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

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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.

Keywords

Mass spectrometry; Absolute quantification; Proteins; Tumor cells

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

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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 Scientific (Fair Lawn, NJ). Dulbecco's phosphate buffered saline (PBS), Urea, ammonium bicarbonate, ammonium chloride, octyl-glucoside (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 40W 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% H₂O) 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 Inc., 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 μ L 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 μ L of 3% ACN in 0.1% formic acid. 5 μ L of 2 fmol Hi3 is added to the sample as the internal standard for absolute quantification. For Pool-2 to Pool-22, 20 μ L of digest is taken from each fraction, then mixed with 10 μ L of 1 fmol Hi3. A total 50 μ L 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 μ L 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 μ L of 3% ACN in 0.1% formic acid. 10 μ L of 2 fmol Hi3 is added to the sample as the internal standard for absolute quantification. For Pool-2 to Pool-22, 15 μ L of digest is taken from each fraction, mixed with 10 μ L of 2 fmol Hi3. A total of 10 μ L 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 μ m $\times$ 20 mm, 5 μ m) and a Waters HSS-T3 C18 nanoAcquity analytical column (75 μ m $\times$ 150 mm, 1.8 μ m). The column oven temperature is set at 50 $^{\circ}$ C, and the temperature of the tray compartment in the auto-sampler is set at 6 $^{\circ}$ C. For each LC-HDMS^E run, approximately 1 μ g of protein digest is loaded onto the trap-column through a 20 μ L sample-loop using 98% mobile phase A (0.1% formic acid in 2% ACN) at a flow rate of 8 μ L/min for 5 min. The desalted peptides are eluted from the analytical column at a flow rate of 500 nL/min with a gradient elution consisting of an increase from 3% to 25% mobile phase B (0.1% formic acid in 98% ACN) over 100 min, 25–85% mobile phase B for 7 min, a wash step held at 85% mobile phase B for 3 min, and a re-equilibration step at 3% mobile phase B for 10 min. The lock mass, 300 fmol/ μ L of [Glu¹] fibrinopeptide solution prepared with 0.1% formic acid in 30% ACN, is delivered from the auxiliary pump of the nanoAcquity UPLC at a flow rate of 0.2 μ L/min to the reference sprayer of the NanoLockSpray source.

2.7. LC-HDMS^E data acquisition

LC-HDMS^E Data is acquired in resolution mode with SYNAPT G2-S using Waters Masslynx (version 4.1, SCN 851). The analyzer (time of flight, TOF) with ion-reflectron has three modes: sensitive, resolution, and high resolution. The mass spectrometer is operated in resolution mode with a typical resolving power of at least 20,000 FWHM at m/z 785.8427 (doubly charged positive ions, Glu-fibrinopeptide B). All analyses are performed using positive mode ESI using a NanoLockSpray source. The lock mass channel is sampled every 60 s. The mass spectrometer is calibrated with a [Glu¹] fibrinopeptide solution (300 fmol/ μ L) delivered through the reference sprayer of the NanoLockSpray source. Accurate mass LC-HDMS^E data is acquired in an alternating, low energy (MS) and high energy (MS^E) mode of acquisition with mass scan range from m/z 50 to 1800, using a capillary voltage of 2.8 kV, a source temperature of 100 °C and a cone voltage of 30 V. The mass measured accuracy is <5 ppm when the instrument operated with the lock mass, and the accuracy is same between low energy and high energy MS scans. The spectral acquisition time in each mode is 1.0 s with a 0.1-s inter-scan delay. In low energy HDMS mode, data is collected at constant collision energy of 2 eV in both Trap cell and Transfer cell. In high energy HDMS^E mode, the collision energy is ramped up from 25 to 55 eV in the Transfer cell only. The RF applied to the quadrupole mass analyzer is adjusted such that ions from m/z 300 to 2000 are efficiently transmitted, ensuring that any ions observed in the LC-HDMS^E data less than m/z 300 arise from dissociations in the Transfer collision cell.

2.8. Data processing

The continuum LC-HDMS^E data is processed using ProteinLynx Global Server (PLGS, version 3.0.1, Waters, Milford, MA). The low-energy (HDMS) and high-energy (HDMS^E) data are automatically smoothed, background subtracted, centered, deisotoped and charge-state reduced to the corresponding mono-isotopic peaks. Each mono-isotopic peak is then lock-mass corrected to yield the accurate mass measurement. The lock mass for charge 2 is 785.8426 Da with a lock mass windows of 0.25 Da. Fragment ions and their corresponding precursor ions are aligned together based on the profile of mobility drift time as well as the chromatographic retention time. Aligned LC-HDMS^E precursor and product ions were considered for identification provided that the deconvoluted, protonated precursor ion mass were within ± 10 ppm. A low energy threshold of 250 counts, and a high energy threshold of 50 counts, and a total ion intensity threshold of 1250 counts are chosen for generating spectra for protein identification and quantification.

