iTRAQ-based quantitative proteomic analysis and bioinformatics study of proteins in retinoblastoma

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Abstract. The aim of the present study was to analyze proteins in the aqueous humor (AH) of patients' retinoblastoma (RB), and investigate their potential role in RB using the comparative proteomic technique of isobaric tags for relative and absolute quantitation (iTRAQ) coupled with offline two-dimensional liquid chromatography-tandem mass spectrometry. A total of 0.1 ml AH was collected from 10 children with RB (mean age, 3.8 years; range, 2-5 years) and patients with senile cataracts (mean age, 70.4 years; range, 65-79 years), which was used as the control. iTRAQ was used to analyze proteins in the AH of patients and controls. Proteins with a fold change of >1.20 or <0.83 were considered to be significantly differentially expressed (with corrected P<0.05). The identified proteins were subjected to subsequent gene ontology (GO) analysis using the DAVID database. A total of 83 proteins that were expressed differently between the controls and patients' AH samples were identified using iTRAQ analysis. Of these proteins, 44 were upregulated and 39 were downregulated. On the basis of biological processes in GO, the identified proteins were primarily involved in glycoprotein, amyloid acute-inflammatory and defensive responses. Among these proteins, pigment epithelium-derived factor serves a potential role in the treatment of RB, and stimulated by retinoic acid 6 may serve as a potential protein involved in RB development. To the best of our knowledge, the present study is the first to identify 83 proteins associated with RB using iTRAQ technology. The results of the present study will aid in furthering the understanding of RB and developing novel therapy targets in the future.

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Introduction

Retinoblastoma (RB) is the most common primary type of intraocular malignancy among children. It originates from the primitive stem cells in the nuclear layer of the retina. Its prevalence is between 1/15,000 and 1/18,000 cases/people, with 95% of cases occurring before the age of 5 years (1). The main symptoms of RB are leukokoria and strabismus. The RB transcriptional corepressor 1 (RB1) gene located on chromosome 13 is associated with RB. The RB1 gene, which produces the RB protein, serves an important role in regulating and controlling the cell cycle (2). Loss-of-function mutations in RB1 disrupt the cell cycle and have been revealed to be an important initiating event prior to the development of RB (3).

Besides the RB1 gene, other genes and proteins have been observed to serve important roles in the pathogenesis of RB. For example, 1% of all cases of RB have high levels of MYCN proto-oncogene bHLH transcription factor amplification and no RB mutations. Furthermore, a previous study demonstrated that proteins associated with the redox signaling pathways are involved in RB pathogenesis (4).

In order to further investigate the pathogenesis of RB, the present study performed the quantitative proteomic strategy of using isobaric tags for relative and absolute quantitation (iTRAQ) coupled with two-dimensional liquid chromatography-tandem mass spectrometry (MS) in order to identify associated proteins. The main advantage of the iTRAQ technique for proteomic analysis is that its multiplexing capability allows various protein samples to be simultaneously quantified with a control-standard sample that is processed in the same run (5). The insights the present study have gained may be used for further research of RB in pursuit of a novel therapy target.

Materials and methods

Subjects. Patients and control subjects were recruited from the Department of Ophthalmology, Peking University People's

Hospital (Beijing, China). The present study was approved by the Clinic Institutional Review Board of Peking University People's Hospital and complied with the Declaration of Helsinki. Written informed consent was obtained from all patients prior to enrollment in the present study. A total of 10 patients (2 women and 8 men; mean age, 3.8 years; range, 2-5 years) diagnosed with RB between September 2014 and March 2015 were included and 10 patients with cataracts (3 women and 7 men; mean age, 70.4 years; range, 65-79 years) were recruited as controls. The inclusion criteria were as follows: Diagnosis of group D RB in accordance with the International Classification of Retinoblastoma (6) with clear optical media in poor responders to chemotherapy, laser or cryotherapy. Patients with RB or control subjects with a history of any systemic or ocular disorder or condition (including ocular surgery, trauma and disease) were excluded from the current study.

