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Thyroid-associated orbitopathy and tears: A proteomics study

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ABSTRACT

To date, Thyroid-Associated Orbitopathy (TAO), an autoimmune inflammatory disease affecting the eye, remains poorly characterised and its diagnosis challenging. The aim of this study was to investigate the tears of the TAO patients in order to identify potential biomarkers. Two independent quantitative Tandem Mass Tag™ 6-plex experiments were done. After in-solution digestion and isoelectric fractionation, the 12 fractions were analysed with a LTQ Orbitrap Velos coupled to a liquid chromatography. Raw files were searched against Swiss-Prot-AC database using Proteome Discoverer software, with a false discovery rate of 1% at peptide and protein levels. The differential proteins were then verified using orthogonal approaches in independent patients. Globally, 712 tear proteins were quantified with 2 unique peptides. Interestingly, cystatin c (TAO/controls ratio: 1.53), alpha-1 antichymotrypsin (ratio: 1.70) and retinal dehydrogenase (ratio: 0.68), displaying differential levels in the tears of TAO patients using proteomics experiments emerged as highly promising biomarkers after verification. In conclusion, this proteomics study supports the idea that tears reflect biological modifications occurring in a disease context and can therefore be a promising fluid for biomarker discovery. Moreover, our study identified three candidates that could in the future open new avenues in the diagnosis of TAO disease.

Significance: Thyroid associated orbitopathy (TAO) is the most common disease affecting the orbit. Moreover, the later, severe stages of the disease can be sight threating [1]. On the other hand, the early sign and symptoms can be mistaken with other ocular pathologies [2]. Here we explore the modification of the tear content of the TAO patients using proteomics strategies and we proposed three new biomarker candidates, which could allow the early diagnosis of the disease and prompt action to prevent more severe stages. Moreover, our findings could also help to better understand the pathophysiology of the disease.

1. Introduction

Thyroid-associated orbitopathy (TAO) is the most common autoimmune disorder affecting the orbit [3]. Potential relationship with thyroid dysfunction, also known as Graves' disease (GD), is frequently reported but not compulsory. Clinical manifestations of TAO disease include eyelid retraction, proptosis, chemosis, increased intraocular pressure, and in rare cases vision reduction due to the pressure on the optic nerve [4]. The general course of TAO is well described [5] and exhibits two phases. The first phase corresponds to an inflammatory phase which requires an anti-inflammatory treatment. This phase is followed by a second, less active form of the disease. The disease induced symptoms and sequelae which are often invalidating and disfiguring. Only surgeries, such as orbital decompression by both bone and fat removal and blepharoplasty, can alleviate symptoms [3]. To assess the activity of the disease, the Clinical Activity Score (CAS) based on 7 points of the classic symptoms and signs of inflammation is measured [6]. According to the European Group of Graves Orbitopathy, patients with CAS ≥ 3 should be treated with immunosuppressive agents [7]. Nevertheless, the beginning of the disease, its time course, its worsening and prognosis and the risk of recurrence are to date not really understood. Furthermore, no biomarker is available for helping clinicians and improving the management of the TAO patients.

In this context, we suspected that tears, due to their direct contact with the eye and the fact that lacrimal glands can be also affected by the disease, could give new insights on the mechanisms taking place in TAO disease and reveal potential promising biomarkers.

Until now, only very few studies investigated tears in the context of TAO disease. We can however report an interesting study of Matheis et al. [8] comparing tears of patients suffering from TAO with those of

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control subjects. This study highlighted upregulation of pro-inflammatory proteins and downregulation of protective proteins. Recently, another study from Aass et al. [9] reported 16 proteins including lysozyme C, lacritin and zinc-alpha-2-glycoprotein 1 significantly upregulated in GD patients with ocular complications as compared to GD patients without ocular involvement.

Despite the lack of further investigation on multicentric studies, these proteomics studies showed that the choice of tears seemed relevant for discovering new targets of TAO disease. Nevertheless, the small number of studies available does not allow to clearly validate these biomarker candidates for this disease.

Consequently, the objectives of this study were to identify new potential tear biomarkers useful for the diagnosis of TAO. In order to achieve this goal, two independent quantitative proteomics experiments were carried out, followed by the verification of three promising candidates using independent TAO patients and orthogonal approaches (Western blot and Enzyme-linked immunosorbent assay or ELISA). The concentrations of these three candidates were significantly different in tears of TAO patients compared to control subjects. Furthermore, the level of Alpha-1-antichymotrypsin in tears can even discriminate patients presenting low versus high CAS.

2. Materials and methods

2.1. Sample collection

Subjects were recruited at the Jules-Gonin Eye Hospital (Lausanne, Switzerland) from December 2013 until July 2015. All participants gave their written informed consent and the study was carried out according to the ethical standards (Declaration of Helsinki, Human Research Ethics Committee in Lausanne, No 204/14). Twenty-eight TAO patients without any other ocular pathology (including dry eye syndrome) were included, as well as twenty-five healthy controls who did not suffer from any systemic or ocular pathology.

A sheet form was filled for each patient, summarising the demographic data and recording its medical history, both general and ocular anamnesis.

To measure the inflammatory activity of the disease, the clinical activity score (CAS) [6,7], which is the sum of 7 items of classical signs of inflammation present, was recorded for each eye. The CAS cut-off at 3 discriminates between the mild inflammatory form of the disease and the moderate to severe form of the disease. In addition, a CAS $\geq$ 3, together with other parameters, suggests to the physician to initiate an immunosuppressive treatment.

