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Linköping University Post Print

N.B.: When citing this work, cite the original article.

Original Publication:

Robert Ihnatko, Ulla Edén, Neil Lagali, Anette Dellby and Per Fagerholm, Analysis of protein composition and protein expression in the tear fluid of patients with congenital aniridia, 2013, Journal of Proteomics, (94), 78-88.

<http://dx.doi.org/10.1016/j.jprot.2013.09.003>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-104841>

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

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Running title: Proteomic analysis of tear fluid in aniridia patients

Keywords: aniridia, keratopathy, tear fluid, two-dimensional electrophoresis, LC-MS/MS, α -enolase

4992 words; 49 references; 3 figures; 3 tables

This work was supported by funds from The Swedish Research Council, The Country of Östergötland and Kronprinsessan Margaretas Arbetsnämnd.

The authors declare no conflict of interest.

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Abstract

Aniridia is a rare congenital genetic disorder caused by haploinsufficiency 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- α 2-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 patient 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.

Highlights: • Tear proteome of patients with congenital aniridia was analyzed. • Two-dimensional map of tear proteins in aniridia was established. • The relative abundance of some of the tear proteins was altered in aniridia patients. • These proteins may be new candidate molecules involved in aniridia pathophysiology.

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.

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 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 conjunctivalisation 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 to enter 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 patient 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 tears were identified.

Material and methods

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 ethics committee of University Hospital at Linköping University (Linköping, 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.

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

Tear fluid from left and right eye of ten patients ($n = 10$) with diagnosed aniridia and 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 the 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 sec, followed by additional incubation for 2 h on ice. After centrifugation (15,000 x g, 30 min, 4 °C), which removed debris, the samples were desalted using 3 kDa cut-off centrifugal filter units (Millipore Ireland, Cork, IRL). 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).

Two-dimensional electrophoresis

Isoelectric focusing (IEF) was carried out using ready-to-use immobilized pH gradient (IPG) strips (17 cm) with nonlinear pH range IPG 3-10 NL (Bio-Rad). The individual samples (50 µg of proteins) were loaded during rehydration of IPG strips in 300 µl (total volume) of IEF buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 10 mM 1,4-dithioerythritol, 1.25 % IPG buffer pH 3-10 and traces of bromophenol blue,

Sigma-Aldrich) for 18 h. IEF was carried out for a total 60 kVh (50 μ A/IPG strip) using Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). After IEF, the strips were stored at -80 °C until run in the second dimension (SDS-PAGE). Prior to SDS-PAGE, the IPG strips were incubated in equilibration (EQ) solution [(6 M urea, 0.375 M Tris-HCl pH 8.8, 2 % SDS, 20 % glycerol (Sigma-Aldrich) containing 2 % dithiothreitol (DTT, Sigma-Aldrich)] and subsequently in EQ solution containing 2.5 % iodoacetamide (Sigma-Aldrich) for 15 minutes each and immediately applied on the top of 13 % polyacrylamide gels (200 x 200 mm). SDS-PAGE was performed using Protean Plus Dodeca cell (Bio-Rad) at 15 mA/gel for the first 1 h followed by 30 mA/gel for the rest of the separation.

Image Capture Spot Quantitation and Statistical Analysis

Gels were fixed in 50 % methanol, 7 % acetic acid (Sigma-Aldrich) for 2 h followed by staining with SYPRO Ruby (Bio-Rad) for 12 h. Excessive dye was removed by incubation of gels in solution containing 10 % methanol, 7 % acetic acid and gels were scanned and the images were captured as 16-bit TIFF files for further analysis. The experimental dataset comprised of 36 two-dimensional gel images of tear fluid proteome from 36 eyes divided into patient group (20 eyes) and control group consisting of healthy individuals (16 eyes). Two gel images (obtained from two tear samples (left and right eye of the patient A4) were excluded from analysis due to remarkable different protein pattern caused probably by arterial leakage. Analysis of the gel images, including background subtraction, spot volume normalization, and differences in protein expression among the groups, was performed using PDQuest 8.01 software (Bio-Rad). All images were visually inspected for artifactual spots, and merged or missed spots. Each matched spot was manually inspected and confirmed on each individual gel. For the statistical analysis, the only spots which were present in all gels from the same group were included. The amount of protein in a spot was normalized in each gel by dividing the raw quantity of each spot by the total intensity value of all the pixels in the image. The normalized spot volume is referred to as abundance. Data are presented as mean $\pm$ SEM. The Anderson-Darling test of normality [18] was used to find the data that differed from normal distribution. Statistical differences between the groups were assessed using the Student's *t*-test or the Mann-Whitney test with $P < 0.01$ considered as significant. The Mann-Whitney test was used for cases where data were not normally

distributed. The statistical analysis was performed with commercial software SYSTAT 11 (Chicago, IL, USA). The Pearson correlation between proteomic data and clinical parameters for the patient group was performed with software Excel 2010 (Microsoft Inc., Redmond, WA, USA).

In-gel digestion and mass spectrometric identification

Excised protein spots from SYPRO Ruby stained gels were destained with 100 mM ammonium bicarbonate in 50 % acetonitrile (AcN, Sigma-Aldrich), washed with 100 % AcN and dried on SpeedVac (Christ, Osterode am Harz, Germany). In-gel reduction and alkylation reactions were carried out with 10 mM DTT in 100 mM NH_4HCO_3 (45 min, 56 °C) and subsequently with 50 mM iodoacetamide in 100 mM NH_4HCO_3 (30 min in the dark, 20 °C). Dried gel pieces were incubated in solution containing 0.1 µg of sequencing grade modified trypsin (Promega, Madison, WI, USA) in 40 mM NH_4HCO_3 /10 % AcN for 20 hours at 37 °C. Extraction of the resulting peptides was performed with 2 % formic acid (FA) in AcN (Sigma), and twice with 100 % AcN for 30 min at room temperature. The extracted peptides were dried on SpeedVac (Christ) and dissolved in 5 µl 0.1 % FA in 50 % AcN. The resulting peptide mixtures were analyzed by LC-MS/MS using nano-flow HPLC system (EASY-nLC; Bruker Daltonics, Bremen, Germany) on a 20 mm × 100 µm (particle size 5 µm) C18 pre-column followed by a 100 mm × 75 µm C18 column (particle size 5 µm) at a flow rate 300 nl/min, using a linear gradient starting with 0.1% FA (solvent A) and ending with 0.1% FA in 100% AcN (solvent B) for 45 min. The HTCultra PTM Discovery System (Bruker Daltonics, Bremen, Germany) was used for data acquisition. Raw data from tandem MS analyses were processed using DataAnalysis 3.4 software (Bruker Daltonics) and the resulting Mascot generic files were used for the search in NCBI protein database on the Mascot server (www.matrixscience.com). The search parameters were: Taxonomy: "*Homo sapiens*"; Enzyme: trypsin with permission of one missed cleavage site; Fixed modification: "Carbamidomethyl (C)"; Variable modifications: "Protein acetylation" and "Oxidation (M)"; Peptide tolerance: ± 0.8 Da for MS data and ± 0.8 Da for MS/MS data. The significance threshold for MASCOT ion identification was set to $P < 0.05$. Proteins with at least one peptide passing the required bold red criteria were considered to be positively matched.