Sample collection. Aqueous humor (AH) samples from patients with RB and control subjects were collected. The whole procedure was performed using a microscope viewing through a dilated pupil, followed by intravitreal injections. As previously described (7), anterior chamber paracentesis was performed through a clear cornea limbus track created with a 25G MVR blade without perforating the Descemet's membrane. A 32G needle mounted on a tuberculin syringe was then introduced through the track tangentially into the anterior chamber periphery, parallel to the iris. A volume of 0.1 ml aqueous fluid was aspirated, registered and stored at -80˚C until processing. For patients with RB, three cycles of freeze and thaw (6 sec each) were applied at the injection site at the time of removal of the needle.

Protein extraction and digestion. Total AH protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol and as described in a previous study (8). Each sample (200 μg) was reconstituted in SDS-PAGE sample buffer with 5% β-mercaptoethanol, and electrophoresed on a (10-14.5%) SDS-PAGE precast gel (Criterion; Bio-Rad Laboratories, Inc.). A total of nine gel slices were excised from each lane. In-gel digestion was performed as previously reported (8). Briefly, the excised bands were de-stained using 40 mM ammonium bicarbonate in 50% acetonitrile (ACN) solution (45˚C for 20 min). The de-stained gel bands were then subjected to reduction using 5 mM dithiothreitol (60˚C for 45 min), followed by alkylation using 10 mM iodoacetamide (56˚C for 60 min). The gel pieces were dehydrated using 100% ACN, followed by digestion with trypsin (modified sequencing grade; Promega Corporation, Madison, WI, USA) at 37˚C for 12-16 h. The peptides were extracted from the gel fragments by treating the gel bands twice with 0.4% formic acid and 3% ACN solution once with 0.4% formic acid and 50% ACN solution and finally with 100% ACN solution (all at room temperature for 15 min each). The samples were labeled with iTRAQ® reagents by adding the contents of the iTRAQ Reagent-8Plex Multiplex kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to the sample solutions. The two samples were iTRAQ-labeled as follows: R0 (RB) and C4 (Control).

Two-dimensional liquid chromatography-electrospray ionization MS. Sample analysis was performed using a QTRAP 5500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) to generate MS and tandem MS (MS/MS) data. Peptides were loaded onto a Kinetex 100.0x2.1-mm C18 column (300 Å, 2.6 μm; Phenomenex, Torrance, CA, USA) and then submitted to mobile-phase elution in buffer A (0.1% formic acid in water) and buffer B (0.1 % formic acid in acetonitrile), according to the manufacturer's protocol. The peptides were eluted at a flow rate of 400 μl/min. The liquid chromatography eluent was directed to an electrospray ionization source for quantitative time-of-flight MS analysis. Electrospray ionization was performed for information-dependent acquisition in positive-ion mode with a spray voltage of 1.8 kV and a selected mass range of 350-2,000 *m/z*. The QTRAP 5500 system was operated in data-dependent acquisition mode. The three most abundantly charged peptides above a 5-count threshold were selected for MS/MS.

Database search. All MS/MS samples were analyzed using Mascot (version 2.4.1; Matrix Science, London, UK). Mascot was set up to search the Uniprot2014_human database (www.uniprot.org; accessed January 2014). The analysis and search parameters were as follows: Trypsin as the digestion enzyme with allowance for a maximum of one missed cleavage; Carbamidomethyl (C) as a fixed modification; Oxidation (M), Gln→Pyro-Glu (N-term Q) and iTRAQplex modification (K, Y and N termini) as a variable modification; peptide mass tolerance of 15 ppm; and fragment mass tolerance of 20 mmu.

Expression changes of the identified peptides in human AH were determined and compared with the controls using the iTRAQ reporter ion intensities. Based on the relative quantification and statistical analysis, a 1.2-fold change cut-off was selected to categorize proteins as significantly altered. Therefore, proteins with iTRAQ ratios >1.2 were considered to be upregulated, whereas those with iTRAQ ratios <0.83 were considered to be downregulated.