Protein identification is done by searching the processed spectra against the complete proteome set of *H. sapiens* from Uniprot (release 2013_10) to which amino acid sequence from the internal standard protein (Chaperone protein ClpB, P63284) is appended. The mass tolerance allowed for the low-energy precursor ions is 20 ppm, while the mass tolerance of elevated-energy fragment ions is set to 50 ppm. A fixed propionamide modification for Cysteine, and a variable oxidation modification for Methionine are specified. Peptide identification is performed using the trypsin digestion rule with one missed cleavage. The search thresholds used are: minimum fragment ion matches per peptide, 3; minimum fragment ion matches per protein, 7; minimum peptides per protein, 1; and false positive rate

for protein identification, 5. The inputs used for quantification measurement are: calibration protein, P63284; calibration protein concentration: 10 fmol.

2.9. Label-free quantification

The LC-HDMS^E data is used to identify and quantify the proteins in the mixture using a proven label-free quantification technique (“Hi3” method) developed by Silva and his co-workers [9]. It has been reported that the concentration of a protein in a complex mixture can be estimated using the added ESI-MS signal of the top three best responding peptide precursors [9–12]. The three most abundant peptides of all proteins detected in an LC–MS dataset are compared to the three most intense peptides of a spiked-in reference protein of known concentration. The quantification method calculates the molar amounts loaded onto the column for all identified proteins (including the spiked ones). Muntel et al. used this approach for a study of comprehensive absolute quantification of the cytosolic proteome of *Bacillus subtilis* [11]. Muntel also performed a comparative analysis with heavily labeled peptides (AQUA approach) and showed the use of only one standard digest is sufficient for global quantification. Doneanu and colleagues applied this “Hi3” method for quantification analysis of host-cell proteins in biotherapeutic proteins, and then subsequently developed target peptides and transitions for monitoring the concentration of selected HCPs by MRM analysis [13]. This “Hi3” approach has been applied for the analysis of different mutants of *Staphylococcus aureus* SCV [14] and the probing of the exoproteome of *Corynebacterium pseudotuberculosis* [15]. Recently, Weinhold and coworkers quantified the overexpressed novel antimicrobial peptides (AMPs) from the leaf apoplast of *Nicotiana attenuate* using the “Hi3” technique [16]. Norais et al. applied the “Hi3” methodology to qualitatively and quantitatively characterize the outer-membrane-vesicle (DOMV) proteins of the Bexsero vaccine for the study of Meningococcal disease [17]. Using nonparametric hypothesis testing and complementary log–log linear regression, they demonstrated that the “Hi3” quantification methodology is robust for a broad range of proteins. This technique can be used with ion trap based instruments with data dependent acquisition (DDA) mode [18–20]. A LTQ-FT-ICR mass spectrometer with DDA mode was used for absolute quantification of proteins from complex yeast extracts with T3PQ (Top 3 Protein Quantification) [19], an implementation of the “Hi3” method.

In the present study, we applied this “Hi3” label-free quantification technique based on calculation of 10 fmol loading of spiked internal standard (Hi3, Chaperone protein ClpB, P63284) to quantify proteins from tumor cells. The intensity response under ESI conditions of the three most intense peptides is a function of the molar amount infused in the mass spectrometer. Using the ‘Hi3’ peptide intensity of a spiked internal standard as reference, the absolute amount of every identified protein from one LCHDMS^E analysis can be calculated according to Eq. (1),

$$y = \frac{\sum_{i=1}^3 \text{peptide intensity [Protein of interest]}}{\sum_{i=1}^3 \text{peptide intensity [Protein spike]}} \times 10 \text{ fmol} \quad (1)$$

whereby y is the protein quantification value in fmol. The final protein quantification value from the analysis of 24 pooled fractions is calculated according Eq. (2),

$$y = \sum_{i=1}^{24} x_i \quad (2)$$

whereby x_i is protein quantification value (fmol) measured from LC-HDMS^E analysis of one pooled fraction, y is the total protein quantification value (fmol) after combining the measurements from the analysis of 24 pooled fractions.

