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NANO-LC MASS SPECTROMETRY PROTEOMIC TEAR SECRETION ANALYSIS IN PATIENTS WITH SECONDARY SJÖGREN'S SYNDROME

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Sjögren's syndrome (SS) is a systemic autoimmune disorder characterized by dry eye and mouth as a result of exocrine glands destruction due to lymphocytes and plasma cells infiltration. The aim of this study was to compare the protein profile in the tears of healthy subjects and patients with SS associated with rheumatoid arthritis (RA) in an effort to reveal potential biomarker candidates. The study groups consisted of subjects with SS associated with RA, patients with RA without SS and healthy volunteers. Tear fluids were collected by Schirmer method. The tear proteins were separated by electrophoresis and analysed by Nano-LC-nano-ESI-MS/MS. The results demonstrated that the patients with SS have significantly reduced values of Schirmer tests. The Coomassie colloidal blue staining evidenced only the most abundant peptides present in the tears from all groups analyzed with significant quantitative differences in the abundance of some peptides. The trypsin digestion and Nano-LC-nano-ESI-MS/MS sequencing were performed for a total of 27 different proteins identified in all tear samples. The proteomic technology proved to be a good strategy to characterize tear proteins patterns in normal subjects and in patients with different ocular dysfunction for the identification of potential biomarkers associated with chronic systemic diseases.

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1. Introduction

Sjögren's syndrome (SS) is a systemic autoimmune disorder characterized by dry eye and dry mouth. The disease can exist in association with a connective tissue disorder, in that case it is termed secondary Sjögren's syndrome (s-SS), or without definable associated connective tissue disorder, in which it is termed primary Sjögren's syndrome [1]. The disease is characterized by infiltration of exocrine glands, such as lacrimal and salivary glands, with lymphocytes and plasma cells [2, 3]. The inflammatory cells recognize unknown self-antigens, produce autoantibodies and contribute to glandular destruction [4]. This process ultimately results in a characteristic loss of tearing and saliva. The most common connective tissue disease associated with s-SS is rheumatoid arthritis (RA) [5]. The preliminary European classification criteria for s-SS, include one or more sicca symptoms and at least two objective findings: one pathological finding indicating eye involvement and one for oral involvement [6]. The tear film is a complex liquid structure resting on the exposed corneal and conjunctive surfaces of the eye. It acts to smooth out irregularities in the corneal epithelium and serves as the anterior refractive surface of the eye.

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The tear film consists of a three-layer structure, with two fluid layers resting on a semisolid mucin layer. The outermost layer has a lipid composition and originates primarily from the meibomian glands of the eyelid. The middle aqueous layer arises from the main lacrimal glands. It contains inorganic salts, glucose, urea, proteins, and trace elements, all of which are secreted by the main and accessory lacrimal glands [7]. Proteins secreted by the lacrimal glands include lysozyme, lactoferrin, lipocalin and secreted IgA [8]. The innermost mucin layer rests on the underlying surface epithelium. Mucin consists of hydrated glycoproteins [7]. In s-SS the destruction of the lacrimal glands leads to the decrease of the aqueous component of the tear film that can be revealed by decreased level of the Schirmer test. By this test we can evidence the qualitative modifications in tears composition. The qualitative changes in tear protein profile associated with s-SS can be further evaluated only by complex proteomic techniques.

The aim of this study was to display and compare the protein profile in the tears of healthy subjects and patients with s-SS associated with RA. The current study aimed to identify the qualitative modification in tear protein patterns in the subjects with s-SS.

2. Materials and methods

All the subjects participating in this study were from Clinical Eye Emergency Hospital of Bucharest and from Department of Rheumatology of Dr. I. Cantacuzino Clinical Hospital of Bucharest. Informed consent for the tears collection and analysis was obtained from all donors. The research performed followed the rules of the Declaration of Helsinki and were approved by the appropriate institutional review board of regulations of the ethic Committee of ICBP "N. Simionescu" and Romanian Law no.471/2002.

