



# Progress and impact of clinical phosphoproteomics on precision oncology

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**Abstract:** Advances in phosphoproteomic technologies have enabled a step-change in the ability to make precise measurements of global protein phosphorylation states within tumours, providing critical information on the activation status of key oncogenic pathways driving disease. As an ever increasing number of molecularly targeted drugs enact their effect through modulation of such pathways, translational phosphoproteomic studies represent an avenue for biomarker discovery that provides insight into the direct functional impact of drug treatment whilst uncovering previously unrecognised mechanisms of drug response and resistance. Array- and mass spectrometry (MS)-based techniques allow for the characterisation of hundreds to thousands of phosphorylation sites that in principle can be readily integrated with genomic and transcriptomic datasets to produce highly detailed molecular portraits of human cancers. However, in practice, the routine application of phosphoproteomics to clinically-derived material has been limited by factors relating to sample integrity, assay resolution and computational challenges. In this perspective, we highlight a number of recent studies that have applied phosphoproteomics to the analysis of tumour specimens and discuss their impact on our knowledge of dynamic pathway biology and response to targeted therapies. We provide examples of pioneering studies that have integrated phosphoproteomic data with genomic methods to demonstrate the utility of these approaches in identifying clinically important biology for applications in personalised medicine. Collectively, these studies suggest that the increasing use of phosphoproteomics in translational precision oncology holds the promise of contributing to practice-changing discoveries over the coming decade.

**Keywords:** Cancer; phosphoproteomics; mass spectrometry (MS); precision medicine; biomarkers; drug resistance

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## Introduction

Cancer initiation and progression is driven by the dysregulation of signalling pathways that control fundamental cellular processes including growth, survival, differentiation and migration. Aberrations in cell signalling resulting in unrestrained phosphorylation of proteins is critical to the process of oncogenesis, which is consistent with the high rate of mutations in kinases and phosphatases

in cancers (1,2). Such mutations can act as key disease drivers, as seen in the activating mutants of EGFR and BRAF in non-small cell lung cancer and melanoma respectively, and may predict heightened sensitivity to targeted drugs that selectively inhibit these mutant kinases. However, the development of acquired resistance to such kinase inhibitors is near-universal and involves mechanisms that are varied and incompletely understood (3,4).

Meanwhile, there are also a number of kinase inhibitors that are approved for use in cancers without recognised oncogene addiction or predictive biomarkers. Again, in most instances the mechanisms of resistance to these agents have not been delineated, despite over a decade of genomic and transcriptomic research.

Techniques based on antibody arrays and mass spectrometry (MS) enable the characterisation of phosphorylation states in hundreds to thousands of proteins within a single experiment, and represent a means of comprehensive profiling of cancer pathway activation (5,6). Phosphoproteomic analyses in preclinical models have produced important insights into cancer biology, including delineating the functional consequences of cancer-associated abnormalities within the genome, epigenome and transcriptome (7,8). However, issues relating to sample input requirements, the need for specialist equipment and personnel, and challenges posed by data processing and analysis have meant that the application of phosphoproteomics to clinical and translational medicine is in its relative infancy (9). Nevertheless, technological and methodological advances in phosphoproteomic techniques (the subject of a number of recent reviews) (10-12) are likely to translate into a rapid growth in the number of clinical phosphoproteomics studies over the next 5-10 years. In this perspective, we highlight a selection of recent studies that demonstrate the feasibility, promise and current challenges of phosphoproteomic approaches in identifying drivers and resistance mechanisms in clinically-derived tumour material.

### Identifying biomarkers and mechanisms of treatment resistance

Translational studies that obtain tumour material from patients both before and after initiation of a given treatment represent a powerful approach for biomarker discovery (13). The correlation of biological features of pre-treatment tumour with therapeutic response allows for the identification of potential mechanisms of sensitivity or primary resistance, forming the basis for predictive biomarkers and alternative molecular targets. Meanwhile, examining the alterations between baseline and post-treatment exposure provides insight into mechanisms of tumour response and escape, which in turn may inform strategies for preventing or overcoming acquired drug resistance. A small number of reported studies have demonstrated the feasibility of applying phosphoproteomic techniques to clinical tumour samples collected before and

after treatment with molecularly-targeted therapies as a tool for discovery of resistance mechanisms and biomarkers for response.

