Relevant phosphoproteomic and mass spectrometry: approaches useful in clinical research

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Abstract

Background: “It’s not what we do, it’s the way that we do it”. Never has this maxim been truer in proteomics than now. Mass Spectrometry-based proteomics/phosphoproteomics tools are critical to understand the structure and dynamics (spatial and temporal) of signalling that engages and migrates through the entire proteome. Approaches such as affinity purification followed by Mass Spectrometry (MS) have been used to elucidate relevant biological questions disease vs. health. Thousands of proteins interact via physical and chemical association. Moreover, certain proteins can covalently modify other proteins post-translationally. These post-translational modifications (PTMs) ultimately give rise to the emergent functions of cells in sequence, space and time.

Findings: Understanding the functions of phosphorylated proteins thus requires one to study proteomes as linked-systems rather than collections of individual protein molecules. Indeed, the interacting proteome or protein-network knowledge has recently received much attention, as network-systems (signalling pathways) are effective snapshots in time, of the proteome as a whole. MS approaches are clearly essential, in spite of the difficulties of some low abundance proteins for future clinical advances.

Conclusion: Clinical proteomics-MS has come a long way in the past decade in terms of technology/platform development, protein chemistry, and together with bioinformatics and other OMICS tools to identify molecular signatures of diseases based on protein pathways and signalling cascades. Hence, there is great promise for disease diagnosis, prognosis, and prediction of therapeutic outcome on an individualized basis. However, and as a general rule, without correct study design, strategy and implementation of robust analytical methodologies, the efforts, efficiency and expectations to make biomarkers (especially phosphorylated kinases) a useful reality in the near future, can easily be hampered.

Keywords: Phosphoproteomics, Mass spectrometry, Clinical research

Findings

Overview

Proteomics and phosphoproteomics clinical research studies imply the comprehensive analysis of the proteins which are expressed in cells or tissues, and can be employed at different stages (e.g. healthy vs. disease). Therefore, comparative proteomics can distinguish small, but relevant changes in protein modifications in their structure -post-translational modifications (PTMs)- at a depth of several thousand proteins to facilitate drug target identification.

Chemical and Biochemical proteomics can be used to identify drug-target interactions and subsequently analyze drug specificity and selectivity. Furthermore, phosphoproteomic approaches can be exploited to monitor changes in phosphorylation events in order to characterize drug actions on cell signalling pathways and/or signalling cascades. In addition, functional proteomic approaches, can be employed to investigate protein-protein and protein-ligand interactions in order to: (i) improve the knowledge or the clarification of the mechanism of drug action, (ii) achieve relevant protein-
identifications of disease-related sub-networks and (iii)
reach the important step of innovation of novel drug
targets.

Furthermore, proteins are currently the major drug
targets, and therefore play a critical role in the process
of modern drug design. This typically involves: (1) the
construction of drug compounds based on the structure
of a specific drug target, (2) validation for therapeutic
efficacy of the drug compounds, (3) evaluation of drug
toxicity, and finally, (4) clinical trial.

Finally, tissue imaging MS is being extended as a cur-
rent promising technique for reproductive research.
Advances in MS imaging will inevitably attract biologists
and clinicians as the advantages and power of this tech-
nology become more widely known. We will detail, in a
simple manner, relevant clues of current proteomic,
phosphoproteomic and MS strategies and techniques
useful for clinical advances [1].

**Phosphoproteomics relevance in signalling transduction
pathways**

It is well known that phosphoproteomics and MS-based
recent advancements have made these approaches the
ideal way by which to study signal transduction
although it implies high speciality and tedious research
studies. In addition, individual protein phosphorylation
events often have important roles and clues in broad
signalling networks within a cell. Unfortunately, while
phosphorylation of kinases frequently, mainly regulates
their own activity, they are commonly under-repre-

sented in phosphoproteomic studies, partly due to their
low expression within the cell. Nevertheless, a viable
solution to this drawback has been successfully proven
via kinase affinity purification techniques. Thus, impor-
tant improvements are helping to achieve relevant data
of phosphorylated kinases - those proteins being the
“key” of signalling pathways and network- connectivity
among different signalling cascades.

Phosphatases are playing equally important roles in
regulating signalling pathways through the removal of
phosphoryl groups from proteins. Indeed, depleting cells
of specific protein phosphatases and employing phos-
phoproteomic approaches, can be used to determine
which proteins are regulated by the phosphatase of
interest, either directly or downstream [2-7].

