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Mapping glycosylation changes related to cancer using the Multiplexed Proteomics technology: a protein differential display approach

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Abstract

The metastatic spread of tumor cells in malignant progression is known to be a major cause of cancer mortality. Protein glycosylation is increasingly being recognized as one of the most prominent biochemical alterations associated with malignant transformation and tumorigenesis. The Multiplexed Proteomics (MP) approach is a new technology that permits quantitative, multicolor fluorescence detection of proteins in two-dimensional (2-D) gels and on Western blots. This methodology allows the parallel determination of both altered glycosylation patterns and protein expression level changes within a single 2-D gel experiment. The linear responses of the fluorescent dyes utilized allow rigorous quantitation of changes in protein expression over a broad 3-log linear dynamic range. Global analysis of changes in protein glycosylation and total protein expression is followed by dichromatic, lectin-based profiling methods for rapidly categorizing glycan branching structures. The MP approach was applied to whole tissue extracts of normal and cancerous liver, so that altered glycosylation modification patterns and protein expression levels could be determined. One prominent glycoprotein determined to be up-regulated in the tumor tissue was haptoglobin, an acute-phase response protein. The detection methodologies associated with the MP technology radically increase the information content of 2-D gel experiments. This new information greatly enhances the applicability of these experiments in addressing fundamental questions associated with proteome-wide glycosylation changes related to cancer.

Keywords: Glycosylation; Multiplexed Proteomics; Differential display; Haptoglobin

1. Introduction

Glycoproteins are polypeptides possessing N- and O-linked carbohydrate chains of different structures and functions [1]. The N-linked glycan chains are attached through the β-amide nitrogen of asparagine residues of polypeptides in the endoplasmic reticulum and the Golgi body of eukaryotic cells by a regulated sequence of glycosyltransferase and glycosidase processing reactions requiring dolichol phosphate intermediates. The O-linked glycan chains are attached through the hydroxyl group of serine or threonine residues of polypeptides in the Golgi body, but without employing dolichol phosphate intermediates. N- and O-glycan chain biosynthesis is regulated at the level of gene expression, transcription, enzyme activity and localization, and through substrate and cofactor concentration fluxes at the sites of glycoprotein biosynthesis [2].

In certain disease states, the relative abundances

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and branching structures of glycans are often altered relative to the normal state, and these alterations in glycosylation may be indicative of the stage of the disease and thus useful for diagnosis. Glycosylation changes in human carcinomas are thought to contribute to the malignant phenotype observed downstream of certain oncogenic events [3]. For instance, increased branching of N-linked oligosaccharides is often associated with metastasis, due, in part, to the regulation of *N*-acetylglucoaminyltransferase V (GlcNAc-T V) [4–7]. This enzyme converts N-linked high mannose glycans on proteins to complex type oligosaccharides [8]. Increased expression of GlcNAc-T V has been noted in a variety of metastatic models of human hepatocellular carcinoma, as well as in surgically resected human carcinoma specimens [5,6,9–13]. Core 2 beta-1,6-*N*-acetylglucosaminyltransferase (C2GnT) is an analogous enzyme to GlcNAc-T V, that increases branching of *O*-glycans during tumor progression [14–16]. The resulting mucin-type *O*-glycans are important substrates for sequential addition of α-2,3-linked sialic acid and α-1,3-linked fucose, resulting in the sialyl Lewis X terminating tetrasaccharide.

One important challenge in proteomics is to develop robust tools for the analysis of protein glycosylation status on a global scale. This study demonstrates the feasibility of utilizing a new fluorescence-based Multiplexed Proteomics (MP) technique to monitor glycosylation patterns in cancer specimens. The MP method involves parallel determination of both glycosylation patterns and protein expression levels in two-dimensional (2-D) gels through serial staining with Pro-Q™ Emerald 300 glycoprotein stain and SYPRO® Ruby protein gel stain, respectively. Computer-assisted differential display techniques are used to highlight differences between normal and diseased tissue. Lectin blotting using concanavalin A and ricin conjugates is then performed to rapidly categorize glycoproteins with respect to their oligosaccharide branching structures.

2. Materials and methods

2.1. Materials

All immobilized pH gradient (IPG) strips (3–10 NL, 18 cm) were obtained from Amersham Biosciences (Piscataway, NJ, USA). Tris, Glycine, and SDS were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Concanavalin A conjugated to alkaline phosphatase, DDAO phosphate, SYPRO Ruby protein gel stain, SYPRO Ruby protein blot stain, and Pro-Q Emerald 300 glycoprotein stain were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Ricin conjugated to alkaline phosphatase was obtained from EY Labs (San Mateo, CA, USA). Normal human liver tissue lysates and human hepatocellular carcinoma tissue lysates were obtained from GenoTechnology (St. Louis, MO, USA). Modified trypsin was obtained from Promega Corporation (Madison, WI, USA). ZipTip® pipette tips were obtained from Millipore Corporation (Bedford, MA, USA). All other chemicals were obtained from Sigma.

2.2. 2-D gel electrophoresis

Isoelectric focusing was performed using a Genomic Solutions™ (Ann Arbor, MI, USA) pHaser isoelectric focusing unit. The liver tissue lysates (150 μg) were mixed with 420 μl 7 *M* urea, 2 *M* thiourea, 65 m*M* DTT, 0.8% carrier ampholytes, 2% CHAPS, 1% zwittergent 3–10. Human plasma samples were treated similarly, except that 10–15 μg of total protein was loaded per gel. The proteins were focused for 60 000 V-h at 110 μA/strip. After isoelectric focusing, the IPG strips were equilibrated in a buffer containing 300 m*M* Tris base, 75 m*M* Tris–HCl, 50 m*M* DTT, 0.01% Bromophenol Blue, 3% SDS, pH 8.8 for 10 min before placing them on the SDS–PAGE gels. The second dimension SDS PAGE gel electrophoresis was conducted using an Investigator™ 2-D unit also from Genomic Solutions. The second dimension separations were performed with a pH 8.3 Tris–glycine gel buffer system using large format 12.5% T, 2.6% C SDS–polyacrylamide gels. %T is the total monomer concentration expressed in g/100 ml and %C is the percentage cross-linker. 2-D gel electrophoresis was performed as described previously [17].

