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# Capturing and identification of differentially expressed fucome by a gel free and label free approach

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ABSTRACT

This research reports a proof-of-concept that describes an instrumental approach that is gel free and label free at both the separation and mass spectrometry ends for the capturing and identification of differentially expressed proteins (DEPs) in diseases, e.g., cancers. The research consists of subjecting/processing equalized and non-equalized (i.e., untreated) disease-free and hepatocellular carcinoma (HCC) human sera *via* a multicolumn platform for capturing/fractionating human serum fucome. The equalization was performed *via* the combinatorial peptide ligand library (CPLL) beads technology that ensured narrowing the protein concentration range, thus allowing the detection of low abundance proteins. The equalized and non-equalized disease-free and HCC sera were first fractionated online onto two lectin columns specific to fucose, namely *Aleuria aurantia* lectin (AAL) and *Lotus tetragonolobus* agglutinin (LTA) followed by the online fractionation of the lectin captured fucome by reversed phase chromatography. The online desalted fractions were first subjected to trypsinolysis and then to liquid chromatography–mass spectrometry (LC–MS/MS) analysis. In comparison with untreated serum, the CPLL treated serum is superior in terms of the total number of identified DEPs, which reflected an increased number of DEPs in a wide abundance range. The DEPs in HCC serum were found to be 70 and 40 in both LTA and AAL fractions for the serum treated by CPLL and untreated serum, respectively. In addition, the platform combined with the CPLL treatment was accomplished with virtually no sample loss and dilution as well as with no experimental biases and sample labeling when comparing the diseased-free and cancer sera using LC–MS/MS.



## 1. Introduction

Glycosylation is an important post-translational modification involving the covalent linkages between oligosaccharides and proteins, which is regulated in various biological processes including birth, differentiation, growth, inflammation, carcinogenesis, and cancer metastasis [1–3]. Fucose is one of a few monosaccharides forming the oligosaccharide chains (i.e., glycans) attached to glycoproteins and glycolipids present in vertebrates, invertebrates, plants and bacteria. Fucosylation refers to the presence of a fucose residue in the *N*-glycans or *O*-glycans attached to glycoproteins [1,4]. Several kinds of fucosyltransferases, guanosine diphosphate (GDP)-fucose synthetic enzymes and GDP-fucose transporters are involved in the regulation of fucosylation [1,4]. The fucosylation level during liver carcinogenesis is relatively high compared to its low level in normal liver. An increase in fucosylated proteins or fucome in sera of the patients with cancer depends on cellular fucosylation of cancer tissues and/or changes in fucosylation states in the liver [1]. Hakomori et al. presented the first paper involving the study of fucosylation in cancer, comparing the fucosylation level of glycolipids in hepatoma cells and normal hepatocytes [5]. Alpha-fetoprotein (AFP) is a well-known tumor marker for hepatocellular carcinoma (HCC) [1,4,6]. Nevertheless, AFP is not specific only for HCC but an increase in the level of AFP is also observed in benign liver diseases such as chronic hepatitis and liver cirrhosis [1,4,7]. Whereas fucosylated AFP or AFP with core fucosylation (AFP-L3) is more specific tumor marker for HCC. Core-fucosylation, comprises the attachment of fucose to the innermost *N*-acetylglucosamine in *N*-glycans, which is catalyzed using alpha-1-6 fucosyltransferase

*Abbreviations:* AAL, *Aleuria aurantia* lectin; AIBN, 2,2′-azobis(isobutyronitrile); CPLL, combinatorial peptide ligand library; DEPs, differentially expressed proteins; GMM, glyceryl methacrylate; HCC, hepatocellular carcinoma; ICAT, isotope coded affinity tags; iTRAQ, isobaric tags for relative and absolute quantification; LAC, lectin affinity chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LTA, *Lotus tetragonolobus* agglutinin; PETA, pentaerythritol triacrylate; RPC, reversed phase chromatography; TFA, trifluoroacetic acid; 2D-DIGE, two dimensional differential gel electrophoresis.

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(Fut8). Although AFP-L3 is more specific marker for HCC, expression of Fut8 during core fucosylation is increased in both HCC tissues and surrounding tissues with liver cirrhosis [8]. Block et al. have reported a fucosylated biomarker for HCC namely Golgi protein 73 (GP73) which has a positive predicative value equal to or greater than the AFP [6]. The use of AFP, AFP-L3 and GP73 as a primary screen for HCC has a limited utility for detecting HCC and more sensitive serum biomarkers for HCC are desired. Therefore, an in-depth profiling of changes in the fucome in HCC is needed for discovering more specific and powerful HCC candidate biomarkers.

