PASEF-DDA enables deep coverage single-shot phosphoproteomics and ion mobility-based elucidation of phosphosite isomers

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Introduction

To elucidate cellular signaling mechanisms, a detailed and highly resolved analysis of phosphorylation sites is crucial. Although LC-MS/MS proved as a powerful tool for in-depth phosphoproteome analysis, challenges remain in the correct determination of the phosphorylation site. Coeluting and isobaric phosphopeptide isomers, harbouring the phosphogroup on different residues, are often impossible to resolve in classical MS/MS analyses. Ion mobility spectrometry (IMS) enables their separation based on their collision cross section (CCS), as the position of the phosphogroup affects the ion geometry in the gas phase. Parallel accumulation serial fragmentation data-dependent acquisition (PASEF-DDA) on the timsTOF Pro mass spectrometer allows the application of IMS on large scale phosphoproteomic studies. Here, we present a high-coverage phosphopeptide dataset from patient-derived osteosarcoma samples [1].

Methods

Tryptic phosphopeptides from osteosarcoma samples before and after treatment were enriched in three replicates from 1 mg lysate each by TiO_2 from GL Science. Enriched phosphopeptide samples were separated within 100 min (2 to 35% B, B: 0.1% FA in ACN, 400nL/min flow rate) on a reversed-phase C18 column with an integrated CaptiveSpray Emitter (25cm x 75µm, 1.6µm, IonOpticks, Australia). After ESI ionization, peptides were analyzed using timsTOF Pro with PASEF enabled at 120Hz. Trapped ion mobility accumulation and elution times were synchronized at 166ms. In addition to high resolution (40,000) accurate mass (<10ppm) the mass spectrometer records mobility (1/K0), and with charge state and m/z deciphers CCS. The data was processed using PEAKS X + (BSI) and MaxQuant v1.6.10.43 (MPI of Biochemistry). The resulting phosphopeptide information was submitted for Integrated Kinase Activity Score analysis (InKA).

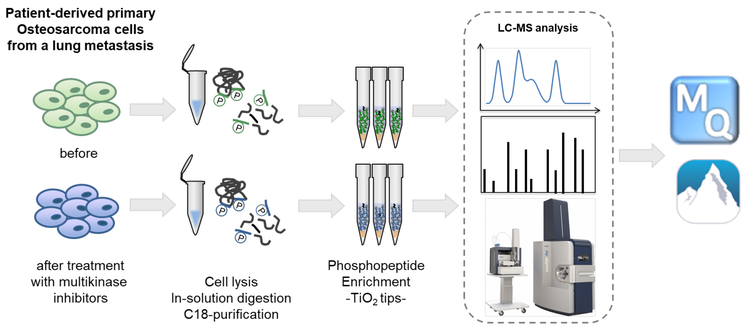

Figure 1

IMS enabled isomer separation

The presented sample preparation procedure and subsequent database search with Peaks X+ resulted in 27,768 identified phosphopeptides without any sequential enrichment or fractionation (fig. 2/ 3). Of these, 11,247 can be classified as *Class I* phosphopeptides.

4,672 phosphopeptide pairs were isobaric positional isomers with p = 0.75 propability of correct phospo-site determination (i.e. AScore > 6) of which a major part was coleuting during RP-LC (ΔRT < 0.5 min).
Of those, 457 phosphopeptide pairs could be separated by IMS (Δ1/k0 > 2%, fig. 3).

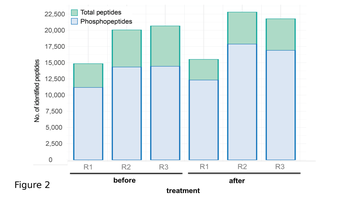

Figure 2

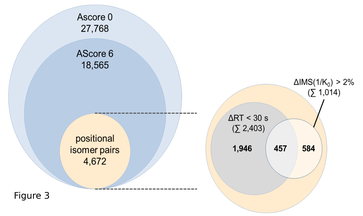

Figure 3

Example 1 - SRRM2

Serine/arginine repetitive matrix protein 2. Aberrant phosphorylation in liver cancer on position S1691 [2]. Multiple coeluting isobaric phosphopeptide isomers were separated by IMS.