Immunoblot analysis of VEGF, NGF, and soluble TrkA

Due to limited protein concentration in tears as well as the limited availability of aniridia patients, for the immunoblot analysis of VEGF, NGF, and TrkA we prepared pooled samples from the same patients and control samples as were used in 2-DE proteomic analysis. These pooled samples were prepared as follows: control sample 1a (30 µg of proteins) by pooling of the equal quantity (7.5 µg of proteins) of the each individual sample from the controls C1-C4 (4 individuals), control sample 1b (30 µg of proteins) by pooling of the equal quantity (7.5 µg of proteins) of the each individual sample from the controls C5-C8 (4 individuals), patient sample 2a (30 µg of proteins) by pooling of the equal quantity (10 µg of proteins) of the each individual sample from the patients A1-A3 (3 individuals), and patient sample 2b (30 µg of proteins) by pooling of the equal quantity (5 µg of proteins) of the each individual sample from the patients A5-A10 (6 individuals). For one-dimensional (1-D) western blot, the samples were prepared in buffer containing 125 mM Tris; 3.3 % SDS; 10 % glycerol and 5 % 2-mercaptoethanol (Sigma-Aldrich) and loaded on 13 % SDS-PAGE gel in the following order: lane 1 - sample 1a; lane 2 - sample 2a; lane 3 - sample 1b; and lane 4 - sample 2b. For two-dimensional (2-D) western blot, samples 1a and 2a (50 µg of proteins, prepared using the same procedure as for 1-D western blot) were loaded during rehydration of IPG strips (7 cm, IPG 3-10, Bio-Rad) in 125 µl (total volume) of IEF buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 10 mM 1,4-dithioerythritol, 1.25 % IPG buffer pH 3-10, Sigma-Aldrich) for 18 h. Isoelectric focusing was carried out for a total 10 kVh (50 µA/IPG strip) using Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). Prior to SDS-PAGE, IPG strips were equilibrated 15 min in solutions containing 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2 % SDS, 20 % glycerol, 2 % DTT and consequently in the same solution with 2.5 % iodoacetamide instead of DTT. After equilibration, the IPG strips were applied on the top of 13 % SDS-PAGE gels. SDS-PAGE was performed using Mini-Protean 3 Cell (Bio-Rad) at 15 mA/gel for the first 15 min followed by 25 mA/gel for the rest of separation. After SDS-PAGE the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane Hybond-P (Amersham, GE Healthcare Life Sci, Uppsala, Sweden) at constant current 100 V for 1.5 h at 4 °C. Membranes were probed with rabbit anti-VEGF antibody (Thermo Scientific, Pierce, Rockford, IL, USA) diluted 1:200 in blocking buffer and anti-rabbit IgG HRP-conjugated secondary antibody (dilution 1:2000, Dako Sweden AB, Stockholm, Sweden). The specific immunoreactivity was visualized using ECL detection kit (GE Healthcare, Uppsala, Sweden). Details for the analysis of NGF and TrkA levels are provided in the

supplemental section since these proteins were not detected in tears of both aniridia patients and healthy subjects.

Clinical examination of patients and in vivo confocal microscopy

The cohort of aniridia patients and control subjects (if applicable, see Table 1) underwent full clinical bilateral corneal examination including data or tests as follows: gender, age, Schirmer's test, pachymetry of the central cornea, corneal sensitivity, and tear break-up time (BUT). Additional data consist of the status of PAX6 mutation (taken from patient records), and ethnicity. The techniques of the clinical tests were described in details previously [2]. Briefly, ultrasound pachymeter (TOMEY SP-2000) was used to measure central corneal thickness. Tear production was assessed by Schirmer's test. Tear samples were excluded from analysis if the Schirmer's strip was moisten less than 10 mm in five minutes. Tear break-up time (BUT) was measured for evaluation the stability of tear film. BUT > 10 sec (measured on the clear part of the cornea) was considered as normal. Corneal sensitivity was measured using Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, France) with a nylon thread length of 60 mm considered as normal. The clinical data are summarized in Table 1. Moreover, a cornea-specific clinical *in vivo* confocal microscope (HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany) was used to detect the presence of blood and lymph vessels in the patients corneas. The technique has been described in details previously [19]. Briefly, the 63x immersion objective lens was placed in contact with the topically anesthetized cornea through a drop of optical coupling medium (carbomer 2mg/g; Viscotears® eye gel, THEA laboratories, France). The microscope field of view was aligned to the central or peripheral cornea, and depth was adjusted by a manual focusing ring. Laser-scanned images were obtained in real time under software control, at various corneal locations and depths.

Results

The presented work provides comprehensive analysis of protein composition in the tears of patients with congenital aniridia and identifies differences in protein levels in patients' tears by comparing with

healthy tears. Thus, two-dimensional map of tear proteome in aniridia has been established using IPG 3-10NL strips and 52 protein spots were analyzed and identified by LC-MS/MS (Fig. 1, Supplemental Table 1). To examine if protein composition and expression in tear fluid is specifically altered in aniridia patients, the 2-D protein patterns of tears in aniridia were compared with 2-D protein patterns obtained from normal tears using IPG 3-10NL strips and 13 % polyacrylamide gels. Separated proteins were visualized by fluorescent SYPRO Ruby stain and detected using PDQuest 2-D analysis software. Image analysis of 34 gels derived from 34 eyes divided into patient group (18 eyes) and normal control group (16 eyes) revealed approximately 143 valid protein spots. All samples with clinical data are summarized in Table 1. Two tear samples from total of 20 patient samples were excluded from analysis due to remarkable different protein pattern observed (sample A4, see Table 1). Only spots present in all gels of the same group (valid spots) were analyzed. Using stringent criteria the image analysis revealed 7 protein spots with $P < 0.01$ to be differently expressed in the tear fluid of aniridia patients compared with healthy individuals (Fig. 2, Table 2). Five of them were more abundant in healthy subjects, in particular α -enolase ($P = 0.0071$), peroxiredoxin 6 ($P = 0.0044$), cystatin S ($P = 0.0041$), gelsolin ($P = 0.0078$), and apolipoprotein A-1 ($P = 0.0073$). Two other spots were more expressed in the tear fluid of aniridia patients and were identified as zinc- α 2-glycoprotein ($P = 0.00997$) and lactoferrin ($P = 0.0053$). The complete list and nomenclature of all the proteins identified in 2-DE analysis, particularly the sequence coverage and the identified peptides is given in the Supplemental Table 1. The differentially expressed proteins were generally in weak correlation with the clinical parameters examined. Moderate correlation was found only in the case of peroxiredoxin 6, apolipoprotein A-1, α -enolase, and cystatin S with disease duration and for peroxiredoxin 6, α -enolase, and cystatin S with corneal sensitivity. The Pearson correlation between proteomic data and clinical parameters is provided in Table 3.

Because the clinical examination of the patient cohort revealed abundant blood and lymph vessels with opaque corneas (Supplemental Fig. 3), as the next step we analyzed expression of potential vascularization factors, such as vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and a soluble form of the high affinity nerve growth factor receptor TrkA in the samples from the same patient and control cohorts as were used in 2-DE analysis. For this aim, highly sensitive and specific western blot analyses were required since we were unable to detect and visualize these proteins using 2-DE based comparative proteomics combined with SYPRO Ruby

staining and consequent LC-MS/MS identification due to their low abundance in tears as they function in very low levels in autocrine and/or paracrine mechanisms. One-dimensional immunoblot analysis revealed elevated VEGF levels in the patients tears. The immunoreactivity was present at the band of ~ 80 kDa (Fig. 3a, b), which is the molecular weight corresponding to c-form of VEGF (VEGF-C). Moreover, the analysis using 2-D western blot has not only confirmed the results from 1-D immunoblot but has also identified two VEGF isoforms, both with higher abundance in the patients' tears. However, the isoform with the isoelectric point about 7.4 was in much higher abundance in the patients' tears than in the controls. Further analysis is needed to identify posttranslational modification responsible for this effect. The expression levels of NGF and TrkA were not detected in tears of both aniridia patients and healthy subjects (data not shown).