The best studies of mitogen activated protein kinase
(MAPKs) are the extracellular signal regulated protein
kinases (ERK). ERKs phosphorylate cytoplasmic targets
migrate to the nucleus where they can activate tran-
scription factors involved in cellular proliferation. As a
general view of the orchestrated signalling pathways, it
is important to know that following the communication
of the signal to different cellular compartments are (1)
signal processing and (2) amplification by plasma mem-
brane proximal events.

The activation of multiple signal cascades by (1)
receptors, (2) different protein PTMs, (3) crosstalk
between signalling pathways and (4) feedback loops to
ensure optimal signalling output, are involved in this
process. Also, the binding of receptor Tyrosine (Tyr)
kinases (RTKs) to their cognate ligands at the cell sur-
face results in receptor dimerization and autophosphory-
lization. Phosphorylated Tyr residues subsequently serve
as docking sites to recruit signalling mediators, such as
growth factor receptor-bound protein 2 (GRB2).

Multiple signalling cascades such as (1) the phospho-
nositide-3 kinase (PI3K)-AKT, (2) Ras-Raf- extracellular
signal-regulated kinase (ERK) mitogen-activated protein
kinase (MAPK), and (3) signal transducer and activator
of transcription (STAT) pathways are activated by the
assembly of these signalling complexes. On the other
hand, (4) Casitas B-lineage lymphoma (CBL)-mediated
ubiquitylation of RTKs controls their endocytosis and
the duration of receptor signalling. In addition, binding
of tumour necrosis factor-α (TNFα) to its receptor,
TNFR1, induces trimerization of the receptor and recruit-
mint of the adaptor protein TNFRI-associated death
domain (TRADD). These functions as a hub to assem-
ble a multi-protein signalling complex containing
TNF-associated factor 2 (TRAF2), receptor interacting
Ser/Thr protein kinase 1 (RIPK1) and nuclear factor-κB
(NF-κB) essential modulator (NEMO). The result is the
activation of different signalling networks, such as the
ERK MAPK, p38 MAPK and NF-κB pathways. Proteins
in the MAPK signalling pathways are activated by both
RTKs and TNFα, which allows cells to integrate multi-
ple signals [8-20].

**Advantages/disadvantages and clues of most used MS-
based tools for the detection of phosphorylated proteins/
peptides**

Several analytical techniques exist for the analysis of
phosphorylation, e.g., Edman sequencing and 32P-phos-
phopeptide mapping for localization of phosphorylation
sites, but these methods do not allow high-throughput
analysis or imply very laborious operations [21], while
using MS, high-throughput analysis of phosphorylated
protein residues can be developed [22,23]. On the other
hand, phosphospecific antibodies are routinely used to
immunoprecipitate and therefore to enrich in phos-
phorylated proteins from complex mixtures [24], but,
currently, there are no antibodies available commercially
suitable for enriching all proteins that are phosphory-
lated, and thus, these proteins must be purified or
enriched from complex mixtures using alternative meth-
ods [25].

When carrying out in-gel or in-solution trypsin diges-
tion of protein complex mixtures, the resulting phos-
hophptides and non-phosphopeptides can be loaded
into different metal ion chromatographies (e.g.
Immobilized Metal ion Affinity Chromatography IMAC (Fe$^{3+}$), and Titanium Dioxide TiO$_2$ [26]) in order to enrich in phosphopeptides. The enriched solution can also be submitted into different reverse-phase chromatographies (e.g. Graphite powder [27], POROS R3) [25] in order to clean and desalt those phosphopeptides previously eluted. Moreover, all these types of chromatographies will reduce the suppression of phosphorylated peptides in the mass spectra.

Using IMAC (Fe$^{3+}$) and also (TiO$_2$) [26], the negatively charged phosphopeptides are purified by their affinity to positively charged metal ions, but some of these methods suffer the problem of binding acidic, non-phosphorylated peptides. Ficarro and co-workers [22], circumvented this problem on IMAC (Fe$^{3+}$) by converting acidic peptides to methyl esters but increasing the spectra complexity and requiring lyophilization of the sample, which causes adsorptive losses of especially phosphopeptides [28]. Ficarro et al., were able to sequence hundreds of phosphopeptides from yeast, including SlT2p kinase, but the level of phosphorylated residues identified from kinases were low compared to the ones from phosphoproteins highly expressed within the cell. Fairly recently, TiO$_2$ chromatography using 2,5-dihydroxybenzoic acid (DHB) was introduced as a promising strategy by Larsen et al. [26]. TiO$_2$/DHB resulted in higher specificity and yield as compared to IMAC (Fe$^{3+}$) for the selective enrichment of phosphorylated peptides from model proteins (e.g. lactoglobulin bovine, casein bovine, etc).