Table 1

#	Peptides	-10lgP	Mass	Obs. *m/z*	RT
1	RS(+79.97)SRSS(+79.97)PELTR	55.08	1434.60	718.31	8.14
2	RSS(+79.97)RSS(+79.97)PELTR	57.48	1434.60	718.31	8.15
3	RSS(+79.97)RS(+79.97)SPELTR	59.34	1434.60	479.21	8.63

Figure 4

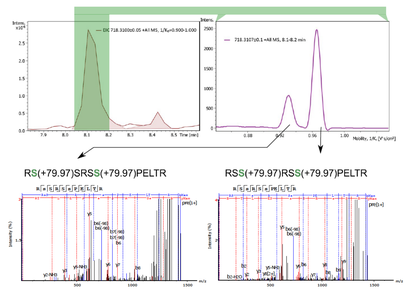

Figure 5

Example 2 - AKAP-12

A-kinase anchor protein 12. scaffold protein for many key signalling factors, such as protein kinase C (PKC), PKA, cyclin as well as F-actin. S286 was identified as significantly increased after treatment, which is descibed as EGF response [3].

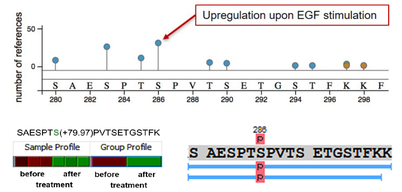

Figure 6

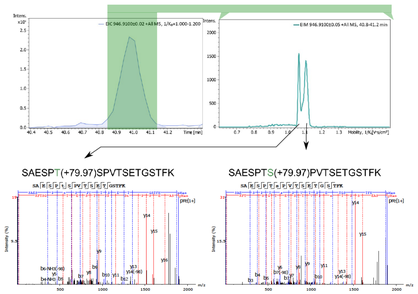

Figure 7

Hyperactive kinase scoring in cancer research requires accurate phosphosite information

timsTOF Pro raw data was analyzed using MaxQuant to facilitate submission to the online interface for InKA score calculation [4]. This score combines information from different sources to obtain a comprehensive picture of hyperactive kinases (fig. 8).

The presented experimental setup enabled the statistical evaluation by t-test to compare before and after treatment status (fig. 9 / 10).

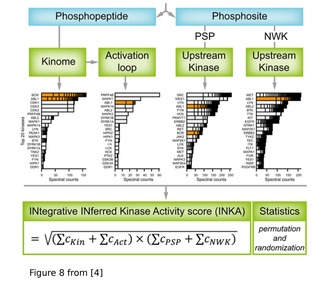

Figure 8 from [4]

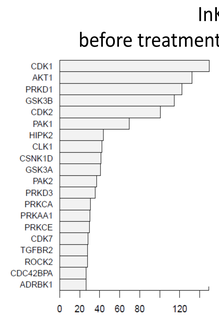

Figure 9

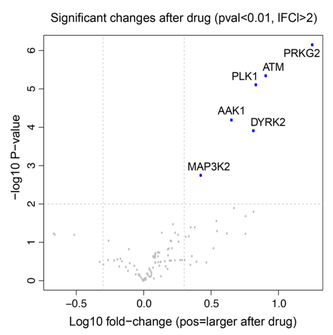

Figure 10

Outlook

With the identification of hyperactive kinases together with whole proteome and biochemical asssays, we hope to shed light on the mode of action of the applied therapy and observed resistance behaviour of the osteosacroma cells towards monotherapy. For more comprehensive analysis, other enrichment techniques like phospho-tyrosine immunoprecipitation (pTyr-IP) and immobilized metal ion affinity chromatography (IMAC) will be applied.

References

[1] Beck, O. Paret, C. Russo, A. *et al.* (2020). Safety and Activity of the Combination of Ceritinib and Dasatinib in Osteosarcoma. Cancers. 12. 793. doi 10.3390/cancers12040793
[2] Zhu, B., He, Q., Xiang, J. *et al.* (2017) Quantitative Phosphoproteomic Analysis Reveals Key Mechanisms of Cellular Proliferation in Liver Cancer Cells. Sci Rep 7, 10908. doi 10.1038/s41598-017-10716-0
[3] Walker-Gray, R. and Klussmann, E. (2020), The role of AKAP12 in coordination of VEGF-induced endothelial cell motility. Acta Physiol, 228. doi 10.1111/apha.13359
[4] Beekhof R., van Alphen C., Henneman A. *et al.* (2019) INKA, an integrative data analysis pipeline for phosphoproteomic inference of active kinases, Molecular Systems Biology 15. doi 10.15252/msb.20188250