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Analysis of Glycoproteins for Biomarker Discovery

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Summary

Glycoproteins play an important role in cell signaling and cell-cell interaction. The alterations of glycoproteins are often relevant to progression of diseases and these changed glycoproteins can be important biomarkers. The lectin-based glycoproteomic technology has extensively been used for high-throughput screening of potential glycoprotein biomarkers. Here we describe a multi-lectin affinity chromatography and label-free quantitative glycoproteomic approach for discovery of glycoprotein biomarkers relevant to differentiation of glioblastoma stem cells.

Keywords

Glycoprotein; Biomarker; Lectin Affinity Chromatography; Mass Spectrometry; Proteomics

1. Introduction

Glycoproteins are a group of proteins with oligosaccharide chains covalently attached to them. These proteins are ubiquitously distributed, and play a critical role in various biological processes, such as cell signaling, cell-cell interaction, immune recognition, cell proliferation and differentiation (1). According to recent findings, the alterations of glycoproteins are often associated with the progression of diseases including cancer (2-4). These altered glycoproteins may be important biomarkers and therapeutic targets.

The proteomic technology provides a powerful tool for discovery of potential biomarkers. The quantitative comparison of the proteome of different samples can result in identification of differentially expressed proteins which are potential biomarkers. Glycoproteomics is a branch of proteomics that studies the entire complement of glycoproteins. Glycoproteomic studies have greatly benefited from lectin affinity chromatography (5-10) which can be used to enrich glycoproteins, as well as from mass spectrometry which plays a vital role in the characterization of glycoproteins (11-14). Lectins are a class of proteins which can specifically bind to the glycan moieties of glycoproteins (15). Currently, hundreds of lectins have been purified and characterized. Concanavalin A (Con A) and wheat germ agglutinin (WGA) are the most commonly used lectins for glycoprotein enrichment where they have high affinity to most of the N-linked glycoproteins.

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In this chapter, we describe a lectin-based glycoproteomic approach for discovery of glycoprotein markers relevant to differentiation of a human glioblastoma-derived stem-like neurosphere HSR-GBM1. A diagram of the workflow of this approach is shown in Fig. 1.

2. Materials

2.1 Protein Extraction

1. Protein extraction buffer: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% (w/v) octyl- β -D-glucopyranoside. Store at 4 °C. Add 1% protease inhibitor cocktail (Sigma-Aldrich) mixture immediately before use.
2. PBS buffer: PBS powder (Sigma-Aldrich) dissolved in 1 L distilled water.
3. Dounce glass homogenizer with a tight-fitting pestle (Wheaton Science)
4. Temperature controlled centrifuge (Thermo Fisher Scientific)
5. 1.5 ml eppendorf tubes (Eppendorf)

2.2 Lectin Affinity Chromatography

1. Agarose-bounded Con A, WGA and peanut agglutinin (PNA) (Vector Laboratories) (**Note 1**)
2. Binding buffer: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM MnCl₂.
3. Elution buffer: 300 mM methyl-R-D-mannopyranoside (Sigma-Aldrich), 300mM N-acetylglucosamine (Sigma-Aldrich) and 200 mM D-galactose (Sigma-Aldrich) in binding buffer.
4. 5 mL disposable screw endcap spin column (Thermo Fisher Scientific)
5. Microcon YM-10 centrifugal filter (Millipore)
6. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), iodoacetamide (IAA) and ammonium bicarbonate are purchased from Sigma-Aldrich

2.3 Digestion with Trypsin and PNGase F

1. TPCK modified sequencing grade porcine trypsin (Promega)
2. PNGase F (Sigma)
3. Speedvac concentrator system (Thermo Fisher Scientific)
4. Formic acid (Sigma-Aldrich)

2.4 LC-MS/MS

1. Paradigm MG4 micropump system (Michrom Biosciences, Inc.)

¹Con A, WGA and PNA have different carbohydrate specificities. Con A is specific to α -linked mannose; WGA is specific to N-acetyl-D-glucosamine; PNA is specific to β -galactose.