Bioinformatics analysis. In order to characterize the function of the proteins identified in the quantitative proteomics analysis, information from the DAVID Bioinformatics website (https://david.ncifcrf.gov/) was applied to the functional enrichment and gene ontology (G) analyses, as previously described (9). In the present study, the GO categories with $P<0.05$ were considered to indicate a statistically significant expression in patients with RB.

Results

Protein identification in AH. Proteins with corrected $P<0.05$ and a fold change of >1.2 or <0.83 were considered to be significantly differentially expressed. In total, 83 proteins were identified in the AH of patients with RB by iTRAQ analysis (Table I). Of these proteins, 44 were upregulated and 39 were downregulated. The proteins with an increased fold change of >2.0 included the following: Vitamin D-binding protein, angiotensinogen, carbonic anhydrase 1, Ig κ-chain V-III region B6, Ig α-1 chain C region, α-1-antitrypsin, prothrombin, anti-thrombin-III stimulated by retinoic acid 6 (STRA6), fibrinogen λ-chain, Ig λ-2 chain C region, thyroxine-binding

Table I. Proteins identified in the aqueous humor of patients with retinoblastoma using iTRAQ analysis.

Fold change	Protein	Description
0.269	CRYGS	β-crystallin S
0.351	PSAP	Prosaposin
0.387	GPX3	Glutathione peroxidase 3
0.389	CTSD	Cathepsin D
0.478	RBP3	Retinol-binding protein 3
0.52	LGALS3BP	Galectin-3-binding protein
0.522	LDHA	L-lactate dehydrogenase α-chain
0.528	CLSTN1	Calsyntenin-1
0.571	C4A	Complement C4-A
0.572	Ig κ chain V-III region SIE	Ig κ-chain V-III region SIE
0.593	Ig κ chain V-I region Roy	Ig κ-chain V-I region Roy
0.594	LACRT	Extracellular glycoprotein lacritin
0.596	FBLN	Fibulin-1
0.598	LUM	Lumican
0.614	SERPING1	Plasma protease C1 inhibitor
0.621	APLP	Amyloid-like protein 2
0.625	PTGDS	Prostaglandin-H2 D-isomerase
0.631	CLEC3B	Tetranectin
0.66	A2M	α-2-macroglobulin
0.681	KRT9	Keratin, type I cytoskeletal 9
0.682	SPON1	Spondin-1
0.687	ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2
0.727	GSN	Gelsolin
0.73	LYZ	Lysozyme C
0.739	KRT14	Keratin, type I cytoskeletal 14
0.743	CP	Ceruloplasmin
0.75	RBP	Retinol-binding protein 4
0.758	Ig λ chain V-I region NEW	Ig λ-chain V-I region NEW
0.773	SERPINF1	Pigment epithelium-derived factor
0.777	IGFBP7	Insulin-like growth factor-binding protein 7
0.787	KRT10	Keratin, type I cytoskeletal 10
0.788	TTR	Transthyretin
0.803	CST3	Cystatin-C
0.814	Ig λ chain V-III region SH	Ig λ-chain V-III region SH
0.82	KRT1	Keratin, type II cytoskeletal 1
0.82	C2	Complement C2
0.82	IGLL5	Immunoglobulin λ-like polypeptide 5
0.832	HP	Haptoglobin
0.833	KRT2	Keratin, type II cytoskeletal 2 epidermal
1.238	Ig heavy chain V-III region GAL	Ig heavy chain V-III region GAL
1.24	AZGP1	Zinc-α-2-glycoprotein
1.261	ORM1	α-1-acid glycoprotein 1
1.261	Ig heavy chain V-I region EU	Ig heavy chain V-I region EU
1.274	OPTC	Opticin OS=Homo sapiens
1.278	AMBP	Protein AMBP
1.288	APOA1	Apolipoprotein A-I
1.293	LRG1	Leucine-rich α-2-glycoprotein
1.306	Ig heavy chain V-III region BRO	Ig heavy chain V-III region BRO
1.321	CHI3L1	Chitinase-3-like protein 1
1.354	VTN	Vitronectin
1.378	HPX	Hemopexin
1.378	IGHG1	Ig γ-1 chain C region

Table I. Continued.