Another important limitation concerning the phosphoenrichment methods is that mainly phosphopeptides from highly expressed proteins within cells can be purified, while the ones from phosphorylated proteins with low level expression (e.g. kinases) do not bind so well to those resins. This is due to the low proportion of this kind of proteins, or on the other hand, their available amount binds to metal ions although it is not sufficient to be detected by MS. The combination of Strong Cation Exchange Chromatography (SCX) with IMAC (Fe$^{3+}$) has been proven on yeast, resulting in a huge number of phosphorylated residues identified (over 700, including Fus3p kinase) [23]. Although more than 100 signalling proteins and functional phosphorylation sites were identified, including receptors, kinases and transcription factors, it was clear that only a fraction of the phosphoproteome was revealed [23].

It is evident that methodologies to enrich for phosphorylated residues from kinases should be improved. However, this is not straightforward for several reasons: (a) the low abundance of those signalling molecules within cells, and (b) the stress/stimulation time-duration, as only a small fraction of phosphorylated kinases are available at any given time as a result of a stimulus. Also, the time adaptation over signalling pathways is a relevant and fast factor for kinases phosphorylation [29], and (c) the current phosphoenrichment methods, which are mainly successful to purify phosphopeptides from highly expressed proteins.

In a simple manner, we will detail the manual validation of the phospho-data (assignments of the phosphate group on specific amino acids) obtained in an MS experiment during CID (Collision Induced Dissociation) operations. When peptide ions are fragmented via CID, series of $y$- and $b$- ions are formed [30,31]. The peptide sequence is obtained by correlating mass difference between peaks in the $y$-ion series or between peaks in the $b$-ion series with amino acid residue masses. The CID fragmentation mainly occurs on the peptide backbone, and sequence information is obtained. In relation to phosphotyrosine residues, partial neutral loss is observed (HPO$_3$, 80 m/z) in MS2 mode, and the phosphate group on tyrosine (Tyr) residues is more stable than on serine (Ser) and threonine (Thr) residues. Also, the phospho-finger-print characteristic of phosphotyrosine is the phosphotyrosine immonium ion (~216 Da) [32,33]. Via MS3 mode, the ion originating from neutral loss (NL) of phosphoric acid (H$_3$PO$_4$) can be selected for further fragmentation. Then, the selected ion is automatically selected for further fragmentation after neutral loss fragmentation. Therefore, it is possible to add extra energy for the fragmentation of peptide backbone.

Nevertheless, the MS3 mode requires that the phosphorylation on Ser and Thr residues are labile and conventional fragmentation via CID commonly resulting in the partial NL of H$_3$PO$_4$ (98 m/z) in MS2 mode. This is due to the gas phase $\beta$-elimination of the phosphoester bond and thus, dehydroalanine (Ser ~69 Da) and dehydro-2-aminobutyric acid (Thr ~83 Da) are generated [32,33].

In addition, as alternative phosphopeptide enrichment strategies, phosphopeptides can be de-protected and collected under acidic conditions and a variety of chemical methodologies have likewise appeared. BEMA ($\beta$-elimination/Michael addition), takes advantage of the ease of $\beta$-elimination of phosphorylated Ser and Thr residues at basic pH and the ability to subject the resulting dehydroalanine/methyl-dehydroalanine products to Michael addition with a desired tag for affinity purification [34-36]. In addition, Calcium phosphate precipitation (CPP) has been proven to be a fast, economical, and simple enrichment technique [37] in exchange for diminished specificity. Moreover, PhosphorAmidate Chemistry (PAC) is another important approach in which phosphopeptides are coupled to a solid-phase support such as an amino-derivatized dendrimer or controlled-pore glass derivatized with maleimide for selection [38,39].
**Tandem MS Methodology - basic issues useful for Phosphoproteomics via ElectroSpray Ionization (ESI)**

It can be taken as a general rule, that during MS-based experiments, a phosphopeptide mixture is separated using capillary liquid chromatography (LC). A typical separation column is 25 to 100 microns in diameter and 5 to 30 cm in length. The eluent is concurrently introduced into the mass spectrometer via electrospray ionization (ESI). ESI is a process that generates multiply protonated gas-phase peptide cations. The mass-to-charge ratio \(m/z\) and intensity \(I\) of the intact peptide precursors are recorded by an initial MS scan - commonly referred to as a full scan MS. Then, \(m/z\) values for peaks (list of masses) with high intensity are automatically selected in order of decreasing abundance for sequencing by tandem MS (MS/MS). This process of precursor selection, dissociation, and fragment ion mass analysis is repetitively performed on analyte species as they elute from the LC column. Ideally, MS/MS interrogation of a phosphorylated peptide generates a series of fragment ions that differ in mass by a single amino acid, so that the peptide primary sequence and position of the phosphorylated modifications can be determined. This necessitates peptide bond cleavage that is not only specific to the peptide backbone, but is robust enough to elucidate differences in peptides whose primary amino acid sequence are the same, yet vary in the site of phosphorylation (e.g., positional isomers) [40].