2.3. Gel staining and imaging

After completion of the second dimension electrophoresis, gels were fixed overnight in 50% methanol, 10% acetic acid (600 ml/gel) to remove the SDS.

Gels were stained for glycoproteins using the Pro-Q Emerald 300 glycoprotein gel and blot stain kit (Molecular Probes) as previously described [18]. All staining and washing steps were performed with continuous, gentle agitation (e.g. on an orbital shaker at 50 rev./min). Gels were washed twice in 3% acetic acid for 15 min each before oxidizing the glycans with 500 ml 1% periodic acid in 3% acetic acid for 1 h. The oxidizing solution was removed using four washes of 3% acetic acid for 15 min each before staining for 2.5 h in 200–300 ml Pro-Q Emerald 300 dye per gel. This solution was prepared by diluting the stock Pro-Q Emerald 300 dye solution 1:50 in Pro-Q Emerald 300 dilution buffer just prior to staining. The diluted reagent degrades upon long term storage, so only the amount required for staining should be prepared. Excess stain was removed by incubating gels in two to three changes of 3% acetic acid. All gels were imaged on a Roche Lumi-Imager™ (Mannheim, Germany) using a 300 nm trans-illumination source and a 520 nm band pass emission filter. After imaging all gels were subsequently stained with SYPRO Ruby protein gel stain overnight as previously described [19–22]. Briefly, gels were incubated in a fixative solution of 7% acetic acid, 10% methanol over night. Minimal staining volumes of 500 ml were used for a 20 cm×20 cm×1 mm large format 2-D gel. This corresponds to a solution volume that is roughly 10 times the volume of the gel. All staining and washing steps were performed with continuous, gentle agitation (e.g. on an orbital shaker at 50 rev./min). After staining, gels were incubated in 7% acetic acid–10% methanol for 30–60 min to wash excess dye out of the polyacrylamide matrix. SYPRO Ruby dye stained gels were scanned on a FUJI FLA 3000 fluorescent image analyzer using a 473 nm excitation laser and a 580 nm emission filter at a sensitivity setting of F1000.

2.4. Generating differential display maps

Computer-generated differential display maps of protein glycosylation and/or protein expression patterns were generated using the Z3 software, version 1.5 (Compugen, Tel-Aviv, Israel) [23,24]. The Z3 software is a Microsoft Windows-based package for analyzing 2-D electrophoresis images. Unlike many commercially available software packages, Z3 software uses raw-image-based computation of registration, region-based matching, and a complementary pseudocolor visualization technique. With the system, spots of the reference gel appear green and those of the comparative gel appear magenta. When images are aligned, similarly expressed spots in the overlay image appear gray or black, while those that differ in expression levels appear green or magenta. This facilitates identification of differentially expressed protein spots by simple visual inspection.

2.5. Western blotting and lectin detection

2-D gels were run as described in Section 2.2 and the proteins were then transferred onto PVDF membrane using a buffer containing 10% methanol, 10 m*M* Tris Base, 96 m*M* glycine. The proteins were electroblotted at 1.25 mA/cm^2 for 1.5 h on a semi-dry blotter (W.E.P., Concord, CA, USA). After transfer, the membranes were stained with SYPRO Ruby protein blot stain according to the manufacturer's instructions and imaged for total protein staining using a FUJI FLA 3000 fluorescent image analyzer [25]. After imaging, the blots were blocked with 50 m*M* Tris, 150 m*M* NaCl, 0.2% Tween 20, 0.25% MOWIOL 4–88, pH 7.5 for 1 h. The lectins (alkaline phosphatase conjugated concanavalin A or ricin) were diluted into a lectin reaction buffer (blocking solution plus 0.5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$) at a final concentration of 1 μg/ml. Again the blot was incubated for 1 h followed by two washes in lectin reaction buffer. In order to remove the Tween 20, the blots were washed twice in 50 m*M* Tris, 150 m*M* NaCl, pH 7.5 before detecting the lectins. Alkaline phosphatase activity was detected with DDAO phosphate (1.25 μg/ml) in 10 m*M* Tris, 1 m*M* $MgCl_2$, pH 9.5 upon incubation for 15 min [26]. After drying the membrane, the DDAO signal was detected using a FUJI FLA 3000 fluorescent image analyzer using the system's 632 nm laser and a 675 nm emission filter.

2.6. Protein identification by mass spectrometry

Proteins were analyzed by MALDI-TOF mass spectrometry as previously described [20,21]. Spots were excised from SYPRO Ruby dye stained gels and stored at −20 °C until they were processed further. The gel pieces were washed twice in 200 μl

(50% acetonitrile, 50 m*M* NH_4HCO_3) for 30 min each. All pieces were dehydrated in 100% acetonitrile before reduction and alkylation. For reduction, the pieces were rehydrated in 100 m*M* DTT, 100 m*M* NH_4HCO_3 for 1 h at 56 °C. After washing off the excess DTT with deionized water the cysteine residues were alkylated with 100 m*M* iodoacetamide for 30 min at room temperature. The excess was removed again with a brief wash in 100 m*M* NH_4HCO_3 before complete dehydration with 100% acetonitrile. For tryptic digestion a solution of 25 ng/ml modified trypsin (Promega, Madison, WI, USA) was prepared in the supplied buffer and 20 μl was added to each gel piece. After 10 min, 50 μl of 50 m*M* NH_4HCO_3, 5% acetonitrile was added to fully cover the pieces. The trypsin was incubated over night at 30 °C. The next day the supernatant was removed to a new tube and the gel pieces were extracted once with 10% acetonitrile, 0.1% trifluoroacetic acid followed by 50% acetonitrile, 0.1% trifluoroacetic acid (20 min each). All supernatants were combined and the peptides were dried in a SpeedVac® (Thermo Savant, Holbrook, NY, USA). The extracted peptides were resuspended in 5% acetonitrile and desalted/concentrated by ZipTip® C^{18} columns according to the manufacturer. Peptides were eluted with 50% acetonitrile 0.1% TFA and mixed 1:1 with 20 mg/ml α-cyano 4-hydroxy cinnamic acid in acetonitrile or methanol before spotting onto a stainless steel MALDI target plate. Spectra were obtained using a Shimadzu Biotech (Kratos) Axima CFR MALDI-TOF mass spectrometer (Columbia, MD, USA).

