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Brief Report

Serum proteomic panel validated for prediction of knee osteoarthritis progression

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ABSTRACT

Objective: To further validate a serum proteomics panel for predicting radiographic (structural) knee OA progression.
Design: Serum peptides were targeted by multiple-reaction-monitoring mass spectrometry in the New York University cohort (n = 104). Knee OA progression was defined as joint space narrowing ≥1 in the tibiofemoral compartment of one knee per study participant over a 24-month follow-up. The discriminative ability of an 11-peptide panel was evaluated by multivariable logistic regression and area under the receiver operating characteristic curve (AUC), without and with demographic characteristics of age, sex, and body mass index. The association of each peptide with OA progression was assessed by odds ratios (OR) in multivariable logistic regression models adjusted for demographics.
Results: The cohort included 46 (44%) knee OA progressors. The panel of 11 peptides alone yielded AUC = 0.66 (95% CI [0.55, 0.77]) for discriminating progressors from non-progressors; demographic traits alone yielded AUC = 0.66 (95% CI [0.55, 0.77]). Together the 11 peptides and demographics yielded AUC = 0.72 (95% CI [0.62, 0.83]). CRAC1 had the highest odds for predicting OA progression (OR 2.014, 95% CI [0.996, 4.296], p = 0.058).
Conclusions: We evaluated a parsimonious serum proteomic panel and found it to be a good discriminator of knee radiographic OA progression from non-progression. Since these biomarkers are quantifiable in serum, they could be deployed relatively easily to provide a simple, cost-effective strategy for identifying and monitoring individuals at high risk of knee OA progression.

1. Introduction

In the Foundation for the National Institutes of Health (FNIH) cohort, we recently identified a set of 30 serum peptides (referred to as a 'stable set') that alone well predicted radiographic joint space loss (rJSL) progression with AUC (95% CI) of 0.726 (0.692–0.731) without, and AUC 0.745 (0.712–0.749) with demographics (sex and baseline knee OA radiographic severity) [1]. Among these, 11 constituted a minimal 'essential set' to discriminate rJSL progressors from non-progressors with AUC 0.698 (0.631–0.735) without, and AUC 0.718 (0.654–0.747) with demographics [1]. The goal of this research was to validate this smaller parsimonious panel of 11 biomarker predictors in the New York University (NYU) cohort with symptomatic radiographic OA and 24-month follow-up.

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2. Patients and methods

2.1. NYU cohort

In total, 183 study participants with symptomatic OA in the signal knee, defined by the American College of Rheumatology clinical OA criteria [2], and Kellgren Lawrence (KL) grade ≥1, were enrolled in a New York University (NYU) 24-month prospective study of knee radiographic OA (rOA structural) progression. A total of 105 participants with complete follow-up data and available serum samples were eligible for this analysis. Participants had no other form of arthritis including gout, diabetes, malignancy, or body mass index (BMI) >32 kg/m^2. Participants were excluded with severely advanced OA (joint space width (JSW) = 0 and/or KL-grade = 4) [3]. Standardized weightbearing fixed-flexion posteroanterior knee radiographs were obtained using the SynaFlexer™ X-ray positioning frame (Synarc) as previously described [3]. The primary outcome was knee rOA progression of the tibiofemoral joint (i.e. either medial or lateral compartment) of one knee (either left or right) defined by change (≥1 unit) over 24 months in categorical joint space narrowing (JSN), scored using the OARSI atlas [4]. Patient demographics, clinical data, and baseline non-fasting blood samples were collected by approval of the NYU School of Medicine Institutional Review Board (IRB, i05-131). This research was conducted in compliance with the Helsinki Convention.

