Low expression of GSTP1 in the aqueous humour of patients with primary open-angle glaucoma

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Abstract
Primary open-angle glaucoma (POAG) is characterized by irreversible neurodegeneration accompanied by visual field defects and high intraocular pressure. Currently, an effective treatment is not available to prevent the progression of POAG, other than treatments to decrease the high intraocular pressure. We performed proteomic analysis of aqueous humour (AH) samples from patients with POAG combined with cataract and patients with cataract to obtain a better understanding of the pathogenesis of POAG and explore potential treatment targets for this condition. Samples were collected from 10 patients with POAG combined with cataract and 10 patients with cataract. Samples from each group were pooled. A high-resolution, label-free, liquid chromatography-tandem mass spectrometry-based quantitative proteomic analysis was performed. In total, 610 proteins were identified in human AH samples from the two groups. A total of 48 up-regulated proteins and 49 down-regulated proteins were identified in the POAG combined with cataract group compared with the control group. Gene Ontology (GO) analysis revealed key roles for these proteins in inflammation, immune responses, growth and development, cellular movement and vesicle-mediated transport in the biological process category. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated the down-regulated expression of glutathione S-transferase P (GSTP1) in the glutathione metabolism signalling pathway in the POAG combined with cataract group. Additionally, certain significantly differentially expressed proteins in the proteomic profile were verified by enzyme-linked immunosorbent assay (ELISA). GSTP1 levels were reduced in the human AH samples from the POAG combined with cataract group, based on the results of ELISA and
proteomic profiling. Therefore, GSTP1, a redox-related marker, may be involved in the pathological process of POAG and may become a treatment target in the future.

**KEYWORDS**
aqueous humour, cataract, GSTP1, inflammation, oxidative stress, primary open-angle glaucoma, proteome

# 1 | Introduction

Primary open-angle glaucoma (POAG) is defined as a chronic progressive optic neuropathy associated with elevated intraocular pressure (IOP) and visual field defects, and this disorder is one of the leading causes of irreversible blindness worldwide. The dynamic balance of IOP is closely related to drainage of the aqueous humour (AH). A high IOP occurs as the result of impaired AH outflow. The AH performs various functions, including maintaining IOP, providing nutrients to the ocular tissues and removing metabolic products from ocular tissues. Changes in the protein composition of the AH are closely related to the metabolism of the anterior segmental tissue, such as the trabecular meshwork (TM).

The TM acts as a static filter that exerts stable resistance to AH outflow. Abnormal expression of extracellular matrix (ECM) components within the TM microenvironment is associated with impaired AH drainage and leads to an elevated IOP. In addition, abnormal expression of ECM proteins in the TM is related to the oxidative stress response in the TM. Many ocular diseases and related treatments are associated with oxidation and inflammation. Some studies have also reported markedly elevated levels of oxidative stress markers in the AH from patients with POAG, along with altered expression of markers of antioxidant defences and an enhanced inflammatory response.

Quantitative proteomics based on mass spectrometry (MS) is an important methodology for biological and clinical research. Label-free MS has several advantages, including the lack of a restriction on the sample size, the lack of a requirement for expensive isotope labelling and the ability to detect a wide range of proteins. Comparison and identification of the changes in proteins in the AH using proteomics help researchers detect differentially expressed proteins in patients with different diseases. In this study, we sought to detect differentially expressed proteins and explore the pathological mechanisms of POAG, which will provide new molecular targets for glaucoma treatment.

# 2 | Materials and Methods

## 2.1 | Subjects

The Institutional Ethics Committee approved this study, and we adhered to the tenets of the Declaration of Helsinki when conducting experiments involving human subjects. Patients were enrolled after providing informed consent at the Tian Medical University Eye Hospital. Samples were collected at the Tianjin Medical University Eye Hospital from October 2016 to December 2017. Twenty subjects, including 10 patients with POAG combined with cataract and 10 age- and sex-matched patients with cataract as a control group, were recruited for this study. All subjects underwent a thorough ophthalmic evaluation by a glaucoma specialist using standard diagnostic criteria, including glaucoma-related measurements such as IOP, visual acuity, gonioscopy examination, fundus photography, preoperative cup-disc ratio and Humphrey visual field analysis (Figure 1). The following inclusion criteria were used for patients with POAG combined with cataract: IOP > 21 mm Hg (1 mm Hg = 0.133 kPa), expanding cup/disc, narrowing visual field and open anterior chamber angle. The patients were also diagnosed with cataract. The criteria for cataract included an opaque lens and a progressive decrease in visual acuity. None of the control subjects had a history of glaucoma. Patients with ophthalmic conditions such as uveitis, ocular trauma; a history of ocular surgery within the previous three months; intraocular inflammation; secondary or neovascular glaucoma; or use of topical or systemic corticosteroids were excluded from the study.

AH samples (25-100 μL) were collected through an anterior chamber paracentesis using a 30-gauge needle at the beginning of the surgical procedures from patients undergoing cataract or trabeculectomy surgery. Samples were transported on ice, centrifuged at 704 g for 10 minutes at 4°C and immediately stored at −80°C until further analysis.