2. Paradigm Platinum Peptide Nanotrap (Michrom Biosciences, Inc.)
3. Magic C18AQ column (0.1 mm × 150mm, C18 AQ particles, 5 µm, 200 Å, Michrom Biosciences, Inc.)
4. Solvent A: 0.3% formic acid in H₂O (HPLC grade)
5. Solvent B: 0.3% formic acid in acetonitrile (HPLC grade)
6. LTQ linear ion trap mass spectrometer (Thermo Electron Corporation)

2.5 Mass Spectrometry Data Analysis

1. Proteome Discoverer software version 1.1.0.263 (Thermo Fisher Scientific)
2. Swiss-Prot database (Swiss Institute of Bioinformatics)

2.6 Western Blotting

1. PBST: 0.05% Tween-20 in PBS.
2. Blocking buffer: 1% BSA in PBST.
3. Mini-PROTEAN 3 system (Bio-Rad)
4. Polyvinylidene difluoride (PVDF) membrane (Bio-Rad)
5. Trans-Blot SD Semi-Dry System (Bio-Rad)
6. Peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (H+L) (Abcam)
7. Supersignal West Pico Chemiluminescent HRP Substrate (Thermo Fisher Scientific)

3. Methods

3.1 Protein Extraction

Carry out all procedures at 4 °C unless otherwise specified.

1. Collect ~ 20 million of undifferentiated and differentiated HSR-GBM1 cells, wash the cells twice with PBS, and centrifuge at 500 × g for 5 min at 4 °C. Store the cell pellets in -80 °C until use.
2. Resuspend the cell pellets in 1 mL of protein extraction buffer, then transfer the suspension to a Dounce glass homogenizer and homogenize the cells with 25 strokes using a tight-fitting pestle. After 10-min incubation on ice, repeat the process.
3. Transfer the cell lysates to 1.5 mL eppendorf tubes (**Note 2**) and centrifuge at 40,000 × g for 30 min at 4 °C. Collect the supernatants to new eppendorf tubes and determine protein concentrations of each sample by the Bradford method (16).

²All the microcentrifuge tubes used in this work are Eppendorf Protein LoBind tubes which can reduce sample-to-tube binding and thus improve sample recovery.

3.2 Glycoprotein Enrichment by Lectin Affinity Chromatography

Glycoproteins were enriched using multi-lectin affinity chromatography essentially as previously described (17). The workflow of this method is shown in Fig. 2.

1. Pack 2 Pierce centrifuge columns with agarose-bound lectin mixtures. Each column contains 0.8 mL of Con A, 0.8 mL of WGA and 0.8 mL of PNA. Wash the columns with 7.2 mL of binding buffer.
2. Dilute cell lysates four times with ice-cold binding buffer, where each sample contains ~ 2 mg of proteins. Load the diluted samples to lectin columns, suspend the beads and incubate for 15 min. Collect the flow-through and repeat the step (**Note 3**).
3. Wash each lectin column with 4×2.4 mL of binding buffer to remove the non-specific binding proteins (**Note 4**).
4. Add 2.4 mL of elution buffer to each column, incubate for 15 min and collect the eluate. Repeat this step twice, and pool the eluates of the same sample.
5. Transfer the eluates to Microcon YM-10 centrifugal filter devices, centrifuge at $5,000 \times g$ for 45 min at 20 °C, and wash each sample with 6 mL of 25 mM ammonium bicarbonate (**Note 5**).
6. Add 200 μ L of 10 mM TCEP to each sample, incubate for 20 min at room temperature. And then add 200 μ L of 30 mM IAA to each sample, incubate in dark for 20 min at room temperature (**Note 6**).
7. Wash each sample with 6 mL of 25 mM ammonium bicarbonate twice. Determine protein concentration and store the glycoprotein samples at -80 °C until further use.

3.3 Digestion with Trypsin and PNGase F

1. Add trypsin to each glycoprotein sample (w/w 1:50) and incubate for 12-16 h at 37°C.
2. Add 1 μ L of formic acid to each sample to stop the enzymatic reaction, and dry the sample with a speedvac concentrator.
3. Suspend the dried sample with 25 mM ammonium bicarbonate, and then add 1 U of PNGase F, mix by vortex and incubate for 16-20 h at 37°C.
4. Add 1 μ L formic acid to the digest to stop the enzymatic reaction, and dry the sample with a speedvac concentrator. Keep the dried sample at -80 °C before analysis by mass spectrometry.

³Do not discard the flow-through until confirming that the enrichment is successful.

