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Feasibility of label-free phosphoproteomics and application to base-line signaling of colorectal cancer cell lines☆

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ABSTRACT

Robust phosphopeptide enrichment methods with minimal fractionation are required to profile signaling network analysis in cancer cell lines and tissues. We assessed performance of single-shot LC-MS/MS label-free phosphoproteomics using TiOx-based phosphopeptide enrichment and report phosphopeptide identification reproducibility (75.8%), depth of identification (6014–6150 phosphopeptides) and reproducibility of label-free quantification (CV 17.8%). Subsequently, we have profiled the baseline global phosphorylation of 8 colorectal cancer (CRC) cell lines representing different CRC prognostic subtypes. Global single-shot phosphoproteomics can distinguish CRC subtypes previously identified by transcriptomics and identifies signaling proteins and processes associated with the CCS3 poor prognosis subtype. Data are available via ProteomeXchange with identifiers PXD001546 and PXD001550.

Biological significance
Label-free single-shot phosphoproteomics is a mature workflow that can be used for global quantitative profiling of biological cell lines and tissues to map signaling networks in comparative analyses. Here we show the feasibility of label-free profiling of CRC cell lines at sample input levels compatible with clinical samples such as tumor biopsies.
This article is part of a Special Issue entitled: HUPO 2014.

1. Introduction

Robust phosphopeptide enrichment methods are required to profile signaling network analysis in cancer cell lines and tissues with minimal fractionation. With recent instrument developments thousands of unique phosphopeptides can be detected by single-shot LC-MS/MS. However, successful phosphoproteomics experiments still rely on efficient phosphopeptide enrichment from a tryptic digest prior to LC-MS/MS analysis. For global phosphopeptide enrichment two popular methods have been described: immobilized metal affinity chromatography (IMAC) using Fe^{3+} as metal ion [1,2] and metal oxide affinity

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chromatography (MOAC) using titanium dioxide (TiO_2 or TiOx) beads [3–5]. For specific enrichment of phosphotyrosine (pTyr)-containing peptides, antibody-based methods have been reported [6,7]. Our preferred method for global phosphopeptide enrichment is TiOx, since it does not show bias towards phosphorylated amino acid, sequence context, peptide length or hydrophobicity as reported recently [8], and is commercially readily available. To minimize interference by acidic peptides, initially dihydroxy benzoic acid was included during peptide binding [9]. Later, hydroxy acids such as lactic acid and glutamic acid have been shown to minimize interference by acidic peptides as well [10,11]. This latter method, described by Ishihama and coworkers, has been termed HAMMOC (hydroxyl acid modified metal affinity chromatography). Here we describe a performance assessment of HAMMOC combined with single shot label-free quantitation at 500 μg peptide input level showing high ID reproducibility, depth of phosphopeptide identification and good label-free quantitation. We apply the method to profile the baseline phosphorylation landscape of a panel of 8 colorectal cancer (CRC) cell lines. These CRC cell lines represent 3 recently reported CRC prognostic subtypes (CCS1, CCS2 and CCS3) identified by large-scale transcriptome analysis [12]. We report an analysis of the phosphoprotein network and processes enriched in the cell lines representing the poor prognosis CCS3 subtype.

2. Materials and methods

Colorectal cancer (CRC) cell lines HT29 and CaCo2 were obtained from the American Type Culture Collection. CRC cell lines HCT116, RKO, SW480, SW1398, Colo205 and DLD1 were kindly provided by Prof. dr. G.F. Peters, Dept of Medical Oncology, VU University Medical Center. Cells were cultured in biological triplicates in DMEM medium (Lonza Biowhittaker, Verviers, Belgium) containing 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml sodium penicillin and 100 μg/ml streptomycin.

