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Phospho-onco-proteomics

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Abstract- Phosphoproteomics is the global analysis of protein phosphorylation, holds great promise for the discovery of cell signaling events that link changes in dynamics of protein phosphorylation to the progression of various diseases, particularly cancer and diabetes. Proteomic research first came for research with the introduction of two-dimensional gel electrophoresis. Proteomics has been increasingly applied to oncology research with the wide-spread introduction of mass spectrometry and protein-chip. Applying proteomics to foster an improved understanding of cancer pathogenesis develop new tumor biomarkers for diagnosis, and early detection using proteomic portrait of samples. The study of Phospho-onco-proteomics provides a better understanding of cancer diagnosis.
Keywords- Phosphoproteomics, Phosphorylation, Analytical technologies, transcriptomics

1. Introduction

Phosphoproteomics is a branch of proteomics that identifies, catalogs, and characterizes proteins containing a phosphate group as a post-translational modification [1]. Phosphorylation is a key reversible modification that regulates protein function, subcellular localization, complex formation, degradation of proteins and therefore cell signaling networks. Compared to expression analysis, phoshoproteomics provides two additional layers of information. First, it provides clues on what protein or pathway might be activated because a change in phosphorylation status almost always reflects a change in protein activity. Second, it indicates what proteins might be potential drug targets as exemplified by the kinase inhibitor Gleevec (STI571/ Imatinib). 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 [2]. Phosphorylation of proteins on specific amino acid residues is a key regulatory mechanism in cells. Protein phosphorylation controls many basic cellular processes, such as cell growth, differentiation, migration, metabolism, and cell death, and is in itself regulated by the activity of kinases and phosphatases. Protein kinases are one of the largest gene families in humans and mice, accounting for 1.7% of the human genome [3, 4], and up to 30% of all proteins may be phosphorylated [5]. Identification of differentially phosphorylated proteins by means of phoshoproteomics therefore increases our insight into the signal transduction pathways that are activated in cells in response to different stimuli, such as growth factor stimulation or exposure to toxicants.

History of Phospho-onco-proteomics

Oncoproteomics is the study of proteins and their interactions in a cancer cell by proteomic technologies. Proteomic research first came to the fore with the introduction of two-dimensional gel electrophoresis. At the turn of the century, proteomics has been increasingly applied to cancer research with the wide-spread introduction of mass spectrometry and protein-chip. There is an intense interest in applying proteomics to foster an improved understanding of cancer pathogenesis, develop new tumor biomarkers for diagnosis, and early detection using proteomic portrait of samples [6]. The analysis of the entire complement of phosphorylated proteins in a cell is certainly a feasible option. This is due to the optimization of enrichment protocols for phosphoproteins and phosphopeptides, better fractionation techniques using chromatography, and improvement of methods to selectively visualize phosphorylated residues using mass spectrometry. Although the current procedures for phosphoproteomic analysis are greatly improved, there is still sample loss and inconsistencies with regards to sample preparation, enrichment, and instrumentation. Bioinformatics tools and biological sequence databases are also

necessary for high-throughput phosphoproteomic studies [7]. The Biological General Repository for Interaction Datasets (BioGRID) database (http://www.thebiogrid.org) was developed to house and distribute collections of protein and genetic interactions from major model organism species. BioGRID currently contains over 1,98,000 interactions from six different species, as derived from both high-throughput studies and conventional focused studies [8]. Phospho-onco-proteomics has the potential to revolutionize clinical practice, including cancer diagnosis and screening based on proteomic platforms as a complement to histopathology, individualized selection of therapeutic combinations that target the entire cancer-specific protein network, real-time assessment of therapeutic efficacy and toxicity, and rational modulation of therapy based on changes in the cancer protein network associated with prognosis and drug resistance. Besides, it is also applied to the discovery of new therapeutic targets and to the study of drug effects. In pace with the successful completion of the Human Genome Project and proteome project, the wave of proteomics has raised the curtain on the postgenomic era. The study of Phospho-onco-proteomics provides mankind with a better understanding of neoplasia and tumorogenesis.