Fold change	Protein	Description
1.388	LCN1P1	Putative lipocalin 1-like protein 1
1.408	APOA4	Apolipoprotein A-IV
1.457	ALB	Serum albumin
1.468	ORM2	α-1-acid glycoprotein 2
1.482	A1BG	α-1B-glycoprotein
1.538	HBD	Hemoglobin subunit delta
1.546	FGA	Fibrinogen α chain
1.547	Ig heavy chain V-III region TIL	Ig heavy chain V-III region TIL
1.549	HRG	Histidine-rich glycoprotein
1.55	IGHM	Ig μ-chain C region
1.552	SMTN	Smoothelin
1.642	EFEMP	EGF-containing fibulin-like extracellular matrix protein 1
1.705	HBB	Hemoglobin subunit β
1.746	Ig heavy chain V-I region HG3	Ig heavy chain V-I region HG3
1.765	NOL6	Nucleolar protein 6
1.768	KNG1	Kininogen-1
1.975	SERPINA3	α-1-antichymotrypsin
2.012	GC	Vitamin D-binding protein
2.089	AGT	Angiotensinogen
2.124	CA1	Carbonic anhydrase 1
2.191	Ig κ chain V-III region B6	Ig κ-chain V-III region B6
2.218	IGHA1	Ig α-1 chain C region
2.267	SERPINA1	α-1-antitrypsin
2.429	F2	Prothrombin
2.437	SERPINC1	Antithrombin-III
2.479	STRA6	Stimulated by retinoic acid gene 6 protein homolog
2.573	FGG	Fibrinogen γ chain
2.605	IGHG2	Ig γ-2 chain C region
2.973	SERPINA7	Thyroxine-binding globulin
2.98	AHSG	α-2-HS-glycoprotein
3.146	HBA1	Hemoglobin subunit α

iTRAQ, isobaric tags for relative and absolute quantitation analysis.

globulin, α-2-HS-glycoprotein and hemoglobin subunit α. The five proteins with a decreased fold change of >0.5 were β-crystallin S, prosaposin, glutathione peroxidase 3, cathepsin D and retinol-binding protein (RBP) 3.

GO analysis. In order to identify the functions of proteins identified using the iTRAQ technique, the present study performed GO analysis with the assistance of DAVID Bioinformatics Resources. The DAVID classification of proteins by biological process demonstrated that the proteins were primarily involved in the defensive (27.3%), inflammatory (27.3%) and acute-phase (27.3%) responses, and the response to wounding (16.7%) (Fig. 1). On the basis of molecular function annotations, the proteins in the present study were implicated in endopeptidase inhibitor activity (21.2%), peptidase inhibitor (21.2%), enzyme inhibitor (21.2%), serine-type endopeptidase inhibitor (15.2%), structural molecule (15.2%), lipid binding (12.1%) and carbohydrate binding (10.6%) activities (Fig. 2). In the cellular component ontology, the present study revealed that the majority of enriched categories were associated with extracellular construction, including extracellular region (75.8%), extracellular region part (56.1%) and extracellular space (50%) (Fig. 3). Functional annotation clustering demonstrated that they belong to glycoprotein, amyloid, acute-inflammatory and defense responses (Fig. 4).