The study groups consisted of subjects with s-SS associated with RA (n=10), patients with RA without s-SS (n=10) and healthy volunteers (n=10). The identification of patient suggested to have s-SS was made by using a sicca symptoms questionnaire [9]. The Schirmer test without topical anesthesia was performed in all subjects. The values of Schirmer test for each subject were recorded and the filter papers containing tears were collected in a centrifuge tube and soaked with 40 µl of buffer solution containing (6% SDS, 4M urea, 4mM EDTA, 50mM Tris-HCl, 0.5% β-mercaptoethanol and 0.1% bromphenol-blue). The samples were stored at -20ºC until the protein electrophoresis was performed. Prior to electrophoresis the samples were heated at 95ºC for 5 min then the tear fluids were eluted by centrifugation at 1000 x g. The tear proteins were separated by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) under reducing conditions. The protein bands were detected by Coomassie colloidal blue staining (1 h in a mixture of 30% methanol, 0.2M orthophosphoric acid and 170 g/l ammonium sulphate, before incubation in the same solution to which 0.66 g/l of G250 brilliant blue had been added). The Coomassie blue-stained protein bands were excised from the polyacrylamide gels. The gel fragments were distained by washing three times with 300µl of 100mM ammonium carbonate/acetonitrile (ACN) 1:1(v/v) solution for 20 min under shaking. The pieces were dehydrated in acetonitrile and the enzymatic cleavage was performed by incubating the fragments in 150 µl ammonium carbonate solution (50 mM) containing 12.5 ng/µl trypsin. After absorption of the protease, 40 µl of 50mM ammonium carbonate solution were added. The trypsin digestion was performed at 37ºC for 12h. The digested peptides were extracted from the gel fragments by elution with solution containing 45% ACN/10% formic acid. The pooled extracts were dried using a Speed Vac-concentrator (Eppendorf, France). Nano-LC-nano-ESI-MS/MS analyses were performed on an ion trap mass spectrometer (LCQ Deca XP^+, Thermo Electron Corp.) equipped with a nano-electrospray ion source coupled with a nano-high pressure liquid chromatography system (LC Packings Dionex). Tryptic digests were resuspended in 4 µl of 0.1% HCOOH, and 1.4 µl were injected into the mass spectrometer using the Famos autosampler (LC Packings Dionex).

The mass spectrometer was operated in positive ionization mode. MS/MS data were acquired using a 2 *m*/*z* unit ion isolation window and 35% relative collision energy. MS/MS.raw data files were transformed in .dta files with Bioworks 3.1 software (Thermo Electron Corp.). The .dta files generated were next merged with merge.bat software to be downloaded in Mascot software (version 2.1) for database searches in Swiss-Prot. Search parameters were the following:

Homo sapiens for taxonomy, one allowed missed cleavage, methionine oxidation as variable modification, carbamidomethylation of cysteine as fixed modification, 2 Da for peptide tolerance and 0.8 Da for MS/MS tolerance. Results were scored using the probability-based Mowse score. The protein score is –10xlog(p) where p is the probability that the observed match is a random event. Protein scores greater than 66 were considered significant ($p < 0.05$). All the identifications were made on the basis of minimum two different peptides detected. For each protein identified, the Swiss-Prot/TrEMBL accession number has been reported in the table. Manual evaluation was performed for all MS/MS spectra to minimize falsely identified proteins.

3. Results and discussion

The characteristics of the study groups are shown in the Table 1.

Table 1: Characteristics of the study groups series.

Parameter	**s-SS (n=10)**	**RA without s-SS (n=10)**	**Healthy volunteers (n=10)**
Sex **M** **F**	 n=1 n=9	 n=2 n=8	 n=4 n=6
Age(years) mean (±SD)	52.9 (3.3)	55.3(5.2)	50.7(4.2)
Schirmer test (mm) mean (±SD)	6.3 (1.2)	15.4 (4.5)	16.2 (2.4)

n = number of patients, SD = standard deviation, M = males, F = females,
RA = rheumatoid arthritis, s-SS = secondary Sjögren's syndrome.