2.1.1. Sample processing and analysis

In total, 105 non-depleted serum samples from the NYU cohort were thawed and randomly split into 4 groups (Set 1, Set 2, Set 3, and Set 4). Sample protein concentrations were determined by Bradford assay in duplicate (50× dilution). Each sample (20 μg of protein) was normalized to 0.50 μg/μL, digested overnight with 0.8 μg of trypsin at 37C, and spiked with a mixture of 178 SpikeTide TQL stable isotope labeled (SIL) peptides (JPT, Hamburg, Germany) representing 109 proteins that was diluted to 5 fmol/μL. This multiple-reaction-monitoring (MRM) proteomic panel for serum-based prediction of knee OA structural progression was based on our prior discovery proteomic studies in synovial fluid, urine, and serum and literature searches [1]. Quantitative liquid chromatography multiple-reaction-monitoring (LC-MRM) was performed in singlicate on 1 μg of protein digest with SILs, using a nanoAcquity UPLC system (Waters Corp) coupled to a Waters Xevo TQ-XS triple quadrupole mass spectrometer via a nanoelectrospray ionization source. To create the MRM assay in Skyline (MacCoss Laboratory, University of Washington), we used both data dependent acquisition (DDA) (QExactive) and SIL peptide mixtures. Data collection was performed in a targeted mode and imported into Skyline (v3.6.1.10482) for peak integration and area calculation. Proteomic data were expressed as a ratio of the endogenous to stable isotope labeled (SIL) peptide quantity; therefore, there were no units associated with the peptide measurements.

2.2. Mass spectrometry-based quantification of CRAC1 protein in articular cartilage

To analyze protein expression of cartilage acidic protein 1 (CRAC1, gene name *CRTAC1*) in the superficial, middle, and deep regions of knee cartilage, cartilage specimens were embedded in Tissue-Tek O.C.T. (Sakura, The Netherlands) for cryosectioning. Serial transverse frozen sections of 12 μm thickness were generated at different depths from the cartilage surface. The first 20 sections were collected and categorized as representing the superficial layer. The subsequent 20 sections were skipped, then 20 sections were collected to represent the intermediate layer, 20 again skipped, and the 20 subsequent deepest sections were collected to represent the deep layer. CRAC1 was analyzed in duplicate by MS-based MRM and Skyline, as previously described [5]. The collection of these surgical waste cartilages (two knees – one non-OA and one OA, and two hips – one non-OA and one OA) was previously described [6]. This study was approved by the IRB of Duke University (PRO00008622).

2.3. Microarray-based gene expression data

2.3.1. Cartilage gene expression datasets

By microarray, we evaluated cartilage expression of genes corresponding to the essential set of proteins previously found to be predictive of joint space loss progression [1]. OA cartilages were acquired at the time of knee joint replacement; after isolation of RNA from each sample, 30 individual RNA samples were pooled into six samples of 5 individuals each (aged 47–78 years, 66.6% female). Control cadaveric cartilages were obtained from the National Disease Research Interchange. After isolation of RNA from each sample, 25 individual RNA samples without OA were pooled into five samples of 5 individuals each (aged 42–91 years, 40% female). All OA specimens exhibited macroscopic evidence of OA, including thinning and loss of cartilage, and focal eburnation; non-OA cartilage had no histologic evidence of degeneration, and was procured from donors with no history of joint disease. Total RNA of high quality for all samples (260/280 ratios 1.7–1.8) was extracted from pulverized cartilage, as described previously [7]. The eleven pooled samples and further methods on sample processing are accessible from publicly available databases: NCBI GEO GSE169077, GSM5176138, GSM5176139, GSM5176140, GSM5176141, GSM5176142, GSM5176143, GSM5176144, GSM5176145, GSM5176146, GSM5176147, GSM5176148.

2.4. Statistical analysis

2.4.1. NYU cohort

For ease of direct comparison, peptide ratios (endogenous/SIL) were converted to z-scores. Outliers were determined by boxplots of biomarker distributions, and clusters depicted from the first two principal components (PCs) computed from all biomarker data (PC1 versus PC2 plot). The corresponding amino acid positions (based on Swiss UniProt database searched on 4/8/21) for the peptide sequence denote the precise peptide identity; for example, the CRAC1 peptide was denoted CRAC1$_{(101\text{-}108)}$.