⁴Monitor the protein concentration of the flow-through. If there is still a significant amount of proteins in the flow-through buffer after this 4×2.4 mL washing step, wash the column using another 2.4 mL of binding buffer.

⁵After this step, the glycoproteins are concentrated, the glycans and salts in the eluates are removed, and meanwhile, the buffer system is changed to ammonium bicarbonate which is suitable for the following tryptic digestion.

⁶IAA is light sensitive. Prepare the IAA buffer freshly before use and keep it away from light.

3.4 LC-MS/MS

1. Dissolve the peptide mixtures in 70 μ l of 0.1% formic acid, centrifuge samples at $15,000 \times g$ for 10 min, and transfer the supernatants to new eppendorf tubes.
2. Inject 10 μ l of each sample using an autosampler, transfer peptides to a Nanotrap column using a Paradigm MG4 micropump system, and separate peptides using a C18 separation column. A 90-min linear gradient of acetonitrile (solvent B) /water (solvent A) containing 0.3% formic acid is used (**Note 7**). The flow rate is 300 nL/min. The gradient is as follows:

0%-10% B for 10 min
10%-40% B for 90 min
40%-100% B for 8 min
100% B for 1 min
100%-0% B for 1 min
0% B for 10 min
3. Analyze the peptides using an LTQ linear ion trap mass spectrometer. Operate the mass spectrometric analysis in positive ion mode and acquire the data with a Xcalibur software. For each cycle of one full mass scan (range of m/z 400-2000), select the five most intense ions in the spectrum for MS/MS analysis unless they appear in the dynamic or mass exclusion lists. Set the ESI spray voltage at 1.5 kV, and the capillary voltage at 30 V. The ion activation was achieved by utilizing helium at a normalized collision energy of 35%.

3.5 Mass Spectrometry Data analysis

1. Search all MS/MS spectra against the Swiss-Prot database using SEQUEST algorithm incorporated in Proteome Discoverer software version 1.1.0.263. The search parameters are as follows: (1) Fixed modification, Carbamidomethyl of cysteine; (2) variable modification, oxidation of M and asparagine to aspartate; (3) allowing two missed cleavages; (4) peptide ion mass tolerance 1.50 Da; (5) fragment ion mass tolerance 1.4 Da; (6) peptide charges +1, +2, and +3.
2. Set false discovery rate (FDR) to be < 0.01 for each database search. The Xcorr values for all the charge states (+1, +2, and +3) are automatically adjusted to achieve the predetermined FDR value. If multiple proteins share the same peptide sequences, report them as a protein group.
3. Export the database search results to excel files, and quantify proteins using label-free approaches such as spectral counting (18) and spectral index (19) (**Note 8**). This will result in a list of differentially expressed proteins.

⁷To identify more proteins by the following mass spectrometry analysis, a longer gradient (for example, a 4-hour gradient) may be used to try to separate the peptides as much as possible.

⁸The quantification is based on comparison of spectral counts or spectral indexes of each protein in different samples.

4. Bioinformatically interrogate the proteins according to the Swiss-Prot annotation to identify a list of differentially expressed glycoproteins which are potential glycoprotein biomarkers.

3.6 Validation of Potential Biomarkers by Western Blotting

1. Separate 12 µg of proteins from undifferentiated and differentiated HSR-GBM1 cells by SDS-PAGE using a Mini-PROTEAN 3 system.
2. Transfer proteins to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot SD Semi-Dry System according to the manufacturer's instruction manual (**Note 9**).
3. Wash the PVDF membrane with PBST and block the membrane with blocking buffer for 1 h at room temperature.
4. Incubate the membrane with various primary antibodies overnight at 4 °C.
5. Wash the PVDF membrane 3 × 10 min with PBST.
6. Incubate the membrane with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (H+L) for 1 h at RT.
7. Wash the PVDF membrane 3 × 10 min with PBST.
8. Visualize the blots with Supersignal West Pico Chemiluminescent HRP Substrate.

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⁹PVDF membrane is hydrophobic and should be pre-wet in methanol before use.

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Fig. 1.
Workflow of lectin-based glycoproteomic technology for biomarker discovery.

Fig. 2.
Illustration of the lectin affinity chromatography method for glycoprotein enrichment.