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Identification of potential pancreatic cancer serum markers: Increased sialyl-Lewis X on ceruloplasmin

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ABSTRACT

Pancreatic adenocarcinoma (PDAC) usually shows an enhanced expression of sialyl-Lewis X (sLe^x) and related epitopes. PDAC may secrete some of the proteins carrying such increased sLe^x determinant into serum, so they could be used as PDAC markers. Previously, we identified acute-phase proteins with increased sLe^x in both PDAC and in chronic pancreatitis patients. In this study, depleted sera from the main acute-phase proteins has been analysed for the search of proteins with increased sLe^x levels in PDAC. Sera from healthy controls, chronic pancreatitis and PDAC patients were depleted, electrophoresed and subjected to sLe^x immunodetection. Proteins that differentially expressed sLe^x in PDAC were trypsin digested and identified by LC-ESI-QTOF mass spectrometry. Five protein bands that differentially expressed sLe^x in PDAC were identified and corresponded to seven different acute-phase proteins. Among them, ceruloplasmin (CP) was selected for further analysis. *N*-glycan sequencing of CP confirmed the increase of sLe^x levels in CP in PDAC patients. Healthy controls, chronic pancreatitis and PDAC patients' sera were immunoprecipitated with anti-CP antibodies, and their sLe^x and CP levels were analysed by western blot. The sLe^x/CP ratio tended to be higher for the PDAC group, which altogether suggests that the sLe^x/CP ratio could be a useful biomarker for PDAC.

1. Introduction

Pancreatic cancer (PDAC) has the lowest 5-year survival rate (about 5%) of all cancer types. Although only representing around 3% of all cancer cases, it was the fourth leading cause of cancer death in Europe and the United States [1]. This poor survival may be attributed to its late diagnosis, usually performed after metastases have occurred. Early detection of pancreatic cancer would improve 5-year survival rate to 20% [1,2].

CA19-9 serum detection is currently used to monitor PDAC patients. However, its use in diagnosis is restricted by its false positive results, as it is also increased in patients with benign pancreaticobiliary disorders such as chronic pancreatitis (ChrP) [3,4]. Thus, the availability of adequate biomarkers for PDAC detection is of major interest.

Glycosylation changes are a universal feature of malignant transformation and tumour progression. These changes can be found either in tumour cell surface or in secreted glycoconjugates. Glycan changes in malignant cells take a variety of forms, usually affecting terminal glycan structures [5]. In particular, sialyl-Lewis X (sLe^x) and related Lewis antigens have been found to be overexpressed in PDAC cell lines [6,7] and tissues [8–10]. An increase of sialylated Lewis antigens and both fucosylation and sialylation of certain glycoproteins have been detected in the sera of PDAC patients compared to healthy individuals and ChrP patients [11–13]. These data suggest that pancreatic tumour may shed into the blood glycoproteins carrying sLe^x, which could be used as PDAC tumour markers.

In a previous work, we identified serum glycoproteins carrying increased sLe^x in both advanced PDAC and chronic pancreatitis patients [14]. However, these proteins corresponded to major acute-phase proteins (APP); alpha-1-acid-glycoprotein, haptoglobin and transferrin, which are produced mainly by the liver. Other APPs were also found to bear increased sLe^x levels only in chronic pancreatitis patients (alpha-1-antitrypsin and fetuin). Although sLe^x on these APPs may be used as cancer prognostic factors, these modifications are not specific enough to be used as PDAC markers.

Abbreviations: A2M, alpha-2-macroglobulin; APP, acute-phase proteins; CP, ceruloplasmin; ChrP, chronic pancreatitis; ITIH4, inter-alpha-trypsin inhibitor heavy chain H4; PDAC, pancreatic cancer; sLe^x, sialyl-Lewis X.

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In the present work, a glycoproteomic strategy to identify potential pancreatic cancer biomarkers based on changes in sLe^x glycan antigen in serum proteins was performed. For this purpose, the most abundant serum proteins were depleted in order to identify other glycoproteins with enhanced sLe^x from PDAC patients and have found ceruloplasmin (CP) as an interesting candidate for further analysis.

CP is an acute-phase protein produced by the liver and secreted in plasma. Its function is related to copper transport in serum and it is suggested to have a role in cancer since it is involved in angiogenesis and neovascularisation [15,16]. CP has 4 described N-glycosylation sites with complex type, bi, tri and tetrantennary structures both sialylated and fucosylated, containing sLe^x epitope mainly in triantennary structures, but also bi- and tetra-antennary [17,18]. In this study, the sLe^x levels on CP from sera of PDAC, ChrP and healthy controls were analysed and tended to be increased in the PDAC group.

2. Materials and methods

2.1. Serum samples

Serum samples were obtained from 13 healthy controls (HC) (7 females and 6 males; age range 44–69 years), 20 PDAC patients (11 females and 9 males; age range 45–70 years, 3 stage IIA, 7 stage IIB, 4 stage III and 6 stage IV) and 14 ChrP patients (6 females and 8 males; age range 46–79 years) from the Hospital Josep Trueta (Girona, Spain) following the standard operating procedures of its Ethics Committee. Patients were diagnosed by biopsy or image examination by the Pathology and Digestive Units.

