Methods 81 (2015) 34–40

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Mass spectrometry based proteomics for absolute quantification of proteins from tumor cells

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 9 March 2015
Accepted 11 March 2015
Available online 17 March 2015

Keywords:
Mass spectrometry
Absolute quantification
Proteins
Tumor cells

ABSTRACT

In-depth quantitative profiling of the proteome and sub-proteomes of tumor cells has relevance to tumor classification, the development of novel therapeutics, and of prognostic and predictive markers and to disease monitoring. In particular the tumor cell surface represents a highly relevant compartment for the development of targeted therapeutics and immunotherapy. We have developed a proteomic platform to profile tumor cells that encompasses enrichment of surface membrane proteins, intact protein fractionation and label-free mass spectrometry based absolute quantification. Here we describe the methodology for capture, identification and quantification of cell surface proteins using biotinylation for labeling of the cell surface, avidin for capture of biotinylated proteins and ion mobility mass spectrometry for protein identification and quantification.

1. Introduction

Mass spectrometry based proteomics allows profiling of protein expression on a genome-wide scale providing an important resource for the development of novel diagnostic and therapeutic targets [1–3]. Advances in mass spectrometry have resulted in the development of a high definition MS (HDMS) technology with data-independent acquisition (DIA) and ion-mobility function. As a result, consistency of peptide identification and protein sequence coverage in complex biological samples has been substantially improved. In the MS^E implementation, the collision energy is alternated between low energy and high energy ramp in order to produce precursor- and product-ion spectra, respectively. However, with complex proteomes there are always co-eluting peptides for which retention time alignment is inadequate to reduce chimerism in MS^E spectra. One approach to address this issue is to include an ion mobility separation prior to peptide fragmentation, providing accurate coordinates for assigning precursor/product ion relationships by aligning the precursor and its fragment ions in mobility drift time as well as chromatographic retention time [1–3]. This approach is designated Ion Mobility-Assisted DIA or $HDMS^E$. It adds an additional orthogonal dimension of separation without increasing analysis time and improves the confidence in measurement of mono-isotopic accurate mass for both peptide and its fragment ions.

Cell-surface proteins, including trans-membrane proteins, membrane attached proteins, and membrane associated proteins, play major roles in signal transduction, cell adhesion, ion transport, as well as in cancer pathogenesis [4–7]. Cell-surface protein profiling could provide a better understanding of the manner in which the cell-surface proteome is regulated and how it responds to a variety of intracellular and extracellular signals. Cell-surface proteins have been recognized as major targets for diagnosis, prognosis, and therapy. For example, the discovery that the gene for a growth factor receptor (HER2) is amplified in breast tumors and its protein product is overexpressed at the cell surface have led to an effective form of therapy for breast cancer utilizing an antibody that targets HER2 [8]. Therefore, comprehensive characterization and quantification of cell-surface proteins of tumor cells are critical for identifying markers for clinical diagnosis and prognosis, and drug targets for therapy. We have developed a discovery-platform that integrates *in vitro* cell-surface protein capture, intact protein fractionation and $HDMS^E$ based label-free quantitative mass spectrometry for analyzing tumor cell proteins. Here we present our detailed methodology as well as some findings from the analysis of a population of leukemia cells.

2. Materials and methods

2.1. Chemicals and materials

Trifluoroacetic acid, Formic acid, acetonitrile, 2-propanol, and water (Optima LC/MS grade) were purchased from Fisher

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http://dx.doi.org/10.1016/j.ymeth.2015.03.007
1046-2023/

Scientific (Fair Lawn, NJ). Dulbecco's phosphate buffered saline (PBS), Urea, ammonium bicarbonate, ammonium chloride, octylglucoside (OG), and acrylamide (ACS reagent) were purchased from Sigma–Aldrich (St. Louis, MO). Dithiothreitol (DTT), Bond-break TCEP, Trypsin (MS-grade), Zeba Spin Desalting Columns (7 k MWCO, 2 mL), Biotin, Lysine, and Sulfo-NHS-SS-BIOTIN were purchased from Thermo Scientific (Rockford, IL). Tris–HCl (ACS reagent) was purchased from Corning Life Sciences (Manassas, VA). Protease inhibitors (complete protease inhibitor cocktail) and phosphatase inhibitors (PhosSTOP) were purchased from Roche Diagnostics (Mannheim, Germany). Amicon Ultracel-3k (regenerated cellulose membrane) concentrator was purchased from Merck Millipore (Darmstadt, Germany).