The Human Oncogenome Project

The emergence of genomics has changed the way we need to think about cancer in radical ways. When the draft sequence of the human genome was published in 2001, we realized that no one had attempted to assemble the broad expertise to educate working scientists and clinicians, graduate and medical students, advanced practice nurses, genetic counselors, and health educators across all of the cancer-relevant disciplines that have been altered by genomics. Cancer is not a single genetic disease but hundreds of diseases consisting of different combinations of genetic alterations. Several types of genetic alterations contribute to neoplastic transformation. Mutator genes that control the fidelity of genome maintenance and checkpoint genes responsible for quality control in cell division cycles are lost. Oncogenes are activated and tumor suppressor genes are lost. To consider the types of alterations required to effect neoplastic transformation, it is useful to delineate several of the properties of normal cells. Normal cells correct spontaneously occurring and induced mutations. Normal cells arrest their division cycles when progression would lead to damaged progeny or to mitotic catastrophes. Normal cells divide in harmony with their environments: those in stem cell populations regenerate while terminally differentiated cells in epithelial layers slough off when they are worn out. Normal cells undergo programmed cell death in response to developmental signals and irreparable damage. Normal cells have tightly defined migratory potential [9].

Phospho-proteomics strategies

- A sample large-scale phosphoproteomic analysis includes steps [10].
- Cultured cells undergo SILAC encoding.
- Cells are stimulated with factor of interest (eg. growth factor, hormone).
- Stimulation can occur for various lengths of time for temporal analysis.
- Cells are lysed and enzymatically digested.
- Peptides are separated using ion exchange chromatography.
- Phosphopeptides are enriched using phosphospecific antibodies, immobilized metal affinity chromatography or titanium dioxide (TiO2) chromatography.
- Phosphopeptides are analyzed using mass spectrometry.
- Peptides are sequenced and analyzed.

SILAC (Stable isotope labelling with amino acids in cell culture) is a mass spectrometry-based technique developed to detect differences in protein abundance between two (or more) samples [11]. It is one of the most popular methods for quantitative proteomics. Two populations of cells are cultivated in cell culture. One of the cell populations is fed with growth medium containing normal amino acids. In contrast,

the growth medium of the second cell population contains amino acids labeled with stable (non-radioactive) heavy isotopes. For example, the medium can contain arginine labeled with six carbon-13 atoms (13C) instead of the normal carbon-12 (12C). When the cells are growing in this medium, they incorporate the heavy arginine into all of their proteins. Therefore, all of the arginine containing peptides are now 6 Da heavier than their normal counterparts. The trick is that the proteins from both cell populations can be combined and analyzed together by mass spectrometry. Pairs of chemically identical peptides of different stable-isotope composition can be differentiated in a mass spectrometer owing to their mass difference. The ratio of peak intensities in the mass spectrum for such peptide pairs accurately reflects the abundance ratio for the two proteins. SILAC has emerged as a very powerful method to study cell signaling, protein-protein interaction and regulation of gene expression [12]. Pulsed SILAC is a variation of the SILAC where the labeled amino acids are added to the growth medium for only a short period. This allows monitoring changes in protein expression rather than raw concentration [13].

Phospho-proteomics Technology

Recent advances in mass spectrometry-based proteomics helped to overcome many of the previous limitations in protein phosphorylation analysis. Improved isotope labeling and phosphopeptide enrichment strategies in conjunction with more powerful mass spectrometers and advances in data analysis have been integrated in highly efficient phosphoproteomics workflows, which are capable of monitoring up to several thousands of site-specific phosphorylation events within one large-scale analysis. Combined with ongoing efforts to define kinase-substrate relationships in intact cells, these major achievements have considerable potential to assess phosphorylation-based signaling networks on a system-wide scale [14].

