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Assessment of global proteome in LNCaP cells by 2D-RP/RP LC–MS/MS following sulforaphane exposure

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ABSTRACT

The phytochemical sulforaphane can induce cell cycle arrest and apoptosis in metastatic prostate cancer cells, though the mechanism of action is not fully known. We conducted a global proteome analysis in LNCaP metastatic prostate cancer cells to characterize how global protein signature responds to sulforaphane. We conducted parallel analyses to evaluate semi-quantitative 1-dimensional versus 2-dimensional liquid chromatography tandem mass spectrometry (LC–MS/MS) and their utility in characterizing whole cell lysate. We show that 2-dimensional LC–MS/MS can be a useful tool for characterizing global protein profiles and identify TRIAP1 as a novel regulator of cell proliferation in LNCaP metastatic prostate cancer cells.

1. Introduction

Sulforaphane (1-isothiocyanato-4-(methylsulfinyl) butane) is a plant derived isothiocyanate that can stimulate cell death in metastatic prostate cancer cells while sparing normal prostate epithelial cells under similar conditions [1]. Although this cytotoxic effect has been known for some time, the underlying proteins and signaling networks that control the response to sulforaphane are not fully characterized. Characterizing the response to sulforaphane in metastatic prostate cancer cells, and defining the biological significance of observed responses, may therefore identify proteins and/or pathways that confer cancer-selective cytotoxicity.

Sulforaphane stimulates a global change in gene transcription in prostate cells that is both dose- and time-dependent [2,3]. Analysis of such global expression data has been used to infer the outcome of sulforaphane treatment; however, there is reason to believe that this type of analysis fails to capture biologically relevant responses to sulforaphane that govern proliferation and cell fate decisions. Gene transcription is known to correlate poorly with protein level in prostate cancer cells [4], which can be a consequence of many factors (e.g. protein stability, degradation rate, mutation, etc), suggesting that drawing conclusions regarding phenotypic outcome based on gene transcription alone may be misleading. Analysis of transcript levels also cannot provide a full picture of immediate responses that influence protein synthesis, stability or activity that is regulated through control of translation or post-translational modification [5]. This is particularly relevant with respect to sulforaphane since previous research has noted a decrease in the mammalian target of rapamycin (mTOR) activity, a master regulator of protein synthesis [6,7], in response to sulforaphane that decreases global protein synthesis in prostate cancer cells [8]. Furthermore, although some changes in gene transcription may lead to rapid protein production, others will take an extended time period to manifest at the protein level. These observations suggest that transcript analyses cannot provide a complete picture of the response to sulforaphane in prostate cancer cells and that alternative methods that directly assess protein levels will provide a clearer and more accurate characterization of the factors that control cell fate following

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sulforaphane exposure. We therefore applied proteomics to characterize the protein profile of LNCaP metastatic prostate cancer cells and how it responds to sulforaphane.

Proteomic approaches have previously been applied to identify sulforaphane-responsive proteins in several cell types [9–12], though none have utilized an analysis of whole cell lysate to assess the global proteome and how it responds to sulforaphane in prostate cancer cells. We therefore subjected control- and sulforaphane-treated LNCaP whole cell lysate to liquid-chromatography tandem mass spectrometry (LC–MS/MS) for unbiased assessment of potential alterations in global protein profile. We evaluated the standard protocol of peptide trapping and cleaning upstream of 1-dimensional (1D) separation in parallel with 2-dimensional (2D) reversed-phased (RP)/RP separation of lysate prior to MS/MS analysis. 2D-RP/RP-LC–MS/MS has been shown to increase proteome coverage relative to standard methods [13–18] and could substantially increase our ability to detect changes in the proteome in response to sulforaphane or identify proteins that have not yet been implicated in prostate cancer cell biology. Two-dimensional separation was found to be superior to standard 1D methods in terms of proteome coverage and protein coverage. Although we observed no global remodeling of the proteome in response to sulforaphane under our experimental conditions, increased proteome coverage by 2D separation methods did identify biologically relevant proteins that influenced cancer cell proliferation, suggesting 2D-RP/RP-LC–MS/MS may be a useful tool for the discovery of novel protein targets for therapeutic evaluation.

2. Materials and methods

2.1. Chemicals and reagents

Sulforaphane was purchased from LKT Laboratories (St. Paul, MN, USA) and resuspended in dimethyl sulfoxide (DMSO) (EMD Millipore, Darmstadt, Germany). Primary antibodies for TRIAP1 (Santa Cruz Biotechnology, Dallas, TX, USA), fibrillarin (Santa Cruz Biotechnology), p-S780-RB (Cell Signaling Technology, Danvers, MA, USA), cyclin B1 (Cell Signaling), cyclin D2 (Santa Cruz Biotechnology), p21 (Santa Cruz Biotechnology), NQO1 (Santa Cruz Biotechnology), cleaved poly-ADP ribose polymerase (cPARP) (Cell Signaling Technology), and GAPDH (Santa Cruz Biotechnology) were used in accordance with the manufacturer's protocol. HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for Western Blotting detection were used in accordance with the manufacturer's protocol. LC/MS grade solvents were purchased form EMD Millipore (Billerica, MA, USA).