Discussion

RB has become curable in the majority of cases in children, so long as there is early diagnosis and accurate prognosis. In order to investigate RB, the present study performed iTARQ analysis to identify proteins that may serve a role in RB in children. A total of 44 upregulated proteins and 39 downregulated proteins were identified. Functional annotation clustering revealed that they belong to glycoprotein, amyloid, acute-inflammatory and defense responses. Among these proteins, the present study

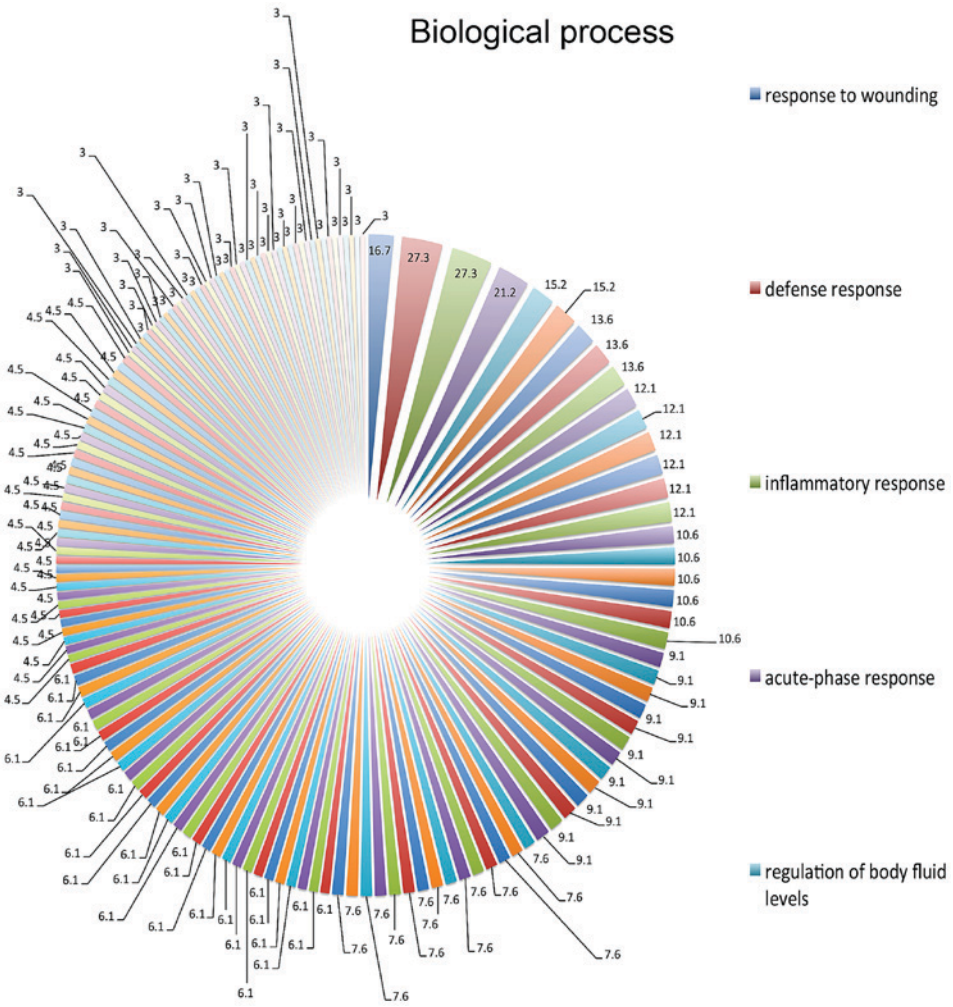

Figure 1. Functional classification of proteomic data by bioinformatics analysis. The biological process categories. Categorizations are based on information provided by the online resource, Gene Ontology, and DAVID Bio-informatics Resources.