3. Profiling the repertoire of cell surface proteins of a leukemia cell population

3.1. RP-HPLC separation of proteins from the cell-surface and TCE

Cell-surface proteins from tumor cells as exemplified by leukemia cells were enriched through *in vivo* labeling with Sulfo-NHSSS-BIOTIN followed by affinity chromatographic capture with Avidin and quantification by label-free mass spectrometry. An established schematic platform is shown in Fig. 1. Given that some proteins released due to cell turnover may become biotinylated, proteins found to be biotinylated and enriched in the cell surface compartment relative to TCE are considered to be contributed by the cell surface compartment. Moreover for cells harvested directly from biological fluids, proteins normally found in biological fluids may also become biotinylated if not thoroughly washed and the contribution of this biological fluid compartment need to be taken into consideration in ascertaining the true source of the biotinylated proteins.

In an illustrative experiment involving leukemia cells, a total of 76 µg of proteins captured with biotinylation and 380 µg of proteins from TCE were separated by RP-HPLC, respectively. Representative chromatograms are shown in Fig. 2. The overall separation profiles are similar except for the difference in peak intensity which reflects the relative protein amount in the sample. A total of 84 fractions were collected from the cell-surface protein separation and from the TCE protein separation. The volume of each collected fraction was 700 µL with a 20 s of collection interval at a flow rate of 2.1 mL/min. As shown in the lower panel of Fig. 2, in this illustrative experiment, 24 pooled fractions were subjected to LC-HDMS^E analysis after in-solution trypsin digestion.

3.2. LC-HDMS^E and data analysis

10 fmol of Hi3 spiked into ~1 µg of protein digest from each of 24 pooled fractions was analyzed by LC-HDMS^E. A 120 min of gradient elution was used for separating the protein digests through the nanoAcquity UPLC system coupled in-line with SYNAPT G2-S mass spectrometer employing the ion-mobility function and alternating acquisition modes of low energy (HDMS) and high energy (HDMS^E). The representative total ion chromatograms with gradient elution curve are shown in Fig. 3. The eluted peptides were acquired by SYNAPT G2-S between 5 and 120 min with a typical of 15 s of peak width in ion chromatogram. In this representative study, a total of 3335 HDMS spectra of the co-eluted peptides and 3335 HDMS^E spectra of the fragment ions from the co-eluted peptides were collected together with a total of 115 HDMS spectra of [Glu¹] fibrinopeptide for accurate mass measurement. The representative HDMS^E spectra are shown in Fig. 4. For each of the

LC-HDMS^E analysis, protein quantification measurement was performed according to “Hi3” method [9] and Eq. (1). The final protein quantification value was obtained according to Eq. (2). After merging the results of protein quantification (5% FDR) from the LC-HDMS^E analysis of the 24 pooled fractions from the cell-surface sample or TCE sample, a total of 3865 protein forms were quantified from the TCE sample, and an even larger total of 5340 of protein forms were quantified from the cell-surface (Supplementary Table 1). Proteins that were identified in the TCE fraction but with lack of enrichment on the cell surface are considered as contaminants resulting from lysis of some cells resulting in biotinylation of their released proteins. Additionally non-specific binding of circulating proteins may account for the large number of biotinylated proteins. These may be distinguished from true cell surface proteins by subtraction of known plasma proteins. Scatter plots for the measured quantification value according to Eq. (2) versus the number of quantified proteins in the cell-surface and TCE are displayed in Fig. 5. The dynamic range of protein quantification was four orders of magnitude, spanning 0.012– 444.51 fmol for the cell surface compartment. A comparison analysis for two major types of plasma membrane proteins between the cell-surface sample and the total cell lysates was performed to evaluate the effectiveness of the biotinylation for targeting specifically the cell surface. The two types of plasma membrane proteins are: CD antigens and HLA class I/II histocompatibility antigens (listed in the Supplemental Table 2). A total of 34 CD antigens were quantified in the cell-surface fraction, compared to 12 CD antigens that were quantified in the TCE fraction. Similarly, a total of 16 HLA class I/II histocompatibility antigens were quantified in the cell surface fraction, compared to 11 HLA class I/II histocompatibility antigens that were quantified in the TCE fraction. Moreover the identified plasma membrane proteins had a higher expression levels in the cell surface compared to the TCE fractions as illustrated (Fig. 6). On average, there an 11.88-fold difference in CD antigens quantifications was observed between cell surface and TCE fractions, and 23.19-fold change in HLA class I/II histocompatibility antigens was observed between cell surface and TCE fractions.