The dominant NL peak in the fragmentation spectra of phosphopeptides obtained via traditional collisionally induced dissociation (CID) has received much attention [41-43]. The NL peak can easily suppress sequence diagnostic ion peaks causing identification of the peptide to become extremely difficult and sometimes impossible. Since the use of ion traps, currently, as the most common mass spectrometers of performing phosphoproteome analyses, there have been various attempts to combat this specific problem. Modified fragmentation regimes have been introduced, such as (a) NL triggered MS3 or (b) multistage activation (MSA), which alleviate the neutral loss issue. NL MS3 and MSA methods allow fragmenting of the NL peak of the precursor ion further, in order to generate more backbone cleavages. These “extra” generated backbone cleavages, then form more diagnostic sources for peptide sequencing [23,44-46].

Alternatively, Electron transfer dissociation (ETD) and electron capture dissociation (ECD) have also shown great promise since the phosphate group remains attached during and after activation. Many detected phosphopeptides contain multiple Ser/Thr/Tyr (serine, threonine, and tyrosine) residues representing the likelihood that there is more than one possible location for the site of phosphorylation within the peptide. The abundant NL observed in low energy CID can hamper the correct assignment of the phospho-sites in such peptides. Thus, a concerted effort has been made to understand, in detail, the rules of phosphopeptide fragmentation [47-51]. Figures 1 and 2 illustrate the flow-through to identify proteins via proteomics-MS, and different phosphoproteomic strategies to ensure high efficiency for clinical research study, respectively.

**Discovering Biomarkers via OMICS Tools**

MS-based proteomics technologies are capable of identifying hundreds to thousands of proteins in cells, tissues, and biofluids. Proteomics may, therefore, provide the opportunity to elucidate new biomarkers and pathways without a prior known association with a specific disease. However, important obstacles remain.

Additionally, improved biomarkers are of vital importance for cancer detection, diagnosis and prognosis. Significant advances in understanding the molecular basis of disease are being made in genomics, while proteomics will ultimately delineate the functional units of a cell: proteins and their intricate interaction networks and signalling pathways in health and disease. Much progress has been made to characterize thousands of proteins qualitatively and quantitatively in complex biological systems by use of multi-dimensional sample fractionation strategies, MS and protein micro-arrays. Comparative/quantitative analysis of high-quality clinical biospecimen (e.g., tissue and biofluids) of the human cancer proteome landscape can potentially reveal protein/peptide biomarkers responsible for this disease by means of their altered levels of expression, PTMs as well as different forms of protein variants. Despite technological advances in proteomics, major hurdles still exist at every step of the biomarker development pipeline [52-63].

The field of proteomics, in the post-genome era, incited great interest in the pursuit of protein/peptide biomarker discovery especially since MS demonstrated the capability of characterizing a large number of proteins and their PTMs in complex biological systems, in some instances even quantitatively. Technological advances, such as protein/antibody chips, depletion of multiple high abundance proteins by affinity columns, and affinity enrichment of targeted protein analytes, as well as multidimensional chromatographic fractionation, have all expanded the dynamic range of detection for low abundance proteins by several orders of magnitude in serum or plasma, making it possible to detect the more abundant disease-relevant proteins in these complex biological matrices [63-71]. Nevertheless, plasma and cell-extract based discovery research studies aimed at identifying low abundance proteins (e.g some kinases) are extremely difficult. Therefore, it is necessary to develop significant technological improvements related to identifying these low abundance, yet high biological
impact molecules. Furthermore, if these protein kinases to be studied contain PTMs, it is important to know that spatial and temporal factors can decrease the efficiency of our study (e.g., many kinases are regulated by phosphorylation of the activation loop, which then directly reflects cellular kinase activity).

Moreover, proteomics has been widely applied in various areas of science, ranging from the deciphering of molecular pathogenesis of diseases, the characterization of novel drug targets, to the discovery of potential diagnostic and prognostic biomarkers, where technology is capable of identifying and quantifying proteins associated with a particular disease by means of their altered levels of expression [72-74] and/or PTMs [75-77] between the control and disease states (e.g., biomarker candidates). This type of comparative (semi-quantitative) analysis enables correlations to be drawn between the range of proteins, their variations and modifications produced by a cell, tissue and biofluids and the initiation, progression, therapeutic monitoring or remission of a disease state.