In our prior study of the FNIH cohort, the top 30 serum peptides with highest selection frequencies by bootstrapped elastic net selection distinguishing rOA progressors were defined as the "stable" set; of these, 11 serum peptides were determined to be a minimum optimized set of peptides that were highly predictive of rOA progression (joint space loss) and these were termed the 'essential set' [1]. These 11 'essential set' peptides were measured in the NYU cohort. The predictive performance of three logistic regression models (JSN progression as outcome) was assessed by AUC: the 11 peptides (Model 1); demographic traits BMI, age, and sex alone (Model 2); and a combination of 11 peptides with demographics (Model 3). Approximate Wald tests and profile CIs were generated for odds ratios (ORs) of the peptides and demographic characteristics.

Protein expression of CRAC1 in OA and non-OA cartilage was plotted using barplots with the three CRAC1 measured peptides, summed to obtain an overall cartilage protein expression value. Raw gene expression data from array scans were pre-processed using the Affy package from Bioconductor, and normalized using the Robust Multi-array Analysis (RMA) method, as described previously [8]. Wilcoxon–Mann–Whitney (WMW) tests were used to compare OA versus control cartilage expression of genes corresponding to the 11 'essential set' peptides. All statistical analyses were conducted using R version 4.1.1.

3. Results

3.1. Cohort characteristics

After removal of one outlier identified from boxplots and PC clusters, the NYU cohort included: n = 104 individuals; median age 61 years; median BMI 26.8 kg/m^2; 65.4% female; 34 (32.7%) experienced JSN progression (Supplementary Table 1).

Table 1
Essential peptide prediction of radiographic knee OA progression in NYU cohort.

Peptide	Model 1: Essential	Model 2: Demographics	Model 3: Essential + demographics
[AUC, 95% CIs]	**0.66 [0.55, 0.77]**	**0.66 [0.55, 0.77]**	**0.72 [0.62, 0.83]**
	Individual peptide odds ratio (95% CIs) for predicting OA progression; p value (results for a univariable context are shown in columns of Model 1 and 2, or for a multivariable context in column of Model 3)		
$C1R_{(683-689)}$	1.131 (0.316, 4.198); 0.85		0.739 (0.194, 2.887); 0.656
$CD14_{(192-210)}$	1.062 (0.54, 2.029); 0.856		1.077 (0.516, 2.146); 0.836
$CRAC1_{(170-178)}$	1.9 (0.989, 3.889); 0.064		2.014 (0.996, 4.296); 0.058
$HRG_{(140-153)}$	0.657 (0.308, 1.344); 0.259		0.587 (0.265, 1.242); 0.173
$IC1_{(287-298)}$	1.401 (0.854, 2.522); 0.204		1.37 (0.805, 2.622); 0.282
$PLF4_{(82-92)}$	1.309 (0.814, 2.152); 0.272		1.214 (0.732, 2.042); 0.452
$RET4_{(185-195)}$	1.385 (0.683, 2.884); 0.37		1.155 (0.531, 2.537); 0.715
$SHBG_{(170-183)}$	0.931 (0.581, 1.466); 0.758		1.043 (0.632, 1.71); 0.867
$VTDB_{(128-149)}$	0.451 (0.059, 3.103); 0.423		0.757 (0.096, 5.868); 0.788
$VTDB_{(364-370)}$	0.66 (0.183, 2.29); 0.515		0.615 (0.165, 2.203); 0.456
$ZPI_{(438-444)}$	1.541 (0.592, 4.129); 0.378		1.759 (0.65, 4.989); 0.273
BMI		1.323 (0.872, 2.053); 0.196	1.405 (0.846, 2.4); 0.197
Age		1.422 (0.951, 2.172); 0.092	1.277 (0.799, 2.068); 0.31
Sex male		1.823 (0.781, 4.301); 0.165	2.275 (0.818, 6.581); 0.12