Current methods for analysis of the phosphoproteome rely heavily on mass spectrometry and 'phosphospecific' enrichment techniques. Emerging technologies that are likely to have important impacts on phosphoproteomics include protein [15] and antibody [16] microarrays, and fluorescence-based single-cell analysis [17]. While these methods have the potential for high sensitivity and high throughput, they require prior knowledge of particular phosphoprotein targets. In contrast, mass-spectrometry-based approaches both allow large-scale analysis and provide the ability to discover new phosphoproteins. The speed, selectivity, and sensitivity of mass spectrometry also provide important advantages over biochemical methods for the analysis of protein phosphorylation [18-20]. Because many phosphoproteins, especially signaling intermediates, are low-abundance proteins phosphorylated at sub-stoichiometric levels, a considerable amount of effort has been devoted to the development of phosphospecific enrichment methods that are compatible with, or directly coupled to, mass spectrometry. These methodological approaches have been described in a number of recent reviews [18, 19, 21-24].

With the recent advances in phosphoproteomic techniques, the large-scale identification of kinase substrates, including their phosphorylation sites, is finally possible. Studies in mainly non-plant systems have demonstrated the high potential of this method by uncovering numerous novel phosphorylation events. There have been also recent developments in the field of phosphoproteomics that are based on phosphopeptide isolation from complex mixtures by immobilized metal-affinity chromatography coupled to sequence identification by mass spectrometry. Combination of these methods with labeling techniques now allows quantitative analysis of phosphorylation between different samples [25].

5.1. Analytical technologies: separation techniques

5.1.1 2D PAGE

Detection of phosphorylated proteins within complex mixtures is usually not possible without prior separation of the proteins such as 2-DE [26]. Phosphoproteins can be visualized [27] either directly in 2-D gels using phosphospecific stains [28] or by western blotting techniques [29]. The most sensitive method is radioactive labelling of the phosphate-groups using 32P or 33P and

subsequent radioimmunoblotting [30, 31]. A major advantage of these techniques is that all kinds of phosphorylations are detected and signals can be quantitated absolutely. Introduction of radioactive phosphate-groups can be done in vivo or in vitro. The latter is carried out by incubating the respective protein or protein mixture with a chosen kinase and [γ-32/33P]-ATP. After an appropriate incubation time the sample can be subjected to downstream analysis steps. Throughout the purification and/or separation process phosphoproteins can be traced by observation of the Cerenkov-radiation. Thus, this technique is also suitable for non-gel-based separations. Major drawbacks of such in vitro labelling techniques are unspecific phosphorylations due to very high reagent concentrations and reaction conditions.

5.1.2 HPLC

5.1.2.1 IMAC

Immobilized metal-ion affinity chromatography (IMAC) was originally introduced [32] for purification of His-tagged proteins and is the most frequently applied technique for phosphopeptide and -protein enrichment nowadays [33]. Thereby, phosphorylated peptides and proteins are bound to the stationary phase by electrostatic interactions with positively charged metal-ions that are on their part bound to the column material via iminodiacetic acid (IDA), nitriloacetic acid (NTA) or Tris-(carboxymethyl)-ethylendiamine (TED) linkers. Unphosphorylated species can be washed away and the phosphopeptides and/or –proteins may be eluted by salt- and/or pH-gradients. Unfortunately, predominantly multiply phosphorylated peptides are enriched this way and also very acidic peptides are bound to the column [34]. The latter problem can be overcome by prior esterification of the acidic side chains of glutamate and aspartate residues [35] using HCl-saturated, dried methanol [36]. However, reaction conditions have to be chosen carefully to avoid both incomplete esterification and side reactions for instance with asparagines because they increase sample complexity and interfere with subsequent mass spectrometric analysis. Thus, various metal-ions such as Fe31, Ga31, Al31 or Zr41 have been used for better selectivity and phosphopeptide recovery [37, 38]. Especially Ga31-ions have proven well [39] in different studies but Fe31-based methods are still used more often. The good compatibility of IMAC-procedures to subsequent separation and detection techniques such as CE [40], LC-MS/MS [41, 42] and direct MALDI-MS of phosphopeptides bound to IMAC-beads on the target [43], will grant this technique even wider spreading.