3. Results

3.1. Glycoprotein detection in normal and cancerous liver tissue extract

Image analysis approaches for the examination of 2-D gel electrophoresis profiles are typically based upon the comparison of at least two different protein profiles. The chief goal of differential display proteomics is to increase the information content of proteomics studies through multiplexed analysis. The Multiplexed Proteomics (MP) technology employs the same dye to measure proteins across all gels in the database, and reserves additional dyes with different excitation and/or emission maxima to highlight specific functional attributes of the sample [27]. The MP approach allows the parallel determination of both protein expression levels and functional attributes of the proteins (e.g. glycosylation, drug-binding capabilities, drug-metabolizing capabilities) within a single experiment.

In this study the principles of MP technology were applied to the analysis of protein glycosylation changes arising from cancer. It should be noted that a feasibility study is presented here, demonstrating the potential of MP technology for clinical studies of glycosylation. An actual clinical investigation would certainly involve many more samples than the ones available to us in this study. Glycoprotein and total protein expression profiles were obtained from the same 2-D gel using a serial dichromatic detection method that employs a fluorescent Schiff's base dye for the labeling of glycoproteins (Pro-Q Emerald 300 dye) and a noncovalent fluorescent dye for the staining of total protein (SYPRO Ruby dye) [18,19,21]. Both dyes allow detection of less than one nanogram of target and both provide a linear response over three orders of magnitude [18,19,21]. Fig. 1 is a schematic diagram that summarizes the basic experimental design of MP as applied to the analysis of protein glycosylation changes. First, a set of 2-D gels is fluorescently stained and imaged to reveal some functional attribute of the proteins, such as glycosylation state. Next, SYPRO Ruby protein gel stain is used as the foundation staining technology to reveal protein levels in the same gels. Differential display analysis is accomplished by computer, using image analysis software. The approach is able to characterize two different properties of a tissue specimen in a single 2-D gel, thus reducing the total number of gels that are required for an analysis by a factor of two.

Glycosylation and protein expression profiles generated from normal human liver tissue extract and tumor liver extract are shown in Fig. 2. A comparison of Pro-Q Emerald 300 dye staining of the glycoproteins clearly demonstrates either an increase in glycosylation of proteins or an increase in the total amount of glycosylated proteins present in tumor liver tissue extracts relative to normal liver tissue extracts (Fig. 2A,C). In order to distinguish between