Different analytical tools such as two dimensional polyacrylamide gel electrophoresis (2DE) [9,10], two dimensional differential gel electrophoresis (2D-DIGE) [10–17], isotope coded affinity tags (ICAT) [18] and isobaric tags for relative and absolute quantification (iTRAQ) [19] have been developed for the identification of DEPs in complex biological sample of HCC. 2DE is a widely used proteomics technology to study proteome as well as differentially expressed proteins in HCC [9,10]. The principle of 2DE is based on separation of proteins according to their charge in first dimension by isoelectric focusing and size in the second dimension by polyacrylamide gel electrophoresis [9]. However, the lack of reproducibility between gels is a major limitation of 2DE. Additional problems with 2DE include its low sensitivity and insufficient resolution to separate multi species originating from a single protein [9,20]. 2D-DIGE has been introduced in order to address some of the drawbacks of 2DE technique. 2D-DIGE is one of the proteomic labeling techniques, which have been employed for separation and identification of DEPs in HCC [10–17]. 2D-DIGE is performed by sample pre-labeling with different fluorescent cyanine (Cy) dyes. These labeled proteins are then mixed and separated simultaneously on the same 2D gel electrophoresis. The different protein extracts labeled with different CyDye DIGE fluor can then be visualized separately by exciting the different dyes at their specific excitation wavelengths [9,20,21]. 2D-DIGE has ability to reduce the effect of gel-to-gel variation, provides more accurate and reliable quantification information of protein abundance because the samples are separated together on the same gel. However, DIGE labeling suffers from the systematic variation because the phenomenon of protein-specific dye bias. Here, certain proteins are preferentially labeled with CyDye over others, despite their similar structures and identical reactive groups [22]. In addition, the labeling chemistry is required for attaching the dye to the proteins. For example, with the minimal dyes, high abundance protein spots in a conventional gel system could be a medium or low abundance protein spot in the DIGE system [20]. This is due to a low percentage of lysine residues in high abundance proteins. And also, 2D-DIGE is not applicable to those proteins without lysine (when labeling with the minimal dyes) or cysteine (when labeling with the saturation dyes) [20]. Non-gel based quantitative proteomic methods have been developed for studying DEPs in HCC, namely ICAT [18] and iTRAQ [19]. These methods are based on the similar physical and chemical properties of isotope labeled compounds to their unlabeled equivalents but with a different mass that can be recognized by mass spectrometer, and quantification is achieved by comparing their respective signal intensities [23,24]. Limitations of using these stable isotope labeling methods include the potential for incomplete labeling, high cost of the labeling reagents, variable labeling efficiency, limitations of available labeling reagents for simultaneous quantification of proteins from multi samples and the requirement for specific quantification software. Furthermore, ICAT is only applicable to proteins containing cysteine [23–25].

In this work, which is a continuation to our recent investigations [26,27], we intend to feature three new elements of the recently developed multicolumn platform: (i) the effectiveness of the platform in mapping the altered fucome in another cancer serum, namely human serum with HCC, (ii) reveal clearly the instrumental aspect of the platform for comparative proteomics that does not involve labeling chemistry and consequently requires much less labor than existing technology, and (iii) demonstrate the superiority of the described method in generating the whole fucome as compared to partial fucome by the combination of other methodologies. Regarding aim (i), since every cancer develops and behaves differently, it is therefore important to challenge the developed strategy in revealing similarities and differences in the altered fucome in HCC with respect to that in breast cancer. Aims (ii) and (iii) are justified by assessing the high throughput of the developed platform with respect to the existing technologies.

In short, this investigation describes the online selective capturing of fucosylated serum glycoproteins (i.e., the human fucome) by LAC followed by fractionation of the captured human fucome by reversed phase chromatography (RPC) in an instrumental multicolumn platform. The fucome in HCC serum was then analyzed with respect to disease free serum using LC–MS/MS. Recently, LAC has been shown very effective in the capturing and differential profiling of glycoproteins in biological matrices [26–30]. Our approach is a label free technique in both the separation stage using LAC and RPC and the identification stage using LC–MS/MS. Therefore, all of the above mentioned drawbacks associated with label-based approaches have been eliminated. In fact, the platform eliminates all experimental biases, centrifugation, multistep dialysis, dilution and transfer from vessel-to-vessel. And also, the platform allows the transfer/processing of proteins from column-to-column in the liquid phase using high precision pumps and valves, and no sample loss as well as zero propagation of experiment errors. To further exploit the potentials of the platform and demonstrate its effectiveness in profiling the altered fucome in HCC, an off line protein equalization *via* the combinatorial peptide ligand library (CPLL) approach was performed prior to sample processing by the platform. The CPLL is a mixture of a multitude of linear hexapeptides, which has been shown to be very effective in the detection of novel proteins, due to the concomitant reduction of high abundance proteins and the concentration of low- or very low abundance proteins in many biological fluids and extracts [31–33], thus allowing an in-depth proteomics profiling. To capture the fucome, two lectin columns were incorporated in the platform. They consisted of immobilized fucose specific lectins namely, AAL and LTA onto the surface of glyceryl methacrylate (GMM)/pentaerythritol triacrylate (PETA) monolith which was recently introduced by Gunasena and El Rassi for performing immuno affinity chromatography at reduced nonspecific interactions [34]. Immobilized AAL has a strong affinity towards glycoproteins with core fucosylated glycans [35], whereas immobilized LTA can bind to glycoproteins with glycans having fucose present in the outer arm. LTA also has an affinity for glycans containing the $Le^x$ determinant [36]. The haptenic sugar for AAL and LTA is α-L-fucose. In order to establish the efficiency of the combination of CPLL technology with the multicolumn platform that facilitates the capturing, enrichment and fractionation of the human fucome prior to LC–MS/MS analysis, the aim of this work is to compare the differentially expressed glycoproteins in HCC with respect to disease free serum in both sera that were treated or untreated by CPLL beads (i.e., ProteoMiner™ treated or untreated serum).

## 2. Materials and methods

### 2.1. Materials

Two fucose specific lectins namely, AAL and LTA were purchased from Vector Laboratories (Burlingame, CA, USA). Pooled human HCC serum from five donors and pooled disease-free