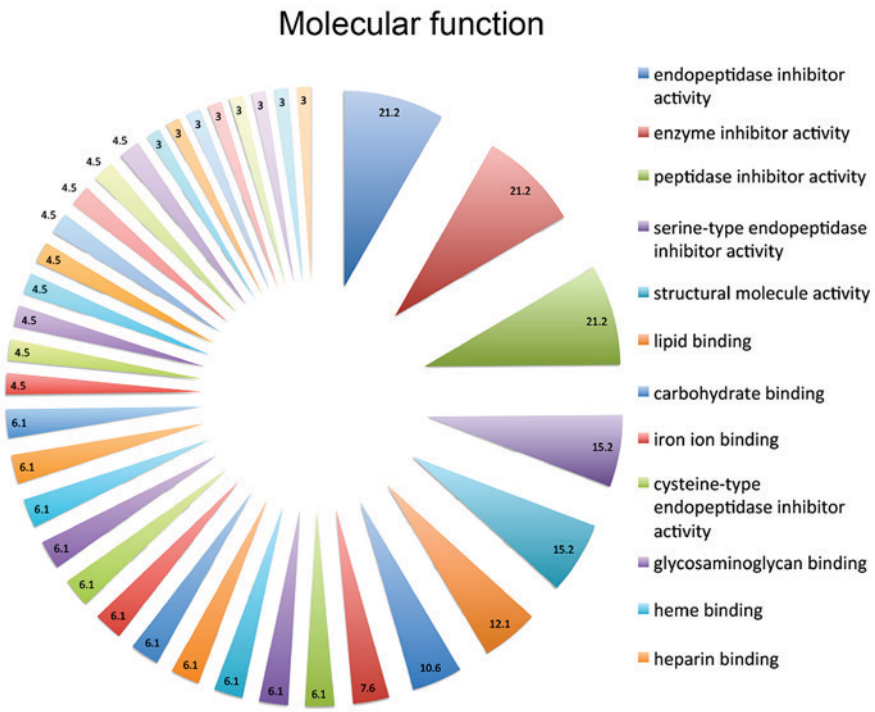

Figure 2. Functional classification of proteomic data by bioinformatics analysis. The molecular function categories. Categorizations are based on information provided by the online resource, Gene Ontology, and DAVID Bio-informatics Resources.

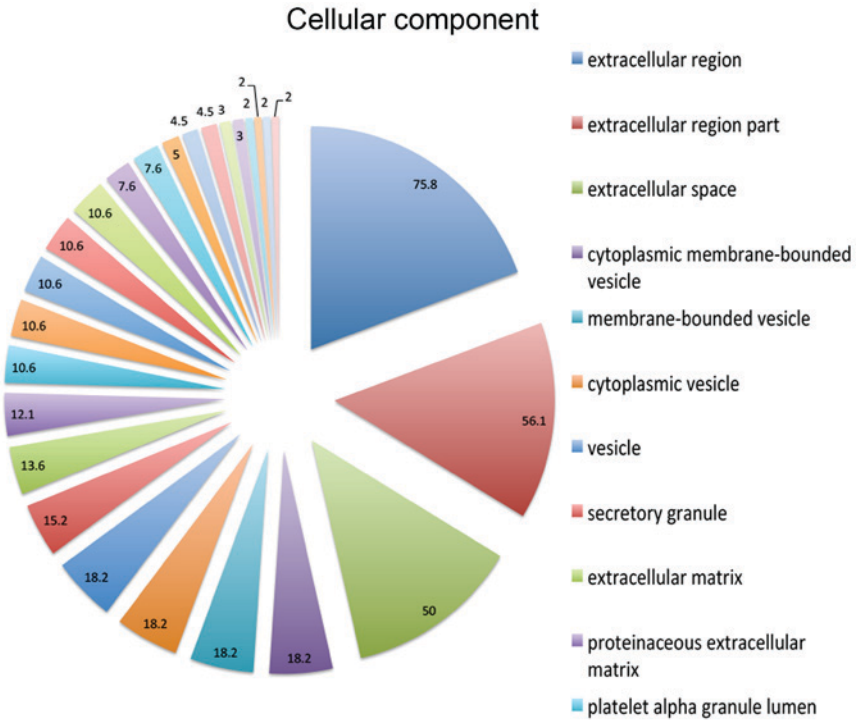

Figure 3. Functional classification of proteomic data by bioinformatics analysis. The cellular component categories. Categorizations are based on information provided by the online resource, Gene Ontology, and DAVID Bio-informatics Resources.