## 2.2 | Estimation of the total protein content using the Bradford method

The total protein concentration in the AH was determined using a Bradford assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 5 μL of AH proteins was added to 200 μL of Bradford reagent. The optical density was measured at 595 nm after a 15 minutes incubation. The protein concentration of each sample was calculated from a standard curve using BSA as the reference.

## 2.3 | Proteolysis

Approximately 5 μg of total AH protein was aliquoted, the volume was brought to 500 μL with 5 mmol/L ammonium bicarbonate
etry (LC-MS/MS) analysis. Trypsin-digested AH proteins from each group (n = 10) were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

2.4 Nano-HPLC-MS (Q-Exactive) proteomic and data analyses

Samples were subjected to MS analysis (Thermo Fisher). Components obtained by high pH reverse-phase chromatography were resolved with 20 μL of 2% methanol and 0.1% formic acid. Samples were centrifuged at 11,269 g for 10 minutes. Then, 10 μL of the supernatant was loaded using the sandwich method. The loading pump flow rate was 350 nL/min for 15 minutes, and the separation flow rate was 350 nL/min. The following separation gradient was used: phase B percentage (%) 4/0 min, 15/5 min, 25/40 min, 35/65 min, 95/70 min, 95/82 min, 4/85 min and 4/90 min. The following ion source parameters were used: spray voltage of 2.1 kV and capillary temperature of 250°C. The full MS were obtained at a resolution of 70,000 FWHM and full scan AGC target of 1e6. The dd-MS2 data were obtained at a resolution of 17,500 FWHM and AGC target of 5e6. The label-free MS analysis was performed using a mass spectrometer, and the raw MS data were processed using MaxQuant software.

2.5 Quantification of significantly differentially expressed proteins using enzyme-linked immunosorbent assay (ELISA)

AH samples from control patients with cataract alone (n = 21) and patients with POAG combined with cataract (n = 20) were collected to determine the levels of the significantly differentially expressed proteins glutathione S-transferase P (GSTP1), C-reactive protein (CRP), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1), transforming growth factor β (TGF-β), growth differentiation factor 11 (GDF11) and tenascin (TNC) using ELISA kits (mlbio). Each individual sample was used independently and individually. The procedure described below was performed according to the manufacturer's protocol. Preparation: The collected AH samples were removed from the −80°C freezer, dissolved at room temperature and centrifuged at 704 g for 30 min. Standard wells and sample wells were established. Each standard well was filled with 50 μL of the standards. Forty microlitres of the sample dilution solution and 10 μL of the sample solution (fivefold final dilution concentration of the sample) were added to each of the sample wells. The samples were gently shaken. One hundred microlitres of enzyme-labelled reagent was added to each well except for the blank wells. The plate was sealed with a membrane and incubated at 37°C for 60 minutes. The plate was washed with X1 washing solution and incubated for 30 seconds, the liquid was discarded, and the plate was dried. This procedure was repeated five times. Fifty microlitres of developer-A was added to each well, followed by 50 μL of developer-B. The samples were gently shaken and incubated at 37°C in the dark for 15 minutes. Fifty microlitres of stop solution was added to each well to stop the reaction. The absorbance (OD value) of each well was measured at a wavelength of 450 nm over 15 minutes. The linear regression equation of the standard curve was calculated using the concentration of the standard and the OD values. The OD value of the sample was input into the equation, the sample concentration was calculated, and then the value was multiplied by the dilution factor of 5 to obtain the actual concentration of the sample.

2.6 Statistical analysis

Protein expression profiles were analysed with MaxQuant software (version 1.6.2.0.). MaxQuant significance A24 was used to evaluate the significance of differences. Differentially expressed proteins were identified from the raw data (fold change > 1.5 and P < .05). The data were processed and analysed using GraphPad® Prism 7 software. Clinical variables and ELISA data were analysed using an unpaired t test followed by the Mann-Whitney U test. Data are presented as means ± SDs, and P < .05 was considered to indicate a statistically significant difference.

3 RESULTS

3.1 Patient information

According to the inclusion and exclusion criteria, patients with cataract had a turbid lens, normal anterior chamber depth, appropriate cup/disc ratio and regular structure of the optic nerve head. Patients with POAG combined with cataract displayed slight turbidity of the lens and a normal anterior chamber depth, abnormal cup/disc ratio and irregular optic nerve head. The present study included 10 eyes from 10 patients with POAG combined with cataract and 10 eyes from 10 patients with cataract. The demographic and clinical characteristics are summarized in Table 1. The mean ages of the patients with POAG combined with cataract and the controls were 72.8 ± 2.6 years and 71.7 ± 2.5 years, respectively (P = .923). No significant differences in the sex distribution, axial
length, corneal thickness, aqueous depth or visual acuity were observed between the two groups (P > .05). As expected, the POAG combined with cataract group had a higher mean IOP and larger cup/disc ratio than the control group (P = .043 and P = .0005, respectively).