5.1.2.2 Hydroxy Acid-Modified Metal Oxide Chromatography (HAMMOC)

A number of phosphopeptide isolation methods have been developed including immobilized metal affinity chromatography (IMAC) and metal oxide chromatography (MOC) using titania (titanium dioxide) and zirconia (zirconium dioxide). However, these methods show different selectivity and isolate a different set of phosphopeptides. Therefore, it is necessary to use these methods complementary to obtain wider coverage of phosphoproteome at this moment [44]. A previously developed a phosphopeptide enrichment method using MOC modified with aliphatic hydroxy acids (HAMMOC) [45]. These approaches made it possible to enrich phosphopeptides directly from cell lysates. As expected, HAMMOC with titania/zirconia enriches phosphopeptides with different physico-chemical properties from those by IMAC, especially in terms of the number of the phosphorylated residues per peptide. This technique has been further optimized for phosphopeptides [46].

5.2. Analytical technologies: detection techniques

5.2.1 Western-blotting

Western-blotting using phosphospecific antibodies is also widely used [47] and is able to detect very low amounts – few femtomoles – of phosphoproteins but specificity and sensitivity of this method is strongly dependent on the respective antibodies [48]. Various phosphotyrosine antibodies of good specificity are available and only little cross-reactivity to unphosphorylated tyrosine or serine-/threonine-phosphorylated residues is observed [49, 50]. However, phosphoserine and –threonine specific antibodies are mostly dependent on consensus sequences

in addition to the phosphorylated residue and are thus more likely to be unable to access a phosphorylation site due to steric hindrance. Therefore, global approaches for the identification of Ser/Thr-phosphorylation sites from 2-D gels have to be accomplished in combination with other methods.

5.2.2 Mass spectrometry (MS)

Mass spectrometry is currently the best method to adequately compare pairs of protein samples. The two main procedures to perform this task are using isotope-coded affinity tags (ICAT) and stable isotopic amino acids in cell culture (SILAC). In the ICAT procedure samples are labeled individually after isolation with mass-coded reagents that modify cysteine residues. In SILAC, cells are cultured separately in the presence of different isotopically labeled amino acids for several cell divisions allowing cellular proteins to incorporate the label. Mass spectrometry is subsequently used to identify phosphoserine, phosphothreonine, and phosphotyrosine-containing peptides [51]. Quantitative methods for mass spectrometry-based phosphoproteomics rely on the use of heavy isotopes and fall into three general categories: in vitro labeling of phosphoamino acids, in vitro labeling of proteins and peptides, and in vivo metabolic labeling. The basic principle of all three involves labeling peptides from one sample (control cells, for example) with a heavy isotope. This sample is then mixed with an unlabeled sample (from stimulated cells, for example) and the two are analyzed simultaneously. The ability of mass spectrometers to resolve the normal and isotopically labeled versions of the same peptide allows direct comparison of the amount of peptide in each sample. If the labeled peptide is a phosphopeptide, this method can be used to determine changes in the level of phosphorylation [52].