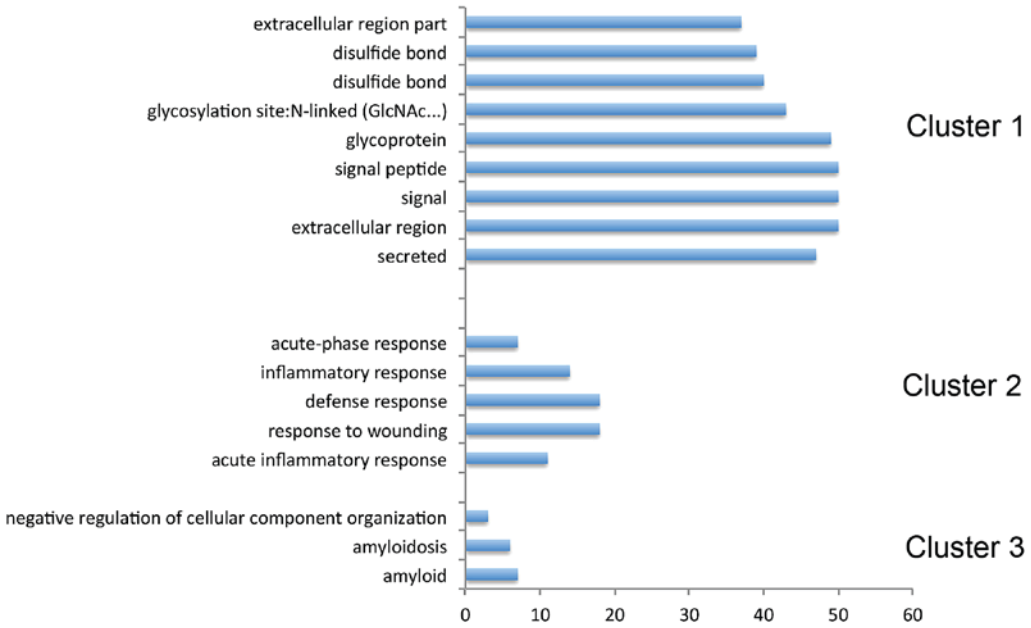

Figure 4. Functional annotation clustering determined using DAVID Bioinformatics Resources with respect to the target proteins. The x-axis represents the number of proteins and the y-axis represents the ontology categories.

speculated that pigment epithelium-derived factor precursor (PEDF) serves a potential role in the treatment of RB and STRA6 may be involved in RB development.

PEDF is a member of the serine protease inhibitor (serpin) superfamily. As a nerotrophic factor, PEDF promotes the differentiation of RB cells and other tumor cells of neuronal origin (10). It has potent anti-angiogenesis activity, and it has been revealed that PEDF may delay tumor growth and decrease the expression level of vascular endothelial growth factor (VEGF) (11). Of note, PEDF has been demonstrated to be an inhibitor of tumor cell invasion, migration and metastasis *in vitro* (12), and in numerous *in vivo* models *(*13-15*)*. It is also known that the Fas cell surface death receptor (Fas)/Fas-L/caspase-8 apoptotic signaling pathway is involved in the apoptosis of PEDF-induced endothelial cells (16). Dawson *et al* (11) identified PEDF as a potent inhibitor of angiogenesis in the eye. A number of studies have identified that PEDF overexpression could prevent ocular

neovascularization, and delay photoreceptor and neural retinal cell death *in vivo* (17-19). Yang *et al* (20) revealed that PEDF inhibited tumor angiogenesis, as microvessel density was demonstrated to have decreased in PEDF-treated tumor tissues. Further research demonstrated that PEDF may down-regulate VEGF expression *in vitro* and *in vivo* by inhibition of hypoxia-inducible factor-1α. The anti-angiogenic effect of PEDF makes it an excellent candidate as a therapy target for RB. The present study revealed that the expression level of PEDF was lower in RB compared with control samples, which was consistent with the results in a previous study (20). These results suggest the potentially pivotal role that PEDF may serve in RB treatment.

