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Proteomic analysis of aqueous humor proteins associated with cataract development

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ABSTRACT

Background: Cataract is one of the most common eye diseases that can further cause blindness. Discovering susceptibility factors contributing to cataract development is helpful in identifying predisposed patients and improving treatment efficacy. Although proteomics technology has been used in study of protein markers related to eye diseases, few were on studies of cataract development.

Methods: To explore cataract-associated susceptibility factors in aqueous humor (AH), a quantitative proteomics study using the iTRAQ methodology was employed to compare AH protein profiles between high myopia patients & controls, glaucoma surgery patients & controls, and vitrectomy surgery patients & controls, respectively.

Results: A total of 445 AH proteins were identified, and 210 proteins were differentially expressed between myopia patients and controls, 262 proteins were differentially expressed between glaucoma surgery patients and controls, and 161 proteins were differentially expressed between vitrectomy surgery patients and controls. Among the 445 identified proteins, 77 were considered to be cataract-associated, and 5 of the 77 proteins were randomly selected and confirmed by ELISA assay. Biological functions of these 77 proteins were annotated by GO/pathways analysis. Additionally, 17 proteins were found to be involved in protein–protein interaction networks, 5 of which were associated with nervous system disease and eye diseases including cataract.

Conclusions: The identified candidate protein biomarkers associated with cataract development may lead to more insights into the underlying mechanisms of cataract disease.

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1. Introduction

Cataract is one of the most prevalent eye diseases, and numerous studies have been conducted to investigate the underlying mechanisms of cataract development for potential prevention and treatment strategies [1–4]. Many evidences have showed that age is the biggest risk factor [5]. There are three types of age-related cataracts, including cortical cataracts, nuclear cataracts, and posterior subcapsular cataracts (PSCs) that differ in optical density, shape, color, age, size, and location.

Besides aging, environmental factors and genetics are also involved in cataract development. Prolonged UV exposure can increase cataract risk [6]. Diabetes, glaucoma, myopia, etc. are also associated with the formation of cataract [7]. In European and African populations, diabetes is found to be associated with age related posterior subcapsular and cortical cataract, but probably not nuclear cataract [8]. Cigarette smoking has consistently been identified as a risk factor for nuclear and possibly posterior subcapsular cataract, but not cortical cataract. In 2013, Pan et al. reported that myopia was associated with nuclear cataract, in addition, especially high myopia, may predispose PSC [9]. The report

showed that the OR of myopia for PSC increased dramatically once myopia was defined as spherical equivalent of more than -5.0 D to -6.0 D.

Recently, proteomic analysis has been performed in various ocular diseases including diabetic retinopathy and cataract, which allows the simultaneous detection of a large number of proteins in specific cells, tissues or body fluids at a given time [10–14]. The main objective of the proteomic approach in these studies is to identify protein or peptide disease biomarkers for study of disease mechanisms, diagnosis and novel therapies. Previous studies of ocular fluids included tears, aqueous humor, and vitreous. Notably, vitreous humor accounts for over 80% of the eyeball volume and is the major fluid where sight of damage happens. Many proteins have been identified in vitreous humor, including albumin, transferrin, IgG, apolipoprotein, transthyretin serum, and a group of enzymes known as matrix metalloproteinases (MMPs) mediating the structure and function of extracellular matrix [15]. Besides vitreous humor, aqueous humor (AH) is also an important intraocular fluid which supply nutrients and remove metabolic wastes from avascular tissues in the eye. An increasing number of studies have identified the correlation between altered AH proteins and prognosis of many eye diseases including wet age-related macular degeneration (AMD) and myopia [16,17]. Previous studies have revealed that some protein groups such as antioxidant proteins, anti-angiogenic proteins, and

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immunoregulatory proteins were abundant in AH [18]. Those proteins have important roles in regulating homeostasis in the eye and are potential biomarkers for identifying eye disease earlier.

Many previous proteomic studies of ocular fluids, including AH, are performed using 2-DE, which required pooling of large amount of samples due to the low protein concentration in ocular fluids [15]. One technique called isobaric tagging for relative and absolute protein quantification (iTRAQ) in combination with mass spectrometry has been proved to be a sensitive quantitative proteomic method for high throughput protein identification and quantification [19]. In this study, we used iTRAQ combined with RPLC/RPLC–MS to conduct comparative proteomic profiling of AH from three patient/control groups—high myopia/control, after glaucoma surgery/control, and after vitrectomy surgery/control. The results revealed remarkable proteomic differences between patients and controls. These findings discovered potential AH biomarkers and susceptibility factors for predicting cataract development, and provide insights into the underlying mechanisms associated with high myopia, glaucoma, and vitrectomy.

2. Materials and methods

2.1. Subjects

Twenty-four subjects were recruited for this study, which were divided into four groups: 6 with high myopia, 6 after glaucoma surgery, 6 after vitrectomy surgery, and 6 controls (Table 1). All 24 subjects met the inclusion criteria of no history or slit-lamp evidence of ocular trauma, no ocular disease other than cataract, and no use of systemic antimetabolites, immunosuppressants, or corticosteroids. The average age of the patients was 58 (ranging from 49–68), and the average control age was 61 (ranging from 53–66). Other information for these subjects including axial length and clinical application of the lens opacities classification system III (LOCSIII) was also listed (Table 1). The study protocol was reviewed and approved by the Ethics Committee of Eye & ENT Hospital of Fudan University, Shanghai, China.

