotrimeric G-protein complex	155	46,564	8.56	24%	11	-21.561
890	CAM25565	Kinesin family member C1	KIFC1	ND	ND	ND	147	22,613	10.33	46%	10	Cs only
893	EAX10319	hCG2040067		ND	ND	ND	26	6,487	11.35	51%	2	Cs only
894	NP_001876	Alpha-crystallin B chain	CRYAB	Cytoskeletal protein binding/ microtubule binding/ protein binding/ protein homodimerization activity/ structural constituent of eye lens/ unfolded protein binding	Aging/anti-apoptosis/ camera-type eye development/glucose metabolic process/ microtubule polymerization or depolymerization/ muscle contraction/ muscle organ development/negative regulation of cell growth/ negative regulation of intracellular transport/ oxygen and reactive oxygen species metabolic process/ protein folding/ protein homooligomerization/ response to estradiol stimulus/response to heat/ response to hydrogen peroxide/stress-activated MAPK cascade	Golgi apparatus/Z disc/ actin filament bundle/ cell surface/ contractile fiber/ cytoplasm/ insoluble fraction/ microtubule cytoskeleton/ plasma membrane/ soluble fraction	180	20,146	6.76	55%	13	Cs only

Table II. Continued.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
896	BAB15368	Growth hormone-regulated TBC protein 1	GRTP1	Rab GTPase activator activity	Regulation of Rab GTPase activity	Intracellular Golgi apparatus/	95	29,398	5.74	25%	7	Cs only
897	NP_006794	AP-3 complex subunit mu-2	AP3M2	Protein binding	Intracellular protein transport/vesicle-mediated transport	Clathrin adaptor complex	207	47,175	7.15	25%	18	Cs only
906	NP_001998	Fibroblast growth factor 4 precursor	FGF4	Growth factor activity/heparin binding	Cell-cell signaling/chondroblast differentiation/fibroblast growth factor receptor signaling pathway/mesenchymal cell proliferation/positive regulation of ERK1 and ERK2 cascade/positive regulation of cell division/positive regulation of cell proliferation/signal transduction	Extracellular region	191	22,148	9.73	61%	12	-19.061
911	NP_004485	Hepatoma-derived growth factor isoform a	HDGF	DNA binding/growth factor activity/heparin binding/nucleotide binding	Cell proliferation/regulation of transcription/signal transduction	Cytoplasm/extracellular space/nucleus	216	26,886	4.7	68%	16	-20.769
920	NP_060011	Beta crystallin S	CRYGS	Structural constituent of eye lens	Lens development in camera-type eye/morphogenesis of an epithelium		68	21,392	6.44	45%	9	Cs only
970	NP_000487	Beta-crystallin B2	CRYBB2	Structural constituent of eye lens	Response to stimulus/visual perception		413	23,479	6.5	75%	30	Cs only
979	NP_000487	Beta crystallin B2	CRYBB2	Protein homodimerization activity/structural constituent of eye lens/structural molecule activity	Camera-type eye development/response to stimulus/visual perception		110	23,479	6.5	65%	13	Cs only
985	AAH20718	CFI protein	CFI	Peptidase activity/scavenger receptor activity/serine-type endopeptidase activity	Complement activation, classical pathway/innate immune response/proteolysis	Extracellular region/extracellular space/membrane	62	44,315	8.49	33%	9	Cs only
1009	BAA20881	HLA-A26	HLA-A26	ND	Antigen processing and presentation/immune response	MHC class I protein complex	162	24,393	9.54	67%	17	Cs only

Table II. Continued.

ID	Accession no. ^a	Definition	Name	Molecular function ^b	Biological process	Cellular component	Mascot score	Nominal mass (Mr)	Calculated pI value	Sequence coverage ^c	No. of matched peptides	Ratio ^d
1011	AAG49447	LYST-interacting protein LIP8 (centrobin)	CNTROB	Protein domain specific binding	Centriole replication/ centrosome separation/ cytokinesis	Centriole	73	13,652	10.43	23%	6	Cs only
1155	Q8NB50	Isoform 2 of Zinc finger protein 62 homolog	ZFP62	Metal ion binding/ nucleic acid binding/ zinc ion binding	Regulation of transcription	Intracellular/ nucleus	62	58,765	9.34	23%	11	5.58146
1218	NP_000468	Isoform 1 of serum albumin precursor	ALB	DNA binding/ antioxidant activity/ chaperone binding/ copper ion binding/ drug binding/ fatty acid binding/ metal ion binding/ oxygen binding/ protein binding/ pyridoxal phosphate binding/toxin binding	Cellular response to starvation/hemolysis by symbiont of host erythrocytes/ maintenance of mitochondrion location/ negative regulation of apoptosis/ negative regulation of programmed cell death/ transport	extracellular region/ extracellular space/ platelet α granule lumen/protein complex	245	71,317	5.92	47%	33	12.2397
1247	CAI56771	Protein-cysteine N-palmitoyltransferase HHAT (hypothetical protein)	HHAT	GTP binding/ acyltransferase activity	Multicellular organismal development	Endoplasmic reticulum membrane/integral to membrane	106	9,436	7.82	40%	7	10.786
1348	NP_000468	Isoform 1 of serum albumin precursor	ALB	DNA binding/ antioxidant activity/ chaperone binding/ copper ion binding/ drug binding/ fatty acid binding/ metal ion binding/ oxygen binding/ protein binding/ pyridoxal phosphate binding/toxin binding	Cellular response to starvation/ hemolysis by symbiont of host erythrocytes/ maintenance of mitochondrion location/ negative regulation of apoptosis/ negative regulation of programmed cell death/ transport	Extracellular region/ extracellular space/ platelet α granule lumen/ protein complex	92	71,317	5.92	25%	16	Cs only
1612	EAW90919	Dynamin 3, isoform CRA_e		ND	ND	ND	377	46,798	8.21	67%	32	-10.998