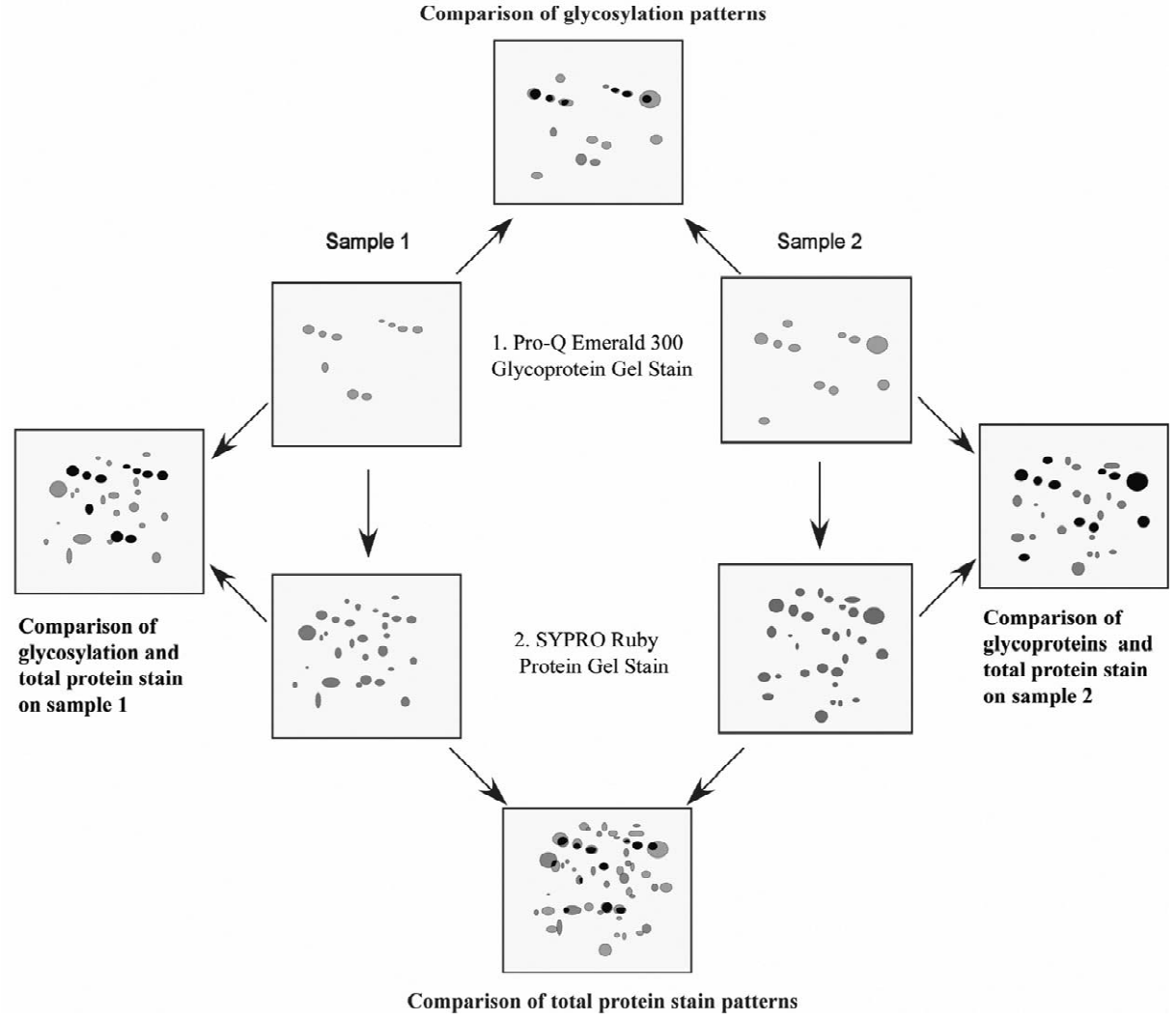

Fig. 1. Schematic diagram illustrating the basic concept of the Multiplexed Proteomics (MP) technology [27]. Two different samples are run on two 2-D gels. Gels are stained for glycosylation using Pro-Q Emerald 300 dye and imaged. Gels are then stained for total protein using SYPRO Ruby dye and imaged again. Images are registered and redisplayed in pseudocolors using Z3 software (Compugen). Differences in glycosylation and protein expression are identified by evaluation of the pseudocolor-coded differential display maps and data spreadsheets.

an increase in the total amount of protein compared with an increase in the levels of glycosylation of the proteins, gels were post-stained with SYPRO Ruby dye. Fig. 2B and D demonstrate that the amount of glycoproteins is generally up-regulated, not the amount of oligosaccharide affixed per unit protein.

The resulting gel images may be automatically matched by computer, with the option of adding some manual anchor points to facilitate the process. Any two images can then be re-displayed as a single pseudo-colored map, allowing visual inspection of differences (Fig. 3). It becomes readily apparent after generating differential display maps using Z3 image analysis software, that only a few newly glycosylated proteins are detectable in the tumor tissue extracts when compared to the normal liver extracts. Though the Z3 software was used in this analysis, other software packages, such as Delta2D

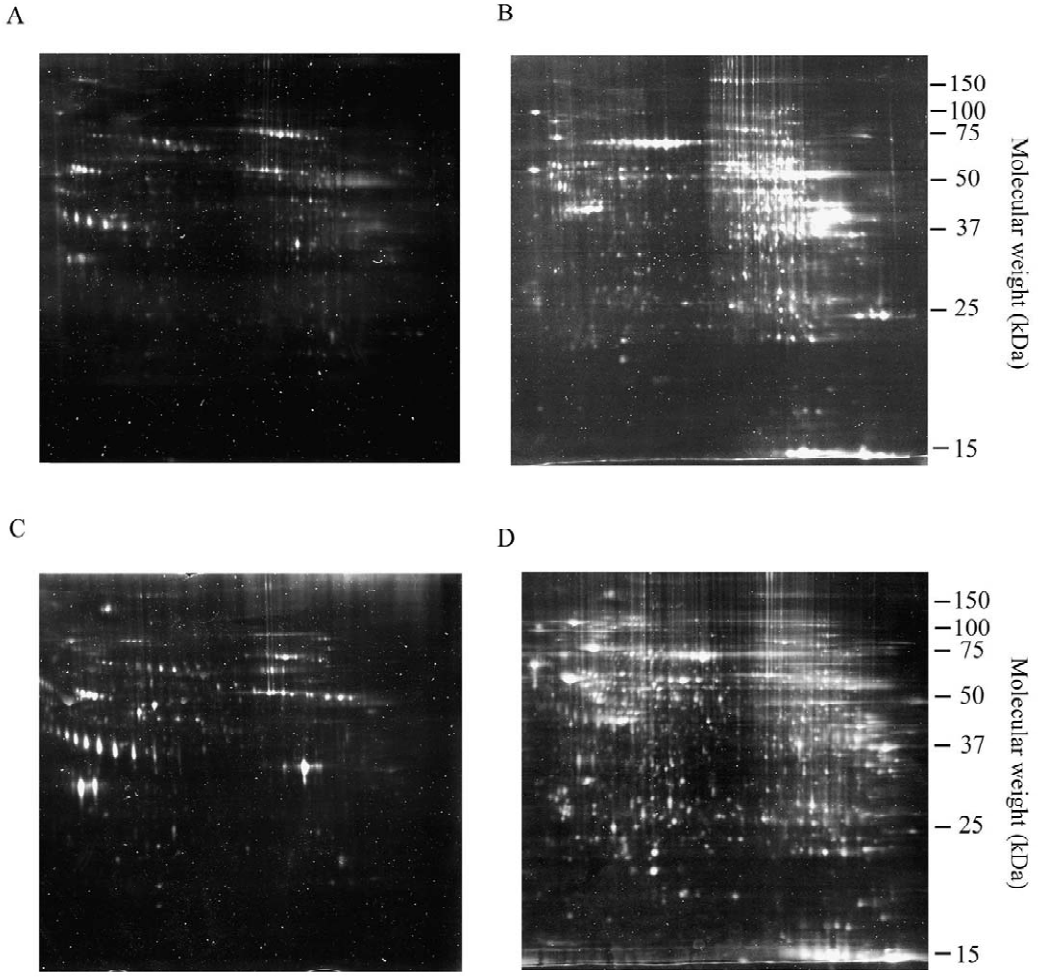

Fig. 2. Dichromatic analysis of protein glycosylation and expression levels in normal human liver and tumor tissue lysates. Proteins were separated by 2-D gel electrophoresis, glycoproteins revealed by Pro-Q Emerald 300 dye followed by total protein staining of the same gel using SYPRO Ruby protein gel stain. (A) Glycoprotein profile obtained from normal human liver tissue. (B) Total protein profile obtained from normal human liver tissue. (C) Glycoprotein profile obtained from human liver tumor tissue. (D) Total protein profile obtained from human liver tumor tissue.

(Decodon, Greifswald, Germany) offer similar capabilities [28].

One of the important advantages of the MP technolgy is that similar profiles, such as total protein patterns, are matched by computer from different gels, while dissimilar ones, such as total protein patterns versus glycoprotein patterns, are matched by computer from the same gel. Thus, though few landmarks are shared between total protein profiles and glycoprotein profiles, the matching of images is facilitated by the process of superimposition (Fig. 3A,B).