3.2 | Data acquisition

The process of the label-free proteomics technology is divided into three main stages: protein sample preparation, MS measurement and data analysis. Ten pooled samples each were available from the POAG combined with cataract group and the cataract group. The protein concentration was 0.15 µg/µL in the POAG combined with cataract group and 0.07 µg/µL in the control group. Proteins in each group were divided into two subgroups, that is, high-density proteins and low-density proteins, to identify proteins with a low density. The test was repeated twice using the same method. From the heatmap, we concluded that the repeatability of the results was sufficient and that noticeable differences were observed between the two groups. Red represents up-regulated proteins and blue represents down-regulated proteins in the comparison of the POAG and control groups shown in Figure 2.

3.3 | Data analysis

All significantly differentially expressed proteins identified in the LC-MS analysis are listed in Table 2, which describes the differentially expressed proteins between the POAG combined with cataract group and the control group. Selected interesting proteins are also labelled in Table 2, and their functions are shown in Figure 4. Gene Ontology (GO) analysis revealed the cellular components, molecular functions and biological processes of all differentially expressed proteins using the UniProt website, and the results are shown in Figure 3. KEGG pathway analysis was conducted on the complete data sets of modulated proteins using the KEGG website (http://www.genome.jp/kegg) to highlight possible molecular mechanisms underlying the differential expression of proteins in patients with POAG. Known mutual interactions among differentially expressed proteins were used to construct protein-protein interaction (PPI) networks with the STRING database.

A total of 610 proteins were detected in the two groups. Ninety-seven significantly differentially expressed proteins were detected in the AH of patients with POAG combined with cataract compared with patients with cataract (proteins with a corrected P < .05 and fold change > 1.5 were considered significantly differentially expressed). Forty-eight of these proteins were up-regulated and 49 were down-regulated. Some of these proteins, such as lipopolysaccharide-binding proteins (LBP), scavenger receptor cysteine-rich type 1 protein M130 (CD163), CRP, carboxypeptidase N catalytic chain (CPN1), GSTP1 and annexin A1 (ANXA1), are associated with inflammation. Some proteins, such as thioredoxin (TXN) and GSTP1, function in redox reactions. Other proteins, such as cadherin 5 (CDH5), cartilage oligomeric matrix protein (COMP), desmocollin-2 (DSC2), mammalian ependymin-related protein 1 (EPDR1), hyaluronan-binding protein 2 (HABP2), laminin subunit beta-2 (LAMB2), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1) and tenasin (TNC), are associated with cell adhesion and movement. Some proteins, such as reelin (RELN), semaphorin-3F

| Characteristics | POAG | Cataract | P-value | Significance |
|-----------------|------|----------|---------|-------------|
| Subjects, n     | 10   | 10       |         |             |
| Male/female     | 8/2  | 8/2      | .9999   | ns          |
| Age, y (mean ± SD) | 72.80 ± 2.60 | 71.70 ± 2.51 | .7644   | ns          |
| Cup/disc ratio (mean ± SD) | 0.62 ± 0.06 | 0.36 ± 0.02 | .0005   | ***         |
| IOP (mean ± SD), mm Hg | 30.50 ± 0.90 | 15.34 ± 0.77 | <.0001  | ****        |
| Axial length (mean ± SD), mm | 23.94 ± 0.50 | 23.66 ± 0.23 | .6243   | ns          |
| Corneal thickness (mean ± SD), µm | 530.80 ± 13.53 | 520.50 ± 6.49 | .5012   | ns          |
| ACD (mean ± SD), mm | 3.03 ± 0.09 | 3.09 ± 0.11 | .6636   | ns          |
| BCVA (mean ± SD) | 0.50 ± 0.09 | 0.31 ± 0.07 | .1153   | ns          |
| Other disease history | –     | –        | –       | –           |

Note: Statistical analysis: nonparametric t test (*** represents P < .001; **** represents P < .0001; ns represents no significant difference).

Abbreviations: ACD, anterior chamber depth; BCVA, best corrected visual acuity; IOP, intraocular pressure; SD, standard deviation.

**FIGURE 1** Patients’ diagnostic examinations. Patients with cataract have a turbid lens, normal anterior chamber depth (A), formal cup/disc ratio (B) and regular structure of the optic nerve head (C). However, patients with POAG combined with cataract display slight turbidity of the lens, a normal anterior chamber depth (D), abnormal cup/disc ratio (E) and irregular optic nerve head (F) [Colour figure can be viewed at wileyonlinelibrary.com]
(SEMA3F) and semaphorin-4B (SEMA4B), are associated with nerve growth; metabolism-related proteins include pyruvate kinase (PKM) and carboxypeptidase N subunit 2 (CPN2).

