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Validation of a new method by nano-liquid chromatography on chip tandem mass spectrometry for combined quantitation of C3f and the V65 vitronectin fragment as biomarkers of diagnosis and severity of osteoarthritis

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ABSTRACT

Microfluidic liquid chromatography coupled to a nanoelectrospray source ion trap mass spectrometry was used for the absolute and simultaneous quantitation of C3f and the V65 vitronectin fragment in serum. The method was first carefully optimized and then validated in serum biological matrix. Stable isotopes for the two biomarkers of interest were used as stable isotope labeled peptide standards. A weighted $1/x^2$ quadratic regression for C3f and a weighted $1/x$ quadratic regression for the V65 vitronectin peptide were selected for calibration curves. Trueness (with a relative bias < 10%), precision (repeatability and intermediate precision < 15%) and accuracy (risk < 15%) of the method were successfully demonstrated. The linearity of results was validated in the concentration range of 2.5–200 ng/mL for C3f and 2.5–100 ng/mL for the V65 vitronectin fragment. Serum samples (n=147) classified in 7 groups [(healthy volunteers, OA with 5 grades of severity and rheumatoid arthritis (RA) patients] were analyzed with our new quantitative method. Our data confirm that C3f and the V65 vitronectin fragment are biomarkers of OA severity, but also that C3f fragment is further related to OA severity whereas the V65 vitronectin fragment is more related to early OA detection.

1. Introduction

Osteoarthritis (OA) is one of the most common chronic joint diseases causing substantial disability to work and public health costs [1,2]. Further, OA is becoming increasingly more prevalent as the population ages [3]. OA is a degenerative disease characterized by a dysregulation of normal joint homeostasis that leads to intra-articular cartilage degradation, bone remodeling and synovial inflammation [4–8]. Articular cartilage is a specific connective tissue covering joint surfaces. It is composed of water, collagen, proteoglycan and a wide range of matrix proteins including chondronectin, fibronectin, vitronectin and thrombospondin [9]. In OA, cartilage is degraded and finally lost as the disease progresses. Chondrocytes are the unique cell type embedded in the articular cartilage. These cells are essential for the production of a large amount of extracellular matrix. In the early stage of OA, cartilage is soft and swollen due to increase hydration. Proteoglycan overexpression enhances this harmful cartilage hyperhydration leading to cartilage breakdown and collagen fibers dissociation [10], and catabolic hyperactivity of chondrocytes ensues [11]. The synovial membrane starts to secrete inflammatory mediators (cytokines and metallo-proteinases) enhancing extracellular matrix degradation in cartilage and releasing cleaved fragments of proteins inside the joint cavity. At an advanced stage, full-depth cartilage may be destroyed exposing the subchondral bone.

Abbreviations: OA, osteoarthritis; 2-DE, two-dimensional electrophoresis; SELDI-TOF-MS, surface enhanced laser desorption/ionization-time of flight mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ ionization-time of flight; K & L, Kellgren and Lawrence; HV, healthy volunteers; RA, rheumatoid arthritis; JSN, joint space narrowing; LC, liquid chromatography; MeOH, methanol; ACN, acetonitrile; FA, formic acid; TFA, trifluoroacetic acid; SIS, stable isotope labeled peptide standards; NH_4OAc, ammonium acetate; NH_4OH, ammonium hydroxide; ESI, electrospray ionization source; *m/z*, mass-to-charge ratio; EIC, extracted ion current; NSAID, non steroidal anti-inflammatory drug; LLOQ, lower limit of quantitation; SPE, solid phase extraction; WCX, weak cation exchange; HLB, hydrophilic-lipophilic balance; MCX, mixed-mode cationic exchange; PE, process efficiency; ME, matrix effect; ER, extraction recovery; EY, extraction yield

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The search of novel biomarkers in OA has considerably increased over the last decade and new strategies have been developed for protein identification and quantification [12]. Several proteomics techniques [e.g. Two-dimensional electrophoresis (2-DE) combined with mass spectrometry [13], the surface enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS) [14,15] or analogous techniques based on matrix-assisted laser desorption/ ionization-time of flight [MALDI-TOF] analysis [16] were used in discovery phases of OA proteomics studies. However, these approaches only provide information about increased or decreased level of newly discovered biomarkers in OA compared to controls, and present therefore a relative quantitation of these biomarkers. Absolute quantitation was truly implemented with the development of stable-isotope based methods in which ratios of 'light' and 'heavy' versions of the same peptides are accurately determined and reported to the calibration curve.

In a previous study, protein expression levels were analyzed in serum samples provided from 284 patients with knee OA [17] and classified according to the degree of severity, as measured by the Kellgren and Lawrence (K & L) score (0–4) [18]. OA serum samples were compared to serum samples provided from healthy volunteers (HV; n=36) and rheumatoid arthritis (RA) patients (n=25). RA is another pathology affecting joints but presenting autoimmune and systemic inflammatory properties while OA is primarily a degenerative joint disease with local inflammation in joints. SELDI-TOF-MS, a semi-quantitative approach, was used to investigate differential expression level of small proteins and cleaved fragments [19]. This study led to the discovery of 4 novel protein fragments, two of which were identified as vitronectin fragment and C3f, both peptides were found to be expressed at higher levels in sera of OA patients at all four K & L scores compared to HV and RA [17].