The patients with s-SS have shown significantly reduced values of Schirmer tests. There were no differences in Schirmer tests values between healthy volunteers and patients with RA without clinical signs of dry eyes. Figure 1 shows a representative aspect of the SDS-PAGE protein electrophoresis from tears of 3 donors (a patient with RA without s-SS, a patient with s-SS and a healthy volunteer). Notable differences could be observed between the control group (line A) and the two pathological groups (lines B and C) and also between the RA and s-SS groups, suggesting that inflamatory process developed in these pathologies have both common as well as specific regulatory mechanisms. In the Coomassie colloidal blue staining only a few protein bands were easily observed. The very high and very low molecular weight proteins were difficult to be separated by SDS-PAGE electrophoresis. The samples from the healthy volunteers and from the patient with RA without s-SS revealed almost the same electrophoretic protein patterns.

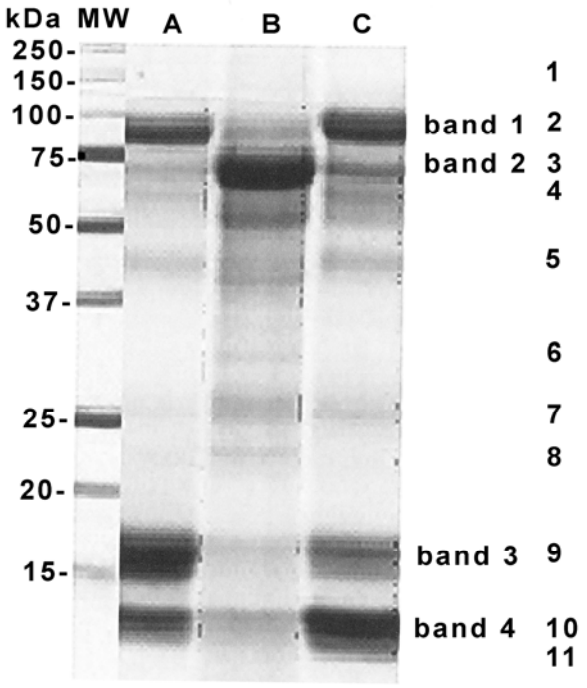

Fig. 1. Representative pattern of the separated tear proteins by SDS-PAGE (Coomassie blue staining) from healthy volunteers (A), patients with s-SS (B) and patients with rheumatoid arthritis without clinical manifestations of s-SS (C).

The methods used in this study (SDS-PAGE and nano-LC-MS-MS) did not allowed a precise quantitation regarding the relative abundance of the detected peptides. However the abundance is significantly altered in the probes harvested from the patients with clinical signs of dry eye. The most abundant bands were of approximately molecular weight of 80 kDa (band 1), 65 kDa (band 2), 20 kDa (band 3) and 16 kDa (band 4). For patients with s-SS the bands 1, 3 and 4 were less prominent than those seen in the normal subjects. Also, six patients with s-SS displayed a more intense band 2 than the healthy donors. A total of eleven bands labeled in Figure 1 were cut, trypsin digested and analyzed by Nano-LC-nano-ESI-MS/MS sequencing. The majority of bands contained multiple protein components displayed in the Table 2.

Table 2. Proteins identified in tears by 1D- electrophoresis followed by nano-LC-nano-ESI-MS/MS, with Mascot protein score ≥ 143

Band no.	**SP-TrEMBL**	**Protein description**	**No. of peptides found**	**Mascot protein score**	**Molecular weight**
1	P02788	Lactotransferrin precursor	15 (9)	663	78338
	P01833	Polymeric-immunoglobulin receptor precursor	5 (3)	202	83262
2	P02788	Lactotransferrin precursor	29 (13)	823	78338
3	P02768	Serum albumin precursor	27 (13)	823	69367
	P02788	Lactotransferrin precursor	10 (6)	482	78338
	P01024	Complement C3	3 (3)	180	187164