Discussion

Analysis of protein profile in tear fluid is important for biomarker identification and potentially useful in the assessment of prognosis or treatment of ocular pathologies. The present study identifies, for the first time, proteins in the tear fluid from patients with congenital aniridia. Using 2-DE analysis and LC-MS/MS we established 2-DE map of tear proteins in aniridia (Fig. 1, Supplemental Table 1) and identified proteins that were differently expressed in the tear fluid of patients when compared with the healthy tears. In order to obtain the most accurate results, no pooling of samples or sample fractionation and pre-enrichment protocols that may result in protein loss were used prior 2-DE analysis with the exception of the 3 kDa cut-off filtration which was required for the sample desalting. Separate analysis of each individual sample has also the advantage of visual evaluation of protein patterns that show variation within the group. Thus, one patient (sample A4 from both eyes, see Table 1) was excluded from data analysis in this manner due to probable vascular leakage that was manifested by higher abundance of plasma proteins, such as serum albumin (Supplemental Fig. 1).

By comparing the global protein expression in tear fluid we found 7 protein spots with $P < 0.01$ with different expression between patients and healthy subjects (Fig. 2, Table 2, and Supplemental Fig. 2). The expression of 5 proteins was greater in the healthy subjects; α -enolase,

peroxiredoxin 6, cystatin S, gelsolin, and apolipoprotein A-1. Two proteins, zink- α 2-glycoprotein and lactoferrin, were found to be more expressed in aniridia patients. Additionally, due to defects in corneal innervation and pathological growth of blood and lymph vessels observed in the corneas of aniridia patients (Supplemental Fig. 3) we examined expression of VEGF, NGF, and soluble TrkA in the same patient and control cohorts using immunoblot analysis. Using the highly sensitive western blot analysis was required due to extremely low abundance of these proteins in the tear samples which prevented their detection in 2-DE gels with SYPRO Ruby stain and consequent LC-MS/MS identification. Using one- and two-dimensional western blot we have found greatly elevated VEGF levels in the tears of aniridia patients with the immunoreactivity present at the band of ~ 80 kDa which is the molecular mass corresponding to VEGF-C. VEGF-C is the member of VEGF family involved more specifically in the regulation of physiological and pathological growth of blood and lymphatic vessels [20]. Using 2-D immunodetection we confirmed the results obtained from 1-D western blot and, additionally, we were able to identify two VEGF isoforms with the similar molecular size of ~ 80 kDa present in higher levels in the patient tears. Moreover, one of these VEGF isoforms with isoelectric point $pI \sim 7.4$ was even in much higher abundance in the patients than in the control tears. However, further investigation is needed to identify the process responsible for the observed posttranslational processing.

Additionally, we have also found that NGF, as another proangiogenic factor [21], and the soluble NGF receptor TrkA are probably not involved in the pathophysiology of aniridia since they were not detected in both patients and control tears (data not shown).

In the text below we discuss, in some details, the possible functional roles of the proteins with altered expression in the patient tears, identified in both approaches, in the context of aniridia pathophysiology.

α -Enolase

α -Enolase is a highly conserved enzyme catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the process of glycolysis. The levels of enolase isozymes may serve as a diagnostic tool for assessment of the severity and outcome of certain pathological conditions, e.g. stroke, cardiac arrest or other neurological disorders, as in the case of neuronal-specific enolase in

plasma [22]. In regard of α -enolase and its function in physiological or pathological conditions of eye, expression of this isozyme has been previously reported to be related to the limbal basal epithelium, as demonstrated by immunofluorescent microscopy [23]. In another study, an up-regulation of α -enolase has been found in the tear fluid of patients with dry eye syndrome [24]. In the present study α -enolase was down-regulated (0.54-fold) suggesting that its down-regulation is not related with tear disturbances often found in this disease but more likely as a result of impaired corneal epithelial function caused by mutations in the *PAX6* gene [25]. However, further studies are needed for evaluation of this finding and usefulness of this potential biomarker for development of symptomatic therapy for aniridia patients.

Peroxiredoxin-6

Another protein which may be involved in maintaining of corneal transparency is a glutation-dependent peroxidase, peroxiredoxin 6. It has been demonstrated that the topical administration of this protein had inhibitory effect on VEGF expression, inflammation, and suppressed neovascularization and apoptosis in ultraviolet irradiated rat corneas [26]. The presence of proteins involved in the metabolism of reactive oxygen species, such as peroxiredoxins and catalase in the normal tear film [27] suggests their function in the defense against these highly reactive and toxic compounds. Moreover, oxidative stress has been demonstrated to be a significant factor contributing to chronic wound state and wound-healing delay of corneas in Pax6^{+/-} mouse model [28]. We have found that peroxiredoxin 6 was down-regulated (0.69-fold) in the tears of aniridia patients when compared with healthy family members. Decreased expression of peroxiredoxin 6 may thus be associated to the elevated expression of VEGF in the tear fluid and to the increased corneal susceptibility to oxidative stress.

Cystatin S and Lactoferrin

Cystatins are naturally occurring inhibitors of cysteine proteinases. This function contributes to prevention of uncontrolled proteolysis and tissue damage [29-31]. The expression of these proteins in tears is also known to be reduced in several pathological conditions [32-35]. Cystatin S and lactoferrin are components of the corneal and the conjunctival epithelial defense against bacterial and viral pathogens. Lactoferrin is an iron-binding protein with bacteriostatic effect by impeding of iron

utilization by bacteria [31]. In the present study we have found the levels of cystatin S decreased in aniridia tears (0.54-fold). On the other hand, the levels of lactoferrin were increased (1.41-fold). It is tempting to hypothesize that the elevated expression of bacteriostatic lactoferrin in aniridia tears may be beneficial in corneal and conjunctival defense against pathogens compensating for a cystatin insufficiency.

Gelsolin

The gelsolin family of proteins is involved in cytoskeletal rearrangement controlling actin organization by binding, severing and capping of actin filaments [36]. In addition to their role in actin filament remodeling, these proteins play roles in many cellular processes, such as cell motility, apoptosis or regulation of gene expression [37]. The levels of gelsolin have been found to be up-regulated in the corneal epithelium of patients with keratoconus [38]. In the present study we found a decreased expression of gelsolin in tears from aniridia patients (0.69-fold). The decreased expression of this protein may contribute to the many defects attributed to the corneal epithelium in aniridia.

Zinc- α 2-glycoprotein

Zinc- α 2-glycoprotein (ZAG) is a 41 kDa protein which is secreted in various body fluids, such as plasma, saliva and tears with multifunctional roles including stimulation of lipid breakdown in adipocytes, and expression of immune response or insulin resistance [39;40]. The altered abundance of this protein in tears was found in several pathological eye conditions. For example, increased abundance of ZAG was found in the tears of keratoconus patients [15], in patients with Grave's ophthalmopathy [41] or keratitis [42]. On the other hand, no significant changes of this protein were noticed in tears from patients with dry eye syndrome [43]. In the present study we have found elevated levels of ZAG in aniridia tears (1.4-fold). The exact role of up-regulated ZAG in aniridia is unknown but the alterations in the tear lipidome profile observed in patients with fungal keratitis [42] may suggest its possible role in the tear lipid degradation. The tear film break-up time in aniridic patients is often short [44] and here ZAG may play a role in changes the qualities of the lipid layer in the tear film.

