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Research article

Proteomics of vitreous in neovascular age-related macular degeneration

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ABSTRACT

Neovascular age-related macular degeneration (nAMD) has been described as a predominantly inflammatory and proangiogenic retino-choroidal disease. Vitreous humor (VH) is the adjacent and accessible compartment which, due to the vicinity to the retina, might best represent changes of protein-based mediators of nAMD. The aim of this clinical-experimental study was to analyze the nAMD associated VH proteome of previously untreated patients whilst taking different groups of nAMD into account, based on their clinical presentation (clinical diagnosis groups). Electrophoresis coupled online to mass spectrometry (CE-MS) as well as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) were used to analyze VH of 108 nAMD patients and 24 controls with idiopathic floaters. A total of 101 different proteins with at least two unique peptides could be identified. Using a stringent statistical analysis with implementation of the closed test principle, we were able to identify four proteins that may be involved in the pathophysiology of nAMD: Clusterin, opticin, pigment epithelium-derived factor and prostaglandin-H2 D-isomerase. Using independent samples, ROC-Area under the curve was determined proving the validity of the results: Clusterin 0.747, opticin 0.656, pigment epithelium-derived factor 0.514, prostaglandin-H2 D-isomerase 0.712. In addition, validation through ELISA measurements was performed. The identified proteins may serve as potential biomarkers or even targets of therapy for nAMD.

1. Introduction

Age-Related Macular Degeneration (AMD) is a complex, multifactorial disease and the major cause of central vision loss among the elderly in western, industrialized countries (Bourne et al., 2014; Klettner et al., 2013). Neovascular or wet AMD (nAMD) with choroidal neovascularization (CNV) is considered more aggressive, due to the rapid disease progression without therapy, than late dry AMD with geographic atrophy (Bhutto and Lutty, 2012; Cheung and Eaton, 2013). It is estimated, that in

Abbreviations: AMD, age-related macular degeneration; AUC, area under the curve; CE, capillary electrophoresis; CE-MS, Capillary electrophoresis coupled online to mass spectrometry; CNV, choroidal neovascularization; CNVwB, choroidal neovascularization with bleeding; CNVw/oB, choroidal neovascularization without bleeding; ELISA, Enzyme-linked Immunosorbent Assay; GO, gene ontology; hCNV, hemorrhagic choroidal neovascularization; LC-MS/MS, liquid chromatography coupled online to tandem mass spectrometry; MS/MS, tandem mass spectrometry; nAMD, neovascular age-related macular degeneration; PEDF, pigment epithelium-derived factor; PH2D, prostaglandin-H2 D-isomerase; ROC, receiver operating characteristic; VEGF, vascular endothelial growth factor; VH, vitreous humor.

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Germany (representing the other western countries) the incidence of AMD, due to the ageing population, will almost double by the year 2030 (Finger et al., 2011).

Intensive experimental and clinical research in recent years expanded our knowledge on the pathophysiology of nAMD. At present dysregulated complement system activation, oxidative stress and inflammatory processes are widely accepted as cofactors to proangiogenic activity mediated by vascular endothelial growth factor (VEGF) (Coleman et al., 2008; de Jong, 2006; Fletcher et al., 2014; Klettner et al., 2013). Nevertheless, there is still a lack of understanding of many pathophysiological components, especially on a molecular level (Bhutto and Lutty, 2012; Coleman et al., 2008; Holz et al., 2014).

The introduction of anti-VEGF agents marked a breakthrough in the therapy of nAMD (Holz et al., 2014). Nevertheless, a consistent dosing scheme is missing, the benefit of anti-VEGF agents for all patients is not predictable and long term anti-VEGF treatment may have severe side effects on ocular tissues (Bora et al., 2015; Cheung and Eaton, 2013; Coleman et al., 2008). Furthermore, it is difficult to predict the development of the disease and the individual outcome in patients. Therefore, research to evaluate potential future biomarkers, allowing optimal therapeutic decision-making, or possible new targets of therapy is warranted and may overcome these obstacles. Although biomarkers in the blood would be desirable, its indirect contact with the target tissue will most likely not provide biomarkers of use for therapeutic decision making. In contrast, the vitreous humor (VH) seems to be a valuable source of such therapeutic decision biomakers in AMD. VH can be obtained in the context of a therapeutic procedure and most likely contains molecules signaling disease-specific alterations within the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex due to the close anatomical proximity (Bhutto and Lutty, 2012; Stefansson, 2009).