Identification of phosphorylated residues within proteins is mostly done by MS these days. For this purpose, “bottom-up” approaches dealing with peptides derived from protein digests are far wider spread than “top down” techniques referring to whole proteins [53]. Phosphorylation analysis of entire proteins revealing the overall modification state is mostly done by FT-ICR-MS because of its superior resolution and mass accuracy [54]. Both MALDI- and ESI-sources may be used but usually localization of the phosphorylation sites is easier using doubly or triply charged ions in MS/MS-mode as produced by ES-ionization. Furthermore, FT-ICR-MS is the only mass spectrometric technique capable of electron capture dissociation (ECD) [55]. ECD measurements are very suitable for the analysis of protein modifications usually labile in MS/MS-experiments; the peptide backbone is cleaved upon electron capture yielding c- and z-ions [56] rather than b- and y-ions produced by collisionally induced dissociation (CID). However, modifications such as phosphorylations remain intact in ECD-experiments. This is particularly interesting in comparison to IRMPD-(infrared multiphoton dissociation)-MS/MS-spectra yielding fragmentation patterns similar to CID-experiments [57]. Nevertheless, “top-down” analysis of phosphoproteins has also been done successfully via IT MS/MS using ion/ion-reactions for reduction of charge state and subsequent CID experiments [58]. Recently, an alternative to the ECD method called electron transfer dissociation (ETD) was established using a modified linear IT-system yielding fragmentation patterns similar to ECD [59]. Thereby, electrons are transferred to the protein/peptide ions from anions generated by a chemical ionization source with methane buffer gas. The possibility to use this technique in IT-MS will surely affect the analysis of PTMs in the near future. A new method combining chemical modification and affinity purification is described for the characterization of serine and threonine phosphopeptides in proteins. The method is based on the conversion of phosphoserine and phosphothreonine residues to S-(2-mercaptoethyl) cysteinyl or beta-methyl-S-(2-mercaptoethyl)cysteinyl residues by beta-elimination/1,2-ethanedithiol addition, followed by reversible biotinylation of the modified proteins. After trypsin digestion, the biotinylated peptides were affinity-isolated and enriched, and subsequently subjected to structural characterization by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Database searching allowed for automated identification of modified residues that were originally phosphorylated. The applicability of the method is demonstrated by the identification of all known phosphorylation sites in a mixture of

alpha-casein, beta-casein, and ovalbumin. The technique has potential for adaptations to proteome-wide analysis of protein phosphorylation [60]. Stable isotope-based quantitative MS is applied to globally monitor the kinetics of complex, ordered phosphorylation events on protein players in the canonical mitogen-activated protein kinase signaling pathway. In excellent agreement with activity assays and phosphospecific immunoblotting with the same samples, the epidermal growth factor-induced changes in nine phosphorylation sites in the extracellular signal-regulated kinase (ERK)/p90 ribosomal S6 kinase-signaling cassette was quantified. Additionally, 14 previously uncharacterized and six known phosphorylation events after phorbol ester stimulation in the ERK/p90 ribosomal S6 kinase-signaling targets, the tuberous sclerosis complex (TSC) tumor suppressors TSC1 and TSC2 were monitored. By using quantitative phosphorylation profiling in conjunction with pharmacological kinase inhibitors a ERK-independent, protein kinase C-dependent pathway to TSC2 phosphorylation was uncovered. These results establish quantitative phosphorylation profiling as a means to simultaneously identify, quantify, and delineate the kinetic changes of ordered phosphorylation events on a given protein and defines parameters for the rapid discovery of important in vivo phosphoregulatory mechanisms [61]. Phosphopeptide identification and site determination are major challenges in biomedical MS. Both are affected by frequent and often overwhelming losses of phosphoric acid in ion trap CID fragmentation spectra. These losses are thought to translate into reduced intensities of sequence informative ions and a general decline in the quality of MS/MS spectra. To address this issue, several methods have been proposed, which rely on extended fragmentation schemes including collecting MS3 scans from neutral loss-containing ions and multi-stage activation to further fragment these same ions. this has been evaluated by the utility of these methods in the context of a large-scale phosphopeptide analysis strategy with current instrumentation capable of accurate precursor mass determination. Remarkably, it was found that MS3-based schemes did not increase the overall number of confidently identified peptides and had only limited value in site localization. We conclude that the collection of MS3 or pseudo-MS3 scans in large-scale proteomics studies is not worthwhile when high-mass accuracy instrumentation is used [62].

