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Evaluating kinase ATP uptake and tyrosine phosphorylation using multiplexed quantification of chemically labeled and post-translationally modified peptides

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ABSTRACT

Cancer biologists and other healthcare researchers face an increasing challenge in addressing the molecular complexity of disease. Biomarker measurement tools and techniques now contribute to both basic science and translational research. In particular, liquid chromatography–multiple reaction monitoring mass spectrometry (LC–MRM) for multiplexed measurements of protein biomarkers has emerged as a versatile tool for systems biology. Assays can be developed for specific peptides that report on protein expression, mutation, or post-translational modification; discovery proteomics data rapidly translated into multiplexed quantitative approaches. Complementary advances in affinity purification enrich classes of enzymes or peptides representing post-translationally modified or chemically labeled substrates. Here, we illustrate the process for the relative quantification of hundreds of peptides in a single LC–MRM experiment. Desthiobiotinylated peptides produced by activity-based protein profiling (ABPP) using ATP probes and tyrosine-phosphorylated peptides are used as examples. These targeted quantification panels can be applied to further understand the biology of human disease.

0. Introduction

Discovery proteomics has myriad uses in biology; one of the most valuable contributions is made through the ability to identify and localize post-translational modifications. However, systems biology now requires quantitative data to elucidate signaling mechanisms and functionally investigate complex biological processes [1]. In cancer, many signaling pathways are controlled by post-translational modifications, like phosphorylation, which mediate protein–protein interactions, enzyme activity levels, and subcellular localization. These molecular cues change cellular phenotype in response to internal and external stimuli. Dynamic kinase activity and changes in protein phosphorylation sites and their levels must be quantified in order to characterize signaling networks and their downstream biological effects. Novel tools are still required to effectively study these changes in model systems and in patients.

One current paradigm for developing the required assays involves translation of discovery proteomics data into quantitative measurements using liquid chromatography–multiple reaction monitoring mass spectrometry (LC–MRM) [2–7]. LC–MRM can quantify molecularly diverse biomarkers in complex biological and clinical samples with high degree of portability between labs [8,9]. Protein quantification using tryptic peptides as surrogates has been used to evaluate the expression, mutation, and phosphorylation of individual proteins [10–17]. The capability for multiplexing has also been demonstrated, making LC–MRM an ideal tool for quantitative systems biology [18,19]. Indeed, the

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technique has already been applied to monitoring the outcomes for individual signaling pathways and multiple components of complex biological processes [20–24]. Large panels of post-translationally modified peptides can also be developed from liquid chromatography tandem mass spectrometry (LC–MS/MS) data, using these previously reported biomarker development strategies. The critical requirement is a viable affinity enrichment technique for the modified peptides.

Two examples illustrate this point: chemically labeled peptides and post-translationally modified. Activity-based protein profiling (ABPP) uses chemical probes as biotinylating reagents to label and enrich classes of target proteins; the use of ATP mimics has provided the ability to enrich kinases for mass spectrometry analysis via desthiobiotinylation of lysines near the ATP-binding pocket and active site (and potentially lysines on substrates as well) [25–28]. Because a broad spectrum ATP-utilizing enzymes can be captured, LC–MRM is highly useful for selectively detecting the kinases from cells and tissues, where the background of other proteins can be overwhelming compared to the lower abundance kinases. These experiments provide insight into the levels of kinase ATP uptake and may be used to infer either the expression or the activity of the kinases in a biological sample (depending on whether the ATP binding pocket is always accessible or tightly regulated). Furthermore, kinase inhibitors can block probe binding; consequently, the reduction in labeled peptide ion signal can be used to determine potential targets and effected downstream kinases. ABPP-LC–MRM approaches with isotope-coded probes and extensive development of stable isotope-labeled standard (SIS) peptides have recently been reported [29,30]. As an example of an endogenous post-translational modification, phosphotyrosine-containing (pY) peptides can be enriched by immunoprecipitation, providing the ability to examine signaling networks and the consequences of kinase inhibitor treatment [21,31,32]. The complement of these two techniques provides additional insight and higher confidence in altered signaling pathways by combining kinase activity and substrate phosphorylation into pathway maps generated from the differentially modified peptides and their proteins of origin.