3.2. Lectin based profiling of glycoproteins

In order to discriminate between branched glycan isoforms, we developed a method, utilizing lectin blotting, to rapidly profile the glycoproteins resolved by 2-D gel electrophoresis. The method is based upon the successful use of lectin affinity chromatog-

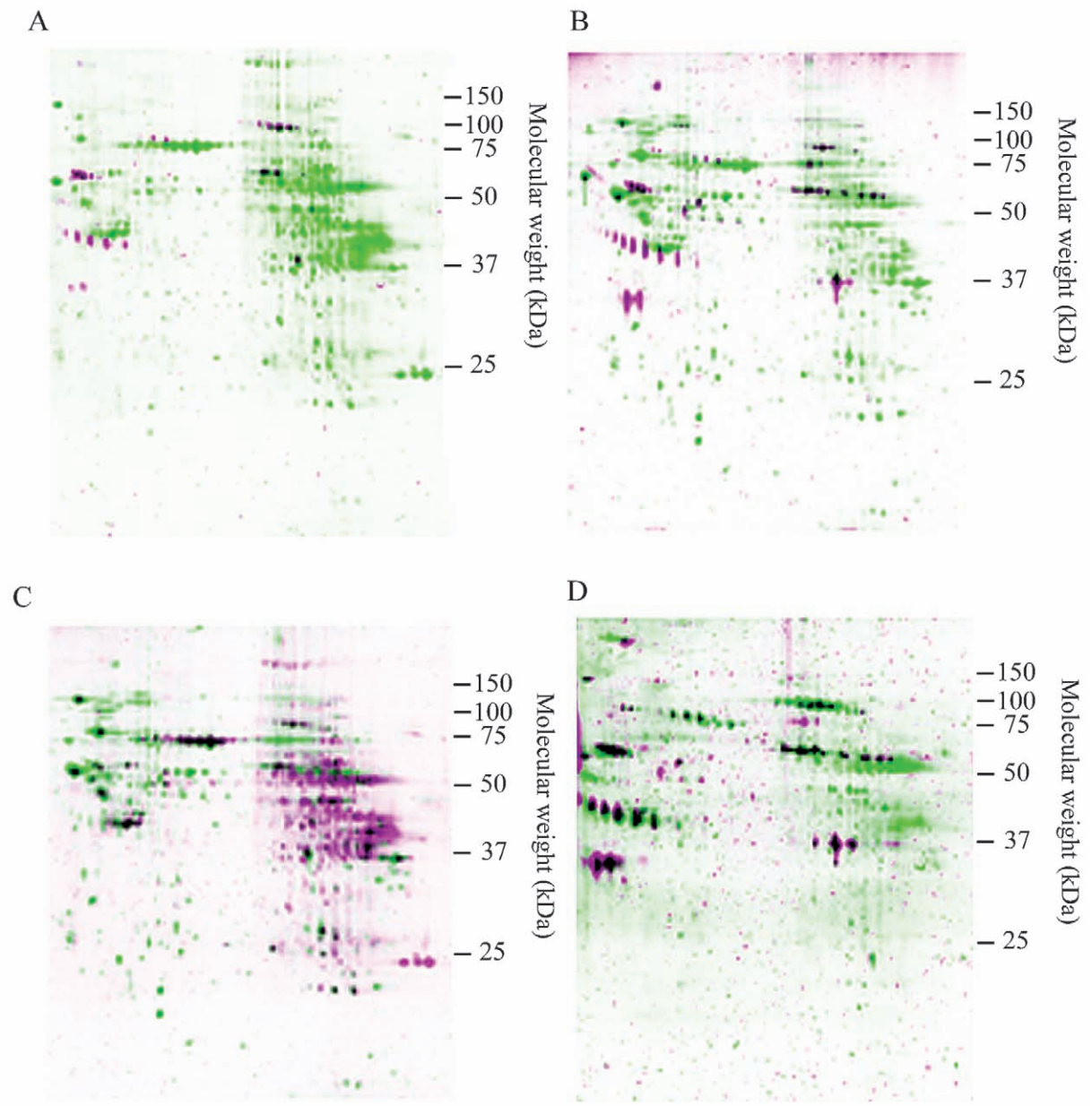

Fig. 3. Differential display maps of normal and tumor human liver tissue extracts. (A) Differential display map demonstrating glycosylated and protein expression levels in normal human liver tissue. Magenta spots depict glycoproteins, while green spots depict total protein. (B) Differential display map demonstrating glycosylated and protein expression levels in human liver tumor tissue. Magenta spots depict glycoproteins, while green spots depict total protein. (C) Differential display map indicating protein expression changes between normal and tumor tissue. Magenta spots depict normal tissue, while green spots depict tumor tissue. (D) Differential display map indicating changes in glycosylation between normal and tumor tissue. Magenta spots depict tumor tissue, while green spots depict normal tissue.

raphy for structural analysis of oligosaccharides from complex mixtures [29–31]. Oligosaccharides have a several thousand-fold higher affinity towards lectins than simple monosaccharides [31]. Certain lectins exhibit a high affinity for N-linked high mannose type, and hybrid type, as well as mono-antennary and bi-antennary complex type glycan structures while others preferentially bind the N-linked tri-, tetra- and penta-antennary complex type structures. The two lectins selected for this study were concanavalin A, which binds avidly to the former class of glycans and *Ricinus communis* II (ricin), which possesses a high affinity for the latter class. Two major types of tri-antennary glycans are generated when a lactosamine unit is enzymatically affixed to the bi-antennary glycan core; 2,4-branched (Tri-1) and 2,6-branched (Tri-2) structures [31]. Based upon affinity chromatography, ricin lectin binds both glycan structures with high affinity. Naturally, tetra-antennary and penta-antennary structures are also bound tightly by the lectin as these structures are produced from bi-antennary cores with 2,4- and/or 2,6- branched substitutions. By contrast, concanavalin A interacts with the common pentasaccharide core structure of N-linked oligosaccharides but fails to interact with Tri-1 and Tri-2 structures.