3.4 | GO, KEGG and PPI analyses

GO analysis mainly evaluates three aspects: cell components, molecular functions and biological processes. The differentially expressed proteins were enriched mainly in the cytoskeleton region, nucleus, cytoplasm, extracellular region, cell membrane, mitochondrial endoplasmic reticulum and vesicles. The main cellular functions of all differentially expressed proteins were rRNA binding, protein binding, metal ion binding, receptor binding, signal transducer and transmembrane transport. All differentially expressed proteins were enriched mainly in the biological processes of immune response, cell death, division, adhesion, movement, growth and development and signal transduction transport (Figure 4). We obtained signalling pathways from the KEGG website by analysing all significantly differentially expressed proteins. These signalling pathways were ranked according to the p-value and enrichment. The IL-17 signalling pathway plays a vital role in the inflammatory response. Glutathione metabolism also exerts an indispensable effect on inhibiting oxidative stress. The signalling pathway and metabolism of xenobiotics by cytochrome P450 have been verified to be involved in the pathology of congenital glaucoma. Most signalling pathways are related to metabolism, such as drug metabolism, glutathione metabolism, propanoate metabolism and sphingolipid metabolism. Axon guidance is
### Table 2

Up- or down-regulated protein between POAG combined cataract and Cataract

| UniProt ID          | Gene name | P/C   | P-value | Up/down |
|---------------------|-----------|-------|---------|---------|
| Q5JWQ4_HUMAN        | NRP1      | 10.078| .034    | 1       |
| M0QZ24_HUMAN        | N/A       | 8.859 | .045    | 1       |
| J3KQ66_HUMAN        | RELN      | 12.436| .021    | 1       |
| H7BYK9_HUMAN        | PROC      | 8.865 | .045    | 1       |
| H3BTN5_HUMAN        | PKM       | 33.962| .001    | 1       |
| G3XAP6_HUMAN        | COMP      | 16.351| .011    | 1       |
| G3V2V8_HUMAN        | NPC2      | 9.069 | .043    | 1       |
| F5H7V9_HUMAN        | TNC       | 11.756| .024    | 1       |
| F5H6I0_HUMAN        | B2M       | 12.512| .021    | 1       |
| F5GZ29_HUMAN        | CD163     | 26.216| .003    | 1       |
| E9PHM6_HUMAN        | DST       | 50.572| .000    | 1       |
| E5RIQ1_HUMAN        | LY6E      | 8.997 | .044    | 1       |
| D6RD58_HUMAN        | LECT2     | 29.651| .002    | 1       |
| A6QRJ1_HUMAN        | ATP6AP1   | 10.509| .031    | 1       |
| A0A0U1RR20_HUMAN    | PRG4      | 15.738| .012    | 1       |
| GNPTG_HUMAN         | GNPTG     | 38.685| .001    | 1       |
| PCSK1_HUMAN         | PCSK1N    | 31.039| .002    | 1       |
| LRC4B_HUMAN         | LRRC4B    | 12.710| .020    | 1       |
| NEUR1_HUMAN         | NEU1      | 12.065| .023    | 1       |
| SFRP2_HUMAN         | SFRP2     | 13.689| .017    | 1       |
| SFPR3_HUMAN         | FRZB      | 9.188 | .042    | 1       |
| SNED1_HUMAN         | SNED1     | 24.553| .003    | 1       |
| CH3L2_HUMAN         | CH3L2     | 11.897| .024    | 1       |
| HABP2_HUMAN         | HABP2     | 24.229| .004    | 1       |
| NID2_HUMAN          | NID2      | 19.434| .007    | 1       |
| COTL1_HUMAN         | COTL1     | 20.392| .006    | 1       |
| MG5T5A_HUMAN        | MGAT5     | 9.576 | .038    | 1       |
| REG3A_HUMAN         | REG3A     | 8.510 | .049    | 1       |
| DSC2_HUMAN          | DSC2      | 38.833| .001    | 1       |
| LAMB2_HUMAN         | LAMB2     | 19.989| .006    | 1       |
| CAPG_HUMAN          | CAPG      | 11.208| .027    | 1       |
| TKT_HUMAN           | TKT       | 27.104| .003    | 1       |
| HGF5_HUMAN          | MST1      | 14.122| .016    | 1       |
| S10A4_HUMAN         | S10A4     | 41.509| .001    | 1       |
| CNP2_HUMAN          | CNP2      | 14.875| .014    | 1       |
| LBP_HUMAN           | LBP       | 21.431| .005    | 1       |
| DESP_HUMAN          | DSP       | 29.863| .002    | 1       |
| CBPN_HUMAN          | CPN1      | 9.070 | .043    | 1       |
| NID1_HUMAN          | NID1      | 27.549| .002    | 1       |
| THIO_HUMAN          | TXN       | 12.533| .021    | 1       |
| LDHB_HUMAN          | LDHB      | 10.637| .031    | 1       |
| GDN_HUMAN           | SERPINE2  | 30.014| .002    | 1       |
| ANXA1_HUMAN         | ANXA1     | 22.100| .005    | 1       |
| CRP_HUMAN           | CRP       | 83.521| .000    | 1       |
| IGHM_HUMAN          | IGHM      | 10.647| .030    | 1       |