Stacchiotti *et al.* used anti-phospho receptor tyrosine kinase (RTK) antibody arrays to investigate phosphoprotein correlates with treatment response to the multi-target tyrosine kinase inhibitor (TKI) sunitinib and identify candidate biomarkers in eight patients with solitary fibrous tumour (14). Pre-treatment specimens demonstrated strong activation of PDGFRB, EGFR and IGF-1R, whilst phosphorylation levels of CSF1R, PDGFRA and VEGFRs were present at low levels. Post-treatment tumour specimens from two of eight patients were also available for analysis. In one patient, the lesions that were resected following sunitinib treatment were a lung metastasis that demonstrated radiological and histological features of progression on treatment, and another lung metastasis that had radiologically stabilised with associated histological features of tumour regression. Here, analysis of pre- and post-treatment samples showed unchanged phospho-RTK profile in the progressing lesion but a reduction in signal for phospho-PDGFRB and IGF-1R in the lesion with apparent drug sensitivity. In the second patient, a progressive peritoneal nodule was resected following mixed radiological response to sunitinib. This lesion showed greater levels of activated PDGFRB and EGFR compared to pre-treatment baseline, whilst RTKs such as IGF-1R that were not phosphorylated in pre-treatment tumour were now activated. These findings lead the authors to suggest that a shift in signalling dependency from PDGFRB to IGF-1R may represent a mechanism of sunitinib resistance. This study represents an example of a phosphoproteomic approach implicating a change in kinase dependency as a resistance mechanism that is potentially amenable to additional salvage targeted therapy.

In a window-of-opportunity study in HER2-overexpressing esophago-gastric cancer, De Silva *et al.* used an antibody array platform to assess the levels of phospho-RTKs in pre- and post-treatment tumour samples to gain insight into the baseline and adaptive pathway signalling that underlies treatment response or resistance (15). Tumour biopsies were obtained at pre-treatment baseline and again after 10 days of monotherapy with the anti-HER2 and EGFR TKI lapatinib. Following this, three full cycles of lapatinib in combination with capecitabine and oxaliplatin were administered. On completion of this neoadjuvant treatment, tumours were surgically resected, with the surgical specimen then included in translational

analyses. Phosphoproteomic analyses of these three serial samples demonstrated a significant drop in the ratio of phosphorylated to total amounts of HER2 and EGFR after lapatinib monotherapy in 10/10 and 9/10 patients respectively. This reduction in RTK activation persisted at the time of surgery and was associated with a significant reduction in downstream PI3K phosphorylation and a trend toward a reduction in the levels of phosphorylated AKT and ERK. A possible role for c-Met activation as a mechanism of treatment resistance was suggested by the observation of higher levels of c-Met phosphorylation post-lapatinib in cases with elevated PI3K and ERK activation that persisted after lapatinib treatment, indicative of persistent downstream signalling despite HER2 and EGFR inhibition. Whilst co-expression or co-amplification of c-Met has been shown to be an infrequent event in HER2-overexpressing cancers of the upper gastrointestinal tract (16), Lee *et al.* demonstrated by phospho-antibody array that co-activation of these two RTKs occur in 22% of patients (17), revealing a druggable mechanism of resistance to HER2-directed therapy that may be underestimated in the absence of phosphoproteomic analysis. These results support the combination of already available HER2- and c-Met-targeted drugs as a means of overcoming primary resistance to lapatinib in this disease.

Cheraghchi-Bashi *et al.* sought to identify protein-based biomarkers predictive of benefit in the treatment of epithelial ovarian cancers with a novel AKT inhibitor GSK2141795 (18). The authors initially used antibody arrays to assess proteomic and phosphoproteomic changes in *in vitro* and *in vivo* preclinical models when treated with the drug. Through this, an AKT inhibition-related signature was developed, consisting of the changes seen most consistently across the studied models and that included increased phosphorylation of AKT and p38 MAPK, and decreased phosphorylation of ribosomal protein S6, RB1 and PRAS40, a downstream substrate of AKT. This signature was then investigated in paired tumour biopsies taken from patients with ovarian cancer before and after GSK2141795 treatment within a phase 1 trial. The authors found a correlation between CA125 tumour marker response and an induction of the AKT inhibition-related signature during drug treatment. Meanwhile, patients whose tumours exhibited the signature prior to drug exposure experienced less reduction in CA125, suggesting that the absence of the AKT inhibition-related signature in baseline tumours could act as a predictive biomarker for response. This study highlights the potential

for the findings of phosphoproteomic analysis of preclinical models to inform translational research for the discovery of clinically meaningful biomarkers.

