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Changes in the expression of N- and O-glycopeptides in patients with colorectal cancer and hepatocellular carcinoma quantified by full-MS scan FT-ICR and multiple reaction monitoring

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ABSTRACT

Alternations in the glycosylation of proteins have been described in connection with several cancers, including hepatocellular carcinoma (HCC) and colorectal cancer. Analytical tools, which use combination of liquid chromatography and mass spectrometry, allow precise and sensitive description of these changes. In this study, we use MRM and FT-ICR operating in full-MS scan, to determine ratios of intensities of specific glycopeptides in HCC, colorectal cancer, and liver metastasis of colorectal cancer. Haptoglobin, hemopexin and complement factor H were detected after albumin depletion and the N-linked glycopeptides with fucosylated glycans were compared with their non-fucosylated forms. In addition, sialylated forms of an O-linked glycopeptide of hemopexin were quantified in the same samples. We observe significant increase in fucosylation of all three proteins and increase in bisialylated O-glycopeptide of hemopexin in HCC of hepatitis C viral (HCV) etiology by both LC-MS methods. The results of the MRM and full-MS scan FT-ICR analyses provide comparable quantitative readouts in spite of chromatographic, mass spectrometric and data analysis differences. Our results suggest that both workflows allow adequate relative quantification of glycopeptides and suggest that HCC of HCV etiology differs in glycosylation from colorectal cancer and liver metastasis of colorectal cancer.

Significance: The article compares N- and O-glycosylation of several serum proteins in different diseases by a fast and easy sample preparation procedure in combination with high resolution Fourier transform ion cyclotron resonance mass spectrometry. The results show successful glycopeptides relative quantification in a complex peptide mixture by the high resolution instrument and the detection of glycan differences between the different types of cancer diseases. The presented method is comparable to conventional targeted MRM approach but allows additional curation of the data.

1. Introduction

Protein glycosylation fulfills many biochemical and physiological functions including antigen recognition, cell-cell interaction, cell signal regulation, and protein stability [1–4]. It is known that under pathological conditions such as inflammation, the glycan composition is altered mostly due to changes in the activity of enzymes responsible for building the oligosaccharide components [5–8]. Several types of glycan modifications are commonly detected in cancer diseases depending on the type, activity, and localization of specific glycosyltransferases [9,10]. We and others have described changes in the glycosylation of liver secreted glycoproteins in hepatocellular carcinoma [11–16]. The best known examples in this context represent changes in glycan structures related to the increased expression of fucosyltransferases FUT-8 or FUT-6 which result in elevated core or outer arm fucosylation of complex glycans, respectively. Formations of Lewis type epitopes such as SLeX, LeX, or LeY were recently observed on liver secreted glycoproteins [17–21], in addition to the well-known increase in core fucosylated α-fetoprotein in hepatocellular carcinoma [22–24]. These alterations in the glycosylation of proteins were extensively studied in human tissues and especially in serum [13,15,25–27]. Other described alterations of glycans include increased branching, sialylation, agalactosylated glycans or polylactosamine chains [16,28,29].

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Recent developments in mass spectrometry and separation techniques have enabled characterization and quantification of glycopeptides in complex biological matrixes by the detection of released glycans [30–34], detection of glycopeptides [35–38], or top-down mass spectrometry of intact proteins [39]. Each of the approaches has its benefits and the methods offer complementary view of protein glycosylation. Major benefit of the quantification of glycopeptides is its ability to quantify site-specific glycoforms because the knowledge of glycan distribution within a protein is important for its function [40–42]. We therefore describe two LC-MS workflows for quantitative analysis of glycopeptides and document their applicability to serologic analysis of glycoproteins in cancer diseases.

Quantitative analysis by multiple reaction monitoring (MRM) is typically performed on triple quadrupole instruments in combination with liquid chromatography (LC). We and others have recently shown that this workflow can be used for quantification of glycopeptides [13,25,43–46]. Collision induced dissociation of glycopeptides (CID) yields high intensity oxonium ions that originate from the glycans and less intense peptide-glycan or peptide fragments. The highly sensitive but less specific oxonium ions are typically used as quantitative transitions in the MRM mode [13,25,47]. In complex sample mixture, the most intense peptide-glycan fragments are often selected as specific transitions used for glycopeptide confirmation.

Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry provides the highest mass resolution and accuracy measurements of all analyzers. The high resolving power (>100,000 at m/z 400) and accuracy bellow 1 ppm in combination with high separation efficiency of HPLC allow accurate mass detection of analytes in complex sample matrices. We have used the 12T FT-ICR with ParaCell (solariX XR, Bruker Daltonics) to evaluate high resolution quantification of the glycopeptides. The 12T magnet provides a high magnetic field, which improves the dynamic range, mass accuracy, mass selectivity and signal-to-noise level [48,49]. The ultra-high resolution MS survey scan without data dependent acquisition (DDA) performed on such a high magnetic field instrument enables precise mass determination and quantification of peptides or glycopeptides [50]. Here, we demonstrate, for the first time, quantification of glycopeptides from partially depleted human sera by ultra-high resolution full-MS scan analysis performed by nano-capillary reversed phase chromatography coupled with the FT-ICR mass spectrometer and compare these data with results obtained by our previously optimized LC-MS/MS-MRM workflow [11,13,27].