Apolipoprotein A-1

Apolipoproteins are lipid-binding proteins which transport the lipids through the lymphatic and blood circulatory systems. Besides their lipid-transporting function, an anti-inflammatory effect of apolipoproteins has also been demonstrated [45;46]. Increased secretion of apolipoprotein A-I by lacrimal gland was reported in patients with advanced diabetic retinopathy [47] as well as in tears of patients with fungal keratitis [42]. In the present study, we have found a decreased content of apolipoprotein A-1 in the tear fluid of patients with aniridia (0.53-fold) contributing to an insufficiency in the anti-inflammatory potential of tear film in patients with aniridia. This may be one possible explanation for the elevated numbers of dendritic immune cells in the central cornea, even in the absence of evident signs of inflammation, suggesting on a subclinical form of inflammation in early-stage of aniridia associated keratopathy [2].

Vascular endothelial growth factor (VEGF)

Pathological corneal vascularization or aniridia associated keratopathy is an associated ocular abnormality in aniridia patients (Fig. 3). Corneal avascularity, which is a prerequisite for intact vision, is achieved by maintaining of low corneal levels of VEGF expression and strong expression of vascular endothelial growth factor receptor 3 (VEGFR-3) by corneal epithelium [48]. Up-regulation of both VEGF-C and VEGFR-3 has also been demonstrated in inflamed rat corneas [49]. In the present study we found greatly elevated expression of VEGF in the tear fluid of aniridia patients identified as the immunoreactive band of ~ 80 kDa, which is the molecular size corresponding to VEGF-C (Fig. 3a, b), suggesting a breakdown of balance between pro- and anti-angiogenic factors and stimulation of angiogenesis and lymphangiogenesis. Moreover, analysis of VEGF expression by two-dimensional western blot revealed that posttranslational processing of this protein may play a significant role in regulation involved in aniridia pathophysiology since we found highly up-regulated isoform with pI around 7.4 in the patient tears (Fig. 3b).

Conclusion

The proteomic analysis of tears in patients with aniridia has identified proteins with significant differences in abundance when compared to healthy tears. Five proteins were down-regulated: α -

enolase, peroxiredoxin 6, cystatin S, gelsolin, and apolipoprotein A-1. Two proteins were up-regulated: zinc- α 2-glycoprotein and lactoferrin. Likewise 80 kDa VEGF isoform corresponding to VEGF-C was found up-regulated using 1- and 2-dimensional western blot. NGF and the receptor TrkA were not detected. Further studies are needed to clarify the involvement of the identified proteins in aniridia pathophysiology and their potential as targets for the symptomatic therapy of this severe ocular condition.

Acknowledgement

We thank aniridia patients, their families, and healthy volunteers for their valuable cooperation in this study.

Competing interests

The authors declare that they have no competing interests. All authors read and approved the final manuscript.

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Figure legends

Fig. 1

2-D protein reference map of tear film in aniridia. The protein spots which were identified by LC-MS/MS are numbered and the differentially expressed spots with $P < 0.01$ are labelled in red (see Table 2 and Suppl. Table 1 for more details).

Fig. 2

A. Proteins differentially expressed in the tear fluid between aniridia patients and healthy controls with $P < 0.01$. Each bar represents the mean of the relative spot volume intensity $\pm$ SEM.

B. Microphotographs of the proteins differentially expressed in the tears from patients with aniridia vs. normal tears.

Fig. 3

Increased levels of VEGF in the tear fluid from patients with aniridia.

A. One-dimensional western blot analysis of VEGF in aniridic and healthy tears. Lane 1 and lane 3: control sample 1a and control sample 1b respectively; lane 2 and lane 4: patient sample 2a and patient sample 2b respectively.

B. Two-dimensional western blot analysis of VEGF in patient and healthy tears. Tear samples used for 2-D western blot were as follows: Controls: sample 1a; Patients: sample 2a. (see Material and methods for more details)

Figure 2

Figure 3.

Table 1 List of analyzed patient and control samples with clinical data

Sample ID	Gender	Age	Shirmer test		Pachymetry		Corneal sensitivity		BUT		Note
			R	L	R	L	R	L	R	L	
A1	F	17	26	35	641	657	50	50	6	8	1,a,b,c,n
A2	M	55	35	35	663	677	35	20	9	9	1,a,b,c,n
A3	F	21	35	35	602	608	60	60	9	16	1,a,b,c,n
A4	F	30	10	12	646	701	35	35	0	0	1,a,b,c,n
A5	M	33	29	35	587	580	60	60	5	5	1,a,b,c,n
A6	F	36	20	35	660	667	10	20	9	9	1,a,b,c,n
A7	M	34	30	35	599	604	35	50	5	15	1,a,b,c,n
A8	M	21	25	28	683	642	50	60	11	11	1,a,b,c,n
A9	F	20	30	35	671	649	60	60	5	15	1,a,b,c,n
A10	M	19	35	30	1135	622	25	45	9	10	1,a,b,c,n
C1	F	55	35	35	554	554	60	60	11	11	2,a,n
C2	M	56	11	16	526	526	60	60	15	16	2,a,n
C3	F	26	30	35	597	597	60	60	11	14	2,a,n
C4	F	49	25	17	497	497	60	60	13	14	2,a,n
C5	F	24	27	17	N/A	N/A	60	60	14	13	2,a,n
C6	M	35	31	31	N/A	N/A	N/A	N/A	N/A	N/A	3,a,n
C7	M	41	11	10	N/A	N/A	N/A	N/A	N/A	N/A	3,a,n
C8	F	31	29	34	N/A	N/A	N/A	N/A	N/A	N/A	3,a,n

Abbreviations: A1-A10 = patients; C1-C8 = controls; F/M - female/male; R - right eye; L - left eye; N/A - not applicable; BUT - tear breakup time

Notes: 1 - patient; 2 - healthy relatives with the same genetic background but without *PAX6* mutation; 3 - volunteers; a - ethnicity: Caucasian; b - disease duration is the same as the age of each of the patient due to inherited feature of congenital aniridia; c - confirmed *PAX6* mutation; n - no therapy

Table 2 Summary of differentially expressed tear proteins in aniridia identified by 2-DE

Spot nr.	Protein name	Database nr. NCBI	Theor. <i>Mr</i>	Theor. <i>pI</i>	MASCOT score (MS)	Matches/ Sequence coverage	Protein abundance (mean AU)		Fold change	<i>P</i> - value
							aniridia tears (<i>n</i> = 18)	healthy tears (<i>n</i> = 16)	healthy vs. aniridia tears	healthy vs. aniridia tears
5904	gelsolin isoform A	gi 4504165	86043	5.9	449	8(6)/ 12%	148.41	214.28	0.7	0.0078
6205	peroxiredoxin 6	gi 4758638	25133	6.0	463	12(5)/ 55%	519.58	754.84	0.7	0.0044
2208	apolipoprotein A-1 precursor	gi 4557321	30759	5.56	707	17(5)/ 44%	778.77	1478.08	0.5	0.0073
7510	alpha-enolase, isoform 1	gi 4503571	47481	7.01	641	10(9)/ 28%	1030.93	1902.97	0.5	0.0071
0007	cystatin S precursor	gi 4503109	16489	4.95	268	6(3)/ 25%	1506.45	2811.34	0.5	0.0041
2302	Zn-alpha2-glycoprotein	gi 38026	34942	5.71	542	16(7)/ 38%	23349.83	16687.19	1.4	0.0100
8801	lactoferrin	gi 186833	80228	8.56	2249	54(38)/ 53%	68084.26	48241.02	1.4	0.0053

Abbreviations: AU = arbitrary units, Theor. *Mr* = theoretical molecular weight, Theor. *pI* = theoretical isoelectric point.