(Continues)
| UniProt ID    | Gene name     | P/C  | P-value | Up/down |
|--------------|---------------|------|---------|---------|
| FA10_HUMAN   | F10           | 10.586 | .031    | 1       |
| SLIK3_HUMAN  | SLITRK3       | 13.532 | .017    | 1       |
| VTM2B_HUMAN  | VSTM2B        | 9.157  | .042    | 1       |
| X6R5A3_HUMAN | TDP2          | 0.406  | .012    | -1      |
| S4R460_HUMAN | IGHV3OR16-9   | 0.334  | .005    | -1      |
| MOQ8X0_HUMAN | LSM4          | 0.584  | .048    | -1      |
| J3QS39_HUMAN | UBB           | 0.342  | .005    | -1      |
| I3L1J2_HUMAN | CDH5          | 0.076  | .000    | -1      |
| H0Y3I0_HUMAN | GDF11         | 0.396  | .011    | -1      |
| H0Y332_HUMAN | STXBP5        | 0.026  | .000    | -1      |
| G3V164_HUMAN | GRIA4         | 0.568  | .043    | -1      |
| F5GZS6_HUMAN | SLC3A2        | 0.579  | .046    | -1      |
| C9JPG5_HUMAN | SEMA3F        | 0.339  | .005    | -1      |
| C9JP14_HUMAN | ADH7          | 0.240  | .001    | -1      |
| B7WNR0_HUMAN | ALB           | 0.373  | .008    | -1      |
| B3KTY4_HUMAN | SLITRK2       | 0.311  | .003    | -1      |
| A0A0C4DH36_HUMAN | IGHV3-38 | 0.527  | .033    | -1      |
| EPDR1_HUMAN  | EPDR1         | 0.044  | .000    | -1      |
| NTRI_HUMAN   | NTM           | 0.423  | .014    | -1      |
| KRT82_HUMAN  | KRT82         | 0.033  | .000    | -1      |
| SEM4B_HUMAN  | SEMA4B        | 0.361  | .007    | -1      |
| CBPB2_HUMAN  | CPB2          | 0.532  | .034    | -1      |
| AEBP1_HUMAN  | AEBP1         | 0.140  | .000    | -1      |
| SBP1_HUMAN   | SELENBP1      | 0.521  | .032    | -1      |
| FSTL1_HUMAN  | FSTL1         | 0.576  | .045    | -1      |
| PLOD1_HUMAN  | PLOD1         | 0.508  | .029    | -1      |
| KRT85_HUMAN  | KRT85         | 0.057  | .000    | -1      |
| TPI5_HUMAN   | TPI1          | 0.166  | .000    | -1      |
| CRBB1_HUMAN  | CRYBB1        | 0.010  | .000    | -1      |
| CRBA4_HUMAN  | CRYBA4        | 0.294  | .003    | -1      |
| CRBB2_HUMAN  | CRYBB2        | 0.055  | .000    | -1      |
| SPB6_HUMAN   | SERPINB6      | 0.487  | .025    | -1      |
| S10A7_HUMAN  | S10A7         | 0.228  | .001    | -1      |
| CRBS_HUMAN   | CRYGS         | 0.234  | .001    | -1      |
| ID5_HUMAN    | IDS           | 0.392  | .010    | -1      |
| GSTP1_HUMAN  | GSTP1         | 0.430  | .015    | -1      |
| CRGD_HUMAN   | CRYGD         | 0.364  | .007    | -1      |
| S10A9_HUMAN  | S10A9         | 0.566  | .043    | -1      |
| CRBA1_HUMAN  | CRYBA1        | 0.057  | .000    | -1      |
| K2C6G_HUMAN  | KRT6B         | 0.464  | .020    | -1      |
| APOB_HUMAN   | APOB          | 0.317  | .004    | -1      |
| ANG_HUMAN    | ANG           | 0.564  | .042    | -1      |
| CRYAA_HUMAN  | CRYAA         | 0.398  | .011    | -1      |
| HV205_HUMAN  | IGHV2-5       | 0.151  | .000    | -1      |
| LV140_HUMAN  | IGLV1-40      | 0.222  | .001    | -1      |
a subfield of neural development concerning the process by which neurons send out axons to reach the correct targets. The Hedgehog signalling pathway participates in the differentiation of embryonic cells. The Ras signalling pathway is very important in regulating cell proliferation, survival, growth, migration, and differentiation and cytoskeletal dynamics. These signalling pathways cover a broad range of molecular biological processes. S100A8 in the IL-17 signalling pathway was up-regulated in patients with POAG combined with cataract. The expression of GSTP1 in the glutathione metabolism signal pathway was down-regulated. PPI networks explain the mutual connections between different proteins. We input significantly differentially expressed proteins into the STRING website and constructed the network with Cytoscape software. Red represents up-regulated proteins, and green represents down-regulated proteins. These differentially expressed proteins are involved mainly in inflammation, oxidative stress, metabolism and remodelling of ECM proteins. Among these differentially expressed proteins, GSTP1 regulates the activity of glutathione and prevents neurodegeneration. Tissue inhibitor of metalloproteinase 3 (TIMP3) may play a role in tissue remodelling induced by acute stimulation. Nidogen 1 (NID1) and nidogen 2 (NID2) are involved in the formation of tight junctions of the basement membrane and the transmission of ECM signals. LAMB2 is involved in the adhesion, migration and reconstruction of cellular tissues. Apolipoprotein B (APOB) and apolipoprotein M (APOM) are involved in lipid metabolism. Many other proteins, such as S100-A7 protein (S10A7), S100-A8 protein (S10A8), S100-A9 protein (S10A9), S100-A4 protein (S10A4) and ANXA1, are involved in the inflammatory response. Keratin-82 (KRT82), keratin-85 (KRT85), cytokeratin17 (K1C17), cytokeratin-1B (K2C1B) and cytokeratin-6B (K2C6B) are related to the formation of keratin and keratinization of the epithelium (Figure 5).