Model 1 includes all the essential peptides that were previously found to be predictive of joint space loss progression in the FNIH cohort. Model 2 are the demographic traits, BMI, age, and gender. Model 3 includes the essential peptides plus the demographic traits. Cells include odds ratio (95%CI) and P-value for each individual peptide for prediction of radiographic knee OA JSN progression. DeLong CIs are utilized for AUCs. The NYU cohort included 104 participants (46 JSN progressors, 58 non-progressors). The amino acid numbers denote the position of the measured peptide within the protein based on Swiss UniProt searched on 4/8/21.

3.2. Validation of essential set of OA progression predictors

Model 1, based on the minimal set of 11 essential peptides alone, yielded AUC 0.66 for JSN progression; a similar AUC was yielded in Model 2 by demographics alone (Table 1). Model 3, including both essential peptides and demographics, yielded the highest discriminative capability with AUC 0.72. With the exception of $SHBG_{(170-183)}$, the peptides were all moderately positively correlated (Supplementary Fig. 1). Although none of the individual peptides were independently associated with OA progression in these multivariable models at a threshold $p < 0.05$, CRAC1 demonstrated the highest odds (OR 2.014, 95% CI [0.996, 4.296], $p = 0.058$) for predicting OA progression in the adjusted multivariable Model 3. This indicates that a 1 standard deviation increase in $CRAC1_{(170-178)}$ was associated with twice the likelihood of JSN progression. On its own (univariable analysis), CRAC1 yielded OR 1.186 (0.804, 1.766) for OA progression.

3.3. Joint tissue gene expression in OA and non-OA cartilage

Five of the 11 genes corresponding to the essential proteins (CD14, C1R, HRG, RBP4 [protein RET4]), and SERPING1 (protein IC1), were significantly ($p < 0.05$) differentially expressed in OA vs non-OA cartilage (Supplementary Table 2). Compared to non-OA, higher gene expression in OA included complement C1 subcomponent R (C1R), CRTAC1, and SHBG, and lower expression of the remaining genes. Congruence of serum proteomic and cartilage genomic direction of expression was notable for CRAC1/CRTAC1 (both elevated protein and gene expression), and HRG and VTDB (both reduced protein and gene expression), in sera of OA progressors (compared to non-OA progressors), and in OA cartilage (compared to non-OA cartilage).

CRAC1 protein was highest in the cartilage surface and lowest in deep zones of cartilage (Fig. 1); results were consistent based on quantification of three separate CRAC1 peptides, both OA and non-OA cartilages, and both knee and hip joints, suggesting the potential for it to be a prognostic biomarker of early stages of cartilage pathology.

4. Discussion

In the NYU cohort we further validated the newly reported essential set of serum peptides described by Zhou et al. as prognostic for two separate radiographic knee OA progression cohorts, the FNIH and Biomarker Factory cohorts [1]. Given that the BMI and percentage of female participants in the NYU and FNIH cohorts were somewhat different—slightly lower mean BMI (NYU 26.8, FNIH 30.7 kg/m^2) and higher percentage of females (NYU 65%, FNIH 59%) in NYU—good model performance here suggests robustness in the original selection of the essential set of peptides. Results for this serum proteomic panel alone (AUC 0.64, 95% CI [0.53, 0.74]) compare favorably with plasma IL-1Ra alone (AUC = 0.63, 95% CI [0.53, 0.74]), previously evaluated in the NYU cohort [3,9] as a predictor of JSN progression. Congruence of serum proteomic and cartilage genomic direction of expression was notable for CRAC1/CRTAC1, HRG and VTDB.