5.3. Phospho-proteomics in practice

Phosida allows retrieval of phosphorylation data of any protein of interest. It lists phosphorylation sites associated with particular projects and proteomes or, alternatively, displays phosphorylation sites found for any protein or protein group of interest. In addition, structural and evolutionary information on each phosphoprotein and phosphosite is integrated.Importantly, Phosida links extensive peptide information to the phosphorylation sites, such as several peptides implicating the same site and temporal profiles of each site in response to stimulus (e.g., EGF stimulation). A phosphorylation site predictor was constructed from the phosphorylation sites of large-scale study on human phosphorylation sites on the basis of a support vector machine (SVM). To create a negative set of the same size,randomly sites were chosen from human proteins that were not present in the phosphoset. SVMs attempt to partition true from false sites by separating them in a high dimensional vector space with the help of hyperplanes and kernel functions. The primary sequence comprised the site and its twelve surrounding residues as features.The accuracies of the prediction based on primary sequences are very high Phosphoserines: 90% and Phosphothreonines: 75% [63].

Quantitative proteomics methods have been used in a targeted way to monitor phosphorylation of individual proteins. A combination of SILAC and IMAC was used to analyze agonist-induced phosphorylation of the 2-adrenergic receptor [64]. The simultaneous monitoring of multiple sites allowed identification of the relevant in vivo phosphorylation sites and the discovery that different agonists (for example, isoproterenol and dopamine) induce differential phosphorylation of individual sites. SILAC was also used to monitor in vivo the kinetics

of EGF-induced phosphorylation of six phosphotyrosine residues in the EGF receptor [65]. The results showed that the kinetics of phosphorylation of the tyrosine residues correlated with the preferential association of the receptor with individual binding partners, such as growth factor receptor bound protein 2 (Grb2) and Src homology 2 domain-containing transforming protein (Shc).

5.4. Technology development in Phospho-proteomics and approaches

5.4.1 Phospho-proteomics in signal transduction

A major application of quantitative phosphoproteomics has been in studying the dynamics of phosphorylation and the assembly of signaling complexes. A combination of SILAC and anti-phosphotyrosine immunoprecipitation was used to examine phosphotyrosine-dependent signaling networks induced by EGF stimulation of HeLa cells [66]. Of the 202 proteins detected, which were either phosphotyrosine proteins or proteins that co-precipitated with phosphotyrosine proteins, the levels of 81 were elevated by 1.5-fold or more following EGF stimulation. In addition to monitoring the activation of tyrosine phosphorylation, these experiments detected and quantitated proteins that associate with phosphotyrosine proteins through Src homology 2 (SH2) domains and other binding motifs. For example, temporal changes in the phosphorylation of the EGF receptor correlated with the co-precipitation of proteins known to interact with it, such as Grb2 and Shc. While nearly all of the proteins known to be associated with EGF receptor signaling were identified in these experiments, many additional proteins that were not previously known to be associated with this pathway were also identified. For example, the time-dependent recruitment of a set of RNA-binding proteins suggested a novel role for EGF receptor signaling in mRNA processing and transport. Six novel EGF-dependent proteins with no known function were also identified in these experiments. Quantitative mass spectrometry was used to compare the time courses of their association with the anti-phosphotyrosine complex with the time course of EGF receptor activation; this comparison allowed the assignment of functions for these proteins in early, membrane-proximal events or in later events such as cytoskeletal reorganization or endosomal trafficking.

Recent advances in mass spectrometry-based proteomics helped to overcome many of the previous limitations in protein phosphorylation analysis. Improved isotope labeling and phosphopeptide enrichment strategies in conjunction with more powerful mass spectrometers and advances in data analysis have been integrated in highly efficient phosphoproteomics workflows, which are capable of monitoring up to several thousands of site-specific phosphorylation events within one large-scale analysis. Combined with ongoing efforts to define kinase-substrate relationships in intact cells, these major achievements have considerable potential to assess phosphorylation-based signaling networks on a system-wide scale [67].