With a similar rationale to the above studies, Dazert *et al.* applied MS-based phosphoproteomics to a single case of hepatocellular carcinoma (HCC) from whom paired biopsies of tumour and adjacent normal liver were obtained before and after 7 weeks of treatment with sorafenib, a multi-target TKI (19). Radiological assessment of the patient at week 8 post-sorafenib initiation demonstrated tumour progression which, when considered alongside levels of the circulating tumour marker alpha-fetoprotein that continued to rise throughout treatment, indicated either pre-existing or rapidly acquired sorafenib resistance. Small needle biopsies were used as input for both proteomic and phosphoproteomic MS analysis compared to a super-SILAC reference consisting of five HCC cell lines. Comparison of proteins differentially phosphorylated between normal and tumour tissues led to the identification of a HCC-specific phospho-signature, while candidate biomarkers for sorafenib sensitivity, intrinsic or acquired resistance were indicated by phosphosites that were respectively downregulated, unchanged or upregulated following sorafenib therapy. Furthermore, effective on-target kinase inhibition by the drug was demonstrated by the observed downregulation of MAPK pathway components in the post-treatment tumour. Ontology enrichment analysis of putative resistance phosphoproteins identified epithelial-mesenchymal transition (EMT) and cellular adhesion pathways as candidate processes involved in sorafenib resistance. This study is the first to use quantitative MS-based phosphoproteomics on serial patient biopsies and provides a proof-of-concept of the viability of this approach in identifying biological signatures that may reflect mechanisms of treatment sensitivity and resistance.

Taken together, these studies highlight the potential for phosphoproteomic-based analysis to be embedded into translational research that aims to identify predictive biomarkers for kinase inhibitor response. Although the amount of assessable tissue that is available from needle biopsies is often significantly limited, these studies show that such specimens are adequate for phosphoproteomic analysis and can produce meaningful data.

### **Integrating phosphoproteomics with genomic analysis**

Next generation sequencing technologies allow for routine

genome and transcriptome-wide tissue profiling that can be incorporated into translational research in an increasingly affordable manner. The integration of phosphoproteomics alongside genomic and transcriptomic data offers comprehensive profiling of tumour biology and improves the likelihood for successfully identifying mechanisms of treatment sensitivity and resistance as well as new biomarkers. This has been illustrated by a series of recent studies by the National Cancer Institute Clinical Proteomics Tumor Analysis Consortium (NCI-CPTAC). In one study which included quantitative MS-based proteomic and phosphoproteomic characterisation of 105 breast cancers comprising a representative balance of intrinsic subtypes, an average of >11,500 proteins and >26,000 phosphosites per tumour were identified (20). These cases had existing genomic and transcriptomic annotation courtesy of The Cancer Genome Atlas (TCGA) Consortium and subsequent alignment of copy number altered (CNA) genes with mRNA, protein and phosphoprotein levels provided insight into the potential functional consequences of CNA genes. This analysis found that compared to mRNA levels, the penetrance of CNA through to the phosphoprotein level was more pronounced in known tumour-associated genes compared to genes without known oncogenic roles, suggesting that determination of phosphoprotein alterations that accompany CNA is likely to be the better approach of assessing the functional impact of CNA. The authors went on to define phosphoproteomic signatures associated with each breast cancer intrinsic subtype and in tumours harbouring mutations of PIK3CA or TP53. From this, they were able to propose novel subtype- or mutation-specific effectors. One example in basal breast cancer is SPEG, a kinase associated with severe dilated cardiomyopathy not previously implicated in breast cancer. Such studies highlight the potential of integrated Omics analysis as a means to gain novel insights into tumour biology and identify potential new therapeutic targets that may not be detectable at DNA or transcript level.

Drake *et al.* utilised a novel computational pipeline known as the TieDIE algorithm to find protein and gene interactions related to disease for use in the integration of phosphoproteomic and transcriptomic datasets derived from metastatic castrate-resistant prostate cancer (21). This approach also integrated somatic mutation and CNA data from prostate cancer patient samples into the analysis. The integrated phosphoproteome-transcriptome network was then applied to the study of tumour samples obtained from 6 patients at autopsy to identify case-specific transcriptional

and kinase master regulators. This patient-specific network analysis demonstrated that whilst the inferred transcriptional master regulators were similar across all cases, the levels of phosphorylated kinases significantly differed. The authors termed this patient-specific network analysis as phosphorylation-based cancer hallmarks using integrated personalised signatures (pCHIPS). This study also implicated several signalling proteins such as PRKDC, PRKAA2, PTK2, RPS6KA4 and CDK family members as potential new therapeutic targets and/or biomarkers in prostate cancer. The fact that the possible therapeutic strategies proposed by the phosphoproteomic data differed in nearly every patient suggests that there is a likely need to approach future phosphoproteomic translational studies in an individualised manner.