2. Experimental section

2.1. Chemicals and materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and water for chromatography were from Merck (Darmstadt, Germany) and from Fisher Scientific (Fair Lawn, NJ). Trypsin was obtained from Promega (Madison, WI). Neuraminidase was from New England Biolabs (Ipswich, MA).

2.2. Study population

All participants were enrolled under the protocols approved by the Georgetown University Institutional Review Board and by the Pilsen Faculty Hospital Board. The HCC/HCV patients (n = 10) and healthy individuals (n = 10) were enrolled into the study in collaboration with the Department of Hepatology and Liver Transplantation, Georgetown University Hospital, Washington D.C., USA. The diagnosis of HCC was made by the attending physician based on liver imaging and/or liver biopsy. All The HCC/HCV patients had early stage disease according to the 7th Edition of the American Joint Committee on Cancer Staging manual and had chronic hepatitis C virus infection as the primary diagnosis. The HCC patients (n = 10), colorectal cancer patients (n = 10), and colorectal cancer/liver metastasis patients (n = 10) were enrolled into the study with the Laboratory of Immunoanalysis, Faculty Hospital in Pilsen, Pilsen, Czech Republic. The basic characteristics of the study population are summarized in Supplemental Table 1.

2.3. Albumin depletion

Albumin was depleted according to a previously described method with some modifications [51]. Ten microliters of human serum was diluted with 290 μL of water and centrifuged at RT for 15 min at 15,000 × g. Lipids floating on the top were removed; the de-lipidated serum (150 μL) was mixed with 300 μL of 150 mM NaCl followed by addition of 350 μL of ice cold 100% ethanol. After 1 h incubation at 4 °C, samples were centrifuged for 45 min at 4 °C and 16,000 × g. Supernatant was removed and the pellet was re-suspended in 200 μL of ice cold 42% ethanol and centrifuged for 15 min at 4 °C and 16,000 × g. Sample pellet was dried and stored at −80 °C until further usage.

2.4. Sample preparation for MS analysis

The pellet was re-suspended in 100 μL of buffer containing 4 M Guanidine-HCl in 50 mM ammonium bicarbonate, pH 7.8. After the pellet was dissolved, the sample was further diluted with 50 mM ammonium bicarbonate to final concentration of 1 M Guanidine-HCl and the protein concentration was measured by BCA assay using the NanoDrop system (Thermo Scientific, Wilmington, DE). The protein concentration of each sample was adjusted to 2 μg/μL by addition of 50 mM ammonium bicarbonate. Ten microliters (20 μg) of total protein mixture was reduced by dithiothreitol (DTT), final concentration 10 mM, and incubated for 45 min at 60 °C. Free –SH groups were modified by iodoacetamide, final concentration 30 mM, for 30 min in the dark. The alkylation reaction was quenched by addition of DTT, final concentration 30 mM and the proteins were digested by trypsin (0.4 μg per sample) over night at 37 °C. Trypsin was inactivated by heating the samples for 5 min at 90 °C and half of each sample was transferred to new tube for treatment with 100 units of α-2/3,6,8 neuraminidase from *Clostridium perfringens* overexpressed in *Escherichia coli* (New England Biolabs) for 24 h at 37 °C. Both neuraminidase-treated and non-treated samples were desalted on a MicroTrap peptide cartridge (Michrom Bioresources, Auburn, CA) as follows: cartridge was equilibrated with 2 × 250 μL of 0.1% trifluoroacetic acid (TFA). Samples were diluted with 150 μL of 0.1% TFA and gently loaded on the cartridge and desalted with 4 × 250 μL of 0.1% TFA. Peptides were eluted with 80% acetonitrile (ACN) containing 0.1% TFA, and dried using a SpeedVac concentrator. To document robustness and reproducibility of the sample preparation procedure, three technical replicates of a control serum sample were processed as described above and analyzed by FT-ICR.

2.5. Detection and quantification of glycopeptides by FT-ICR

One microgram of tryptic digest was injected on a nano-capillary system (Ultimate 3000 RSLC, Thermo Scientific, Waltham, MA) coupled with 12T solariX XR FT-ICR mass spectrometer equipped with Captive Spray ion source (Bruker Daltonics, Billerica, MA). For identification of glycoproteins in depleted serum, 1 μg of protein digest was injected on reversed phase C18 column (Acclaim PepMap 100, 100 μm × 2 cm, nanoViper C18, 5 μm, 100 Å, Thermo Scientific) and trapped for 3 min at a flow rate of 10 μL/min using an aqueous loading solution of 0.1% formic acid (FA). Peptides were further separated on an analytical reversed phase C18 column (Acclaim PepMap RSLC, 75 μm × 15 cm, nanoViper C18, 2 μm, 100 Å, Thermo Scientific) at a flow rate 0.5 μL/min and ACN gradient: 3–45% ACN, 0.1% FA for 5–60 min; 50–98% ACN, 0.1% FA for 60–65 min; 98% ACN, 0.1% FA for 65–70 min. Solvent A consisted of 2% ACN + 0.1% FA and solvent B of 98% ACN + 0.1% FA. Both trap and analytical columns were heated to 60 °C. The FT-ICR instrument was operated in data dependent mode. All spectra were acquired in broadband detection mode, number of data points were set

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