• Brief Report •

Proteome alterations in aqueous humour of primary open angle glaucoma patients

Hanhan Liu[1,2], Fabian Anders[2], Sebastian Funke[2], Karl Mercieca[3], Franz Grus[2], Verena Prokosch[1]

[1]Department of Ophthalmology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz 55131, Germany

[2]Experimental and Translational Ophthalmology, Department of Ophthalmology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz 55131, Germany

[3]Royal Eye Hospital, School of Medicine, University of Manchester, Manchester M202UL, United Kingdom

Correspondence to: Verena Prokosch. University Eye Hospital Mainz, Johannes Gutenberg University of Mainz, Langenbeckstrasse 1, Mainz 55131, Germany. vprokosch@gmx.de

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Abstract

● **AIM:** To unravel the primary open angle glaucoma (POAG) related proteomic changes in aqueous humour (AH).

● **METHODS:** Totally 35 patients listed for cataract surgery (controls: *n*=12, age: 67.4±13.6y) or trabeculectomy for POAG (*n*=23, age: 72.5±8.3y) were included. AH samples of those patients were obtained during cataract surgery or trabeculectomy. AH samples were subsequently pooled into the experimental groups under equal contribution in terms of protein amount of each individual patient. Protein samples were analyzed by a linear trap quadrupol Orbitrap Mass Spectrometry device with an upstream liquid chromatography system. The obtained raw data were analyzed using the Maxquant proteome software and compared. Proteins with a fold-change ratio higher than a cut-off of 2 were considered as noticeably altered.

● **RESULTS:** A total number of 175 proteins could be identified out of the AH from POAG and cataract by means of quantitative mass spectrometric analysis. Apolipoprotein D (fold change, 3.16 times), complement C3 (2.96), pigment epithelium-derived factor (2.86), dickkopf-related protein 3 (2.18) and wingless-related integration (Wnt) inhibitory factor 1 (2.35) were significantly upregulated within the AH of glaucoma compared to cataract serving as controls.

● **CONCLUSION:** AH provides a tool to analyze changes in glaucoma and shows striking changes in Wnt signaling inhibitory molecules and other proteins.

● **KEYWORDS:** primary open angle glaucoma; aqueous humor; proteomics; Wnt signaling pathway

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INTRODUCTION

Glaucoma is a leading cause of vision loss and blindness worldwide, characterized by retinal ganglion cell (RGC) loss and axonal degeneration, resulting in irreversible loss of vision. Elevated intraocular pressure (IOP) is one of the main risk factors and until now the only treatable one. However, RGC loss proceeds despite IOP control[1] and the pathogenesis remains still obscure. Loads of investigational research has been done in the last decades to elucidate the mechanisms taking place in glaucoma with nothing being ground-breaking. Proteomics provides a reasonable tool to look into the pathogenesis of a disease and ample proteomic research has been done in animal models of glaucoma. However animal models of glaucoma do not always reflect the disease state in humans and it is thus needed to look into human tissue as well, which is difficult to get. The aqueous humour (AH) is well-accessible. Besides this, its protein composition alters depending on ocular diseases. Thus the change in AH protein may provide an insight into involved molecular mechanisms of glaucoma and help us understand the molecular changes in the context of the disease.

SUBJECTS AND METHODS

Ethical Approval Informed consent was obtained by the patients and the local Ethical Committee of Rhineland Pfalz was asked for permission.

Patients Selection Patients that were listed for cataract surgery, who served as controls or for trabeculectomy, who served as the primary open angle glaucoma (POAG) group in a tertiary eye care centre in the second term of 2016 were included in the study. AH samples from 35 patients were used for the investigation (controls: *n*=12, age: 67.4±13.6y; POAG: *n*=23, age: 72.5±8.3y). Each patient underwent a thorough ophthalmologic examination including a review of medical

history, best-corrected visual acuity, slit-lamp biomicroscopy, IOP measurement, gonioscopy and dilated fundoscopic examination. Patients were diagnosed with POAG when a reproducible visual field defect or a reproducible deterioration in the appearance of the optic disc was visible excluding any other reason for it, the angle was open and no signs of pigment exfoliation or pseudoexfoliation were present. Exclusion criteria were the following: 1) any ongoing ocular infection or within the previous 3mo; 2) any onsite retinopathy or other retinal abnormalities; 3) any onsite or history of ophthalmic trauma.