Table 3 Correlation analysis of proteomic data with the clinical parameters for the patient group

Pearson's correlation						
Spot nr.	Protein name	Disease duration	Schirmer test	Pachymetry	Corneal sensitivity	BUT
5904	gelsolin isoform A	0.1320	0.1134	-0.1972	-0.0838	-0.1934
6205	peroxiredoxin 6	0.4280	0.2978	0.1630	-0.3773	-0.0402
2208	apolipoprotein A-1 precursor	0.3659	0.1226	-0.1170	-0.1840	-0.2441
7510	alpha-enolase, isoform 1	0.3441	0.2712	-0.0228	-0.3314	-0.0499
0007	cystatin S precursor	-0.5479	-0.1360	0.0875	0.3178	0.2143
2302	Zn-alpha2-glycoprotein	0.1466	-0.2072	0.0815	-0.1535	-0.1610
8801	lactoferrin	0.1449	0.0346	-0.3781	0.1044	-0.1065

Immunoprecipitation and western blot analysis of NGF and soluble TrkA in aniridic and healthy tears

Immunoprecipitation (IP) method was used for detection of NGF and soluble form of the high affinity NGF receptor TrkA. Two samples (200 µg of total protein) were prepared by pooling of an appropriate volume of individual samples from all samples of the patients group (samples A1-A10 excepting of sample A4) or from all samples from the control group (samples C1-C8). Samples prepared in 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) were diluted in 50 mM Tris-HCl, pH 7.5 in order to decrease the concentration of denaturing agents that can disturb IP reaction. After centrifugation (14,000 x g, 15 min, 4 °C), the supernatants were incubated with NGF or TrkA antibody (4 µg) for 1 h at 4°C with gentle shaking followed by incubation with 40 µl of Protein G sepharose beads (GE Healthcare Life Sci, Uppsala, Sweden) for 1 h at 4 °C on orbital shaker. After centrifugation (5000 x g, 1 min, 4 °C), the beads with immunocomplex were washed 3 times with 1 ml buffer containing 25 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, and 0.1 % sodium dodecyl sulphate (SDS). The last wash was performed with 50 mM Tris-HCl, pH 7.5 and the samples were prepared for SDS-PAGE by incubating of beads in reduction buffer containing 125 mM Tris-HCl, pH 6.8; 3.3 % SDS, 10 % glycerol, 5 % 2-mercaptoethanol for 5 min at 95 °C.

Immunoprecipitated proteins were separated on polyacrylamide gels (18 % for NGF and 8 % for TrkA), transferred onto PVDF membrane and incubated with the specific antibodies, rabbit anti-NGF (1:1000, Cell Signaling, Danvers, MA, USA) or rabbit anti-TrkA (extracellular domain; 1:1000, antibodies-online GmbH, Aachen, Germany) for NGF or TrkA detection respectively. Consequently, the membranes were incubated with anti-rabbit IgG HRP-conjugated secondary antibody diluted 1:2000 in blocking buffer (Dako Sweden AB, Stockholm, Sweden) followed by ECL detection

Suppl. Table 1 Summary table from MASCOT identification for all the identified proteins in the tear fluid of aniridia patients using 2-DE based proteomics.

Spot no.	Protein name	Database nr. (NCBI)	Theor. Mr	Theor. pI	Matches/ Sequence coverage	MASCOT score MS	MASCOT score MS/MS	MASCOT peptides identified by MS/MS
0007	cystatin-S precursor	NCBI/ gi 4503109	16489	4.95	6(3)/ 25%	168	22 29 20 119 117 84	ALHFAISEYNK ALHFAISEYNK ATEDEYYR EQTFGGVNYFFDVEVGR EQTFGGVNYFFDVEVGR EQTFGGVNYFFDVEVGR
0107	prolactin-inducible protein precursor	NCBI/ gi 4505821	16847	8.26	11(6)/ 60%	469	21 86 87 60 35 23 101 62 17 39 64	NFDIPK SVRPNDEVTAFLAVQTELK SVRPNDEVTAFLAVQTELK TYLISSIPLQGAFNYK YTACLDDNPK TVQIAAVVDVIR TVQIAAVVDVIR ELGICPDAAVPIK FYTIEILK FYTIEILK FYTIEILKVE
0225	immunoglobulin J chain	NCBI/ gi 114319027	20046	5.27	6(4)/ 21%	181	29 93 48 71 11 49	IVLVDNK SSEDPNEDIVER SSEDPNEDIVER SSEDPNEDIVER IIVPLNNR FVYHLSDLCK
1003	cystatin S	NCBI/ gi 4503109	16489	4.74	3(1)/	165	34	ALHFAISEYNK

	precursor				19%	131	EQTFGGVNYFFDVEVGR
						40	EQTFGGVNYFFDVEVGR
1301	prolactin-inducible protein precursor	NCBI/ gi 4505821	16847	8.26	10(7)/53%	419	18 NFDIPK 55 SVRPNDEVTAVLAVQTELK 98 SVRPNDEVTAVLAVQTELK 55 TYLISSIPLQGAFNYK 61 TYLISSIPLQGAFNYK 97 TVQIAAVVDVIR 31 TVQIAAVVDVIR 86 ELGICPDDAAVIPIK 59 FYTIEILKVE 19 FYTIEILKVE
2205	extracellular glycoprotein lacritin precursor	NCBI/ gi 15187164	14237	5.43	5(2)/21%	110	11 QELNPLK 22 SILLTEQALAK 63 SILLTEQALAK 70 SILLTEQALAK 31 QFIENGSEFAQK
2208	apolipoprotein A-I precursor	gi 4557321	30759	5.56	17(5)/44%	707	64 VKDLATVYVDVLK 87 DLATVYVDVLK 62 DSGRDYVSQFEGSALGK 93 DYVSQFEGSALGK 39 DYVSQFEGSALGK 12 QEMSKDLEEVK + Oxidation (M) 13 DLEEVK 40 VQPYLDDFQK 16 VEPLRAELQEGAR 33 AELQEGAR 42 LSPLGEE <u>M</u> R + Oxidation (M) 23 LSPLGEE <u>M</u> RDR + Oxidation (M) 28 THLAPYSDEL R

[illegible]