STRA6 is widely expressed during embryonic development and in adult organ systems (21). STRA6 is the receptor of RBP and transports retinol from extracellular RBP into cells (22). It also serves as a cytokine and transduces a signaling cascade via Janus kinase 2, and the transcription factors, signal transducer and activator of transcription (STAT)3 and STAT5, leading to induction of STAT target gene expression that promote oncogenic transformation (23). Overexpression of STRA6 has been observed in numerous types of human cancer, including Wilm kidney tumors, melanomas, and colorectal, ovarian and endometrium cancer (24). *In vitro*, STRA6 may facilitate cell proliferation, migration and invasion in cells. However, it was recently reported that STRA6 contributes to p53-induced apoptosis in response to DNA damage (25). The present study demonstrated that STRA6 was upregulated in patients with RB, and further research is required in order to identify the function of STRA6 in the development of RB.

The GO analysis of the present study revealed that the identified proteins were involved in glycoprotein, amyloid, acute-inflammatory and defense responses. Glycosylation serves an important role in posttranslational modifications, and modulates the physical, chemical and biological properties of a protein (26,27). Glycan structures are important for numerous processes, including protein-protein interactions, protein trafficking and folding, immune recognition, cell adhesion, and migration and inter-cellular signaling (28). A number of studies have identified that aberrant glycans may serves an essential role in cancer biology by mediating tumor cell adhesion, motility and invasiveness (29-31). The first group identified in the functional clustering analysis involved glycoproteins. α-1B-glycoprotein (A1BG) is a plasma glycoprotein that has sequence similarity to the variable regions of certain immunoglobulin supergene family member proteins. Recent proteomics studies have revealed that A1BG may act as a biomarker for numerous types of tumors, including bladder (32) and pancreatic (33) cancer. Leucine-rich α-2-glycoprotein (LRG1) is a secreted glycoprotein of the leucine-rich repeat family. It was reported that LRG1 is associated with cancer metastasis and poor prognosis, resulting from its effects on promoting cell invasion, angiogenesis, and migration (33). Chitinase 3-like 1 (CHI3L1) is a member of the glycosyl hydrolase 18 family. CHI3L1 is considered to serve a role in the process of inflammation and tissue remodeling (34). A previous study demonstrated the association of high serum CHI3L1 expression level with poor patient prognosis and short survival time in human solid tumors, including lung cancer and liver cancer (35).

Additionally, the present study indicated that proteins involved in acute phase responses are associated with RB. The acute phase response is a rapid inflammatory response that provides protection against microorganisms using non-specific defense mechanisms (36,37). It was observed that inflammation may serve a dominant role in the pathogenesis of various types of cancer (38,39). Fibrinogen γ, serpin family A member 1 (SerpinA1) and orosomucoid 2 (ORM2) are all acute-phase associated proteins. Fibrinogen is involved in numerous processes, including blood clotting, fibrinolysis, the inflammatory response and wound healing. Previously, the role of fibrinogen and fibrinogen degradation products in carcinogenesis of certain tumor types has been suggested (40,41). Proteomic analysis has also demonstrated that fibrinogen γ is overexpressed in patients with pancreatic cancer (42). SerpinA1 is produced in the liver and secreted into serum. It has been reported to exhibit an invasive and metastatic capacity in lung cancer, gastric cancer, and colorectal cancer (43,44). Previous studies have revealed that SerpinA1 may serve as a biomarker for gastric cancer as it induces the invasion and migration of gastric cancer cells, and its expression is associated with the progression of gastric cancer (45). ORM2 is an important acute phase plasma protein due to its increased abundance during acute inflammation. ORM2 may function in the modulation of the immune system in the acute phase. Upregulation of ORM2 has been reported in patients with colorectal cancer (46).

In conclusion, to the best of our knowledge, the present study is the first to identify proteins associated with RB using iTRAQ technology. The results of the present study may aid in providing an improved understanding of RB and contribute to developing a novel therapy target in the future. Further studies are required to explore the function of proteins in RB development.

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