Of the 11 peptides, CRAC1 yielded the highest odds (OR 2.014) of OA progression in the context of the other 10 peptides in multivariable Model 3, but yielded lower OR (1.186) used on its own. A larger sample size is likely needed to adequately evaluate the merits of CRAC1 and the other peptides on their own. Taken together these results suggest that CRAC1 has a synergistic effect in predicting OA progression in the context of the other peptides and the demographics. Our cartilage proteomic analysis confirmed the presence of CRAC1 protein in OA and non-OA knee and hip cartilage; and consistent with a prior murine study [10], enrichment of CRAC1 protein in superficial compared to deep zones of knee and hip cartilage. Moreover, the CRTAC1 gene was differentially expressed in knee cartilage (OA greater than non-OA). These data support a joint tissue origin for this serum biomarker. Taken together with the recent knowledge that serum CRAC1 is associated with overall OA burden and OA-related knee pain [11], and can predict knee and hip replacement [12], these data suggest the potential for CRAC1 to be a prognostic biomarker predicting early through to late stages of cartilage pathology. The function of CRAC1 in joint tissues is not clear. Genetic deletion of CRTAC1 in female, but not male mice, reduced OA in response to destabilization of the medial meniscus and reduced the ratio of bone volume to total volume by 17% [10]. Conditional knockout would be needed to mitigate developmental defects given that CRTAC1 (also known as CEP-68) is expressed in bone [13], cartilage (as shown here), and synovium [14].

There were several limitations of this study. Although OR 2.014 for CRAC1 in the NYU cohort is greater than the original OR in the FNIH cohort (OR 1.42, 95% CIs [1.11, 1.82]) [1], the NYU result ($p = 0.058$) did not pass the $p < 0.05$ level of significance; this may be due to study power limitations in the NYU sample of only modest size. Nevertheless, this serum proteomic panel was successfully validated in the NYU cohort