PTMs including phosphorylation, glycosylation, acetylation and oxidation, in particular, have been of great interest in this field as they have been demonstrated as being linked to disease pathology and are useful targets for therapeutics.

In addition to MS-based large-scale protein and peptide sequencing, other innovative approaches including self-assembling protein microarrays [78] and bead-based flow cytometry [79,80] to identify and quantify proteins and protein-protein interaction in a high throughput manner, have furthered our understanding of the molecular mechanisms involved in diseases.

Utilities of Matrix-assisted laser desorption/ionization tissue imaging MS
Matrix-assisted laser desorption/ionization (MALDI) tissue imaging mass spectrometry is particularly promising among the numerous applications of mass spectrometry. It is used for testing and analyzing the spatial arrangement of a wide range of molecules including proteins, peptides, lipids, drugs and metabolites, directly in thin
slices of tissue. In the field of proteomics, the technology avoids tedious and time-consuming extraction and fractionation steps classically required for sample analysis. Furthermore, MALDI imaging MS is increasingly recognized as a powerful method for clinical proteomics, particularly in cancer research. This recent technology has particular potential for the discovery of new tissue biomarker candidates, for classification of tumors, early diagnosis or prognosis, elucidating pathogenesis pathways and therapy monitoring. Over recent years, MALDI imaging MS has been used for molecular profiling and imaging directly in male and female reproductive tissues.

In summary, the wealth of advances in MS imaging will inevitably attract experts in OMICS (e.g. genomics, proteomics, bioinformatics) and clinicians, as the advantages and power of this technology become more widely known. In addition, it is important to point out for efficient clinical studies, that the identification of protein biomarkers in easily accessible biological fluids has potential for the development of minimally invasive procedures for early diagnostics, but the analysis of body fluids such as plasma, serum and urine is complicated by their wide dynamic range of protein expression, the variation in their composition and their sensitivity to sample handling [81-83].

Concluding remarks and future needs
Phosphoproteomics is a branch of proteomics that identifies, catalogs, and characterizes proteins containing a phosphate group as a PTM. Furthermore, phosphoproteomics

Figure 2 Flow-through of Current Phospho-proteomic Analysis. Using phosphoenrichments (e.g. IMAC, TiO2, SIMAC) we are capable of isolating phosphorylated peptides and discard un-phosphopeptides. The isolated phosphopeptides have to be cleaned and desalted via chromatography (e.g. POROS R3, Disks C18 or graphite, which isolate hydrophilic peptides) before the MS analysis. Finally, the desalted and cleaned phosphopeptides are injected into the mass spectrometer. Different types of ionization can be used (e.g. Matrix-Assisted Laser Desorption/Ionization MALDI or ElectroSpray Ionization ESI). Also, different kinds of fragmentations can be used (e.g. CID, ETD, ECD). In addition, different MS modes can be useful, for example: MS/MS, MSA, MS3NL. As a general rule, positive MS mode is currently more efficient than negative mode for phosphoproteomic studies. It is always necessary to test and combine different phosphoenrichments together with different MS strategies to recover and identify the maximum level of phosphopeptides. This will imply a high efficiency for your clinical research study. The resulting data (phosphorylated proteins identified) must be coupled to bioinformatic tools (software) in order to improve the biological understanding.
provides clues on which protein or pathway might be activated because a change in phosphorylation status almost always reflects a change in protein activity. Indeed, it can indicate which proteins might be potential drug targets as exemplified by the kinase inhibitor. While phosphoproteomics will greatly expand knowledge about the numbers and types of phosphoproteins, its greatest promise is the rapid analysis of entire phosphorylation based signalling networks. Nevertheless, methodologies to enrich for phosphorylated residues from kinases should be improved, especially due to their low abundance of those signalling molecules within cells.

To summarize, clinical proteomics-MS has come a long way in the past decade in terms of technology/platform development, protein chemistry, and together with bioinformatics and other OMICS tools to identify molecular signatures of diseases based on protein pathways and signalling cascades. Hence, there is great promise for disease diagnosis, prognosis, and prediction of therapeutic outcome on an individualized basis. In addition, imaging MS will have a major impact in reproductive research by opening new avenues to the understanding of various molecular mechanisms and the diagnosis of reproductive pathologies. However, and as a general rule, without correct study design, strategy and implementation of robust analytical methodologies, the efforts, efficiency and expectations to make biomarkers (especially phosphorylated kinases) a useful reality in the near future, can easily be hampered.

