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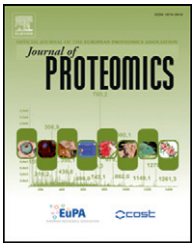

A lectin-coupled, targeted proteomic mass spectrometry (MRM MS) platform for identification of multiple liver cancer biomarkers in human plasma

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ABSTRACT

Aberrantly glycosylated proteins related to liver cancer progression were captured with specific lectin and identified from human plasma by multiple reaction monitoring (MRM) mass spectrometry as multiple biomarkers for hepatocellular carcinoma (HCC). The lectin fractionation for fucosylated protein glycoforms in human plasma was conducted with a fucose-specific aleuria aurantia lectin (AAL). Following tryptic digestion of the lectin-captured fraction, plasma samples from 30 control cases (including 10 healthy, 10 hepatitis B virus [HBV], and 10 cirrhosis cases) and 10 HCC cases were quantitatively analyzed by MRM to identify which glycoproteins are viable HCC biomarkers. A1AG1, AACT, A1AT, and CERU were found to be potent biomarkers to differentiate HCC plasma from control plasmas. The AUROC generated independently from these four biomarker candidates ranged from 0.73 to 0.92. However, the lectin-coupled MRM assay with multiple combinations of biomarker candidates is superior statistically to those generated from the individual candidates with AUROC more than 0.95, which can be an alternative to the immunoassay inevitably requiring tedious development of multiple antibodies against biomarker candidates to be verified. Eventually the lectin-coupled, targeted proteomic mass spectrometry (MRM MS) platform was found to be efficient to identify multiple biomarkers from human plasma according to cancer progression.

1. Introduction

Protein glycosylation plays an important role in adhesion, metastasis, and signaling through cell-to-cell interactions in biological systems. As such, glycoproteomics targeting glycoproteins and protein glycosylation has become a major focus of proteomics. Abnormal protein glycosylation patterns are closely associated with cancer progression [1–3]. Thus, efficient identification of these patterns and reliable differentiation between levels of aberrant protein glycoforms in healthy and cancerous individuals would be useful for understanding the pathological mechanism of cancer and for developing specific cancer biomarkers [4–9]. Since aberrantly glycosylated proteins can either be secreted into the bloodstream from cancer cells via conventional pathways or shed from the cell membrane through abnormally enhanced protease activity,

Abbreviations: HCC, hepatocellular carcinoma; AAL, aleuria aurantia lectin; MRM, multiple reaction monitoring; CV, coefficient of variation; MB, magnetic bead; LOQ, limit of quantitation; ESI, electrospray ionization.

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serological aberrant protein glycoforms in the bloodstream can reflect abnormal states in cancer patients. Thus, aberrant glycoprotein levels in the bloodstream may be used as serological cancer biomarkers [10–13]. Therefore, an efficient quantitative method based on these glycoforms would be important in biomarker development.

Direct identification and quantitation of low-abundant serological glycoproteins remains difficult because of the severe masking effects of highly abundant blood proteins. Furthermore, each aberrant glycoform of a given glycoprotein is present in low, sub-stoichiometric abundance due to glycan micro-heterogeneity. Thus, the abundance of each glycoform is far lower than that of the total glycoforms for a given protein. Fractionation tools are required to efficiently identify and quantify these sub-stoichiometric, aberrant protein glycoforms from complex blood proteomes. Various lectins have been used to fractionate lectin-active glycoforms from complex glycoproteomes prior to mass spectrometric or immunological analysis [14–20]. A variety of lectins are available, most exhibiting a narrow specificity for a given glycan structure. Therefore, lectins act as ideal selectors for capturing aberrant glycoforms with cancer-related glycan structures. A typical example is the capture of fucosylated alpha-fetoprotein (AFP) by a fucose-specific lectin. AFP is a potent biomarker for hepatocellular carcinoma (HCC) [1]. AFP-L3, an aberrantly glycosylated glycoform created via fucosylation, exhibits an increased affinity for fucose-specific lectins, such as lens culinaris agglutinin (LCA), and has been reported to show improved specificity as a biomarker for HCC [8,9,21–23].