Four well characterized model glycoproteins were evaluated to determine whether the detection specificities of concanavalin A and ricin, as defined by lectin blotting, are similar to those reported with high-performance affinity chromatography. Electroblotting of fetuin, horseradish peroxidase (HRP), ovalbumin (Oval) and Tamm-Horsfall glycoprotein (THG), followed by detection of oligosaccharides with alkaline phosphatase conjugates of the two lectins and the fluorogenic substrate DDAO phosphate confirms that the expected glycan structures are detected (Fig. 4 and Table 1) [32–39]. Horseradish peroxidase and ovalbumin are primarily detected by concanavalin A, while Tamm-Horsfall glycoprotein and fetuin are primarily detected by ricin.

Next, normal human liver tissue extract and liver tumor tissue extract were evaluated using the two lectins after 2-D gel electrophoresis and Western blotting (Fig. 5). Differences between normal and tumor tissue extracts with respect to the expression of concanavalin A-binding glycoproteins were found to be quite pronounced. The differences were indeed so numerous that an overlay of the two images could not reliably be achieved. Changes in ricin-binding glycoprotein expression were more modest. Principally, an increase in the level of glycosylation of glycoproteins or an increase in the expression levels of glycoproteins in the tumor tissue lysates relative to the normal tissue lysates was observed. Additionally, it is readily apparent that the two lectins detect completely different classes of glycoproteins with very little overlap between the two classes. Interestingly the blotting results also suggest that ricin-binding glycoproteins are more readily visualized than concanavalin A-binding glycoproteins using Pro-Q Emerald 300 dye. Only the most prominent concanavalin A-binding glycoproteins were visualized using Pro-Q Emerald dye. This might be expected, since tri-, tetra- and penta-antennary glycans contain more terminal residues susceptible to periodate oxidation, a key step in the labeling of glycoproteins with Pro-Q Emerald 300 dye.

3.3. Glycosylation changes associated with hepatocellular carcinoma

A number of the glycoproteins present in the human liver tumor tissue extract are known to correspond to proteins synthesized by the liver and subsequently secreted into the bloodstream. Though identifiable by peptide mass profiling using MALDI-TOF mass spectrometry, glycoproteins present certain challenges in analysis as numerous isoforms of polypeptides are generated on the gel with differing isoelectric points and molecular masses [40]. Glycosylation can protect proteins from proteolytic digestion, making identification by peptide mass profiling more difficult. Glycopeptides substituted with N-linked glycans are difficult to observe by MALDI-TOF mass spectrometry because their combined mass along with the peptide is generally greater than 3500 Da [41]. Finally, glycosylated peptides are often heterogeneous with variable site occupancy, leading to peak broadening in mass spectrometry. Thus, we examined the feasibility of using a Multiplexed Proteomics approach to rapidly landmark the major secreted plasma glycoproteins in the liver tissue extracts.

Human plasma and human tumor liver extract were compared to one another using the MP ap-

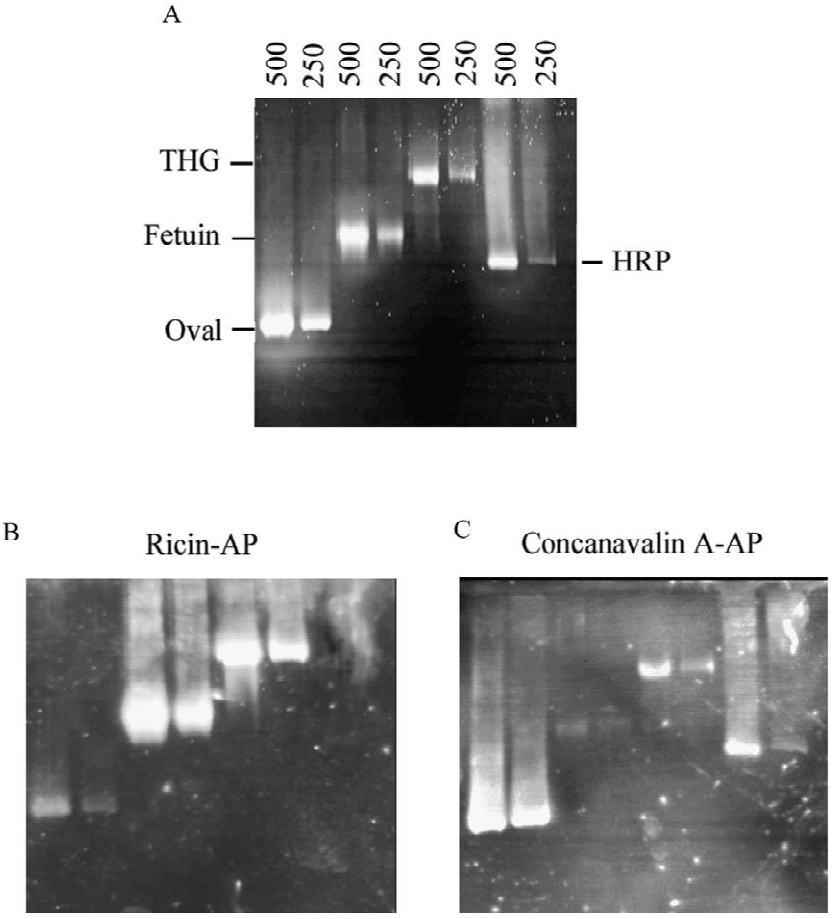

Fig. 4. Dichromatic lectin-based profiling of model glycoproteins. Total protein was visualized using SYPRO Ruby protein blot stain, while concanavalin A- and ricin-binding proteins were visualized using an alkaline phosphatase-based reporter system and DDAO phosphate. (A) Total protein profile of the electroblotted proteins demonstrating similar amounts of each model glycoprotein. (B) Ricin-binding glycoproteins. (C) Concanavalin A-binding glycoproteins.