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Authors’ contributions
Authors EL, SRM, JLP and LM carried out Clinical Proteomics-MS studies for this short-review, in order to develop future Clinical Proteomic-Phosphoproteomic MS research studies and publish this article. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. Lopez E, Wesselink JJ, Lopez I, Mendieta J, Gomez-Puertas P, Rodriguez-Munoz S: Technical Phosphoproteomic and Bioinformatic Tools useful in Cancer Research. J Clin Bioinforma 2011, 1:26. doi:10.1186/2043-9113-1-26.
2. Dengjel J, Akimov V, Olsen JV, Bunkenborg J, Mann M, Blagoev B, Andersen JS: Quantitative proteomic assessment of very early cellular signalling events. Nat Biotechnol 2007, 25:566-568.
3. Kruger M, Kratchmarova I, Blagoev B, Tseng YH, Kahn CR, Mann M: Dissection of the insulin signalling pathway via quantitative phosphoproteomics. Proc Natl Acad Sci USA 2008, 105:2451-2456.
4. Pan C, Olsen JV, Daub H, Mann M: Global effects of kinase inhibitors on signalling networks revealed by quantitative phosphoproteomics. Mol Cell Proteomics 2009, 8(12):2796-808.
5. Daub H, Olsen JV, Barlein M, Gnad F, Oppermann FS, Komer R, Greff Z, Keri G, Steimann O, Mann M: Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol Cell 2008, 31:438-448.
6. Mertins P, Ebel HC, Renkawitz J, Olsen JV, Tremblay ML, Mann M, Ullrich A, Daub H: Investigation of protein-tyrosine phosphatase 18 function by quantitative proteomics. Mol Cell Proteomics 2008, 7:1763-1777.
7. Hilger M, Bonaldi T, Gnad F, Mann M: Systems-wide analysis of a phosphatase knock down by quantitative proteomics and phosphoproteomics. Mol Cell Proteomics 2009, 8(1):1908-1920.
8. Dolado I, Nebreda AR: AKT and oxidative stress team up to kill cancer cells. Cancer Cell 2008, 14(6):427-429.
9. Malumbres M, Barbacid M: To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001, (1):222-231.
10. Swat A, Dolado I, Rojas JM, Nebreda AR: Cell density-dependent inhibition of epidermal growth factor receptor signalling by p38alpha mitogenactivated protein kinase via Sprouty2 downregulation. Mol Cell Biol 2009, 29(12):3332-3343.
11. Ventura JJ, Tenbaum S, Perdiguero E, Huth M, Guerra C, Barbacid M, Pasparakis M, Nebreda AR: p38alpha MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. Nat Genet 2007, 39(6):750-758.
12. Hult J, Sayama K, Chung S, Keren R, Agiostratidou G, Shan W, Dong X, Williams TM, Lisanti MP, Knudsen K, Hazan RB: N-cadherin signalling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation. Cancer Res 2007, 67(7):3106-3116.
13. Chen LL, Ji Y, Xu JF, Lu SH, Hou YY, Hou J, Suje A, Zeng HY, Yan YS: Focal nodular hyperplasia of liver: a clinicopathologic study of 238 patients. Zhonghua Bing Li Xue Za Zhi 2011, 40(1):17-22, Chinese.
14. Muto L, Arbini AA, Marea E, Greco M: Constitutive activation of MAP/ERK inhibits prostate cancer cell proliferation through upregulation of BRC2. Int J Oncol 2007, 31(1):217-224.
15. Zebrisch A, Czenilfofsky AP, Keri G, Smigelskaite J, Sill H, Troppmair J: Signalling through RAS-RAF-MEK-ERK: from basics to bedside. Curr Med Chem 2007, 14(5):601-623.
16. Matsuura K, Nohno Y, Hijya N, Uchida T, Tsukamoto Y, Moriyama M: Extracellular signal-regulated protein kinase is activated in cervical intraepithelial neoplasms but inactivated in invasive cervical carcinoma. Pathol Int 2006, 56(7):368-374.
17. Gerke V, Moos SF: Annexins: from structure to function. Physiol Rev 2002, 82(2):331-371.
18. Alldridge LC, Harris HJ, Plevin R, Hannon R, Bryant CE: The annexin protein lipocortin 1 regulates the MAP/ERK pathway. J Biol Chem 1999, 274(53):37620-37628.
19. Sudo T, Hidaka H: Regulation of calcyclin (S100A6) binding by alternative splicing in the N-terminal regulatory domain of annexin XI isoforms. J Biol Chem 1998, 273(11):6351-6357.
20. Araki R, Fukumura R, Fujimori A, Taya Y, Shishk Y, Kurimasa A, Burma S, Li GC, Chen DJ, Kato K, Hoki Y, Tatsumi K, Abe M: Enhanced...
phosphorylation of p53 serine 18 following DNA damage in DNA-dependent protein kinase catalytic subunit-deficient cells. Cancer Res 1999, 59(15):3543-3546.

