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Technical note

Evaluation of different phospho-tyrosine antibodies for label-free phosphoproteomics☆

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ABSTRACT

Background: Mass spectrometry based phosphoproteomics emerged as advantageous approach for the analysis of tyrosine phosphorylation on proteins and tyrosine kinase signaling. Immunoaffinity purification is required for comprehensive analysis. Here we compared the performance of two antibodies for label-free phosphotyrosine-based phosphoproteomics.

Methods: Phosphopeptide immunoprecipitation of six technical replicates corresponding to 10 mg protein from HCT116 cells was performed using agarose bead-coupled phosphotyrosine antibodies P-Tyr-1000 (N = 3) and 4G10 (N = 3). NanoLC–MS/MS was performed using a Q Exactive mass spectrometer. For relative quantitation of protein phosphorylation, spectral counts of phosphoproteins and ion intensities of phosphopeptides were determined using MaxQuant.

Results: From the 3 samples incubated with P-Tyr-1000 a total of 689 phosphopeptides were identified with 60% ID reproducibility. The phosphopeptide capture using 4G10 resulted in a total of 421 at 46% ID reproducibility. The P-Tyr-1000 was applied to EGFR mutated U87 cells. Erlotinib reduced EGFR phosphorylation with 59% at y978, y1125, y1138, y1172, and y1197. EGFR inhibition was accompanied by enhanced phosphorylation of FYN, MET, PTK2, DYRK1A, MAPK1 and EPHA2.

Conclusion: The P-Tyr-1000 phosphotyrosine antibody performs superiorly when compared to 4G10 antibody for label-free phosphotyrosine-based phosphoproteomics. This workflow allows evaluation of drug target phosphorylation and may give insights in the pharmaco-dynamic effects of tyrosine kinase inhibitors.

Clinical significance:
In the past decade multiple tyrosine kinase inhibitors (TKIs) have been implemented in standard treatment regimens for patients with cancer. Unfortunately the majority of

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patients develops resistance to these drugs. Reliable tools for analysis of pharmacodynamic effects and drug resistance mechanisms are therefore warranted. Phosphoproteomic analyses have meanwhile emerged as a sophisticated approach for the determination of protein phosphorylation. These analyses rely on antibodies for enrichment of tyrosine-phosphorylated peptides. Here we compared two commercially available phosphotyrosine antibodies and show that P-Tyr-1000 yields 64% more phosphopeptides than 4G10 antibody, while including almost all 4G10 captured phosphopeptides. The workflow can be reproducibly performed at intermediate protein input levels of 10 mg. Furthermore, application of the P-Tyr-1000 antibody in a standardized phosphoproteomics workflow allows relative quantitation of drug target inhibition and provides insights in alternative signaling pathways in cancer cells.

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1. Report

Receptor tyrosine kinases (RTKs) are enzymes that phosphorylate proteins and are known to be critical for tumor growth and metastasis formation. In parallel to their discovery, multiple targeted therapeutic agents were introduced and are now implemented in clinical practice [1]. Mass spectrometry based phosphoproteomic analyses emerged the past decade as a novel technology for the study of protein phosphorylation and RTK signaling. Numerous studies were published that showed great potential of the technology for target discovery and pharmacodynamic studies [2–4]. In contrast to serine and threonine, phosphorylations on tyrosine are less abundant, representing approximately 1% of the total cellular phospho-proteome. Phosphoproteomics experiments 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 [5,6] and metal oxide affinity chromatography (MOAC) using titanium dioxide (TiO_2) beads [7,8]. For specific enrichment of phosphotyrosine (pTyr)-containing peptides, antibody-based methods have been reported [2,9]. For deep phosphoproteome profiling combinations of global and pTyr enrichment methods have been reported [10,11]. For immunoaffinity purification a phosphotyrosine antibody is required [9]. Using this approach in a pioneering study, novel phosphokinase targets were identified in a large series of NSCLC cells and tumors [2]. Here we compared the performance of two commonly used commercial antibodies for enrichment of tyrosine-phosphorylated peptides using label-free phosphoproteomics.