60	FEELNADLFR
47	SQIHDIVLVGGSTR
33	LLQDFFNK
58	SINPDEAVAYGAAVQAAILSGDK
31	NSLESYAFN <u>M</u> K + Oxidation (M)

2901	polymeric immunoglobulin receptor precursor	NCBI/ gi 31377806	84429	5.58	30(20)/ 28%	1144	49	GGCITLISSEGYVSSK
							95	GGCITLISSEGYVSSK
							40	CGLGINSR
							20	VYTVDLGR
							39	TVTINCPFK
							35	TVTINCPFK
							46	IRLDIQGTGQLLFSVVINQLR
							41	IRLDIQGTGQLLFSVVINQLR
							93	LDIQGTGQLLFSVVINQLR
							73	LDIQGTGQLLFSVVINQLR
							40	LDIQGTGQLLFSVVINQLR
							50	LSDAGQYLCQAGDDSNSNK
							82	LSDAGQYLCQAGDDSNSNKK
							71	NADLQVLKPEPELVYEDLR
							88	GSVTFHCALGPEVANVAK
							69	GSVTFHCALGPEVANVAK
							45	QSSGENCDVVVNTLGK
							52	QSSGENCDVVVNTLGK
							24	ILLNPQDK
							51	ILLNPQDKDGSFSVITGLR
							81	ILLNPQDKDGSFSVITGLR
							34	DGSFSVITGLR
							90	DGSFSVITGLR
							47	GVAGGSVAVLCPYNR
							54	CPLLVDSEGWVK
36	IIEGEPNLK							
15	IIEGEPNLK							
79	LVSLTLNLVTR							

54 QGHFYGETAAVYVAVEER
62 QGHFYGETAAVYVAVEER

3001	protein S100-A9	SwissProt/ S10A9_HUMAN	13291	5.71	2(1)/ 20%	39	39 7	KDLQNFLK VIEHIMEDLDTNADK + Oxidation (M)
3106	proline-rich protein 4 isoform 2 precursor	NCBI/gi 154448886	15088	6.5	1(1) 11%	94	94	FPSVSLQEASSFFQR
3210	glutathione S-transferase	NCBI/ gi 2204207	23595	5.43	7(5)/ 31%	339	47 134 60 123 64 36 20	PPYTVVYFPVR FQDGDLTLYQSNTILR FQDGDLTLYQSNTILR DQQEAALVDMVNDGVEDLR + Oxidation (M) DQQEAALVDMVNDGVEDLR + Oxidation (M) ALPGQLKPFETLLSQNQGGK ALPGQLKPFETLLSQNQGGK
3301	lipocalin-1 isoform 1 precursor	NCBI/ gi 4504963	19409	5.39	23(14)/ 52%	391	92 94 58 31 84 68 73 24 48 33	EFPEMNLESVTPMTLTTLEGGNLEAK EFPEMNLESVTPMTLTTLEGGNLEAK + Oxidation (M) EFPEMNLESVTPMTLTTLEGGNLEAK + 2 Oxidation (M) EFPEMNLESVTPMTLTTLEGGNLEAK + 2 Oxidation (M) EFPEMNLESVTPMTLTTLEGGNLEAK + 2 Oxidation (M) EFPEMNLESVTPMTLTTLEGGNLEAK + 2 Oxidation (M) VTMLISGR VTMLISGR VTMLISGR + Oxidation (M)

4	VT <u>M</u> LISGR + Oxidation (M)
42	VT <u>M</u> LISGR + Oxidation (M)
32	TDEPGKYTADGGK
45	TDEPGKYTADGGK
31	HVAYIIR
33	HVAYIIR
45	DHYIFYCEGELHGKPV
62	NNLEALED FEK
51	NNLEALED FEK
46	NNLEALED FEK
24	GLSTESILIPR
3	GLSTESILIPR
63	GLSTESILIPR

3303	actin, cytoplasmic 1	NCBI/ gi 4501885	42052	5.26	9(7)/ 28%	500	52	AVFPSIVGRPR
							41	VAPEEHPVLLTEAPLNPK
							48	DLTDYLMK + Oxidation (M)
							23	DLTDYLMK + Oxidation (M)
							37	GYSFTTTAER
							146	LCYVALDFEQEMATAASSSLEK + Oxidation (M)
							60	SYELPDGQVITIGNER
							118	DLYANTVLSGGTTMYPGIADR + Oxidation (M)
							87	DLYANTVLSGGTTMYPGIADR + Oxidation (M)

4001	protein S100-A9	NCBI/ gi 4506773	13291	5.71	7(5)/ 63%	261	68	NIETIINTFHQYSVK
							45	KDLQNFLK
							29	DLQNFLK
							43	VIEHIMEDLDTNADK + Oxidation (M)
							46	QLSFEEFIMLMAR + 2 Oxidation (M)
							75	QLSFEEFIMLMAR + 2 Oxidation (M)
							5	.MHEGDEGPGHHHKPGLGEGTP + Acetyl (N-term)

4010	protein S100-A11	NCBI/ gi 5032057	11847	6.56	4(3)/ 34%	194	81 27 86 80	CIESLIAVFQK DGYNYTLSK TEFLSFMNTELA ^M AFTK + Oxidation (M) TEFLSFMNTELA ^M AFTK + Oxidation (M)
4113	proline-rich protein 4 isoform 2 precursor	NCBI/ gi 154448886	15088	6.5	3(3)/ 11%	117	112 73 117	FPSVSLQEASSFFQR FPSVSLQEASSFFQR FPSVSLQEASSFFQR
4114	heat shock protein beta-1	NCBI/ gi 4504517	22826	5.98	3(1)/ 18%	128	34 66 33 30	AQLGGPEAAK LFDQAFGLPR SSLSSAQADFNQLAELDR LATQSNEITIPVTFESR
5106	heat shock protein 27	NCBI/ gi 662841	22427	7.83	6(4)/ 41%	278	20 70 44 76 67 46	RVPFSLLR LFDQAFGLPR VSLDVNHFAPDELTVK KYTLPPGVDPTQVSSSLSP ^M EGTLTVEAP ^M PK) + Oxidation (M) LATQSNEITIPVTFESR LATQSNEITIPVTFESR
5111	proline-rich protein 4	SwissProt/ PROL4_HUMAN	15088	6.5	1(1)/ 11%	120	120	FPSVSLQEASSFFQR
5112	proline-rich protein 4 isoform 2 precursor	NCBI/ gi 154448886	15088	6.5	2(2)/ 11%	128	54 128	FPSVSLQEASSFFQR FPSVSLQEASSFFQR
5113	fatty acid-binding protein	NCBI/ gi 4557581	15497	6.6	1(1)/ 6%	58	58	ELGVGIALR

[illegible]

64	VFDEFKPLVEEPQNLIK
67	VFDEFKPLVEEPQNLIK
83	QNCELFEQLGEYK
49	QNCELFEQLGEYK
9	FQNALLVR
64	FQNALLVR
74	KVPQVSTPTLVEVSR
47	KVPQVSTPTLVEVSR
59	RMPCAEDYLSVVLNQLCVLHEK
	+ Oxidation (M)
32	CCTESLVNR
63	RPCFSALEVDETYVPK
78	RPCFSALEVDETYVPK
78	EFNAETFTFHADICTLSEK
48	KQTALVELVK
15	QTALVELVK
43	QTALVELVK
100	AVMDDFAAFVEK + Oxidation (M)
46	AVMDDFAAFVEK + Oxidation (M)
16	LVAASQAALGL
85	LVAASQAALGL

5705	Ig alpha-1 chain C region	SwissProt/ IGHA1_HUMAN	38486	6.08	8(7)/ 28%	224	66	DASGVFTWTPSSGK
							41	TFTCTAAYPESK
							48	TPLTATLSK
							56	SGNTFRPEVHLLPPPSEELALNELVTLTCLAR
							75	WLQGSQELPR
							14	YLTWASR
							59	QEPSQGTTTTFAVTSILR
							56	QEPSQGTTTTFAVTSILR
5904	gelsolin isoform a precursor	NCBI/ gi 4504165	86043	5.9	8(6)/ 12%	449	76	FDLVPVPTNLYGDFFTGDAYVILK
							18	EVQGFESATFLGYFK