21. McLachlin DT, Chart BT. Analysis of phosphorylated proteins and peptides by mass spectrometry. Curr Opin Chem Biol 2001, 5(5):591-602.

22. Flaccaro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat Biotechnol 2002, 20(3):301-305.

23. Gruhlker A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen OH. Quantitative phosphoprotein applications to the yeast phosphoregulation signalling pathway. Mol Cell Proteomics 2005, 4(3):310-327.

24. Pande A, Fernandez MM, Steen H, Blagoev B, Nielsen MM, Roche S, Mann M, Lodish HF. Identification of a novel immunoreceptor tyrosine-based activation motif-containing molecule, STAM2, by mass spectrometry and its involvement in growth factor and cytokine receptor signalling pathways. J Biol Chem 2000, 275(39):38633-38639.

25. Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol 2002, 20(6):261-268.

26. Larsen MR, Thimholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol Cell Proteomics 2005, 4(7):873-886, Epub 2005 Apr 27.

27. Larsen MR, Cordwell SJ, Roepstorff P. Graphite powder as an alternative or supplement to reversed-phase material for desalting and concentration of peptide mixtures prior to matrix-assisted laser desorption/ionization-mass spectrometry. Proteomics 2002, 2(9):1227-1287.

28. Speicher K, Kolbas O, Harper S, Speicher DW. Systemic analysis of peptide recoveries from in gel digestion for protein identifications in proteome studies. J Biomol Tech 2000, 11:74-86.

29. Martin H, Flandez M, Nombela C, Molina M. Protein phosphatases in MAPK signalling: we keep learning from yeast. Mol Microbiol 2005, 58(1-6):16.

30. Biermann K. Contributions of mass spectrometry to peptide and protein structure. Biomed Environ Mass Spectrom 1988, 161-1299-111.

31. Roepstorff P, Fohlman J. Proposal for a common nomenclature for phosphoproteins. J Mass Spectrom 1993, 18(9):1048-1057.

32. Steen H, Küster B, Mann M. Quadrupole time-of-flight versus mass spectrometry. J Am Soc Mass Spectrom 1993, 4:710-717.

33. Xia Q, Cheng D, Duong DM, Gearing M, Lah JJ, Levey AI, Peng J. Phosphoprotein mapping by mass spectrometry of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. Proteomics 2005, 4(3):782-790.

34. Wiener MC, Sachs JR, Deyanova EG, Yates JA. Multiplexed proteomics for multiplexed cell signalling analysis by Quadrupole Ion Trap Mass Spectrometry. J Proteom Res 2005, 4(3):782-790.

35. Benschop JJ, Mohammed S, Olfaherty M, Heck AJR, Sliper M, Menke FLH. Quantitative Phosphoproteomics of Early Elicitor Signalling in Arabidopsis. Mol Cell Proteomics 2007, 6:1198-1214.

36. Schroeder MJ, Shabanowitz J, Schwartz JG, Hunt DF, Coon JJ. A Neutral Loss Activation Method for Improved Phosphopeptide Sequence Analysis by Quadrupole Ion Trap Mass Spectrometry. J Proteom Res 2004, 7:591-598.

37. Lopez E, Lopez I, Sequi J, Ferreira A. Discovering and validating unknown phosphoproteins. J Clin Bioinforma 2011, 1(1):16.
COX2-EGFR signalling activation as a potential prognostic pathway biomarker. Oncol Colorectal C 2009, 8:110-117.

63. Ramachandran N, Raphael JV, Hainsworth E, Demirkan G, Fuentes MG, Rufus A, Hu Y, LaBaer J. Next-generation high-density self-assembling functional protein arrays. Nat Methods 2008, 5:535-538.

64. Beirne P, Pantelidis P, Charles P, Wells AU, Abraham DJ, Denton CP, Welsh KJ, Shah PL, du Bois RM, Kelleher P. Multiplex immune serum biomarker profiling in sarcoidosis and systemic sclerosis. Eur Respir J 2009, 34:1376-1382.

65. Kelleher MT, Fruwirth G, Patel G, Ofo E, Festy F, Barber PR, Ameer-Beg SM, Voy!novic B, Gillet C, Coo!n A, Keri G, Ellis PA, Ng T. The potential of optical proteomic technologies to individualize prognosis and guide rational treatment for cancer patients. Target Oncol 2009, 4:235-252.