Phosphotyrosine peptides were enriched using P-Tyr-1000 (Cell signaling Technology, Rabbit mAb-agarose conjugate, #8803) and 4G10 (Millipore, 4G10® mouse mAb-agarose conjugate #16-101) agarose conjugate. A large amount (100 mg) of HCT116 colorectal cancer cells (80% confluent) was lysed (20 mM HEPES pH 8.0, 9 M Urea, 1 mM sodium vanadate). Reduction of cysteine bonds, alkylation and in-solution-digestion (1:40, trypsin:protein ratio) were performed according to standard practice. Peptides were purified on Sep-Pak C18 columns, eluted in 80% acetonitrile (ACN) in 0.1% trifluoro acetic acid (TFA) and lyophilized. Subsequently, this sample was divided into 10 aliquots of 10 mg protein for direct comparison of the immunoprecipitation procedure (N = 3 replicates per antibody). Agarose bead conjugates were washed with 4 × 1 ml cold phosphate-buffered saline (PBS). The peptides were dissolved in 700 μl immunoaffinity (IP) buffer (20 mM Tris–HCl pH 7.2, 10 mM sodium phosphate and 50 mM NaCl) at room temperature for 10 min. After cooling, they were incubated with 40 μl 50% (vol/vol) bead slurry of pTYR-1000 or 4G10 agarose beads, respectively, at 4 °C for 2 h in a head-over-tail rotation wheel. Equal antibody-bead conjugate volumes (20 μl beads and 40 μl bead slurry) were used for pTYR-1000 and 4G10 tests. The 4G10 antibody concentration was 1 mg/1 ml protein A beads (20 μg 4G10 antibody per 10 mg peptides). No antibody concentration was provided for the pTYR-1000 conjugate. The beads were washed with 2 × 1 ml IP buffer followed by 3 × 1 ml MQ, and were collected by centrifugation (2000 x *g*, 30 s). Peptides were eluted in 0.15% trifluoroacetic acid (TFA) in two steps of 30 μl and 25 μl, respectively, which were pooled. Peptides were desalted using 20 μl C18 StageTips (Thermo #SP201), eluted in 20 μl 80% ACN in 0.1% TFA, speed-vac dried and dissolved in 35 μl loading solvent (4% ACN in 0.5% TFA). For nanoLC–MS/MS, database searching, MaxQuant and statistical analysis see Supplementary methods. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [12] via the PRIDE partner repository with the dataset identifier PXD001565.

Phosphopeptides were relatively quantified by their extracted ion intensities ('Intensity' in MaxQuant). This method allows for comparative analysis of cellular signaling states, but linearity of response was not assessed. Phosphopeptide intensities detected are well within the dynamic range of the mass spectrometer used. For each sample the phosphopeptide intensities were normalized on the median intensity of all identified peptides (from the MaxQuant Evidence table) in the sample ('normalized intensity'). Overall kinase phosphorylation was calculated by summing all identified phosphopeptide MS/MS spectra for a kinase (spectral-counting). For both antibodies, three peptide samples originating from 10 milligram protein were processed (P-Tyr-1000 *A-B-C*; 4G10 *D-E-F*) on the same day, using the same processing solutions. Beads coated with P-Tyr-1000 yielded 653, 526 and 496 phosphopeptides with 60% of the phosphopeptides being identified in all 3 samples (Fig. 1A). The 4G10 beads resulted in 342, 337 and 225 phosphopeptides, with 46% of the IDs identified in all replicates (Fig. 1B). Average CV values for phosphopeptides detected in 3/3 replicates were 46.5% and 56.2% for pTyr-1000 and 4G10 enrichments, respectively. In total 689 phosphopeptides were detected in all 3 samples