4. Discussion

Here we presented methodology for enrichment of cell surface proteins and their in-depth identification and quantification using ion mobility mass spectrometry. The current approach which included, HPLC separation of intact biotinylated proteins and label-free nanoUPLC-HDMS^E to perform absolute quantification of proteins has allowed identification of several thousand proteins from the cell surface compartment and is vastly improved compared to our prior approach in part due to the use of ion mobility mass spectrometry [4,21]. The fractionation of intact proteins prior to their digestion allows assessment and identification of protein isoforms that may result from alternative splicing or other types of modifications which may not be readily captured by shot-gun based LC-MS/MS analysis of digested proteins from the initial sample. Another aspect of our study is that we combined the “Hi3” label-free method with the advanced HDMS^E data independent acquisition to achieve the absolute quantification of proteins. Currently, label-free quantitative proteomics is used to compare either the MS peak intensity for sequenced peptides or the number of acquired spectra for the identified peptide. The described approach allows absolute quantification information by spiking internal standards. A key advantage of employing ion mobility with

data independent acquisition is a substantial improvement in protein identification and quality since ion mobility increases analytical peak capacity and reduces chimeric interference due to the additional dimension of separation without increasing analysis time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymeth.2015.03.007>.

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Fig. 1. Quantitative MS based protein biomarker discovery platform employing the integration of *in vivo* cell-surface protein labeling, intact protein RP-HPLC fractionation and LC-HDMS^E for quantifying proteins from patient with AML.

Fig. 2.

Reversed-phase HPLC fractionation of proteins prepared from leukemia cells. After trypsin digestion, 24 pooled fractions were analyzed by LC-HDMS^E. 380 μ g of protein from TCE and 76 μ g of protein from cell-surface were separated by a high performance liquid chromatography system (Shimadzu) controlled by EZ Start workstation (version 7.4 SP3) and equipped with UV detection at 280 nm. Proteins were separated on a RPGS reversed-phase column (4.6 mm I.D. $\times$ 150 mm, 15 μ m, 1000 Å, Column Technology Inc.). The separation was performed via gradient elution of two mobile phases: 0.1% TFA in 95% H₂O and 0.1% TFA in 95% ACN at a constant flow rate of 2.1 mL/min. Fractions were collected at 20 s intervals. 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.

Fig. 3. TIC Chromatograms of RPLC-HDMS^E. (A) RPLC gradient elution; (B) low energy TIC chromatogram; (C) elevated energy TIC chromatogram. Approximately 1 μ g of protein digest from each of 24 pooled fractions was loaded onto the trap-column (C18, 180 μ m $\times$ 20 mm, 5 μ m) through a 20 μ L sample-loop using 98% mobile phase A (0.1% formic acid in 2% ACN) at a flow rate of 8 μ L/min for 5 min. The desalted peptides eluted from the analytical column (C18, 75 μ m $\times$ 150 mm, 1.8 μ m) at a flow rate of 500 nL/min with a 120-min of gradient elution: from 3% to 25% mobile phase B (0.1% formic acid in 98% ACN) over 100 min, 25–85% mobile phase B for 7 min, a wash step to hold at 85% mobile phase B for 3 min, and a re-equilibration step at 3% mobile phase B for 10 min.

Fig. 4.

Mass spectra of HDMS^E. (A): Low energy mass spectrum (peptide ions, HDMS^E); (B): high energy mass spectra (fragment ions, HDMS^E). LC-HDMS^E data was acquired in resolution mode with SYNAPT G2-S. The capillary voltage was set to 2.80 kV, sampling cone voltage to 30 V, source offset to 30 V, and source temperature to 100 °C. Mobility utilized high-purity N₂ as the drift gas in the IMS TriWave cell. IMS wave velocity was 600 m/s, helium cell DC was 50 V, Trap DC bias was 45 V, IMS TriWave DC bias was 3 V, and IMS wave delay was 1000 μ s. The mass spectrometer was operated in V-mode with a resolving power of at least 20,000. Argon was used as the collision gas. In low energy HDMS mode, data was collected at collision energy of 2 eV in both Trap cell and Transfer cell. In high energy HDMS^E mode, the collision energy was ramped from 25 to 55 eV in the Transfer cell.

Fig. 5. Absolute quantification of proteins from two different cellular compartments (cell-surface and total cell extract, TCE) of a leukemia cell population. (A) Patient #1_Cell surface; (B) Patient #2_Cell surface; (C) Patient #1_TCE; (D) Patient #2_TCE. Axis X is the number of quantified proteins, axis Y is the quantified protein amount (fmol on column) combined from the LC-HDMS^E analysis of 24 pooled fractions.

Fig. 6. Comparison of two types of plasma membrane proteins quantified from cell-surface sample and total cell extract (TCE) sample. (A): CD antigens; (B): HLA class I/II histocompatibility antigens.