2.1. Cell lysis and digestion

Cells were lysed in lysis buffer containing 9 M urea, 20 mM HEPES pH 8.0, 1 mM Na_3VO_4 (orthovanadate), 2.5 mM $Na_4P_2O_7$ (pyrophosphate), and 1 mM $Na_2C_3H_7PO_6$ (ß-glycerophosphate) by scraping and subsequently by sonication. After lysis the protein concentration was determined using the BCA method (ThermoPierce, Rockford, IL). Cell lysate was reduced in 4 mM dithiotreitol (DTT) for 20 min at 60 °C, cooled to room temperature and alkylated in 10 mM iodoacetamide for 15 min in the dark. Subsequently, the cell lysate was diluted to 2 M urea using 20 mM HEPES buffer pH 8.0 and digested overnight with trypsin (10 μg/mg protein) at 37 °C. Digestion was stopped in 0.1% trifluoroacetic acid (TFA) and samples were stored at −80 °C.

2.2. Phosphopeptide enrichment

500 μg tryptic digests were acidified by adding TFA to an end concentration of 0.1% and were desalted on a 100 mg Seppak C18 cartridge (Waters, Milford, MA) using a vacuum system. After cartridge activation in acetonitrile (ACN) (1 ml, −0.1 bar) (Biosolve, Valkenswaard, The Netherlands) and equilibration in 0.1% TFA (2 ml, −0.2 bar), peptides were bound, washed in 0.1% TFA (2 ml) and eluted in 80% ACN, 0.1% TFA (1 ml, −0.2 bar). Desalted peptides were enriched for phosphopeptides using TiO_2 beads based using aliphatic hydroxy-acid modified metal oxide chromatography [11]. Briefly, 500 μg peptides (1 μg/μl) were mixed with 500 μl washing buffer (80% ACN, 0.1% TFA containing 300 mg/ml lactic acid) and applied to 2.5 mg TiO_2 beads (GL sciences, 10 μm) packed in a 200 μl STAGE tip containing a 16G empore C8 membrane plug (3 M, St Paul, MN). The STAGE tip was washed with 200 μl washing buffer followed by 200 μl 80% ACN, 0.1% TFA. Phosphopeptides were eluted in two steps in 50 μl 0.5% and 5% piperidine (Fisher scientific) and were quenched in 100 μl 20% H_3PO_4. All steps were performed by centrifugation (1500 ×*g*, 4 min). Phosphopeptides were desalted using a 200 μl STAGE tip containing a 16G empore SDB-XC membrane plug (3 M, St Paul, MN) using the same solvents as used for the Seppak cartridge (20 μl, 1000 ×*g*, 1 min). Desalted phosphopeptides were dried in a vacuum centrifuge and redissolved in 30 μl 4% ACN, 0.5% TFA; 15 μl was injected on column.

2.3. LC-MS/MS

Peptides were separated by an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 20 cm × 75 μm ID fused silica column custom packed with 3 μm 120 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 μl/min on a 10 mm × 100 μm ID trap column packed with 5 μm 120 Å ReproSil Pur C18 aqua at 2% buffer B (buffer A: 0.5% acetic acid (Fischer Scientific), buffer B: 80% ACN, 0.5% acetic acid) and separated at 300 nl/min in a 10–40% buffer B gradient in 90 min (120 min inject-to-inject). Eluting peptides were ionized at a potential of +2 kV into a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured at resolution 70.000 (at *m/z* 200) in the orbitrap using an AGC target value of 3×10^6 charges. The top 10 peptide signals (charge-states 2^+ and higher) were submitted to MS/MS in the HCD (higher-energy collision) cell (4 amu isolation width, 25% normalized collision energy). MS/MS spectra were acquired at resolution 17.500 (at *m/z* 200) in the orbitrap using an AGC target value of 2×10^5 charges and an underfill ratio of 0.1%. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

2.4. Protein identification

MS/MS spectra were searched against the Uniprot human reference proteome FASTA file (release February 2013, 70136 entries) using MaxQuant 1.4.1.2. [13]. Enzyme specificity was set to trypsin and up to two missed cleavages were allowed. Cysteine carboxamidomethylation (Cys, +57.021464 Da) was treated as fixed modification and serine, threonine and tyrosine phosphorylation (+79.966330 Da), methionine oxidation (Met, +15.994915 Da) and N-terminal acetylation (N-terminal, +42.010565 Da) as variable modifications. Peptide precursor ions were searched with a maximum mass deviation of 4.5 ppm and fragment ions with a maximum mass deviation of 20 ppm. Peptide, protein and site identifications were filtered at an FDR of 1% using the decoy database