2.2. Surgical sample collection and preparation

Collection of AH was conducted as previously reported [10]. After collection, AH samples were immediately transferred to dust-free

Table 1
Summary of human aqueous humor samples.

Group (labeled ID)	Patient ID	Gender	Age (years old)	Axial length	LOCSIII
Controls (114)	1	Male	53	24.75	C2N2P3
	2	Female	57	23.8	C3N2P2
	3	Male	60	24.47	C3N3P3
	4	Male	64	24.8	C3N3P4
	5	Female	66	24.04	C3N3P2
	6	Female	66	21.69	C2N3P3
Patients with high myopia (116)	1	Female	49	26.38	C2N3P4
	2	Male	53	28.68	C3N3P3
	3	Female	53	30.16	C4N5P3
	4	Female	58	28.84	C3N5P2
	5	Male	59	25.38	C2N3P4
	6	Male	62	27.14	C4N4P5
Patients after glaucoma surgery (115)	1	Male	50	24.51	C2N2P2
	2	Male	60	23.8	C3N3P3
	3	Female	61	27.22	C3N2P2
	4	Female	63	24.47	C3N3P1
	5	Female	64	23.09	C4N4P3
	6	Male	68	24.55	C3N3P3
Patients after vitrectomy surgery (113)	1	Male	52	27.92	C3N3P5
	2	Male	53	25.17	C3N3P3
	3	Female	54	27.71	C4N3P3
	4	Female	60	23.91	C2N3P2
	5	Female	60	25.33	C3N3P4
	6	Male	66	24.45	C2N2P4

Eppendorf tubes containing 3 μ L 50 \times protease inhibitor cocktail, mixed, and stored for a maximum of 4 h at 4 $^{\circ}$ C. Samples were then centrifuged at 1600 \times g in 4 $^{\circ}$ C for 15 min in a refrigerated centrifuge (Eppendorf 5804R). The supernatant was collected and centrifuged again at 16,000 \times g in 4 $^{\circ}$ C for 15 min to further remove cellular organelles and debris from apoptotic cells. The supernatant was then collected and stored at -80° C until further analysis.

2.3. Proteomic profiling by iTRAQ labeling

The proteomic profiling of all samples was conducted as previously reported [20]. The control group proteins were labeled with reporter tag 114, while the other three group proteins were labeled with reporter tag 116 (high myopia patients), 115 (glaucoma surgery patients), and 113 (vitrectomy surgery patients), respectively. Proteins whose fold changes were more than 1.5 or less than 0.6 between patients and control groups were designated to be differentially expressed.

2.4. Enzyme linked immunosorbent assay (ELISA) assay

Five differentially expressed proteins were randomly selected: spondin-1 (SPON1), lysozyme C (LYSC), chondroadherin-like protein (HOY415), serum amyloid P-component (SAMP), and angiopoietin-related protein 7 (ANGL7). The ELISA assay kits were used to confirm their changes including SPON1 (DRE12753, JRDun Biotech, Shanghai, China), LYSC (DRE10245, JRDun Biotech, Shanghai, China), HOY415 (DRE12760, JRDun Biotech, Shanghai, China), SAMP (DRE11650, JRDun Biotech, Shanghai, China), and ANGL7 (DRE12762, JRDun Biotech, Shanghai, China). Each assay was done with repeat.

2.5. Bioinformatics analyses

The 77 differentially expressed proteins were annotated according to GO database (<http://www.geneontology.org/>). Pathway annotation of these proteins was conducted by searching against the Kyoto Encyclopedia of Genes and Genomes database (<http://www.genome.jp/kegg/pathway.html>). Functional regulatory network analysis of these proteins was performed using web-based tool STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, <http://string.embl.de/>). Statistical analyses including independent t-test were performed using SPSS 17.0 software, and $p \leq 0.05$ or $p \leq 0.01$ were considered to be significant.

3. Results and discussion

3.1. Proteomic analysis of human aqueous humor

To investigate the mechanisms in cataract development, a high throughput quantitative proteomics study using the iTRAQ methodology was employed to profile human aqueous humor from three types of patients and their controls (Table 1). The three types of patients were high myopia, after glaucoma surgery, and after vitrectomy surgery. A total of 445 proteins were identified (Table S1) and this is by far the largest number of identified human aqueous humor proteins in a single proteomic study. Among the 445 proteins, 334 have been reported in aqueous, cornea, vitreous, tears, choroid, and retina, while the rest 111 proteins were first reported in this study according to the human eye proteome project [21]. Notably, 224 proteins were detected for the first time in aqueous, demonstrating the power of proteomic profiling in uncovering new human aqueous humor proteins. These 445 proteins perform catalytic, complement, enzymatic, signaling, structural, transporting, and other functions. As shown in Fig. 1, 15% of the 445 proteins were involved in signaling pathway, 15% were involved in catalytic pathway, while 6% were transporters.

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