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Analysis of protein composition and protein expression in the tear fluid of patients with congenital aniridia☆

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ABSTRACT

Aniridia is a rare congenital genetic disorder caused by haploinsuffiency of the *PAX6* gene, the master gene for development of the eye. The expression of tear proteins in aniridia is unknown. To screen for proteins involved in the aniridia pathophysiology, the tear fluid of patients with diagnosed congenital aniridia was examined using two-dimensional electrophoresis (2-DE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Two-dimensional map of tear proteins in aniridia has been established and 7 proteins were differentially expressed with $P < 0.01$ between aniridia patients and control subjects. Five of them were more abundant in healthy subjects, particularly α-enolase, peroxiredoxin 6, cystatin S, gelsolin, apolipoprotein A-1 and two other proteins, zinc-α2-glycoprotein and lactoferrin were more expressed in the tears of aniridia patients. Moreover, immunoblot analysis revealed elevated levels of vascular endothelial growth factor (VEGF) in aniridia tears which is in concordance with clinical finding of pathological blood and lymph vessels in the central and peripheral cornea of aniridia patients. The proteins with different expression in patients' tears may be new candidate molecules involved in the pathophysiology of aniridia and thus may be helpful for development of novel treatment strategies for the symptomatic therapy of this vision threatening condition.

Biological significance
This study is first to demonstrate protein composition and protein expression in aniridic tears and identifies proteins with different abundance in tear fluid from patients with congenital aniridia vs. healthy tears.

1. Introduction

Aniridia is a severe congenital panocular disorder affecting the cornea, anterior chamber, iris, lens, retina, macula and optic nerve. It occurs as a result of haploinsufficiency in paired box gene *PAX6* which is responsible for a proper eye development. The alterations in retina and development of glaucoma, cataract and keratopathy lead to a progressive deterioration of the visual acuity [1]. The underdeveloped iris and retina are examples of developmental defects. Other

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signs as glaucoma and cataract can be either congenital or develop after birth.

Aniridia associated keratopathy (AAK) typically develops in late childhood or later in life. The reason for this debilitating condition has been attributed to malfunctioning of the corneal epithelial stem cells visualized by a progressive deterioration of the stem cell environment, the palisades of Vogt [2–5]. The break-down of the stem cell niche triggers conjunctivalization and eventually opacification of the cornea [6]. The AAK reduces visual performance further and adds a chronic irritation and photophobia to the symptoms [7]. However, the reason for the development of AAK in patients is still unclear. Of special interest was to establish if surgical procedures triggered the AAK progression. The results however have been inconclusive due to the small amount of material and to the fact that surgery has developed towards less traumatic procedures [8].

It has been shown [9] that vascular endothelial growth factor (VEGF) and its receptors are involved in the maintenance of the stem cell barrier preventing blood vessels from entering the corneal surface. VEGF is also known to influence nerve growth [10,11] which may be a contributing factor to PAX6 since the dosage of PAX6 itself is the main factor contributing to pathological pattern of corneal innervation in aniridia [12]. It has also been suggested [13] that nerve growth factor receptor TrkA is involved in the regulation of the normal stem cell turnover.

The expression levels of the proteins mentioned above in patients' tears with aniridia are unknown. It was thus of interest to find out if these proteins and also other protein components of tear fluid were expressed differently in tears from eyes with aniridia compared to normal controls. In the present study, for the first time, we implemented 2-DE based comparative proteomics and 1- or 2-dimensional western blot for the analysis of changes in tear proteome in aniridia. The findings may help to identify molecules which can be useful for development of novel treatment strategies in symptomatic therapy of aniridia affected eyes. Thus, proteins with different abundance in tears between aniridia patients' and healthy subjects' tears were identified.

2. Material and methods

2.1. Participants

A total 18 participants, 10 patients (20 eyes) with congenital aniridia from a well characterized Swedish cohort of patients (reviewed in [8]) and 16 eyes that belong to 5 healthy family members without *PAX6* mutation (aniridia) or any other ocular diseases and 3 healthy volunteers that belong to the same ethnic group of Caucasian as the other individuals were included in the present study. In order to obtain the best matched patients' samples with controls, the samples in the control group were collected from individuals with the identical genetic background as in the patient group. Therefore similar number of samples used as controls for the patient group was collected inviting healthy family members without aniridia or other eye diseases. In that regard additional parameters, such as age, gender, past and current diagnoses of ocular infection or other ocular pathologies and medication were considered. Due to rare prevalence of aniridia in population it was difficult to obtain larger coherent cohort of patients and even control samples from individuals with that genetically identical background mentioned. Ethical approval was obtained from the ethics committee of the University Hospital at Linkoping University (Linkoping, Sweden) and informed consent was obtained from all participants prior to sample collection. The work described in this article has been carried out in accordance with the Declaration of Helsinki.

2.2. Tear sample collection and extraction of proteins from Schirmer's strips

Tear fluid from the left and right eyes of ten patients (n = 10) with diagnosed aniridia and a group of 8 healthy individuals (n = 8), as described above, was collected using standard Schirmer's strips during clinical examination. Tear collection using Schirmer's strips was chosen as the most convenient method of sample collection for its reproducibility validated by several proteomic studies [14–17]. This method is capable to provide high number of identified tear proteins with function in diverse processes, such as antioxidants, protein-folding proteins, proteins involved in the metabolism, immune response, and also proteins which can be used for the classification of cell structures (reviewed in [17]). Moreover, the Schirmer's test provided important clinical data for the assessment of tear production, thus helping to exclude the samples from individuals with secondary eye pathologies, such as dry eye syndrome. The exclusion criteria in both groups were a Schirmer's test of less than 10 mm of moisture on the strip in 5 min, a current diagnosis of ocular infection, history of ocular or eyelid pathologies with exception of aniridia in the patient group, and the use of current ocular or systemic medication. Congenital aniridia is an inherited disease therefore disease duration for each patient is identical with the patient's age. The clinical data for each patient and healthy individual, if applicable, along with age, gender, and ethnicity are listed in Table 1. After sample collection, wet Schirmer's strips with absorbed tears were immediately snap frozen and kept at −80 °C to prevent protein degradation until analyzed. Before analysis, proteins absorbed on Schirmer's strips were extracted with 100 μl of solubilization buffer containing 20 mM Tris, 7 M urea, 2 M thiourea, 0.1% CHAPS, 10 mM 1,4-dithioerythritol (Sigma-Aldrich Sweden AB, Stockholm, Sweden), 0.5% ampholytes 3–10 (Bio-Rad Laboratories, Hercules, CA, USA), and protease inhibitor cocktail (Complete mini, Roche Diagnostics Scandinavia AB, Stockholm, Sweden) and sonicated three-times for 30 s, followed by additional incubation for 2 h on ice. After centrifugation (15,000 ×g, 30 min, 4 °C), which removed debris, the samples were desalted using 3 kDa cut-off centrifugal filter units (Millipore Ireland, Cork, Ireland). The final sample volume was adjusted to 100 μl with the sample buffer (20 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM 1,4-dithioerythritol (Sigma-Aldrich), 0.5% ampholytes 3–10 (Bio-Rad), and protease inhibitor cocktail (Complete mini) and the protein content was determined using the Bradford reagent (Pierce, Thermo Scientific, Rockford, IL, USA).