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A methodological approach based on gold-nanoparticles followed by matrix assisted laser desorption ionization time of flight mass spectrometry for the analysis of urine profiling of knee osteoarthritis

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ABSTRACT

The aim of this work is to develop a nanoparticle-based methodology to find out biomarkers of diagnostic for knee osteoarthritis, KOA, through the use of matrix assisted laser desorption ionization time-of-flight-based mass spectrometry profiling.

Urine samples used for this study were obtained from KOA patients (42 patients), patients with prosthesis (58 patients), and controls (36 individuals) with no history of joint disease. Gold-nano particle MALDI-based urine profiling was optimized and then applied over the 136 individuals. Jaccard index and 10 different classifiers over MALDI MS datasets were used to find out potential biomarkers. Then, the specificity and sensitivity of the method were evaluated. The presence of ten m/z signals as potential biomarkers in the healthy versus non-healthy approach suggests that patients (KOA and prosthesis) are differentiable from the healthy volunteers through profiling. The automatic diagnostic study confirmed these preliminary conclusions. The sensitivity and the specificity for the urine profiling criteria here reported, achieved by the C4.5 classifier, is 97% and 69% respectively. Thus, it is confirmed the utility of the method proposed in this work as an additional fast, non-expensive and robust test for KOA diagnostic. When the proposed method is compared with those used in common practice it is found that sensitivity is the highest, thus with a low false negative rate for diagnostic KOA patients in the population studied. Specificity is lower but in the range accepted for diagnostic objectives.

1. Introduction

Knee osteoarthritis (KOA) is an articular degenerative pathology defined as the loss of hyaline cartilage, subchondral sclerosis, bone degradation and synovial joint narrowing [1]. At a biochemical level, KOA is characterized by the degradation of several components of the extracellular matrix (ECM), as cartilage [2], as well as an increased local inflammation of the affected joint [3]. Several protein biomarkers such as monocyte chemotactic protein-1 (MCP-1) and fibronectin–aggrecan complex (FAC) correlate with KOA stage [4]. Furthermore, other molecules of cell signaling like IL-1beta, TNF, IL-6, IL-15, IL-17, IL-18, IL-21, leukemia inhibitory factor and IL-8 have been linked to the development of the disease.

OA is the most common articular pathology in developed countries [5]. Prevalence of symptomatic KOA increases from 10% in patients older than 20–14% in patients older than 65, reaching the maximum in the 70–79 age range, and thereafter decreasing [6,7]. KOA is more prevalent in females than in males and seems to be highly associated to elderly [8]. Usual clinical symptoms are pain and the rigidity of the affected joint [9]. Currently, OA is the first permanent impairment cause in the developed world [10].

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This pathology is predicted to increase its incidence dramatically [11], as well as it is predicted that related health care costs will increase."

KOA is commonly diagnosed by radiologic and clinical analysis [12]. The clinical reference for diagnosis is by radiographying the affected articulation and staging by Kellgren–Lawrence scale from 0 to 4 [13]. However, clinicians usually report difficulties in making a correct diagnostic for patients with minimal radiological symptoms referring high pain and others with a clear joint wear that suffer neither pain nor functional alterations. Furthermore, actual diagnostic tests are not useful in early detection [14]. To help in early detection, some biomarkers of inflammation such as high sensitive C-reactive protein (hsCRP) [15] has been proposed as a complement to radiological tests albeit unspecific. Moreover, some other potential biomarkers are being proposed for validation in the last years as well as those that correlate with evolution of the pathology during recommended pharmacologic treatments [16–18].

Within the field of proteomics, methodologies widely used to study the protein content are the chromatographic techniques, mono and two-dimensional electrophoresis and mass spectrometry [19]. Instead of identifying discrete expression biomarkers, a quick and inexpensive way to classify samples is using matrix assisted laser desorption–ionization, MALDI,-mass spectrometry-based fingerprint of peptides [20]. This methodology is applied increasingly by the scientific community due to its versatility and simplicity, even in the field of rheumatology [21]. Peptide fingerprinting only requires an easy analysis per sample and the results are obtained in some tens of seconds [22].

In previous studies we have described a high-throughput method for serum proteomic profiling in patients with multiple myeloma [23]. In the present work such method has been optimized and applied to the complexity of urine samples. The urine´s protein content was concentrated by precipitation using citrate-coated gold nanoparticles (citrate@GNPS). Then, ultra fast ultrasound-assisted in-solution tryptic digestion of the proteins followed by direct peptide profiling by MALDI-MS was used to obtain the profiles of the patients. Then, profiles were used to classify the patients and a pattern of prediction, healthy versus diseased, was obtained.