### 3.5 | Protein validation with ELISA

AH was collected again from patients with POAG combined with cataract and control patients. The patients’ clinical information is shown in Table 3. Figure 6 shows the results of the ELISA verification. Among the proteins, the levels of GSTP1, CRP and TNC were reduced in the AH samples from the POAG combined with cataract group, the GDF11 level was increased in the POAG combined with cataract group, and no significant differences in PLOD1 and TGF-β levels were observed between the two groups. The GSTP1 expression level was consistent with the proteomic data (Figure 6).

### 4 | DISCUSSION

In recent years, the application of omics technology in the field of biomedical research has become increasingly widespread, which has enhanced the data output capability of life science research. Proteomics is an important approach used to explore differentially expressed proteins related to various diseases and has been widely applied in glaucoma, cataract, corneal lesions, macular degeneration, and uveitis and other diseases. Compared with labelled protein profiling, unlabelled MS has the advantages of a lack of limitation on the sample size, the low cost of isotope labelling and the ability to detect a wide range of proteins.

The dynamic balance of the AH is closely related to IOP, which is an important risk factor for POAG. Changes in AH components also reflect tissue metabolism and pathological processes in the anterior segment of the eye. At the same time, because AH is relatively easy to obtain and convenient to store, it is a better sample source for exploring glaucoma. A review of the proteomic data from studies focused on the pathogenesis of POAG that have been conducted over the last decade indicated that studies employing diverse proteomics technologies, such as Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH), label-free proteomics, antibody microarray analysis, RT2 Custom Profiler PCR Array analysis, liquid chromatography-mass spectrometry (LC-MS) and LC-MS/MS, to analyse the AH of patients with glaucoma showed that proteins associated with oxidative stress and inflammation are significantly differentially expressed.

Based on a large number of reports related to POAG research combined with the potential differentially expressed proteins identified in our current proteomic data set, we have been suggested that both inflammation and oxidative stress reactions are involved in the pathological changes in the homeostasis of the AH microenvironment, leading to metabolic dysfunction in the anterior chamber.

### TABLE 2 (Continued)

| UniProt ID   | Gene name      | P/C   | P-value | Up/down |
|-------------|----------------|-------|---------|---------|
| KV133_HUMAN | IGKV1-33       | 0.578 | .046    | −1      |
| CAH1_HUMAN  | CA1            | 0.554 | .039    | −1      |
| FCN3_HUMAN  | FCN3           | 0.515 | .030    | −1      |
| LFTY2_HUMAN | LEFTY2         | 0.366 | .007    | −1      |
| HV103_HUMAN | IGHV1-3        | 0.081 | .000    | −1      |
| HV226_HUMAN | IGHV2-26       | 0.381 | .009    | −1      |
| LV861_HUMAN | IGLV8-61       | 0.529 | .033    | −1      |

Note: Green label represents interesting significant proteins. P/C: Primary open-angle glaucoma combined cataract/Cataract.1: up-regulated proteins in P, −1: down-regulated proteins in P.
tissue. This dysfunction is specifically manifested as ECM disorders, leading to insufficient drainage of the AH and ultimately to an elevated IOP. AH samples were collected from separate patients with POAG combined with cataract and control patients to verify the changes in the levels of inflammatory factors, redox response-related proteins and ECM proteins in the AH using ELISA and to confirm our hypothesis. We subsequently selected six proteins (GSTP1, GDF11, CRP, PLOD1, TGF-β and TNC) that are closely related to the pathogenesis of POAG for subsequent verification using ELISA to exclude the possibility of false positives. As shown in the present study, the change in GSTP1 levels measured by ELISA was consistent with the protein profile; in other words, GSTP1 was expressed at significantly lower levels in patients with POAG than in control individuals. Moreover, the opposite results were obtained for the TNC, GDF11 and CRP levels, and no difference in the PLOD1 and TGF-β levels was detected by ELISA. The potential explanations for these inconsistencies include individual differences, the small sample size and shifts in the protein profile.