Table 1
Lectin-based detection of model glycoproteins

Glycoprotein	Percentage carbohydrate (%)	Carbohydrate structure	Detection with Concanavalin A	Detection with Ricin
Tamm-Horsfall glycoprotein	25–30	Seven N-linked glycans of tri-, tetra- and penta-antennary structure.	Weak	Strong
Fetuin	22	Three N-linked glycans of tri-antennary structure as well as three O-linked glycans.	Weak	Strong
Horseradish peroxidase	22	Eight N-linked glycans of bi-antennary structure.	Strong	Weak
Ovalbumin	3–4	Single N-linked glycan of either high-mannose or hybrid structure.	Strong	Weak

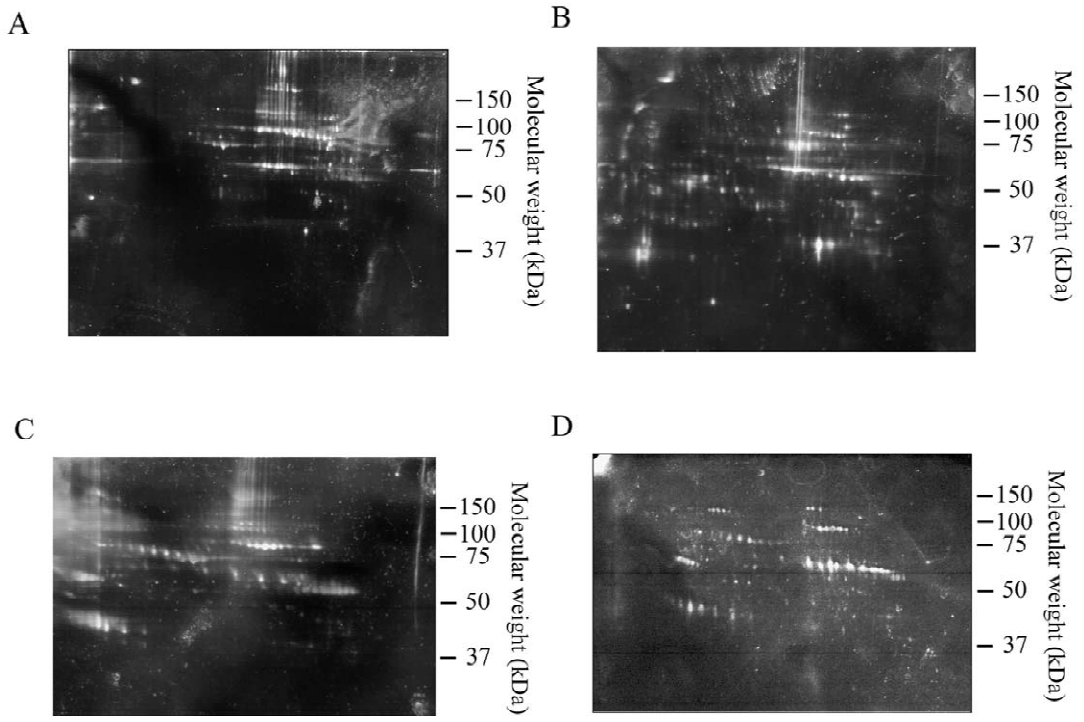

Fig. 5. Lectin-based profiling of normal human liver and tumor tissue after Western blotting. (A) Concanavalin A-binding glycoproteins in normal human liver tissue extract. (B) Concanavalin A-binding glycoproteins in liver tumor tissue extract. (C) Ricin-binding glycoproteins in normal human liver tissue extract. (D) Ricin-binding glycoproteins in liver tumor tissue extract.

proach. Fig. 6 shows an annotated differential display map indicating the more abundant plasma proteins present in the liver tissue extract, based upon well-established maps of human plasma [42]. Image registration was verified by MALDI-TOF mass spectrometry of serum albumin, a nonglycosylated protein present in both specimens. Though certainly not as definitive as peptide mass profiling, the Multiplexed Proteomics approach to rapid protein identification does benefit from the fact that numerous attributes (molecular mass, isoelectric point, isoform morphology, glycosylation status, lectin reactivity) are all taken into consideration when designations are made.

Based upon the MP approach to rapid glycoprotein identification, prominent glycoproteins present in the normal human liver and tumor tissue extracts include α1-protease inhibitor (α1-antitrypsin), haptoglobin, hemopexin and transferrin. Among these proteins, haptoglobin was one of the major glycoproteins determined to be up-regulated in our 2-D gels of tumor tissue extract. The abundance of this protein is roughly 3.5-fold higher in the tumor tissue extract. This acute phase plasma glycoprotein is known to be primarily synthesized in the liver, though it is expressed at low levels in other organs, such as in adipose tissue and the lung [43,44]. Haptoglobin is a tetramer composed of two glycosylated β-chains, each possessing two sites for N-linked oligosaccharides, connected by disulfide bonds with two nonglycosylated α-chains. The 42 600 Da β chains of haptoglobin were detected in this study. The β chains of haptoglobin were readily visualized in gels using the Pro-Q Emerald 300 dye and by Western blotting using alkaline-phosphatase-conjugated ricin and DDAO phosphate, but not by Western blotting using alkaline phosphatase-conjugated concanavalin A and DDAO phosphate. This is consistent with haptoglobin containing roughly 19% carbohydrate by weight, present as N-linked oligosaccharides of complex tri-antennary type.