Sample Collection Surgery was performed under local or general anesthesia according to the special needs of each individual patient. Samples of the AH were taken at the beginning of the cataract surgery or trabeculectomy. After desinfection of the eyeball, a small 1.4 mm corneal incision was made at the 11 o'clock position with a blade just entering the anterior chamber (AC). 100-150 μL AH was drained by means of 30-gauge syringe. The samples were collected in a small container and immediately frozen under -80°C for further biochemical assays.

Quantitative Proteomic Measurements and Software Assisted Proteomic Profiling First of all, the total protein amount of each sample was determined *via* bicinchoninic acid protein assay. All samples were subsequently pooled in the experimental groups. It was taken care that there was an equal contribution of protein amount for each individual patient. The protein mixture was loaded onto a 12% Bis-Tris gel and a sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. After digestion, extraction and cleaning of the generated peptides, the final measurement occurred in a linear trap quadrupol Orbitrap Mass Spectrometry device with an upstream liquid chromatography system. The obtained raw data were analyzed using the Maxquant proteome software and compared.

Statistical Analysis Data were analyzed statistically using the two-independent samples test (SPSS Statistica Version 7) for Gaussian distributions, with the remaining quantitative data analyzed using two-way analysis of variance (Statistica Version 7) with post-hoc analysis using the Turkey HSD test to identify possible differences among the experimental groups. If the distribution was not Gaussian, the Kruskal-Wallis *H* test was used.

RESULTS

The age of the controls and POAG patients were 67.4±13.6y and 72.5±8.3y, respectively. There was no significant age difference. A total number of 175 proteins could be identified out of the AH from POAG and cataract-patients by means of quantitative mass spectrometric analysis. A couple of proteins showed a significant up-regulation in POAG patients compared to the respective control cataract group. Those interesting proteins were afamin (AFM; fold change 1.63, $P<0.005$), apolipoprotein D (ApoD; fold change 3.16, $P<0.005$), complement C3 (C3; fold change 2.96, $P<0.005$), dickkopf-related protein 3 (DKK3; fold change 2.18, $P<0.005$), wingless-related integration inhibitory factor 1 (WIF1; fold change 2.35, $P<0.005$), pigment epithelium-derived factor (PEDF; fold change 2.86, $P<0.005$).

DISCUSSION

Proteomics provides a reasonable tool to look into the pathogenesis of a disease and ample proteomic research has been done in animal models of glaucoma. However animal models of glaucoma do not always reflect the disease state in humans and it is thus needed to look into human tissue as well, which is difficult to get. The AH is well-accessible. Besides this, its protein composition alters depending on ocular diseases. Thus the change in AH protein may provide an insight into involved molecular mechanisms of glaucoma and help us understand the molecular changes in the context of the disease.

Purpose of our study was to find typical glaucoma-related protemic changes in the AH. We had the following findings: We could identify 175 proteins in total. Among those AFM (fold change 1.63, $P<0.005$), ApoD (fold change 3.16, $P<0.005$), C3 (fold change 2.96, $P<0.005$), DKK3 (fold change 2.18, $P<0.005$), WIF1 (fold change 2.35, $P<0.005$), PEDF (fold change 2.86, $P<0.005$) were significantly upregulated.

AFM is a pleiotropic glycoprotein with neuroprotective properties *in vitro*, which might be related to binding vitamin E acting as a radical scavenger[2] in various neurodegenerative diseases[3-4].

ApoD is a small, soluble lipid carrier. It is found in most human tissues, but mostly expressed in glia cells of the central nervous system[5]. ApoD has been described to play a role in various age-related and neurological disorders including glaucoma[6]. A number of studies proved ApoD's ability to protect organisms and cells against both extrinsic and intrinsic stress[6]. This could be partially related to a direct scavenging activity against free radical damage[6]. As oxidative stress has been recognized as one of the main pathogenetic factors in open angle glaucoma, AFM and ApoD might be increased to inhibite oxidative and apoptotic damage in POAG patients.