GSTP1, a member of the glutathione S-transferase (GST) family, catalyses the binding of many hydrophobic and electrophilic compounds to reduced glutathione and has a strong antioxidant capacity. Silencing GSTP1 in patients with chronic obstructive pulmonary disease (COPD) increases reactive oxygen species (ROS) production and DNA damage in cells. Loss of GSTP1 expression in human prostate cells increases DNA damage caused by oxidative stress. Notably, (a) GSTP is a gene downstream of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE)/electrophilic response element (EpRE) transcription pathway. (b) GSTP1 reduces ROS expression and apoptosis induced by oxidative stress through the Nrf2-extracellular signal-regulated protein kinase1/2 (ERK1/2)-mitogen-activated protein kinase (MAPK) pathway and exerts a neuroprotective effect. (c) Oxidative stress may increase methylation of the GSTP1 and thioredoxin reductase 2 (TXNRD2) gene promoters by up-regulating DNA methyltransferase 1 (DNMT1). Increased methylation decreases the transcription of GSTP1 and TXNRD2. Oxidative stress interacts with gene methylation to form a vicious cycle. The redox imbalance promotes the secretion of inflammatory factors and contributes to other reactions induced by inflammation. GST is a multigene family with multiple enzymes that play different roles in anti-oxidation, detoxification and elimination of xenobiotics, including carcinogens, oxidants, toxins and drugs. GSTP1 Ile 105 Val polymorphism results in an absence of their enzyme activity. A meta-analysis on various GST mutations discovered that a polymorphism in the GSTP1 gene was significantly
FIGURE 5  KEGG, PPI and signalling pathway analyses. The main enriched signalling pathways were the IL-17 signalling pathway, the axon guidance signalling pathway and drug metabolism signalling pathway. A, PPI networks reflect the interactions of proteins with other proteins. In the comparison of the POAG combined with cataract group with the cataract group, red represents up-regulated proteins, and green represents down-regulated proteins. The size of the circle represents the amount of the related protein (B). The signalling pathways were acquired from our data analysis and previous articles (C and D) [Colour figure can be viewed at wileyonlinelibrary.com]
TABLE 3 Demographic and clinical characteristics of Cataract, POAG combined cataract subjects

| Characteristics         | POAG     | Cataract | P-value | Significance |
|-------------------------|----------|----------|---------|--------------|
| Subjects, n             | 20       | 21       |         |              |
| Male/female             | 10/10    | 12/9     | >.05    | ns           |
| Age, y (mean ± SD)      | 66.00 ± 10.00 | 69.00 ± 10.00 | >.05    | ns           |
| Cup/disc ratio (mean ± SD) | 0.80 ± 0.20 | 0.30 ± 0.20 | <.0001  | ***          |
| IOP (mean ± SD)         | 33.95 ± 1.20 | 15.59 ± 0.60 | <.0001  | ****         |
| Axial length (mean ± SD) mm | 23.93 ± 0.20 | 23.23 ± 0.49 | >.05    | ns           |
| Corneal thickness (mean ± SD) µm | 510 ± 7.02 | 524.1 ± 6.84 | >.05    | ns           |
| ACD (mean ± SD)         | 3.01 ± 0.03 | 2.98 ± 0.18 | >.05    | ns           |
| BCVA (mean ± SD)        | 0.20 ± 0.10 | 0.40 ± 0.30 | >.05    | ns           |
| Other disease history   | –        | –        |         |              |

Note: Statistical analysis: Nonparametric t test (**P < .01; ****P < .0001; ns: no significant difference).

Abbreviations: ACD, anterior chamber depth; BCVA, best corrected visual acuity; IOP, intraocular pressure; SD, standard deviation.

FIGURE 6 Verification of differentially expressed proteins in the AH of patients with POAG combined with cataract and patients with cataract using ELISAs. Clinical information of the patients is listed in Table 3. The levels of the GSTP1, CRP, GDF11, PLOD1, TGF-β and TNC proteins were verified. **P < .01 and ****P < .0001