2. Material and methods

2.1. Reagents

All reagents were HPLC-grade or higher. Sodium dodecyl sulfate (SDS) and formaldehyde were purchased from Panreac (Barcelona, Spain). β-mercaptoethanol was purchased from Merck (Hohenbrunn, Germany) and bromophenol-blue was purchased from Riedel-de Haen (Seelze, Germany). Trypsin, trifluoroacetic acid, bovine serum albumin, carbonic anhydrase DL-Dithiothreitol (DTT), Iodoacetamide (IAA), acrylamide/bis-acrylamide 30% solution (37.5:1), Tris-base, Coomassie Brilliant Blue R250 (CBB), sodium carbonate, sodium thiosulfate, Na_2-EDTA, Sodium citrate tribasic, silver nitrate solution and the SigmaMarker wide range 6500–200,000 Da were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen tetrachloroaurate (III) hydrate (99,9% Au) (49% Au) at 10% w/v was purchased from Strem Chemicals (Newburyport, MA, USA). α-Cyano-4-hydroxycinnamic acid, ammonium bicarbonate (AMBIC) and formic acid were purchased from Fluka (Steinheim, Germany). ZipTip® was purchased from Millipore. Creatinine urinary kit assay was purchased from Cayman chemicals (Ann Arbor, MI, USA).

Table 1
Data of the samples used in this study.

	Urine samples Control	Arthritis	Prosthesis
Patients	36	42	58
% Women	44	81	72
Age	65 ± 6	67 ± 8	69 ± 8
Age range	54-85	55-84	47-86
Total protein content (μg/ml)			
Concentration	69 ± 26	51 ± 20	57 ± 27
Range	19–138	19–89	11–143
Creatinine normalization (μg/μmol)			
Concentration	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.2
Range	0.03–0.1	0.03–0.8	0.03–0.8

2.2. Samples

Urine samples used for this study, first morning void, were obtained from knee osteoarthritic, KOA, patients (42 patients), patients with prosthesis (58 patients), and controls (36 individuals) with no history of joint disease. Samples were provided by the CHUO (Complexo Hospitalario Universitario de Ourense, Spain). Patients were of both genders and ages ranged from 47 to 86 years. Samples were taken from anonymous patients from the Hospital of Ourense, Spain. All of them signed an informed consent. The study was approved by the ethics committee of Galicia (Spain).

Al patients that volunteered for this study were classified as normoproteinuric, being the average protein concentrations as follows: (i) samples from healthy volunteers: 69.3 ± 25.6 μg/ml, (ii) samples from KOA patients 50.8 ± 19.7 μg/ml and (iii) samples from patients with knee joint prosthetic 56.5 ± 27.0 μg/ml. Table 1 summarizes the characteristics of the patients included in this study.

2.3. Pre-analytical treatment of urine samples and urine normalization

Prior to analysis, 20 ml of urine were centrifuged for 30 min at 5000*g* to remove cells and debris. Then, 3 ml of urine were diluted to 30 ml with ultrapure water, then urine was aliquoted in five subsamples and stored at −60 °C.

Total protein content was determined using a Bradford assay. Urine protein content was normalized to creatinine by the Jaffè assay from Cayman Chemicals (500701 kit, Ann Arbor, MI, USA). Colorimetric absorbance was measured at 492 nm in duplicate; results were expressed in mg protein/mmol creatinine.

2.4. Apparatus

Microplate-reader LT-4000 from Labtech (Uckfield, UK) was used for Bradford and Jaffè colorimetric assays. PowerPac Basic from Bio-Rad (CA, USA) was used as current supplier for SDS-PAGE protein separation. Image gels were obtained using a ProPicII (Digilab-Genomic Solutions, USA). Mass spectrometry analysis was performed using a MALDI Ultraflex from Bruker-Daltonics (Bremen, Germany), equipped with a LIFT cell and a nitrogen laser of 50 Hz. Centrifugation was done in a CM · 50M centrifuge from ELMI (Riga, Latvia).

2.5. Synthesis of citrate-capped gold nanoparticles.

Gold nanoparticles, GNPs, were synthesized by the citrate reduction method in aqueous solution [23]. Briefly, 60 ml of sodium citrate tribasic solution (0.075% w/v) was heated to 100 °C, and then gold was added as 54 μL of 10% w/v of hydrogen