Vitronectin fragment is a 17-amino acids peptide (SQRGHSRGRN QNSRRPS–amino acids 381–397) located at the C-terminal end product of the V65 vitronectin subunit in the heparin-binding domain. Its concentration in blood and synovial fluid was 14-fold increased in OA compared to HV and was correlated with OA severity [17]. Vitronectin is a cell adhesion and spreading factor found in serum and in many tissues including cartilage and synovium [20]. It is mostly known for its interaction with the $\alpha_v\beta_3$ integrin receptor via its arginine-glycine-aspartic acid motif (RGD motif, amino acids 64–66) [21]. The second fragment was identified as complement C3f peptide. Its expression level increased 5.5-fold with OA severity [17]. C3f peptide is a fragment released during the catabolic degradation of C3b by Factor H after C3 activation [22]. It is a 17-amino acids peptide (SSKITHRIHWESASLLR–amino acids 1304–1320).

Diagnosis of OA currently depends on patient-reported pain and disability, on blood biochemistry to rule out any other rheumatic diseases, and imaging (usually plain X-rays) that identify bone features (osteophytes) or cartilage destruction (joint space narrowing, JSN), typical of late stage disease. Therefore many research groups are seeking simple biochemical tests for diagnosis and monitoring OA. Fifteen-years ago, the National Institute of Health defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathogenic process, or pharmacologic responses to a therapeutic intervention" [23]. A recent review confirmed that at present there is no effective biomarker satisfying this definition [24]. Further, they suggested that the scientific community urgently needed to find new investigative biomarkers mainly through advances in "omic" technology [24]. In this study, we have developed a new absolute quantitative approach based on the simultaneous quantitation of C3f and the V65 vitronectin fragment by LC-Chip-MS/MS analysis. The method was fully validated using highly characterized serum samples from HV, OA and RA patients.

2. Material and methods

2.1. Chemicals

Water, methanol (MeOH), acetonitrile (ACN) and formic acid (FA) 99% were all at LC/MS grade and were purchased from Biosolve. Trifluoroacetic acid (TFA) was obtained from Fluka. Ammonium hydroxide (NH_4OH) was purchased from Merck and ammonium acetate (NH_4OAc) 98.9% from VWR. Helium and nitrogen (Alphagaz 2) were obtained from Air Liquide. Human synthetic complement C3f fragment ($_{1304}$SSKITHRIHWESASLLR$_{1320}$), the V65 vitronectin fragment ($_{381}$SQRGHSRGRNQNSRRPS$_{397}$) and the stable isotope labeled peptide standards (SIS) [$^{13}C_6$,$^{15}N_2$]Lys^3, [$^{13}C_6$,$^{15}N_4$]Arg^{17}-Complement C3f, [$^{13}C_6$,$^{15}N_4$]Arg^{3,15}-vitronectin fragment were purchased from Eurogentec (Anaspec peptide). Purity of peptides were > 95% as described in the technical data sheet of Anaspec peptide.

2.2. Instruments

Solid-phase extraction procedure was carried out by Oasis μElution weak cation exchange well plates with a vacuum manifold (Waters Inc.) for peptides extraction and enrichment. Sample evaporation was performed on a vacuum concentrator (Labconco). A 1200 series LC-chip system including nanoflow pump, a capillary pump, a well plate sampler and a LC-chip/MS interface was used for chromatographic separation. ChemStation (Agilent Technologies) is a software package to control Agilent liquid chromatography system. Protonated peptides detection was performed by Ion Trap mass spectrometry combined with a nanoelectrospray ionization source operating in positive mode (Agilent Technologies, Ion Trap LC/MS G6340A). TrapControl (Bruker Daltonik GmbH) determined the mass spectrometry detection parameters. Raw data obtained by mass spectrometry were processed using DataAnalysis and QuantAnalysis softwares (Bruker Daltonik GmbH).

2.3. SIS and calibration standards

All peptides (labeled and unlabeled) were dissolved in a H_2O/ACN/FA (80:20:0.1; v/v/v) solution to reach a concentration of 1 mg/mL, then were aliquoted and stored at −80 °C. Isotopically labeled peptides ([$^{13}C_6$,$^{15}N_2$]Lys^3, [$^{13}C_6$,$^{15}N_4$]Arg^{17}-complement C3f and [$^{13}C_6$,$^{15}N_4$]Arg^{3,15}-vitronectin fragment) were used as SIS for the two biomarkers of interest since they share the same physicochemical properties. For C3f peptide ($_{1304}$SSKITHRIHWESASLLR$_{1320}$), heavy peptides were labeled accordingly: on K^3(Label:13C(6)15N(2)) and R^{17}(Label:13C(6)15N(4)) with a delta mass of 18.0 Da. For V65 vitronectin fragment ($_{381}$SQRGHSRGRNQNSRRPS$_{397}$), heavy peptides were labeled accordingly: on R^{3,15}(Label:13C(6)15N(4)) with a delta mass of 20.0 Da.

SIS at a final concentration of 25 ng/mL and 10 ng/mL for complement C3f and the V65 vitronectin fragment, respectively, were prepared in H_2O/TFA (99:1; v/v) solution. Calibration standards of complement C3f and vitronectin fragments were then diluted in H_2O/TFA (99:1; v/v) solution in the range of 2.5–200 ng/mL at seven concentrations (2.5, 5, 10, 20, 50, 100 and 200 ng/mL) for C3f and in the range of 2.5–100 ng/mL at six concentrations (2.5, 5, 10, 20, 50 and 100 ng/mL) for the V65 vitronectin fragment.

2.4. Solid phase extraction (SPE) procedure

Several Oasis μElution well plates such as hydrophilic-lipophilic balance (HLB), mixed-mode cation exchange (MCX) and WCX were tested for the purification and enrichment of C3f and the V65 vitronectin peptides before chromatographic separation and mass spectrometry analysis (see tested experimental conditions in Supplementary data – Table 1). WCX provided the best results in terms of recovery percentage after elution and was thus selected.