Tear samples were collected with Schirmer paper strips (Biotech Vision Care PVT LTD, Gujarat, India) as described by previous studies [10,11]. Schirmer strips were placed inside the lower eyelid of patient's both eyes for a maximum of 3 min (to avoid any discomfort to patients, time is strictly restricted). No stimulation, anesthetic or eye drops were used before sample collection. External factors such as harsh lighting, background noise and extreme room temperature, all known to affect the content of samples, were strictly supervised in order to ensure satisfactory reproducibility. Care was taken to avoid damage to the conjunctive surface and local eye irritation. Persons collecting tears wore gloves and Schirmer paper was not in contact with the skin of the patient (face) in order to avoid any contamination. The strip was then inserted into 0.5 ml tube with a hole in the bottom, and this assembly was placed into a 1.5 ml tube and centrifuged at 10,000 rpm for 7 min at 4 °C without any additional buffer to obtain the tear samples. After centrifugation, tear samples were immediately stored at − 80 °C until analysis.

2.2. SDS-PAGE

Sample quality control and estimation of the protein concentrations were estimated by silver staining of protein samples [12,13]. For each sample, 1 µl of tears was separated using SDS-PAGE, with a stacking gel of 4% acrylamide (Tris-HCL (pH 6.8) 25%, ammonium persulfate (APS), 0.05%, Temed 0.005%) and a running gel of 12% acrylamide (Tris-HCl (pH 8.8) 25%, APS 0.05%, Temed 0.005%). Two quantities of post mortem cerebrospinal fluid (CSF) (1.5 µg and 3.5 µg, concentration = 0.46 µg/µl) were also loaded on SDS-PAGE as standard samples to estimate protein concentrations in tears. Proteins were fixed with a 30% methanol/7.5% acetic acid/H2O solution then sensitised with a 1% glutaraldehyde solution. SDS-PAGE was stained by silver solution (0.2% silver nitrate/0.28% ammoniac/0.2% NaOH 10N/H2O) and revealed by the mix of 0.005% citric acid/0.02% formaldehyde/H2O. Average intensity of each sample was measured with myImage Analysis v2.0 (Thermo Fisher Scientific, San Jose, US-CA). Protein concentrations were estimated with linear interpolation to the known protein concentration of the CSF and their corresponding average intensity. Similar approaches were applied successfully elsewhere [12].

2.3. Proteomics experiments

2.3.1. Reduction, alkylation, digestion and TMTsixplex™ labeling

Two independent TMTsixplex™ experiments were carried out according to the same protocol. In each experiment, we analysed each time 3 TAO tears and 3 control tears. Appropriate volumes of tears were taken in order to use 10 µg of proteins of each sample. They were dissolved in 33 µl 6 M urea (Merck, Darmstadt, DE) dissolved in 0.1 M Triethylammonium bicarbonate buffer (TEAB; Sigma-Aldrich, St. Louis, US-MO) and 2 µl of 50 mM *tris*(2-carboxyethyl)phosphine (TCEP; Sigma-Aldrich, St. Louis, US-MO) were added. Samples were incubated for 1 h at 37° C. For alkylation, 1 µl of 400 mM iodoacteamid (Sigma-Aldrich, St. Louis, US-MO) was added to each sample and incubated for 30 min at room temperature (RT). After adding 67 µl of 0.1 M TEAB, samples were digested with 10 µl Trypsin and incubated overnight at 37 °C (1:50 ratio, micrograms of enzyme to micrograms of protein; porcine origin; Promega Corporation, Madison, US-WI). Tagging was performed using TMTsixplex™ (Experiment1: Lot OG187738; Experiment 2: Lot OK195064C; Thermo Scientific, San Jose, US-CA). The six tags were dissolved with 42 µl of acetonitrile 190 (ACN; ROMIL ltd, Cambridge, GB); then 40.3 µl of each tag were used to label separately our samples (controls: 126,128,130; TAO: 127, 129, 131). All samples were incubated for 1 h at RT under constant shaking before adding 5 µl of Hydroxylamine 5% (Sigma-Aldrich, St. Louis, US-MO) and incubated again for 15 min with shaking. Finally, the 6 tubes were pooled together and dried in speed-vacuum.

2.3.2. Off-gel electrophoresis (OGE)

After adding 300 µl of a 5% ACN + 0.1% formic acid (FA) solution in the samples, they were purified with C18 Macrospin column (Harvard Apparatus, Holliston, US-MA) according to the manufacturer recommendations. Fractionation was carried out overnight with a 3100 Off-Gel Fractionator (Agilent technologies, Santa Clara, US-CA) using a 13 cm IPG strip (Immobiline DryStrip pH 3–10, 13 cm GE Healthcare, Little Chalfont, UK) and 12 OGE wells. Afterwards, the 12 fractions were collected and technical verification was done (stability of the electrical field during the OGE and linear increase of the pH). Then the fractions were purified with C18 Microspin column (Harvard Apparatus, Holliston, US-MA) according to the manufacturer recommendations and dried under speed-vacuum.

2.3.3. LC-MS/MS

The fractions resulting from OGE were dissolved in 94.9% H2O/5% ACN/0.1% Formic acid. Proteins were identified by Electrospray Ionization (ESI) Linear Trap Quadrupole (LTQ)-Orbitrap (OT) MS that was performed on a LTQ Orbitrap Velos from Thermo Electron (Thermo Scientific, San Jose, US-CA) supplied with a NanoAcquity system from Waters. Peptides were captured on a home-made 5 µm 200 Å Magic C18 AQ (Michrom, Auburn, US-CA) 0.1 × 20 mm pre-column and separated

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