2.2. Cells and culture conditions

LNCaP metastatic prostate cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 media with L-glutamine supplemented with fetal bovine serum (FBS, 50 ml FBS/500 ml media) at 37 °C 5% CO_2. Cell lines were validated by Idexx Radil (Columbia, MO, USA) on December 24, 2012. Subconfluent cells were treated under the indicated conditions prior to harvest. Sulforaphane was used at 15 μM final concentration. DMSO was used for control treatments.

Sample preparation for LC/MS analysis:

LNCaP cells were treated for 24 h with DMSO (control) or sulforaphane prior to harvest. Treatments were performed as 2 independent experiments. Cells were rinsed in 50 mM Tris–HCl pH 7.8 and harvested in 0.5 M urea, 50 mM Tris–HCl pH 7.8, 5 mM DTT. Lysate was heated at 95 °C for 20 min then cleared of insoluble material by centrifugation (10 min, 13,000 rpm, 4 °C). One milligram protein per sample was digested overnight with Trypsin-Gold in accordance with the manufacturer's instructions (Promega). Digestion was terminated with trifluoroacetic acid (TFA). Protein digests were analyzed at the Mass Spectrometry Core Facility at Oregon State University.

2.3. LC/MS analysis

Each sample was analyzed twice (two injections, technical replicates) for downstream determination of treatment response. Methods for 1D LC–MS/MS have been described previously [19]. Waters 2D nano acquity UPLC system (Waters Corp., Milford, MA, USA) in "2D with dilution" configuration was used for the 2-dimentional analysis. System performance tests were conducted according to the manufacturer protocol prior to the experiments. Peptide sample solution (10 μl) was initially loaded onto a XBridge300 (C_{18}. 5 μm, 1.0 × 50 mm) reverse phase column using 20 mM ammonium formate (pH 10) with a flow rate of 2 μl/min for 20 min. Peptide samples were then eluted from the high pH trapping column and loaded onto the analytical trapping column (Symmetry C_{18}, 130 Å, 5 μm, 300 μm × 50 mm) by using varying concentration of acetonitrile (ACN) fractions (11.1, 14.5, 17.4, 20.8, 45 and 65%). Eluted peptides were diluted with 0.1% formic acid at a flow rate of 20 μl/min for 5 min. After this period, the column valve was switched to allow the elution of peptides from the trapping column onto the analytical column. Separation of peptides was achieved by reverse-phase chromatography using a C_{18} column (Agilent Zorbax 300SB-C_{18}, 250 × 0.3 mm, 5 μm) at flow rate of 5 μl/min. Water and ACN with 0.1% formic acid were used as solvent A and solvent B, respectively. The linear gradient employed was 5–35% B in 45 min. Mass spectrometric analysis was carried out in the same manner as the 1D analysis.

Raw data files were analyzed using Mascot and X!Tandem database searching algorithms according to the protocol listed previously [19]. Data comparison and quantification was carried out using Scaffold_3.3.1 (Proteome Software, Portland, OR, USA). Peptide Prophet and Protein Prophet algorithms embedded in Scaffold were used to calculate peptide and protein identification probabilities, respectively [20,21]. Scaffold uses spectral counting for the data normalization. Spectra are summed across fractions for each protein to acquire total spectra count (unweighted spectral count). Sum of the "unweighted spectral count" are then scaled to normalize across samples. The scaling factor for each sample is then applied to each protein group and adjusts its "Unweighted Spectrum Counts" to a normalized "Quantitative value".

For variation analysis, MASCOT and X!Tandem peptide identification and protein assignment were analyzed as groups based on technical replicates or treatment samples from the 2D dataset (Supplemental data file). Spectra were summed across fractions for each protein to acquire total spectra count. Sample #4 was omitted from the technical variation analysis due to failure of Replicate #2. The mean spectra count between technical replicates or treatment samples was then calculated for each protein. Coefficient of variation (CV) for each protein was calculated based on standard deviation and the calculated mean spectra count. X!Tandem thresholds for this analysis: 5% false discovery rate (FDR) for protein identification, 5% FDR for peptide identification, 2 peptide minimum for positive protein identification. Data was plotted and analyzed using GraphPad Prism Software (La Jolla, CA, USA).

2.4. Protein preparation and western blot analysis

Protein lysates were prepared in RIPA protein lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholic acid (NaDOC), 0.1% SDS, 20 mM TRIS pH 8.0) supplemented with protease inhibitor cocktail (Thermo, Waltham, MA, USA). Lysates