2.2. Tumor cell lysate preparation

Tumor cells are obtained from a variety of sources including cell lines, extraction of tumor cells from biological fluids and from tumors. Isolated cells are pelleted and washed with PBS three times. They are lysed in lysis buffer (4 M urea, 2% OG, 0.1 M Tris–HCl, protease inhibitors, and phosphatase inhibitors, pH 8.0). Cell suspensions are sonicated on ice for 5 min total (30 s intervals every 1 min) with Sonifier 250 (Branson Ultrasonics Corporation, Danbury, CT) set to 40 W output energy. Samples are kept on ice for 15 min after sonication and centrifuged at 20,000×*g* at 4 °C for 30 min. The supernatant is collected and protein concentration is measured by Bradford (Bio-Rad, Hercules, CA, USA). A total of ~350 μg of total cell extract (TCE) proteins from each cell population are reduced with TCEP and alkylated with acrylamide before fractionation by reversed-phase high performance liquid chromatography (RP-HPLC) system.

2.3. In vitro labeling for cell-surface protein isolation

Cell surface proteins are *in vitro* biotinylated with 10 mL of 0.25 mg/mL of Sulfo-NHS-SS-BIOTIN in PBS (1×) at room temperature for 10 min. The residual biotinylation reagent is quenched with 10 mM of Lysine. Protein extraction is performed in lysis buffer containing 2% OG in PBS supplemented with protease inhibitors and phosphatase inhibitors. Cells are disrupted by sonication followed by centrifugation at 20,000×*g*. Biotinylated proteins are isolated by affinity chromatography using 2 mL of NeutrAvidin Plus UltraLink Resin following the manufacturer's instructions. Proteins bound to the column are incubated with 2 mL of elution buffer (65 mM of DTT, 1% OG, pH 7.5) overnight at 4 °C, and then centrifuged at 2000 rpm for 10 min. The concentration of collected proteins is measured by Bradford assay before reduction with TCEP and alkylation with acrylamide. A total of ~70 μg of protein is subjected to fractionation by RP-HPLC. An enrichment comparison of proteins located in extracellular space and plasma membrane between TCE preparation and cell-surface protein labeling with biotin is used to evaluate the effectiveness of the biotinylation for targeting cell surface proteins.

2.4. Reversed-phase liquid chromatography (RPLC) fractionation

A Shimadzu HPLC system equipped with two LC-20AD pumps, one dual wavelength SPD-20A UV detector (220 and 280 nm), one FRC-10A fraction collector, one CTO-20A column oven, and one SCL-10 controller is used for protein fractionation. The whole system is controlled with EZ Start workstation (version 7.4 SP3). The protein sample from TCE or cell-surface is loaded onto the RPGS reversed-phase column (4.6 mm I.D. × 150 mm, 15 μm, 1000 Å, Column Technology Inc., Fremont, CA) and desalted for 5 min with 95% mobile-phase A (0.1% TFA in 95% H_2O) at a flow rate of 3 mL/min. Protein elution from the column is done at a flow rate of 2.1 mL/min with a gradient elution consisting of an increase from 5% to 70% mobile phase B (0.1% TFA in 95% ACN) over 25 min, 70–95% mobile phase B for 3 min, a wash step to hold at 95% mobile phase B for 2 min and a re-equilibration step at 95% mobile phase A for 5 min. Fractions are collected into the 1.1 mL Microtube (Dot Scientific In., Burton, MI) at 20 s intervals, and a total of 84 fractions are collected and kept at −80 °C until further used.

