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Analysis of plasma from prostate cancer patients links decreased carnosine dipeptidase 1 levels to lymph node metastasis

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ABSTRACT

There is a need for a better differentiation of aggressive tumors in prostate cancer to design a tailored treatment for each patient, preferably by a minimally invasive analysis of blood samples. In a previous study, we discovered a decrease of plasma levels of carnosine dipeptidase 1 (CNDP1) in association with aggressive prostate cancer. Now this relation has been investigated and characterized further by generating several new antibodies for extended analysis of CNDP1 in plasma. Multi-antibody sandwich assays were developed and applied to 1214 samples from two Swedish cohorts that confirmed decreased levels of CNDP1 in plasma from patients with advanced disease. Therein, data from CNDP1 assays allowed a better differentiation between tumor N stages than clinical tPSA, but did not when classifying T or M stages. Further investigations can now elucidate mechanisms behind decreasing levels of CNDP1 in plasma and primary in regards to lymph node metastasis.

Abbreviations: CAB, commercial antibody; CNDP1, carnosine dipeptidase 1; CV, coefficient of variance; FDR, false discovery rate; HPA, Human Protein Atlas; GLM, general linear model; LOESS, local regression; MAB, monoclonal antibody; MFI, median fluorescence intensity; nMFi, normalized median fluorescence intensity; KW, Kruskal–Wallis one-way analysis of variance; PCa, prostate cancer.

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1. Introduction

In contemporary practice, most patients with prostate cancer (PCa) are diagnosed following a PSA test and are asymptomatic at the time of diagnosis. Although serum PSA has a low specificity for prostate cancer, it can be used to single out patients with advanced disease. Efforts to improve our understanding of disease onset, diagnosis and progression through the analysis of prostate tissue, serum, plasma, urine or seminal fluid offers various entry points for discovery driven analysis. One of these is proteomics that aims at the determination of protein constituents and their isoforms in a give sample [1]. For this type of analysis several technologies are available to allow high-throughput analysis of prostate cancer samples. This includes affinity-based proteomics with a growing number of available binding molecules toward human proteins [2], and combined with microarray assays, multi-parallel immunoassays of many samples can be achieved [3].

In a previous study, we used antibodies from the Human Protein Atlas [4] and suspension bead arrays [5] to protein profile plasma from patients with prostate cancer and respective controls. There we identified the protein carnosine dipeptidase 1 (CNDP1), as a potential marker for aggressive prostate cancer. CNDP1 is a secreted protein of 57 kDa found in human blood and the central nervous system, with an approximate serum concentration of 20 μg/ml in healthy individuals [6,7] and acts as homodimer [8] and displays carboxypeptidase and dipeptidase activity, degrading carnosine and homocarnosine [9]. It is furthermore a glycoprotein that carries N-glycosylation on C-terminal residues 322 and 382 [10] and CNDP1 has been reported to form a complex with protease inhibitor alpha-2 macroglobulin [11]. Thus far, CNDP1 has been mainly mentioned with the susceptibiliy to nephropathy in type 2 diabetes through common genetic variants [12] and carnosine, substrate of the CNDP1, is believed to act as a protective factor in diabetic nephropathy [13]. A first link between CNDP1 and prostate cancer was discovered in our antibody array based analysis that revealed a decreased level of CNDP1 in plasma of patients suffering from an aggressive form of the disease [5].

The aims of this study were to improve the CNDP1 detection in plasma samples by developing multiple sandwich immunoassays and thereby to investigate the association of the decrease in CNDP1 levels with these assays in additional prostate cancer plasma samples. Further, we aimed to analyze whether the reported/predicted glycosylation status [10] or any interacting partner of CNDP1 are causing a differential detection in relation prostate cancer severity.

2. Materials and methods

2.1. Samples

Four sets of plasma samples were studied from three independent collections (see Supplementary Table 2A for details). These samples were analyzed in independent experiments and this is described in four phases (phases I–IV). This included two collections 79 heparin plasma samples (Skåne University Hospital, Sweden, denoted phase I) and 90 EDTA plasma samples (Cancer Prostate in Sweden, phase II) that had been analyzed previously using a single antibody based approach [5]. Phase III was built on 317 additional samples from CAPS. For phase IV, 728 heparin plasma samples were obtained during a collection period of 2004–2010 at Skåne University Hospital.

2.2. Deglycosylation and Western Blot

Plasma samples were diluted 10× in 50 mM $NaPO_4$, 0.1% (v/v) SDS and 1% Triton X100 and incubated at 96 °C for 3 min and 10U PNGaseF (Peptide-N-glycosidase F, Roche Diagnostics) were added for 24 h incubation at 37 °C. Moreover, 300 ng of recombinant CNDP1 (TP310312, Origene) were diluted and prepared as above. The extent of deglycosylation of CNDP1 was then evaluated with Western Blot with HPA-1 as detection antibody. Per lane, 50 ng of recombinant CNDP1 and 2 μg plasma samples depleted from human serum albumin (HSA) and immunoglobulin G (IgG) by the use of Affibody molecules (Affibody AB) coupled to Sulfolink matrix (Pierce) as described elsewhere [10], were loaded to an SDS-PAGE (4–12% Bis Tris, Invitrogen). Proteins were transferred onto membrane (0.45 μm PVDF, Invitrogen) according to the manufacturers protocol and transfer was confirmed with Ponceau (Pierce) staining. Membranes were blocked in 5% milk powder (Semper) in TBS-T for 1 h. Primary antibodies were incubated at optimized concentrations in blocking buffer at 4 °C for 16 h. Membranes were washed in TBS-T for 3 × 5 min followed by incubation with HRP-conjugated polyclonal swine anti-rabbit antibody (Dako) in blocking buffer at RT for 1 h. A final wash was followed by detection with TMBM substrate (Moss Inc.). The antibodies were also directly compared using a multi-screen apparatus (Mini-PROTEAN II, Bio-Rad).

2.3. Antibodies and epitope mapping

For the described immunoassays, different capture antibodies were utilized (Table 1 and supplementary Table 1). Monoclonal antibodies were generated in mice toward antigens 1 and 2 (Fig. 3A) and obtained from Atlas Antibodies AB, Sweden. The polyclonal detection antibody AF2489 (RnD Systems) was labeled with biotin (NHS-PEG4Biotin, Pierce) at a 50-fold molar excess over 2 h at 4 °C and stored after adding Tris-HCl (pH 8.0) at a 250-fold molar excess. All anti-CNDP1 antibodies were epitope mapped on bead arrays using 15-mer peptides with a 10 residue overlap spanning CNDP1 antigens 1 and 2 (Fig. 3A) as described previously [14]. For Alfa-2 macroglobulin, antibodies and protein standard were used from a kit (DY1913, RnD Systems).

2.4. Bead based sandwich assays

Antibodies were coupled to magnetic carboxylated beads (MagPlex, Luminex Corp.) according to the manufacturers protocol and as described previously [5]. The coupling efficiency for each antibody was determined via R-phycoerythrin-labeled anti-rabbit (Jackson ImmunoResearch Laboratories), Alexa Flour 555-labeled anti-goat (Invitrogen) and R-phycoerythrin-labeled anti-mouse (Moss Inc.) IgG