2.2. Serum depletion

Serum samples (20 μL of each) were depleted using the ProteomeLab IgY-12 high-capacity spin column (Proteome Partitioning Kit, Beckman Coulter, Fullerton, CA), following centrifugation using a 0.22 μm Spin-X Centrifuge Tube Filter (Costar, Corning, NY) for 10 min at 2000 rpm according to manufacturer's protocols. This column facilitates the removal of albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid-glycoprotein, α2-macroglobin, apolipoprotein A-I, apolipoprotein A-II and fibrinogen in a single step. The final volume of each serum sample following immunodepletion was concentrated up to 50–100 μL using Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA).

2.3. Protein quantification

Protein concentration was determined by the Bradford protein assay using bovine serum albumin as standard (Quick Start Bradford Protein Assay, BioRad, Hercules, CA).

2.4. SLe^x immunodetection

After immunodepletion and concentration of serum samples, 25 μg of total protein was electrophoresed under reducing conditions on polyacrylamide gels, which were either Coomassie stained or transferred onto a PVDF membrane (Millipore, Billerica, MA). Transferred proteins were Ponceau stained (Ponceau S solution, DIG Glycan Differentiation Kit, Roche Diagnostics, Mannheim, Germany), and after that, sLe^x was immunodetected as previously described [14]. Chemiluminescence was visualised using the imaging system Fluorochem SP (AlphaInnotech, San Leandro, CA) under non-saturating conditions.

2.5. MS analysis

Proteins contained in the bands with specific sLe^x immunodetection for the PDAC patients group were in-gel digested with trypsin, extracted and analysed in an LC-ESI-QTOF mass spectrometer as described by Sarrats et al. [14]. Data were generated in PKL file format and submitted for database searching in the MASCOT server against SwissProt 2010_04 database. The search parameters were human taxonomy, 1 missed cleavage allowed, carbamidomethyl of cysteine as a fixed modification and oxidation of methionine as a variable modification. The peptide tolerance was 200 ppm and 0.25 Da, respectively for MS and MS/MS spectra. The significance threshold was set at $p < 0.05$. In the peptide report, only proteins with at least 2 peptides identified were accepted as positive hits.

2.6. N-glycan analysis

N-glycans were extracted from the gel pieces of CP bands according to the procedure described by Royle et al. [19]. Briefly, the gel pieces were washed and treated with PNGase F to release the N-linked glycans. Afterwards, *N*-glycans were fluorescently labelled with 2-aminobenzamide (2AB) by reductive amination using a Ludger Tag 2-AB labelling kit [20]. The excess of 2AB reagent was removed by ascending chromatography on Whatman 3MM paper in acetonitrile.

The 2AB-labelled glycans were digested in 10 μl of 50 mM sodium acetate buffer, pH 5.5, for 18 h at 37 °C, using arrays of the following enzymes (all purchased from Prozyme, San Leandro, CA) at the indicated concentrations: ABS—*Arthrobacter aureofaciens* sialidase (EC 3.2.1.18), 0.5 U/ml; NAN1—*Streptococcus pneumonia* sialidase (EC 3.2.1.23), 1.7 U/ml; BTG—Bovine testes β-galactosidase (EC 3.2.1.23), 1 U/ml; BKF—bovine kidney alpha-fucosidase (EC 3.2.1.51), 1 U/ml. After incubation, enzymes were removed by filtration through a protein binding EZ filters (Millipore, Billerica, MA), and *N*-glycans were then analysed by HILIC.

2-AB derivatised *N*-glycans were separated by ultra-performance liquid chromatography with fluorescence detection on a Waters Acquity UPLC instrument consisting of a binary solvent manager, sample manager and fluorescence detector under the control of Empower 2 chromatography workstation software (Waters, Milford, MA). Separations were performed using BEH glycan column 2.1 × 150 mm, 1.7 μm BEH particles. Solvent A was 50 mM formic acid adjusted to pH 4.4 with ammonia solution. Solvent B was acetonitrile. The column temperature was set to 30 °C. A 30 min method was used with a linear gradient 70–53% acetonitrile at 0.56 ml/min. An injection volume of 20 μl sample prepared in 60% v/v acetonitrile was used throughout. The fluorescence detection excitation/emission wavelengths were $\lambda_{ex} = 330$ nm and $\lambda_{em} = 420$ nm, respectively. Retention times were converted into glucose unit (GU) values by time-based standardisation against a dextran hydrolysed ladder.

2.7. Ceruloplasmin immunoprecipitation

CP from sera was purified by affinity immunoprecipitation. For each sample, 2.2 μg of streptavidin magnetic beads (Roche Diagnostics, Mannheim, Germany) were washed with buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 10 μL/mL Triton X-100) and incubated for 1 h with 8 μg of biotin-conjugated polyclonal rabbit antibody anti-ceruloplasmin (Abcam, Cambridge, UK) dissolved in buffer B (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1%BSA). Beads were afterwards washed three times with buffer A. Then 50 μL of serum was incubated with the streptavidin magnetic beads conjugated to the antibody anti-ceruloplasmin for 1 h in buffer B. After three washes with buffer A, CP was detached with 100 μL of gentle elution buffer (Pierce Biotechnology, Rockford, IL). All steps were performed at room temperature with shaking. As previously described in Section 2.2, the final volume of CP immunopurified from each serum was concentrated up to 40 μL using Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA).

The protein profile of 25 μL of immunoprecipitated serum was analysed by SDS–PAGE and silver staining. Resolving gel was prepared at 8% of polyacrylamide. Standard CP (0.5 μg), two immunoprecipitated serum samples and a control were reduced and loaded on the gel.

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