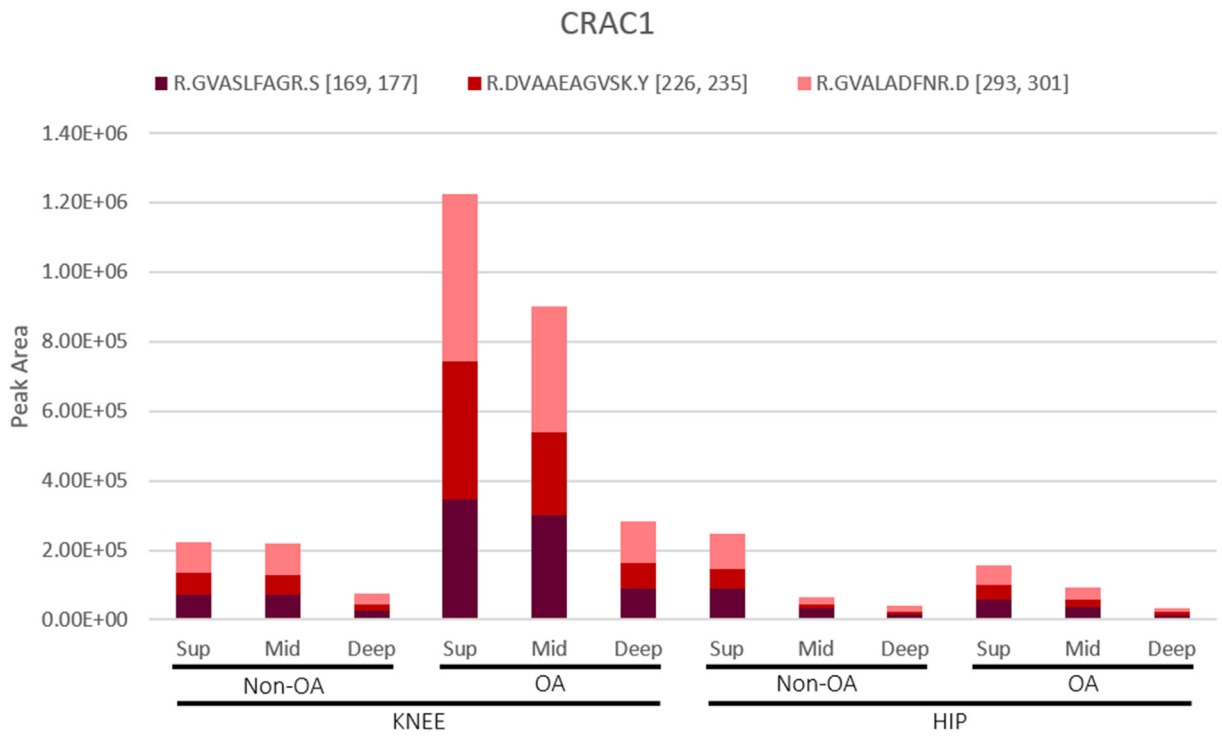

Fig. 1. Cartilage protein expression of CRAC1. Multiple reaction monitoring mass spectrometry analysis demonstrated expression of cartilage acidic protein 1 (CRAC1) in knee and hip, OA and non-OA cartilages. Four cartilages (healthy/OA and knee/hip, one each) were analyzed. All four cartilage samples express all three CRAC1 peptides; the concentration of CRAC1 was markedly higher in the superficial region than in the deep region for all samples. The peptide sequences of the three peptides are GVASLFAGR, DVAAEAGVSK, and GVALADFNR for peptides $CRAC1_{(170\text{-}178)}$, $CRAC1_{(227\text{-}236)}$, and $CRAC1_{(294\text{-}302)}$, respectively. Amounts of CRAC1 protein in cartilage, computed as the sum of raw counts of all three peptides, in superficial, middle and deep regions. CRAC1 was enriched in the superficial regions of cartilage relative to deep regions of all joint types irrespective of OA status. Of note, the amount (raw counts) of CRAC1 was highest in superficial knee OA cartilage and lowest in deep hip OA cartilage.

with progression determined over only 2 years compared with 2–4 years in the FNIH cohort, and a frequency of progressors of 44% in NYU compared with 60% in the FNIH cohort. Although, these differences may also contribute to the more modest results in the NYU compared to the FNIH cohort, they suggest the potential generalizability of the serum proteomic panel to other OA cohorts. Although the combination of proteomic biomarkers and demographics yielded the higher AUC overall, the lower end of the CI encompassed the AUC estimates of biomarkers and demographics used alone, so the combination was not demonstrated to be statistically significantly increased with the modest sample size available here. Finally, more research, including with use of more sensitive outcome measures of structural progression, such as can be generated by magnetic resonance imaging, is needed to validate the direction of the effects of the peptide biomarkers.

In conclusion, we further validated an essential set of proteomic peptide serum biomarkers as predictors of knee radiographic OA progression. Since these biomarkers are found in serum, they could be deployed relatively easily and serially to provide a simple, cost-effective strategy for identifying and monitoring individuals at high risk of knee OA progression.

Author contributions

Conceptualization: VBK, EJS, MAM, MGA.
Methodology: AR, YJL, EJS, MAM, MFH, MGA, VBK.
Investigation: MGA, SBA, JS.
Visualization: AR, MGA, VBK.
Supervision: VBK, YJL, MAM, MGA.
Writing—AR, VBK.
Writing—review & editing: AR, YJL, EJS, MAM, MFH, MGA, JS, SBA, VBK.

VBK takes responsibility for the integrity of the work as a whole, from inception to finished article.

Declaration of competing interest

VBK, ES, and MAM are named inventors in a pending patent related to this work. No other author has conflicts related to this work.

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Data and materials availability

All proteomic data for this study are available at Massive.ucsd.edu under the identifier MSV000089202. The code used in the analyses is available upon reasonable request of the lead statistical analyst for this project (YJL).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2023.100425.

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