5.4.2 Phospho-proteomics and transcriptomics

This emerging field has changed numerous static pathways into dynamic signaling networks, and revealed protein kinase networks that underlie adaptation to environmental stimuli. Mass spectrometry enables high-throughput and high-quality analysis of differential phosphorylation at a site-specific level. Although determination of differential phosphorylation between treatments is analogous to detecting differential gene expression, the large body of statistical techniques that has been developed for analysis of differential gene expression is not generally applied for detecting differential phosphorylation. We suggest possible improvements for analysis of quantitative phosphorylation by increasing the number of biological replicates and adapting statistical tests used for gene expression profiling and widely implemented in freely available software tools [68].

Applications of Phospho-proteomics in oncogenomics

The challenge to use obtained networks for novel drug development has been initiated. Mutations in RTKs are involved in many cancers and are successful drug targets [69]. This allows dissection of drug actions and perhaps aid in further drug optimization and development. EGFR and c- MET are

RTKs that share part of an extensive signaling network involving many tyrosine phosphorylation events, which collapse by application of their inhibitors [70]. EGFRvIII is a hyperactive EGF receptor mutant that plays an important role in glioblastoma (aggressive brain tumor) behavior, and confers resistance against the EGFR inhibitor gefitinib. Huang et al. [71] dissect the phosphotyrosine signaling network triggered by EGFRvIII. The authors carefully examined the network and identified c-MET as being activated in EGFRvIII-carrying glioblastomas. The c-MET amplification bypasses inhibition of hyperactive EGFR mutants by gefitinib through activating a cell survival pathway [72]. Resistance against EGFR inhibitors occurs in most glioblastomas and lung cancers with activating EGFR mutations, but can be defeated by a combinatorial application of both EGFR and c- MET inhibitors [74]. Thus, chemoresistance can be caused by coexpression of different active RTKs, and phosphorylation profiling of RTKs in cancer cells can reveal targets for such therapies [73]. These systematic approaches are thus capable of providing handles to combat cancer [75]. Many chemical kinase inhibitors have been used to treat cancers. Although several have been used successfully for many years now, major questions have remained about the mechanisms underlying side effects and drug resistance. Quantitative analysis of kinase inhibitors has recently shown that most clinically used kinase inhibitors are aspecific [76]. A large-scale, semi-quantitative approach to profile tyrosine phosphorylation showed the expression of active RTKs in different lung cancers, implicating these RTKs in the disease. As mentioned above, these kinases are attractive targets for therapeutic use. In conclusion, these studies gave first insights into the specificities of these cancer drugs and how they affect downstream signaling pathways and may ultimately serve the treatment of cancers.

Limitations

While phosphoproteomics has greatly expanded knowledge about the numbers and types of phosphoproteins, along with their role in signaling networks, there are still a few limitations to these techniques. To begin with, isolation methods such as anti-phosphotyrosine antibodies do not distinguish between isolating tyrosine-phosphorylated proteins and proteins associated with tyrosine-phosphorylated proteins. Therefore, even though phosphorylation dependent protein-protein interactions are very important, it is important to remember that a protein detected by this method is not necessarily a direct substrate of any tyrosine kinase. Only by digesting the samples before immunoprecipitation can isolation of only phosphoproteins and temporal profiles of individual phosphorylaiton sites be produced. Another limitation is that some relevant proteins will likely be missed since no extraction condition is all encompassing. It is possible that proteins with low stoichiometry of phosphorylation, in very low abundance, or phosphorylated as a target for rapid degradation will be lost [77].

Conclusion

The techniques and research methodology use obtained networks for novel drug development has been initiated. Recent advances in analytical-based proteomics helped to overcome many of the previous limitations in protein phosphorylation analysis. New highly efficient phosphoproteomics workflows are capable of monitoring up to several thousands of site-specific phosphorylation events within one large-scale analysis of oncogenes. The study of Phospho-onco-proteomics provides mankind with a better understanding of oncoproteomics.

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