66. Wang P, Whiteaker JR, Paulovich AG. The evolving role of mass spectrometry in cancer biomarker discovery. Cancer Biol Ther 2009, 8:1083-1094.

67. Whiteaker JR, Zhang H, Eng JK, Fang R, Peining BD, Feng LC, Loen!zen TD, Schoenherr RM, Keane JP, Holzman T, Fitzgibbon M, Lin C, Zhang H, Cooke K, Liu T, Camp DG, Anderson L, Watts J, Smith RD, McIntosh MW, Paulovich AG. Head-to-head comparison of serum fractionation techniques. J Proteome Res 2007, 6:828-836.

68. Ermou J, Bouroue A, Gamelin E, Guette C. A proteomic approach for plasma biomarker discovery with iTRAQ labeling and OFFGEL fractionation. J Biomed Biotechnol 2010, 2010:927917, Epub 2009 Nov 1.

69. Nirmalan NJ, Hughes C, Peng J, McKenna T, Langridge J, Cairns DA, Ernoult E, Bourreau A, Gamelin E, Guette C. Initial development and validation of a novel extraction method for quantitative mining of the formalin-fixed, paraffin-embedded tissue proteome for biomarker investigations. J Proteome Res 2010, 10:896-906.

70. Krishnan W, Khan IH, Luciw PA. Multiplexed microbead immunoassays by flow cytometry for molecular profiling: basic concepts and proteomics applications. Crit Rev Biotechnol 2009, 29:29-43.

71. Cha S, Imielinski M, Rejtar T, Richardson EA, Thakur D, Sgro! DC, Karger BL. In situ proteomic analysis of human breast cancer epithelial cells using laser capture microdissection: annotation by protein set enrichment analysis and gene ontology. Mol Cell Proteomics 2010, 9:2529-2544.

72. Anderson KS, Sibani S, Wallstrom G, Guo J, Mendoza EA, Raphael J, Hainsworth E, Mortor WR, Wong J, Park JG, Lokko N, Logvinenko T, Ramachandran N, Godwin AK, Marks J, Engstrom P, Labaer J. Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer. J Proteome Res 2011, 10:835-96.

73. Bateman NW, Sun M, Hood BL, Flint MS, Conradis TP. Defining central themes in breast cancer biology by differential proteomics: conserved regulation of cell spreading and focal adhesion kinase. J Proteome Res 2010, 9:5311-5324.

74. Kristiansen TZ, Harsha HC, Granberg M, Maira A, Pandey A. Differential membrane proteomics using 18O-labeling to identify biomarkers for cholangiocarcinoma. J Proteome Res 2008, 7:4670-4677.

75. An HJ, Lebrilla CB. A glycomics approach to the discovery of potential cancer biomarkers. Methods Mol Biol 2010, 600:199-213.

76. Choudhary C, Mann M. Decoding signalling networks by mass spectrometry-based proteomics. Nat Rev Mol Cell Biol 2010, 11:427-439.

77. Madian AG, Regnier FE. Profiling carboxylated proteins in human plasma. J Proteome Res 2010, 9:1330-1343.

78. Iwabata H, Yoshida M, Komatsu Y. Proteomic analysis of organ-specific post-translational lysine-acetylation and -methylation in mice by use of anti-acetyllysine and -methyllysine mouse monoclonal antibodies. Proteomics 2005, 5:4653-4664.

79. Ceroni A, Sibani S, Baker A, Pothinenni VR, Bailer SM, LaBaer J, Haas J, Campbell CJ. Systematic analysis of the IgG antibody immune response against varicella zoster virus (VZV) using a self-assembled protein microarray. Mol Biotechnol 2010, 6:1604-1610.

80. Wong J, Sibani S, Lokko NN, LaBaer J, Anderson KS. Rapid detection of antibodies in sera using multiplexed self-assembling bead arrays. J Immunol Methods 2009, 350:171-182.

81. McDowell LA, Corthals GL, Willems SM, van Remoortere A, van Zeijl RJ, Deelder AM. Peptide and protein imaging mass spectrometry in cancer research. J Proteomics 2010, 73:1921-1944.

82. Seeley EH, Caprioli RM. MALDI imaging mass spectrometry of human tissue: method challenges and clinical perspectives. Trends Biotechnol 2011, 29:136-143.

83. Hays JL, Kim G, Guroiu I, Kohn EC. Proteomics and ovarian cancer: integrating proteomics information into clinical care. J Proteomics 2010, 73:1864-1872.