Band no.	SP-TrEMBL	Protein description	No. of peptides found	Mascot protein score	Molecular weight
		precursor			
4	P02788	Lactotransferrin precursor	7 (5)	403	78338
	P01876	Ig alpha-1 chain C region	10 (3)	274	37655
	P01009	Alpha-1-antitrypsin precursor	4 (4)	307	46737
5	P25311	Zinc-alpha-2 glycoprotein precursor	15 (5)	437	33872
	P30740	Leukocyte elastase inhibitor	6 (5)	314	42742
	P01877	Ig alpha-2 chain C region	3 (3)	216	36508
6	P04083	Annexin A1	2 (2)	150	38583
7	P80188	Neutrophil gelatinase-associated lipocalin precursor	4 (2)	254	22588
	P02647	Apolipoprotein A-I precursor	5 (2)	208	30778
8	P01876	Ig alpha-1 chain C region	2 (2)	145	37655
	Q06830	Peroxiredoxin 1	4 (3)	145	22110
9	P31025	Von Ebner's gland protein precursor (Tear prealbumin)	17 (3)	250	19250
	P12273	Prolactin-inducible protein precursor	6 (3)	236	16572
10	P06702	Calgranulin B	3 (3)	203	13242
	P61626	Lysozyme C precursor	7 (2)	149	16537
11	P06702	Calgranulin B	5 (4)	266	13242
	P26447	S100 calcium-binding protein A4	4 (2)	143	11729

After database searching and manual examination of the results, a total of 27 different proteins were identified in all tear samples. Proteins detected by our method included lacrimal gland proteins such as lysozyme C, lactotransferrin, tear specific prealbumin (lipocalin) and serum protein such as serum albumin and immunoglobulin. These proteins were previously identified by 1D/2D gel electrophoresis [10, 11, 12, 13] and/or by high performance liquid chromatography (HPLC) techniques [14, 15, 16, 17]. Other proteins detected as complement C3 precursor, zinc-alfa2-glycoprotein precursor, alpha-1-antitrypsin, extra cellular glycoprotein lacritin precursor, clusterin precursor, haptoglobin precursor were also reported by HPLC followed by nano-LC-nano-ESI-MS/MS [18] or by LC-MALDI MS/MS [19] methods. Leukocyte elastase inhibitor, a protein with antimicrobial or antiviral activity [20, 21] was also identified by our technique. Figure 2 shows the LC-MS/MS spectrum and the amino acid sequences of a peptide fragment originating from peroxiredoxin 1 (Prx 1), a protein found in all tear samples. Prx 1 is an antioxidant enzyme involved in regulating many cellular processes including cell proliferation, differentiation and also

characterizes inflammatory disease and/or cellular apoptotic processes in many cellular disorders [22]. This enzyme was reported to be overexpressed in breast cancer tissues as well.

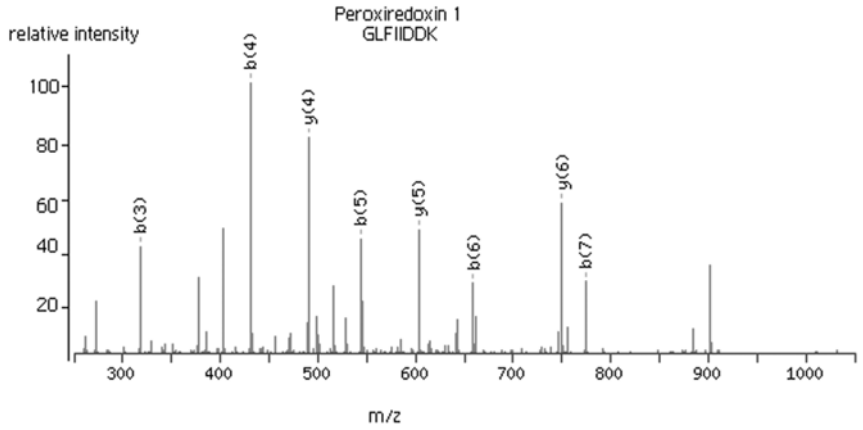

Fig. 2 - LC-MS/MS spectrum of peptide fragment originating from peroxiredoxin 1 (Q06830, GLFIIDDK).