C3 is a protein of the complement system. The activated complement system clears cell and tissue debris. There is accumulating knowledge that complement dysregulation is responsible for numerous immune-mediated and inflammatory disorders. Imbalances in complement regulation and oxidative stress may play a role as a risk factor contributing to the dysregulation of complement activation in glaucoma[7]. Most interestingly a group of wingless-related integration (Wnt)

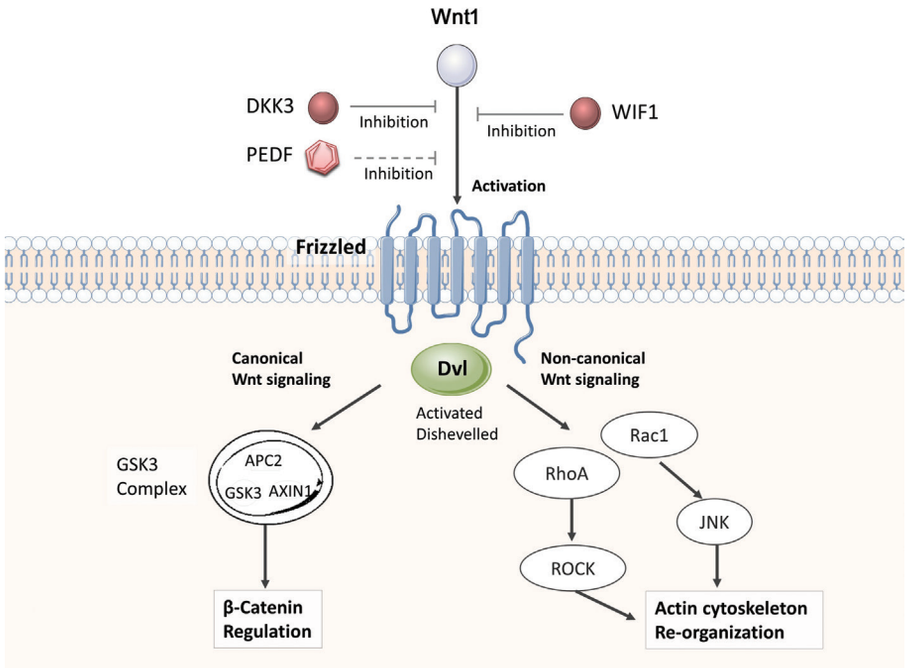

Figure 1 Overview of the chonical and non-chonical Wnt-signaling pathways, generated with a pathway analysis software Up-regulated aqueous humor proteins are indicated in red and might have dramatic effects on Wnt-signaling in the context of POAG. JNK: c-Jun N-terminal kinases; GSK3: Glycogen synthase kinase-3; APC2: APC regulator of Wnt signaling pathway 2; Rac: Rho family of GTPases.

signaling pathway regulating proteins were observed and increased in POAG patients: DKK3, WIF1 and PEDF (Figure 1).

Wnt comprises a diverse and well-conserved family of secreted lipid-modified signaling glycoproteins that are 350-400 amino acids in length. Wnts signaling is involved in plenty processes in evolution, maturation and disease. The Wnt signaling pathways maintain tissue homeostasis and regeneration[8], promote axonal remodeling and synaptic differentiation[8], and participate in the maturation, homeostasis and function of mature neurons. Thus, regulation of Wnt signaling is crucial and can protect against neurodegeneration[8]. Dickkopf (DKK) family and WIF1 are known as Wnt signaling antagonists[9].

Among those Wnt signaling molecules DKK family is a major class of Wnt signaling regulators. DKK3 is demonstrated as an antagonist of Wnt signaling. There are studies indicating that DKK3 may also play a protective role by inhibiting caspase activity in response to retinal injury[10]. Unlike DKK1 and DKK2, its role is yet not well studied in dysfunctional Wnt signaling.

PEDF is a multifunctional protein, which plays a crucial role in various physiological and pathophysiological conditions[11]. Studies show a significant age-related decrease in PEDF levels[12]. It has been seen in *in vitro* and *in vivo* that PEDF can inhibit RGC apoptosis exerting potential neuroprotective features[13]. In addition to this, PEDF has been recognized as a novel Wnt pathway antagonist[13].

Wnt activity plays a positive role in neurodegeneration and regulation of IOP. In our study, three Wnt pathway antagonists, PEDF, DKK3 and WIF1 were found up-regulated in POAG patients, indicating a possible role of Wnt signaling in the pathophysiology of glaucoma. Whether Wnt pathway is involved in neurodegeneration and/or regulation of IOP is still unclear and requires further study.

In correlation with our findings, AFM, ApoD, DKK3 and PEDF were found up-regulated in the AH of POAG patients after implantation of a shunt device[14-16] backing our findings. Thus exploring Wnt signaling in glaucoma patients more in detail might provide some new prospective for further studies.

In conclusion, the AH provides a tool to analyze and possibly better understand the pathophysiology of glaucoma. We could find striking changes in Wnt signaling inhibitory molecules and other proteins, which are known for their importance in neurodegenerative conditions. This might help to understand and diagnose the disease much better in the future and find novel treatments[17-20].

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