Though, like haptoglobin, α1-protease inhibitor is

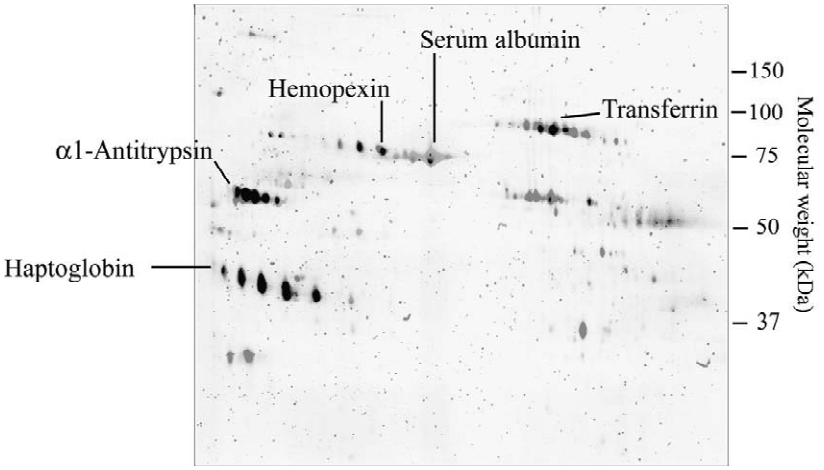

Fig. 6. Annotated differential display map of portions of 2-D gels of human plasma compared with human liver tissue extract. Glycoprotein profiles as detected with Pro-Q Emerald 300 dye are displayed. Magenta spots depict human plasma proteins, while green spots depict human liver tumor tissue extract. The identities of key glycoproteins in the liver tissue extract are indicated, based upon maps of human plasma [42]. Serum albumin, as identified by MALDI-TOF mass spectrometry was used to verify correct registration of the images. Similar maps of total protein using SYPRO Ruby Protein gel stain confirm that apolipoprotein A1 is not a glycoprotein.

an acute phase plasma glycoprotein secreted by the liver, it was not significantly up-regulated in the tumor tissue extract investigated in this study. While hemopexin and transferrin also did not appear to be significantly up-regulated in the cancer tissue, both glycoproteins demonstrated changes in glycosylation profiles as established by the generation of new charge isoforms. These glycoproteins were also found to be reactive with ricin, but not concanavalin A.

4. Discussion

2-D gel electrophoresis is an important tool for proteome-wide analysis and especially for the comparison of changes in protein expression levels due to disease stages or treatment modalities. The capability of 2-D gel electrophoresis to resolve post-translational modifications is considered an especially important strength of the technology and the development of new detection techniques specific to such modifications, such as recently developed fluorescent glycoprotein detection reagents, promise to leverage this advantage further [18,27]. Traditionally, in order to study specific changes in glycosylation, as well as overall changes in protein expression levels, the standard 2-D gel method requires running two separate gels. Typically, one gel is used for the detection of glycoproteins after electroblotting using periodic acid/Schiff (PAS) staining with acid fuchsin dye, PAS labeling with digoxigenin hydrazide followed by immunodetection with anti-digoxigenin antibody conjugated to alkaline phosphatase, or PAS labeling with biotin hydrazide followed by detection with horseradish peroxidase or alkaline phosphatase conjugated to streptavidin (reviewed in Ref. [45]). The second gel is then employed for total protein staining using silver stain or SYPRO Ruby protein gel stain. This approach, however, suffers from some inherent technical difficulties. In order to map the glycoprotein profile to the total protein profile, both gels must run with very high spot positional reproducibly. Often, however, the overall dimensions of the gel change during fixation and staining, making it difficult to directly superimpose it with the electroblot. In fact, we had difficulty directly superimposing Pro-Q Emerald 300 dye stained gels with electroblots used for visualizing concanavalin A- or ricin-binding proteins due to changes in gel dimensions during their processing. Additionally, heavily glycosylated proteins usually are of relatively high molecular mass and are difficult to transfer by electroblotting, thus leading to their loss from the

analysis. Another major drawback is that since numerous manipulations are involved in electroblotting, reliable quantitation is difficult to achieve.

We describe an alternative fluorescence-based approach, the Multiplexed Proteomics (MP) technology, for analysis of glycosylation status and protein expression levels. The feasibility of the MP approach was demonstrated by assessing changes in protein glycosylation as well as overall protein expression levels in normal human liver tissue extracts and human hepatocellular carcinoma tissue extracts. The development of cancer is often accompanied by increased glycosylation and alterations in complex glycan branching structures. Such changes were readily revealed using the Multiplexed Proteomics technique. A more detailed analysis of the glycan branching structure was then achieved using lectin-based Western blot detection methods employing alkaline phosphatase-conjugated ricin and alkaline phosphatase-conjugated concanavalin A. This approach allows one to rapidly categorize the glycoproteins into classes with respect to the complexity of the glycan structures.

One prominent glycoprotein observed in this study to be up-regulated in the tumor tissue compared with the normal liver tissue extract was haptoglobin β chain. Plasma levels of this acute phase protein are known to be elevated under conditions of inflammation, infection, trauma, tissue damage and malignant proliferation [44]. Haptoglobin is known to be synthesized in the liver, with maximum expression of the protein requiring cytokines such as interleukin-1, interleukin-6, tumor necrosis factor-α, and transforming growth factor-β, as well as glucocorticosteroids. Haptoglobin readily and specifically combines with hemoglobin to form a stable complex that may play a role in preventing hemoglobin-mediated generation of hydroxyl radical and lipid peroxide in areas of inflammation. Abnormal changes in the fucosylation of haptoglobin have been observed in patients with ovarian or breast cancer [46]. In addition, increased hepatic expression of haptoglobin and other acute phase proteins has been noted upon administration of peroxisome proliferators in rat models of carcinogenesis [47]. Haptoglobin and the haptoglobin–hemoglobin complex have also been shown to induce apoptosis of hepatocarcinomatous Hep 3B cells in tissue culture [48]. It is reasonable to speculate that haptoglobin may function in association with the immune system to protect an organism against a broad spectrum of agents that elicit a host acute phase response.

Multiplexed fluorescence capabilities have only recently been applied to the analysis of proteins resolved by 2-D gel electrophoresis (reviewed in Ref. [45]). MP permits the parallel analysis of thousands of proteins in a single 2-D gel profile with respect to a number of functional attributes. As illustrated in this manuscript, this new capability should facilitate the use of 2-D gel electrophoresis for the analysis of global changes in protein glycosylation. Information obtained using the MP technology concerning disease-specific glycosylation patterns might potentially be utilized therapeutically. For example, glycoproteins uncovered by the technology might be suitable targets for immunotherapy aimed at preventing cell adhesion or interfering with other binding or biological processes in order to actively interfere with the disease process.

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