### Challenges and future perspectives

A number of key challenges have so far limited the broader adoption of phosphoproteomics in translational cancer research. Prominent among these challenges are issues relating to clinical sample integrity. The phosphorylation status of cellular proteins within tissue begins to change within minutes of removal from the body, posing a significant challenge to capturing representative tumour biology. This issue has been systematically evaluated in a series of studies by the NCI-CPTAC. In one study, resected tissue from patients with ovarian cancer was subjected to variable lengths of cold ischaemia prior to fixation by flash freezing (22). Whilst the global proteome level measurements were largely unaffected, dramatic changes in the phosphoproteome were seen, often within 5 minutes, with up to 4% of phosphoserine or phosphothreonine sites and 62% of phosphotyrosine sites altered. As a potential alternative or adjunct to prospectively collected fresh tissue in clinical/translational phosphoproteomic studies, the wide availability of large and well-annotated institutional FFPE tumour archives represents an attractive resource. However, while good correlation between the total proteome of matched fresh-frozen and FFPE tissues has been demonstrated in a number of MS-based studies (23-25), the use of FFPE samples for the study of the phosphoproteome remains contentious. In addition to the effect that non-controlled variation in tissue handling and warm fixation may have on phospho-profiles in FFPE tissue, it is currently unclear to what degree phosphorylation sites are altered by formalin fixation or the process of heat and/or enzymatic reversal of formalin-induced protein cross-linking required

prior to tissue analysis (26). Reported studies have identified several thousand phosphosites from recently prepared FFPE tissue samples, with approximately 60% overlap with matched fresh frozen tissue—the replicability of these data has yet to be shown. It is currently unclear whether loss or chemical modification of phosphosites by formalin in FFPE tissue occurs at random or whether certain sites may be recurrently altered, potentially giving rise to phosphoproteome “blind spots” in FFPE tissue analysis (27,28). Although the effect of years of storage may have on FFPE tissue are difficult to model in a controlled experiment, fixation and degradation-related artefact signatures could potentially be developed and used to correct phosphoproteomic profiles. For example, the NCI-CPTAC identified a recurrent ischaemia-related phospho-signature that could serve such purpose, although the transferability of such a signature requires further assessment (29).

As is the case for the use of genomic and transcriptomic profiling in precision oncology, temporal and spatial intra-tumour heterogeneity have significant implications for phosphoproteomic profiling of tumours. Reprogramming of pathway signalling in response to specific kinase blockade is a recognised mechanism of acquired resistance to TKIs in multiple cancers, indicating that archival samples collected prior to intervening therapies may not reflect the current pathway activation status of a tumour (30). Meanwhile, significant variation in the phosphoproteome between different tumour areas has been shown in a single case of colorectal cancer and is likely a widespread phenomenon that at least matches and potentially exceeds the degree of genomic heterogeneity (22). Repeat biopsies, potentially from multiple disease sites, are desirable as a way of addressing spatial and temporal heterogeneity but are met with perennial logistical issues. Additionally, the sample input requirements for enrichment of phosphoproteins and subsequent MS must be met and may exceed what is available from percutaneous biopsies. The study by Dazert *et al.* does, however, demonstrate the feasibility of providing biologically meaningful phosphoproteomic data of potential clinical significance from small needle biopsies (19). Exciting avenues that may help to address the issue of heterogeneity and need for repeat biopsies include single cell phosphoproteomics and analysis of circulating tumour-derived proteins found in exosomes, both increasingly possible as a result of improving sensitivity and resolution provided by microfluidics and

high resolution antibody arrays or MS-based techniques (31,32). Lee *et al.* demonstrated the feasibility and potential utility of phosphoproteomic analysis of circulating tumour cells (CTCs) in a translational study in gastric cancer (17). Here, antibody array-based analysis of the phosphorylation status of RTKs in CTCs and ascites-derived tumour cells taken from patients with metastatic disease revealed heterogeneous pathway activation that reflected what was observed in fresh frozen tumour samples taken at resection of primary disease. Furthermore, ascites-derived cells showed changes in pathway activation when challenged with TKI *ex vivo*. This study highlights the potential application of phosphoproteomics to circulating tumour material as an avenue for the minimally invasive and dynamic monitoring of disease response to molecularly targeted drugs. This would represent a powerful tool for biomarker discovery, particularly when coupled with MS-based discovery experiments.

The technical and computational demands of MS-based phosphoproteomics require specialist equipment and expert personnel, impacting upon the expense and availability for translational research and potential clinical application. This challenge can however be overcome by subjecting the unbiased global phosphoprotein profiling data from discovery experiments to data reduction strategies to identify key, recurrent nodal proteins that may then be incorporated into larger validation experiments (33), potentially as part of companion diagnostics that utilise antibody-based technologies which can be integrated with corresponding genomic and/or transcriptomic markers (34).

In summary, the accurate mapping of molecular processes that dictate therapeutic sensitivity is required to deliver on the fundamental promise of precision oncology. Although genomic and transcriptomic profiling have revealed a large amount of clinically-actionable information, the underlying causes of variation in patient response to targeted therapies remain poorly understood. Pioneering studies have shown early promise for phosphoproteomics as a means of directly profiling the activity of targeted agents such as kinase inhibitors with the potential to guide novel therapeutic strategies and clinical decisions through the identification of predictive biomarkers and new drug targets. Whilst clinical application of phosphoproteomics is in its infancy, a wealth of preclinical experience and improving technologies indicates a central role for this approach in translational cancer research and precision medicine over the next decade.

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