107 EVQGFESATFLGYFK
16 TASDFITK
65 QTQVSVLPEGGETPLFK
73 AGALNSNDAFVLK
69 TGAQELLR
45 DSQEEKTEALTSK

6104	IgG kappa chain	NCBI/ gi 4176418	23690	6.92	7(3)/ 33%	213	84	FSGSGSGTDFLTISR
							27	TVAAPSVFIFPPSDEQLK
							16	TVAAPSVFIFPPSDEQLK
							47	TVAAPSVFIFPPSDEQLK
							53	VDNALQSGNSQESVTEQDSK
							39	VDNALQSGNSQESVTEQDSK
							29	VYACEVTHQGLSSPVTK
6201	malate dehydrogenase, cytoplasmic isoform 2	NCBI/ gi 5174539	36631	6.91	5(2)/ 17%	174	46	DVIATDKEDVAFK
							28	DLDVAILVGSM ^{PR} + Oxidation (M)
							46	GEFVTTVQQR
							26	FVEGLPINDFSR
							28	ESAFEFLSSA
6205	peroxiredoxin-6	NCBI/ gi 4758638	25133	6.00	12(5)/ 55%	463	101	PGGLLLGDVAPNFEANTTVGR
							46	DFTPVCTTELGR
							47	LPFPIIDDR
							48	ELAILLGM ^{LDPAEKDEK} + Oxidation (M)
							20	VVFVFGPDK
							36	VVFVFGPDKK
							25	LSILYPATTGR
							34	NFDEILR
							21	VVISLQLTAEK
							32	VVISLQLTAEKR
							10	VATPVDWKDGDSVMVLPTIPEEEAK + Acetyl (N-term)

						43	DGDSVMVLPTIPEEEAK + Oxidation (M)
6207	protein DJ-1	NCBI/ gi 31543380	20050	6.33	5(2)/ 25%	199	39 GAEEMETVIPVDVMR + 2 Oxidation (M)
							8 DVVICPDASLEDAK
							15 DVVICPDASLEDAKK
							71 GPGTSFEFALAIVEALNGK
							137 GPGTSFEFALAIVEALNGK
6302	alpha-enolase isoform 1	NCBI/ gi 4503571	47481	7.01	5(4)/ 14%	295	86 AAVPSGASTGIYEALRL
							88 LAMQEFMILPVGAANFR + 2 Oxidation (M)
							44 LAMQEFMILPVGAANFR + 2 Oxidation (M)
							81 VVIGMDVAASEFFR + Oxidation (M)
							40 YISPDQLADLYK
6319	annexin A1	NCBI/ gi 4502101	38918	6.57	20(13)/ 50%	958	57 .AMVSEFLK.Q + Acetyl (N-term); Oxidation (M)
							36 .AMVSEFLK.Q + Acetyl (N-term); Oxidation (M)
							40 GGPGSAVSPYPTFNPSSDVAALHK
							93 GVDEATIIDILTK
							65 AAYLQETGKPLDETLK
							66 ALTGHLEEVVLLLK
							49 TPAQFDADELRL
							92 TPAQFDADELRL
							90 GLGTDEDTLIEILASR
							78 DITSDTSGDFR
							51 NALLSLAK
							37 NALLSLAK
							107 SEDFGVNEDLADSDAR
							62 SEDFGVNEDLADSDAR
							100 KGTDVNVFNTILTTR
							71 GTD VNVFNTILTTR
							26 VLDLELK

							22	VLDLELKGDIEK
							43	VLDLELKGDIEK
							30	ILVALCGGN
6405	serum albumin	NCBI/ gi 28592	71316	6.05	10(5)/ 16%	400	32	LVNEVTEFAK
							26	LCTVATLR
							24	YLYEIAR
							64	AAFTECCQAADK
							44	YICENQDSISSK
							49	DVFLGMFLYEYAR + Oxidation (M)
							10	TYETTLEK
							50	FQNALLVR
							29	QTALVELVK
							81	AVMDDFAAFVEK + Oxidation (M)
6703	transferrin	NCBI/ gi 115394517	79190	6.97	11(5)/ 15%	169	28	SVIPSDGPSVACVK
							50	DSGFQMNQLR + Oxidation (M)
							12	DGAGDVAFVK
							65	DGAGDVAFVK
							34	HSTIFENLANK
							30	ADRDQYELLCLDNTR
							45	EFQLFSSPHGK
							26	DLLFK
							40	DSAHGFLK
							68	FDEFFSEGAPGSK
							56	EGYYGYTGAFR
7002	protein S100-A8	NCBI/ gi 21614544	10885	6.51	7(4)/ 53%	192	22	MLTELEK + Oxidation (M)
							27	MLTELEK + Oxidation (M)
							9	ALNSIIDVYHK
							49	ALNSIIDVYHK
							52	LLETECPQYIR
							64	ELDINTDGAVNFAQEFLILVIK

							57	ELDINTDGAVNFQEFLILVIK
7106	triosephosphate isomerase 1	NCBI/ gi 17389815	26910	6.45	4(2)/ 16%	183	80	QSLGELIGTLNAAK
							26	RHVFGESDELIGQK
							22	HVFGESDELIGQK
							55	IYGGSVTGATCK
7107	keratin 1	NCBI/ gi 7331218	66149	8.16	2(1)/ 4%	79	37	FSSCGGGGGSFGAGGGFGSR
							45	FLEQQNQVLQTK
7114	Peroxiredoxin 5 chain A	NCBI/ gi 15826629	17060	6.96	3(1)/ 20%	109	55	VNLAELFK
							18	THLPGFVEQAEALK
							36	LLADPTGAFGK
7203	annexin A2 isoform 2	NCBI/ gi 4757756	38808	7.57	13(10)/ 38%	643	10	AYTNFDAERDALNIETAIK
							17	DALNIETAIK
							55	DALNIETAIK
							77	GVDEVTIVNILTNR
							52	GVDEVTIVNILTNR
							67	SALSGHLETIVILGLLK
							53	GLGTDEDSLIEIICSR
							60	TNQELQEINR
							85	TDLEKDIISDTSGDFR
							72	RAEDGSVIDYELIDQDAR
							92	AEDGSVIDYELIDQDAR
							28	SLYYYIQQDTK
							47	ALLYLCGGDD
7321	isocitrate dehydrogenase 1	NCBI/ gi 89573979	42091	6.19	4(3)/ 19%	227	66	ATDFVVPGP GK
							33	VEITYTPSDGTQK
							41	VTYLVHNFEEGGGVAMGMYNQDK
								+ 2 Oxidation (M)
							87	ELAFFANALEEVSIETIEAGFMTK

+ Oxidation (M)