correlated with increased POAG risk in a Caucasian population.46 The GSTM1 null/GSTP1, Ile/Val or Val/Val genotypes were associated with increased IOP and more advanced defect of the right eye optic nerve and visual field.47 The frequency of the GSTT1 and GSTP1 mutation was not statistically different between POAG patients and healthy controls group based on genomic DNA from peripheral blood.48 In conclusion, the relationship between GSTP1 and POAG remains undetermined. Further, the above-mentioned studies made their conclusions based on gene level from patients’ blood. Our research found that GSTP1 protein expression was decreased
| PMID          | Mean age (POAG/cataract) | Nation/ethnicity | Tissue | Size (POAG/cataract) | Method | Significant protein | Similar gene name | Oxidative stress-related proteins | Protein function                                                                 |
|---------------|--------------------------|------------------|--------|----------------------|--------|----------------------|-------------------|----------------------------------|---------------------------------------------------------------------------------|
| Our results   | 73/72                    | Tianjin, Chinese | HAH    | 10/10                | LFQ    | 97                   | GSTP1             | GSTP1, TXN                      | Inflammation, redox reaction, cell adhesion and movement, nerve growth, metabolism |
| 27788204      | 75/78                    | Aarau, Switzerland | HAH    | 5/5                  | SWATH  | 87                   | TKT, GSTP1, CRYAA  | PRDX1, CAT                      | Cholesterol-related, inflammatory, metabolic, antioxidant, proteolysis-related     |
| 26265374      | 58/61                    | Shanghai, Chinese | HAH    | 6/6                  | iTRAQ  | 262                  | SERPING1, NP C2, FBLN1, S PINK4, CA1, F CQR3, APO F, RELN, DSP, FCGR3A, PC SKIN, IGHM, CRYBB1, CR YBA4 | -                                                                                | Catalytic, complement, enzymatic, signalling, structure, transporting            |
| 27193151      | 77/68                    | Moscow, Russia   | HAH    | 7/11                 | LFQ    | 36                   | ALB, SERPIN1 (PEDF) | –                                | Lipoproteins, immunoglobulins, carrier proteins, neurotrophic, development of the neural system |
| 29332228      | 56/55                    | New Delhi, India | HAH    | 9/9                  | LC-MS/MS | 97             | GSTP1             | SOD, GPx, TRAP                    | Tissue and vascular remodelling, immune response, blood coagulation Antioxidant activity |
| 29847670      | 66/65                    | Augusta, America | HAH    | 15/32                | LC-MS  | 33                   | NPC2              | –                                | Signalling, immune response, molecular transporting, lipid metabolism           |
| 22974818      | 75/73                    | Genoa, Italy     | HAH    | 10/10                | Antibody microarray | 4            | SOD1/2, GST1, NOS2, GS | GS, NOS, SOD, GST | Aqueous humour oxidative stress proteomic levels in primary open-angle glaucoma |
| 20666514      | 75/73                    | Caucasian        | HAH    | 10/14                | Antibody microarray | 30           | CDH5              | PRKCE, PRKCD, PRKACA, PRKCQ, NOS2, SOD1/2, MGST1DNCL1 | Oxidative damage, mitochondrial damage, neural degeneration and apoptosis         |
| 30994369      | 60/63                    | Chennai, India   | HAH    | 90/78                | LC-MS/MS | 87             | IGHV3OR16-9       | –                                | Complement and coagulation cascade, regulation of wound healing, inflammatory response and extracellular matrix organization |
| 32246983      | 74/71                    | Florida, America | HAH    | 23/35                | NMR, IROA | 5              | –                 | –                                | Metabolism: lysine, arginine, cysteine and glycine                             |
in aqueous humour of POAG patients. So, we have reason to believe that GSTP1 may be a possible biomarker in POAG pathogenesis.

CRP has been widely used in clinical practice as an indicator of inflammation. Changes in its level suggest the existence of an inflammatory reaction in the AH microenvironment.\textsuperscript{49} According to previous studies, inflammation is associated with changes in the ECM of patients with glaucoma.\textsuperscript{50-52} UniProt data show that GDF11, also called BMP11, regulates cell development and mutations to influence ganglion cell formation and eye morphogenesis.\textsuperscript{53} The GDF11 gene encodes a secreted ligand of the TGF-β superfamily. Ligands of this family bind various TGF-β receptors, leading to the recruitment and activation of SMAD family transcription factors that regulate gene expression.\textsuperscript{54} The GDF11 level in the body decreases with increasing age, and its use in individuals with renal ischaemia-reperfusion injury promotes kidney repair. By activating the ERK1/2 pathway in vitro, the addition of recombinant GDF11 to primary renal epithelial cells promotes the regeneration of luminal cells.\textsuperscript{55} A high IOP may lead to an insufficient blood supply to the retinal layer and a disturbance in optic nerve metabolism, which may be related to the disruption in GDF11 expression. Although the data for CRP and GDF11 from our ELISAs were not supported by our Nano-HPLC-MS data, this study provides a potential target or a meaningful insight into the association between POAG and GDF11, as well as CRP regulation, which is also of considerable significance.

Compared with a single multi-facility study, we further examined the literatures in search for proteins with functions similar to those we found. They analysed aqueous humour, used different testing methods, and studied those of different races. From these studies, we noted the differentially expressed proteins in POAG related to oxidative stress. The GST family was also implicated in these studies. Other proteins, such as those involved in neuronal protection, have also attracted our attention. We believe that inflammation, oxidative stress and neuroprotection are candidate signalling pathways for future therapeutic development.

All patients were enrolled in strict accordance with the inclusion criteria to reduce bias. Based on clinical reality and medical ethics, we were unable to collect AH samples from healthy people at the clinic. Therefore, in this experiment, patients with POAG combined with cataract were enrolled as the study group and compared with patients with cataract alone as the control group. We must not completely ignore the changes in the AH of the cataract group compared with the healthy group. In addition, the medication status of the patients in the POAG group should not be ignored. Different patients took different medications but generally used β-adrenergic receptor agonists and neuroprotective drugs. Drug interference cannot be ruled out, mainly for reasons of patient safety. An imbalance in redox reactions is caused by various factors, leading to increased inflammation. Other factors promote changes in the levels of ECM proteins and increases in the ECM cross-linking of TM cells, resulting in blocked AH release, increased IOP and retinal nerve disruption, which eventually lead to the onset or progression of POAG. GSTP1, a redox-related protein, is expected to become a target for preventing the onset of or treating glaucoma.

Our follow-up study will focus on the biological functions of GSTP1 in TM cells under oxidative stress.

**CONFLICTS OF INTEREST**
The authors confirm that no conflicts of interest exist.

**AUTHOR CONTRIBUTION**
Aihua Liu: Funding acquisition (equal); Resources (equal); Visualization (equal); Writing-original draft (equal).
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**DATA AVAILABILITY STATEMENT**
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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