2.5. Protein in-solution digestion

Collected protein fractions are dried by lyophilization with a Freeze-Dryer (Labconco, Kansas City, MO). 50 μL of 8 ng/μL trypsin (50 mM ammonium bicarbonate in 2% ACN, pH 8.0) is added to each protein fraction, mixed thoroughly and incubated at 37 °C for 5 h. Digestion is quenched by adding 10 μL of Quench-buffer (3% ACN in 1% TFA). The quenched digested protein fractions are pooled based on RP-HPLC chromatogram profile recorded at 280 nm. The internal standard Hi3 (Waters, Milford, MA), consisting of a set of six synthetic peptides from E coli chaperone protein ClpB (P63284), is spiked into the pooled fractions.

A total of 24 pools are made for each of cell surface and TCE by combining individual fractions based on their RP-HPLC chromatogram profiles recorded at 280 nm (Fig. 2). Of fractions is based on their peak intensities. In brief, Pool-1: fraction # 1–22; Pool-2: fraction # 23–24; Pool-3: fraction # 25–26; Pool-4: fraction # 27–28; Pool-5: fraction # 29–30; Pool-6: fraction # 31–32; Pool-7: fraction # 33–34; Pool-8: fraction # 35–36; Pool-9: fraction # 37–38; Pool-10: fraction # 39–40; Pool-11: fraction # 41–42; Pool-12: fraction # 43–44; Pool-13: fraction # 45–46; Pool-14: fraction # 47–48; Pool-15: fraction # 49–50; Pool-16: fraction # 51–52; Pool-17: fraction # 53–54; Pool-18: fraction # 55–56; Pool-19: fraction # 57–58; Pool-20: fraction # 59–60; Pool-21: fraction # 61–62; Pool-22: fraction # 63–64; Pool-23: fraction # 65–72; Pool-24: fraction # 73–84.

For Surface Protein, the Pool-1, Pool-23 and Pool-24 are made as follow: 20 μL of digest is taken from each fraction and transferred to a low protein binding tube (1.5 mL). The combined fractions are mixed, frozen and then dried in lyophilizer. The dried digest is solubilized in 45 μL of 3% ACN in 0.1% formic acid. 5 μL of 2 fmol Hi3 is added to the sample as the internal standard for absolute quantification. For Pool-2 to Pool-22, 20 μL of digest is taken from each fraction, then mixed with 10 μL of 1 fmol Hi3. A total 50 μL of pooled sample is used for LC–MS analysis.

For TCE Protein, the Pool-1, Pool-23 and Pool-24 are made as follow: 15 μL of digest is taken from each fraction and transferred to a low protein binding tube (1.5 mL). The combined fractions are mixed, frozen and then dried in lyophilizer. The dried digest is solubilized in 30 μL of 3% ACN in 0.1% formic acid. 10 μL of 2 fmol Hi3 is added to the sample as the internal standard for absolute quantification. For Pool-2 to Pool-22, 15 μL of digest is taken from each fraction, mixed with 10 μL of 2 fmol Hi3. A total of 10 μL of pooled sample is used for LC–MS analysis.

2.6. Nano RPLC of digested proteins

NanoAcquity UPLC system coupled in-line with SYNAPT G2-S mass spectrometer (Waters, Milford, MA) is used for the separation of pooled digested protein fractions. The system is equipped with a Waters Symmetry C18 nanoAcquity trap-column (180 μm × 20 mm, 5 μm) and a Waters HSS-T3 C18 nanoAcquity analytical column (75 μm × 150 mm, 1.8 μm). The column oven temperature is set at 50 °C, and the temperature of the tray compartment in the auto-sampler is set at 6 °C. For each LC-HDMS^E run, approximately 1 μg of protein digest is loaded onto the trap-column through a 20 μL sample-loop using 98% mobile phase A