7510	alpha-enolase isoform 1	NCBI/gi 4503571	47481	7.01	10(9)/ 28%	641	50	GNPTVEVDLFTSK
							51	GNPTVEVDLFTSK
							91	AAVPSGASTGIYEALER
							58	IDKLMIE M MDGTENK + 2 Oxidation (M)
							72	LAM Q E F M ILPVGAANFR + 2 Oxidation (M)
							37	LAM Q E F M ILPVGAANFR + 2 Oxidation (M)
							109	DATNVGDEGGFAPNILENK
							106	VVIGM D VAAASEFFR + Oxidation (M)
							54	YISPDQLADLYK
							101	VNQIGSVTESLQACK
8109	annexin A1	NCBI/ gi 4502101	38918	6.57	13(8)/ 38%	698	44	GGPGSAVSPYPTFNPSSDVAALHK
							30	AAYLQETGKPLDETLK
							88	TPAQFDADELK
							85	GLGTDEDTLIEILASR
							64	DITSDTSGDFR
							20	NALLSLAK
							7	GDRSEDFGVNEDLADSDAR
							58	GDRSEDFGVNEDLADSDAR
							38	SEDFGVNEDLADSDAR
							126	SEDFGVNEDLADSDAR
							84	KGTDVNVFNTILTTR
							79	GTDVNVFNTILTTR
							30	VLDLELKGDIK
8112	immunoglobulin light chain	NCBI/ gi 2894829	23542	5.95	8(4)/ 35%	293	40	LLIYGASSR
							76	FSGSGSGTDFLTISR
							22	TVAAPSVFIFPPSDEQLK
							46	TVAAPSVFIFPPSDEQLK
							33	TVAAPSVFIFPPSDEQLK
							57	TVAAPSVFIFPPSDEQLK

							113	VDNALQSGNSQESVTEQDSK
							8	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK
8201	beta-2 microglobulin	NCBI/ gi 34616	12905	5.77	3(1)/ 38%	107	56	SNFLNCYVSGFHPSDIEVDLLK
							24	VEHSDLSFSK
							27	VNHVTLNQPK
8301	phosphoglycerate kinase 1	NCBI/ gi 4505763	44985	8.3	3(1)/ 6%	92	62	VLNNMEIGTSLFDEEGAK + Oxidation (M)
							10	VLPGVDALSNI
							30	VLPGVDALSNI
8502	serum albumin	SwissProt/ALBU_HUMAN	71317	5.92	10(5)/ 16%	165	32	LVNEVTEFAK
							26	LCTVATLR
							24	LYEIR
							64	AAFTECCQAADK
							44	YICENQDSISSK
							49	DVFLGMFLYEYAR + Oxidation (M)
							10	TYETLEK
							50	FQNALLVR
							29	QTALVELVK
							81	AVMDDFAAFVEK + Oxidation (M)
8801	lactoferrin	NCBI/ gi 186833	80228	8.56	54(38)/ 53%	2249	75	SVQWCTVSQPEATK
							82	DSPIQCIQIAENR
							57	DSPIQCIQIAENR
							54	ADAVTLDGGFIYEAGLAPYK
							62	ADAVTLDGGFIYEAGLAPYK
							36	ADAVTLDGGFIYEAGLAPYK
							51	ADAVTLDGGFIYEAGLAPYK
							73	ADAVTLDGGFIYEAGLAPYK
							114	ADAVTLDGGFIYEAGLAPYK
							70	ADAVTLDGGFIYEAGLAPYK
							69	ADAVTLDGGFIYEAGLAPYK

84	ADAVTLDGGFIYEAGLAPYK
108	ADAVTLDGGFIYEAGLAPYK
64	LRPVAAEVYGTERT
37	THYYAVAVVK
36	THYYAVAVVK
90	KGGSFQLNELQGLK
50	GGSFQLNELQGLK
84	GGSFQLNELQGLK
82	FFSASCVPGADK
12	GQFPNLCR
31	GQFPNLCR
97	CAFSSQEPYFSYSGAFK
67	DGAGDVAFIR
35	DGAGDVAFIR
78	DGAGDVAFIR
42	ESTVFEDLSDEAER
29	EDAIWNLLR
52	EDAIWNLLR
40	FQLFGSPSGQK
31	FQLFGSPSGQK
30	DLLFK
24	DSAIGFSR
34	DSAIGFSR
16	IDSGLYLGSGYFTAIQNLR
129	IDSGLYLGSGYFTAIQNLR
80	VVWCAVGEQELR
101	GEADAMSLDGGYVYTAGK
110	GEADAMSLDGGYVYTAGK + Oxidation (M)
82	SQQSSDPDPNCVDRPVEGYLAVAVVR
36	RSDTSLTWNSVK
70	RSDTSLTWNSVK
27	SDTSLTWNSVK
92	FDEYFSQSCAPGSDPR
87	SNLCALCIGDEQGENK

45	YYGYTGAFR
90	CLAENAGDVAFVK
42	LADFALLCLDGK
104	LADFALLCLDGK
53	FCLFQSETK
38	FCLFQSETK
96	NLLFNDNTECLAR
83	YLG PQYVAGITNLK
84	CSTSPILLEACEFLR

9001	Lysozyme C	SwissProt/ LYSC_HUMAN	16982	9.38	10(4)/ 36%	132	17	LGM <u>D</u> GYR + Oxidation (M)
							31	LGM <u>D</u> GYR + Oxidation (M)
							70	GISLANW <u>M</u> CLAK + Oxidation (M)
							13	ATNYNAGDR
							40	ATNYNAGDR
							17	STDYGIFQINSR
							39	STDYGIFQINSR
							71	STDYGIFQINSR
							28	DPQGIR
9102	gluthione S-transferase subunit 1 (GST,EC 2.5.1.18)	NCBI/ gi 187132	25733	8.84	5(1)/ 15%	100	19	QYVQGCGV
							3	AILNYIASK
							47	AILNYIASK
							7	YFPAFEK
							25	SHGQDYLVGNK
9201	cystatin-SN precursor	NCBI/ gi 19882251	16605	6.73	5(4)/ 32%	182	26	FLQPGSPR
							52	ALHFAISEYNK
							68	ALHFAISEYNK
							98	QQTVGGVNYFFDVEVGR
							66	QQTVGGVNYFFDVEVGR
							19	SQPNLDTCAFHEQPELQK

9404	peroxiredoxin-1	NCBI/ gi 4505591	22324	8.27	9(5)/ 36%	349	24	DISLSDYK
							28	KQGGLGPMNIPLVSDPK + Oxidation (M)
							50	QGGGLGPMNIPLVSDPK + Oxidation (M)
							32	TIAQDYGVLK
							52	ADEGISFR
							43	GLFIIDDK
							53	QITVNDLPVGR
							34	LVQAFQFTDK
							68	LVQAFQFTDK
9501	chain A, C3b in complex with a C3b specific Fab	NCBI/ gi 224983659	71372	6.82	16(10)/ 35%		15	LESEETMVLEAHDAQGDVPVTVVHDFPGK + Oxidation (M)
							37	VVLVSLQSGYLFIQTDK
							96	VVLVSLQSGYLFIQTDK
							34	TIYTPGSTVLYR
							54	TVMVNIENPEGIPVK + Oxidation (M)
							74	AYYENSPQQVFSTEFVK
							77	EYVLPSFEVIVEPTEK
							83	KVEGTAFVIFGIQDGEQR
							44	VLLDGVQNPR
							52	YFKPGMPFDLMVFVTNPDGSPAYR + 2 Oxidation (M)
							59	VPVAVQGEDTVQSLTQGDGVAK
							28	TELRPGETLNVNLLR
							78	TELRPGETLNVNLLR
							32	YYTYLIMNK + Oxidation (M)
							111	EPGQDLVVLPLSITTDIFPSFR
							34	EPGQDLVVLPLSITTDIFPSFR