Proteome analysis is one of the most advanced exploratory techniques for the discovery of new protein biomarkers of clinical significance (Stalmach et al., 2015). A number of studies have already employed proteome analysis for the study of age-related macular degeneration. Most of them used aqueous humor aspirates, were performed *ex-vivo* or used animal samples (Kang et al., 2014; Kim et al., 2012; Okamoto et al., 2010; Yao et al., 2013; Yuan et al., 2010; Zhang et al., 2014). Two publications, one a prior work of our team without consideration of clinical diagnosis groups, involved samples of VH. In this previous study we were able to identify 19 proteins with differential abundance in VH of AMD patients leading to some additional insight in the pathophysiology of AMD (Koss et al., 2014). Ecker et al. (2012) showed in their study on VH that matrix metalloproteinase 9 might be a protein based biomarker in nAMD.

In this study, we directed our attention to proteins in the VH considering different clinical diagnosis groups of nAMD. The studied groups included choroidal neovascularization without signs of bleeding (CNVw/oB), with signs of bleeding (CNVwB) and hemorrhagic choroidal neovascularization (hCNV). To the best of our knowledge, this is the first VH-based proteomics study involving different nAMD types based on clinical presentation. The aim of the present study was to identify new biomarkers associated with the disease which may also serve as therapeutic targets for treatment of nAMD.

For the analysis of the VH protein content a combination of capillary electrophoresis coupled online to a time-of-flight mass spectrometer (CE-MS) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was employed. CE-MS, due to its high reproducibility (Latosinska et al., 2013; Mischak et al., 2013; Stalmach et al., 2015), is used for detection and semi-quantification of peptides, while LC-MS/MS was used for the determination of peptide amino acid sequences.

2. Material and methods

2.1. Study design

This was a retrospective, clinical-experimental study approved by the local institutional review board and adhering to the tenets of Declaration of Helsinki. Informed consent was signed by all participants.

2.2. Patient characterization

In this study a total of 132 undiluted vitreous body samples from previously untreated patients were analyzed: 108 from patients suffering from nAMD and 24 from patients with idiopathic floaters (controls). Exclusion criteria were previous intravitreal treatment with anti-VEGF, intraocular steroids, coagulation with photo laser, vitrectomy or previous intraocular operations, such as cataract operation at the included or not included eye in the last six months. Patients with systemic diabetes, nephropathy and uncontrolled hypertension were excluded as well as patients with compromising ocular conditions like diabetic retinopathy or uveitis. Furthermore, patients suffering from complications including rubeosis iridis, vascularizations of the iridocorneal chamber, the papilla or in the retina perimeter were also considered ineligible. Personal data including age and gender were obtained for all participants as well as information regarding clinical subgroup of nAMD (CNVw/oB, CNVwB and hCNV), lens status and eye location.

2.3. Clinical diagnosis groups

Indirect ophthalmoscopy and slit-lamp examination, as well as optical coherence tomography and fluorescein angiography were used to examine the patients and to assign them to the different clinical diagnosis groups. Patients showing no signs of bleeding were pooled in the group CNVw/oB. A subretinal bleeding smaller than three times the papillary diameter was the feature of CNVwB, one larger than three times the feature of hCNV. Therefore, these groups differ regarding the amount of subretinal blood and disease severity.

2.4. Sample acquisition

nAMD patients were treated with a combination therapy containing of a core pars plana vitrectomy and intravitreal injections of triamcinolone (8 mg), bevacizumab (1.25 mg) and balanced salt solution (Koss et al., 2009). Samples were taken via single site core vitrectomy using Intrector technology (Koch and Koss, 2011). 1.5 mL undiluted vitreous was aspirated before application of any drugs.

Patients with idiopathic floaters were served with the same surgical technique, substituting balanced salt solution. After collection all samples were immediately frozen and stored at −80 °C until further use.

2.5. Tryptic digestion

Samples were prepared and analyzed as described in Koss et al. (2014). Briefly, ten µl of thawed sample was added to 90 µL 0.1% SDS, 20 mM DTT and 0.1 M Tris−HCl (pH = 7.6). Subsequently the samples were sonicated at room temperature for 30 min followed by denaturation at 95 °C for 3 min. In the