Quantitative analyses of these lectin-active aberrant glycoforms have primarily been performed using lectin-blotting techniques and lectin–antibody sandwich arrays [16,24–28]. The development of an antibody to target a glycoprotein is a prerequisite for immunoassays and antibody development is typically expensive and labor-intensive. In addition, the number of antibodies that can be developed for an immunoassay is limited relative to the number of glycoproteins that have been newly discovered by a variety of biomarker-developing processes. Therefore, antibodies for a specific target antigen may be unavailable. These limitations require the development of a more general analytical method featuring a simple, rapid, cost-effective, and multiplexed analytical system, in order to conduct rapid preliminary verification of potential biomarker candidates [29].

Mass spectrometry has played central roles in identifying new target proteins from various biological media. Multiple reaction monitoring (MRM) mass spectrometry, a multiplexed, targeted proteomic platform, has been recognized as a rapid and cost-effective protein biomarker measurement technology for preclinical verification [30]. Nevertheless, there are relatively few reports detailing MRM-based quantitative analyses of protein glycoforms that focus on lectin-captured materials instead of total protein glycoforms [17,31–34]. Our previous works confirmed that comparative MRM-based analyses are useful for measuring the abundance of aberrant glycoforms of target glycoproteins lectin-captured from the culture media of colorectal cancer cell lines [31], and the serum proteomes of colorectal cancer patients [32]. Our group has also evaluated the lectin-coupled, MRM-based method using samples that were lectin-fractionated from a large number of human plasma [33]. The plasma samples exhibit high complexity, a wide dynamic range of protein abundance, and dramatic variation between individuals. This preliminary study confirmed that lectin-coupled, MRM-based analyses are highly reproducible and exhibit sufficient precision to distinguish differences in lectin-captured protein glycoform levels between multiple individual plasma samples. In addition, this study demonstrated that lectin fractionation effectively removed the masking effects that arise in samples containing high abundances of serological proteins. This allowed the identification of many peptides from less abundant proteins by one-dimensional LC/MSMS, even without a depletion step to remove the highly abundant serological proteins. This lectin-coupled, MRM-based approach can be expanded into a multiplex, lectin-channel monitoring (LCM) method, which employs multiple lectins that fractionate the same proteome sample in parallel [34]. This allows one to quantitatively monitor the glycosylation diversity of glycoproteins in a proteome sample. Additionally since the MRM-based quantification method shows excellent feasibility in multiplexing analytes to be measured, the lectin-coupled, MRM-based approach can also be easily configured to measure a number of target peptides in one assay. Therefore combination of individual potent biomarker candidates observed by the multiplexed assays for many biomarker candidates will give an increasing reliability to the assay results [18,35]. Here the lectin-coupled/MRM-based approach, using lectin-specific fractionation to fucosylated glycoforms of target glycoproteins and MRM-based analysis to tryptic digests of the lectin-captured fraction, was applied to verification of multiple HCC biomarker candidates (Fig. 1).

2. Experimental procedures

2.1. *Lectin-fractionation of plasma*

Human plasma samples were prepared at the Severance Medical Center at Yonsei University (Seoul, Korea) with the consent of the blood donors and in accordance with IRB guidelines. Healthy plasma was collected from volunteers that were not infected with HBV and showed no evidence of cancer. HBV-infected plasma was collected from patients chronically infected with HBV. Cirrhosis plasma was obtained from patients with chronic HBV infections that had been diagnosed with cirrhosis. HCC plasma was collected from HCC patients with chronic HBV infections. Lectin-fractionation of the plasma samples was performed as described previously with minor modifications [33]. K_2EDTA-treated plasma (15 μL) was diluted five-fold with 50 mM ammonium bicarbonate. The diluted plasma samples were spiked with solutions of $CaCl_2$ and $MgCl_2$ to a final concentration of 5 mM each. The plasma solutions were then mixed with aleuria aurantia lectin (AAL)-immobilized magnetic beads (MBs, 800 μg) and incubated overnight at 10 °C with gentle shaking. The AAL-immobilized MBs were prepared in advance by mixing biotinylated AAL (Vector Lab) with streptavidin–magnetic beads (Invitrogen) for 1 h at room temperature, as per the manufacturer's instructions. The lectin-bound proteins were washed three times with phosphate buffered saline (PBS) and