The protein name, Swiss-Prot protein database accession number and Mascot score are shown, followed by molecular mass (Mr), isoelectric point (pI), number of matching peptides, sequence coverage and ratio. ^aReference for the protein identification; ^bND, no description; ^csequence coverage of the matched peptides in protein; ^dabundance ratio between BRVO-induced macular edema and controls. A ratio >2 or <2 indicates statistical significance at p<0.01. The fold change of downregulation is shown as a negative number, and the fold change of upregulation is shown as a positive number. Bs, proteins identified only in patients with BRVO; Cs, proteins identified only in the controls.

IL-6 (5,18,19). However, the surgical harvesting of vitreous fluid is associated with the risk of vitreous haemorrhage, retinal tears and retinal detachment, whereas it is difficult to obtain vitreous samples for diagnostic or investigative purposes without performing surgery. On the other hand, obtaining AH samples is a far easier and less risky procedure. AH can be collected directly from patients and AH proteins may manifest discrete changes in patients with BRVO.

To date, only a few studies have examined the changes in AH protein expression in patients with BRVO. The exact changes in protein expression that occur in AH in patients with BRVO are unclear. Therefore, it was considered of importance to study the changes in AH protein expression in patients with BRVO. Such information may provide new insight into the mechanisms of BRVO and identify potential biomarkers of this condition. AH is valuable for understanding eye disorders and certain studies have tried to identify the majority of the proteins in AH of patients with cataract (7,23-25). Previous studies have suggested that AH proteins activate signaling cascades, which subsequently regulate cellular functions, including mitosis, differentiation, motility, apoptosis and angiogenesis. Such proteins may play a vital role in the pathology of macular edema secondary to BRVO. Noma *et al* found that the aqueous level of VEGF reflected its vitreous level (26); thus, in this study, we investigated the pathogenesis of macular edema induced by BRVO by measuring alterations in protein expression in AH samples from patients with BRVO.

Proteomic analysis is a valuable method for elucidating the molecular nature of AH. Some studies have used this technology to explore the pathogenesis of various eye diseases (6,8-12). In this study, we conducted proteomic analysis of AH from patients with macular edema induced by BRVO and age-matched patients with cataract (controls). The abnormal expression and distribution of proteins in AH were identified. The patterns of 2-DE gels in the patients with BRVO differed from the controls, which indicated that the protein content in the AH changes with the development of BRVO. Proteomics revealed that 56 protein spots were altered by >2-fold, which suggests that there are complex mechanisms involved in the pathogenesis of BRVO.

Several of the proteins identified by proteomics have been implicated in angiogenesis, oxidative stress and collagen synthesis. Such proteins may play a vital role in the pathogenesis of macular edema induced by BRVO.

FGF-4 was found to be downregulated in this study. FGF-4 is a member of the fibroblast growth factor family and it induces the proliferation, migration and survival of several cell types, including endothelial cells. FGF-4 induces vascular permeability, therapeutic angiogenesis and arteriogenesis comparable to that of VEGF (27). Other results point to an indirect angiogenic activity of FGF-4 through the autocrine induction of VEGF secretion (28,29). In a previous study, the induction of the angiogenic morphotype and the parallel modulations of the biosynthetic phenotype in human umbilical vein endothelial cells were completely suppressed by a neutralizing antibody directed against VEGF (28).

HDGF was found to be downregulated in this study, which is mitogenic for vascular smooth muscle and aortic endothelial cells. HDGF is a highly expressed vascular endothelial cell protein *in vivo* and is a potent endothelial mitogen and

regulator of endothelial cell migration by mechanisms distinct from VEGF. As previously demonstrated, with the chick chorioallantoic membrane (CAM), a bioassay for angiogenesis, exogenous recombinant HDGF significantly stimulated blood vessel formation and a dose-dependent reorganization of cells within the CAM into a more compact, linear alignment reminiscent of tube formation (30).