Using these molecularly-specific enrichment techniques and recently developed strategies for LC–MRM assay development, ABPP-LC–MRM and pY-LC–MRM panels containing hundreds of peptides have been prepared. Each of the two experiments requires only a single LC–MRM analysis. Due to the cost of synthesizing and characterizing standards, this method relies on a small pool of commercially available unmodified standard peptides, similar to the labeled reference peptide strategy [33]. Because of the large number of peptide targets and the limited sampling ability of the triple quadrupole mass spectrometer, scheduled methods are required. Data from multiple discovery proteomics representing different tumor types are integrated into a single LC–MRM for each type of post-translationally modified peptide and scheduled using *in silico* retention time calculations (iRT) [34].

In the methods described here, ABPP-LC–MRM and pY-LC–MRM experiments are developed and used to interrogate the kinome of lung cancer cell lines and tissues. The steps in the workflow for peptide selection and LC–MRM analysis are described and applied to the cell lines and frozen tumor and normal specimens from patients. Ultimately, these LC–MRM platforms will be applied for multiplexed analyses of multiple signaling pathways in fresh or frozen patient tissues to elucidate dominant cancer signaling pathways with the goal of directing personalized therapy strategies. Finally, an example of the promise of parallel reaction monitoring mass spectrometry, PRM [35,36], in minimal amounts of total cellular protein is shown to illustrate the ability to translate selected targeted measurements into biopsy specimens.

1. *In silico* data processing and LC–MRM experiment development

This part of the method requires three steps: collection of tandem mass spectra into spectral libraries (Section 1.1), selection of peptides and transitions from the existing data (Section 1.2), and mapping discovery data to reversed phase liquid chromatography retention times to enable scheduling for the LC–MRM data acquisition (Section 1.3).

1.1. Spectral library construction

LC–MRM is performed on triple quadrupole mass spectrometers and relies on the instrument's ability to select the intact *m*/*z* ratio in the first quadrupole, fragment the intact peptide using collisions with background gas in the second stage of the instrument, and then sequentially mass select a series of structural fragments (typically *y* ions for tryptic peptides) using the third quadrupole prior to ion detection. Combined with reversed phase liquid chromatography, this experiment provides three degrees of separation to isolate the signal of interest. The critical step in assay development is the selection of appropriate peptides and transitions (pairs of intact *m*/*z* and fragment *m*/*z* with optimized collision energy values), which uniquely report on the expression, mutation status, chemical labeling, or modification of the protein. General guidance and *in silico* prediction rules include restrictions on peptide length (7–25 a.a.) as well as avoidance of amino acids that can be artifactually modified (*e.g.* Cys or Met) and certain motifs (*e.g.* consensus glycosylation sequences or peptides with adjacent/neighboring tryptic cleavage sites). However, empirical data from discovery proteomics are often the best resource to pick peptides and fragment ions. In cases with synthetic stable isotope-labeled standard (SIS) peptides, the data generation and transition selection can be performed on the QqQ-MS used for LC–MRM by infusion of the peptide standard. However, in cases with hundreds of peptides as described here, costs are prohibitive and standards are not developed for each target molecule. Discovery proteomics data from liquid chromatography–tandem mass spectrometry peptide sequencing experiments can also be relied on, because studies have shown high correlations between fragmentation patterns observed in LC–MS/MS and LC–MRM analyses [4,37]. To rapidly translate discovery proteomics into targeted measurements, a spectral library can be either downloaded or constructed. Spectral libraries are available for unmodified peptides from PeptideAtlas (http://www.peptideatlas.org/speclib/), the National Institute of Standards and Technology (NIST, http://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:start) and the Global Proteome Machine (GPM, ftp://ftp.thegpm.org/projects/xhunter/libs/) *inter al.* However for these studies of chemically labeled and post-translationally modified peptides, custom libraries for ABPP and pY peptides (which are available in the Supplemental Materials) were built in-house using previously acquired discovery data. All raw data files from discovery LC–MS/MS on LTQ-Orbitrap or Q-Exactive Plus mass spectrometers were searched against human entries in the UniProt database (downloaded 05/01/2013) using Sequest HT inside Proteome Discoverer 1.4 (Thermo) with Percolator analysis. In our case, the use of vendor software is straightforward, because conversion of data and search result file formats is not necessary. For Thermo instrument platforms, Proteome Discoverer (Thermo) is also capable of building spectral libraries using the Crystal library module. An alternative approach is to use the open source, freely-available Trans-Proteomic Pipeline (TPP, http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP) [38]. In TPP, the raw data files are converted into mzXML format after database searching, the search results are then converted to pepXML format. Peptide identifications can