The authors suggested that Prx 1 has a proliferative effect and in particular conditions may be related to cancer development or progression [23]. The present high resolution proteomic approach showed that this enzyme is also associated with secondary Sjögren's syndrome (s-SS) suggesting an active participation in the inflammatory process present in the dry eye. More than that, in few tear samples the heat-shock protein beta-1 (HSPB1) was also identified demonstrating that the tear components reflect changes in the health of the ocular surface associated with the Sjögren's syndrome. Figure 3 shows the LC-MS/MS spectrum and the amino acid sequences of a peptide fragment originating from HSPB1. The HSPB1 has been reported as biomarker in breast cancer [24] as well.

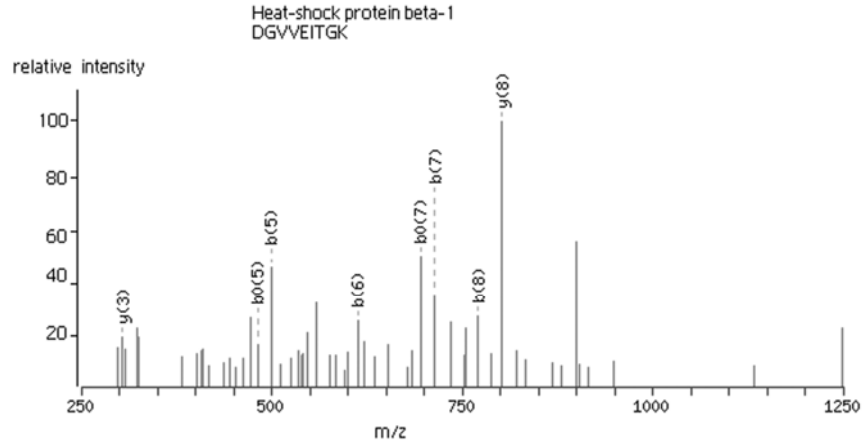

Fig. 3 - LC-MS/MS spectrum of peptide fragment originating from heat-shock protein beta-1 (P04792, DGVVEITGK).

4. Conclusions

In this study was applied the 1D-electrophoresis followed by Nano-LC-nano-ESI-MS/MS to characterize the tear proteome profile in healthy subjects and patients with s-SS. Thus, 27 different proteins were identified in the analysed bands in tears collected from the experimental groups. The HSPB1 was identified only in a few tear samples. The procedures applied did not identified important qualitative differences in tear protein pattern between normal donors and

patients with s-SS. There were only quantitative differences for some peptides shown by SDS-PAGE gels. The proteins identified by this technique in tears were previously reported as biomarkers in breast cancer and in inflammatory disease as well. The relevance of the presence of these cancer biomarkers in the tear of s-SS patients should be further investigated to identifie the signaling pathway and citokines involved in the pathological process. The proteins found in the tears play an important role in maintaining the ocular surface and changes in tear protein components may reflect changes in the health of the ocular surface. 1D-electrophoresis followed by Nano-LC-nano-ESI-MS/MS proved to be a good tool to characterize tear proteins patterns in normal subjects and in patients with different ocular diseases. Recent tear proteomics and immunobloting data [25] support our results showing that in evaporative dry eye disease some proteins had a significant decreased levels (lactoferin, lipocalin-1 and lipophilen A-C), while others (serum albumin) had an increased level or demonstrate no changes in their abundance (as lysozyme and zinc α-2 glycoprotein). More studies are necessary to identify in tears potential biomarkers for cancer or for other systemic diseases.

In summary proteomics provides a comprehensive approach to identify all the proteins of the tear proteome, which will help to elucidate disease pathogenesis, make clinical diagnosis and evaluate the influence of medications on the structure, composition and secretion of tear proteins.

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