Some crystallins were identified to be downregulated in this study. Alpha crystallins are chaperones belonging to the small heat shock protein family. Certain studies have suggested that the expression of alphaA and alphaB crystallin is related to oxidative stress (31,32). AlphaB-crystallin (CRYAB) plays an important role as a chaperone for VEGF-A in angiogenesis. The attenuation of intraocular angiogenesis has been observed in CRYAB knockout [CRYAB (-/-)] mice in 2 models of intraocular disease: oxygen-induced retinopathy and laser-induced choroidal neovascularization. VEGF-A protein expression was low in CRYAB (-/-) mouse retinas compared with wild-type mouse retinas. CRYAB (-/-) retinal pigment epithelial (RPE) cells showed low VEGF-A secretion under serum-starved conditions compared with wild-type cells. CRYAB can bind to VEGF-A but not transforming growth factor- β in cultured RPE cells. CRYAB and VEGF-A are co-localized in the endoplasmic reticulum in RPE cells under chemical hypoxia. Endothelial cell apoptosis in newly formed vessels was greater in CRYAB (-/-) than wild-type mice (33). Ghosh *et al* found that human CRYAB peptides have strong interactions with FGF-2 and VEGF, which are both related with angiogenesis. Chaperone assays confirmed the ability of CRYAB to protect against the aggregation of FGF-2 and VEGF (34). α - and β -crystallin isoforms are overexpressed with diabetes, as shown by proteomics and confirmed by immunoblotting (35). These data suggest that crystallins may function together with VEGF during angiogenesis.

Complement factor I (CFI) was also downregulated in our study. In a previous study, CFI was found to be increased in proliferative diabetic retinopathy vitreous compared with non-diabetic vitreous by a comprehensive proteomic analysis [one-dimensional SDS-PAGE and nano-liquid chromatography (LC)/MS/MS] (36). Thus, CFI may also play a role in angiogenesis.

FGF-4, platelet-derived growth factor (PDGF), crystallins and CFI, which are tightly associated with angiogenesis, were all downregulated in this study. This may be due to the fact that the patients in this study had a long course of disease and the angiogenic tissues had become quiescent. Further investigations are required.

Albumin, anaphase promoting complex subunit 5 (ANAPC5) and β -actin were found to be all upregulated in this study. Human serum albumin (HSA) is the most abundant protein in the circulatory system, and one of its principal functions is to transport fatty acids (37). Albumin is also a very abundant and important circulating antioxidant (38). HSA inhibits endothelial apoptosis in a highly specific manner (39). Increased vascular disease occurs with low albumin, possibly reflecting the specific inhibition of endothelial apoptosis reported for tissue culture (40). Serum albumin has been significantly associated with the severity of retinopathy and neuropathy in patients with type 2 diabetes (41). It has been reported that the expression of ANAPC5 may represent an

important event in the pathogenesis of vascular proliferative diseases (42).

Actins are highly conserved proteins that are involved in cell motility, structure and integrity. β -actin is a major constituent of the contractile apparatus and one of the two non-muscle cytoskeletal actins. β -actin as a transcription factor also stimulates endothelial nitric oxide synthase (eNOS) expression (43).

Of note, some proteins identified in this study may participate in collagen synthesis, the cytoskeleton and organization of the actin cytoskeleton, such as prolyl 4-hydroxylase, alpha polypeptide III (P4HA3); spectrin, alpha, non-erythrocytic 1, alpha-fodrin (SPTAN1); actin related protein 2/3 complex, subunit 2, 34 kDa (ARPC2, also known as PNAS-139). These events are critical for driving a wide range of cellular processes, including motility, endocytosis and intracellular trafficking. Previous studies have demonstrated that the level of IL-6 is increased in the AH of diabetic patients with macular edema and in patients with macular edema induced by BRVO (5,44). IL-6 can induce an increase in endothelial permeability *in vitro* by rearranging actin filaments and by changing the shape of endothelial cells (45). Thus, actin filaments may participate in the pathogenesis of macular edema due to BRVO.

Other proteins identified play crucial roles in photoreceptor or retina functions. Some proteins identified in this study may control cell cycle progression and/or apoptosis, immune responses and oxidative stress. Further investigations are required to fully understand the exact association between changes in AH protein expression and BRVO.

Although a comparison of AH samples from patients with BRVO with and without macular edema may be more useful to analyze the proteins in patients with BRVO that may be involved in the development of macular edema, it is not easy to obtain AH samples from patients with BRVO without macular edema. Moreover, AH from normal healthy adults cannot be obtained ethically. Therefore, we used patients with cataract as the controls in this study, as previously done by others (6,8-11). Therefore, it is possible that some of the proteins identified in our study are present due to the underlying cataract condition. However, the patients with BRVO were all senile and age-matched with the controls and all had cataract which did not require surgery. Thus, the 2 groups were comparable.

Due to the use of pooled samples, the results in this study do not provide any information as to the variation between patients within a group. Thus, it is possible that patient-to-patient variability exists within the current study. However, the use of pooled samples should reduce the component of patient-to-patient variation and reveal overall differences between patients and controls, as previously described (45). The use of lesser numbers of pooled AH is a disadvantage in this aspect. The influence of patient-to-patient variation will be addressed in a subsequent study. Our results may prove valuable for future research in the pathogenesis in BRVO.

In conclusion, the results of the present study revealed that the proteomic composition of AH was differed significantly between the patients with macular edema with BRVO and the controls. The proteins identified may serve as potential biomarkers for macular edema induced by BRVO.

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