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Review

Cancer Research

Kinome Profiling of Clinical Cancer Specimens

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Abstract

Over the past years novel technologies have emerged to enable the determination of the transcriptome and proteome of clinical samples. These data sets will prove to be of significant value to our elucidation of the mechanisms that govern pathophysiology and may provide biological markers for future guidance in personalized medicine. However, an equally important goal is to define those proteins that participate in signaling pathways during the disease manifestation itself or those pathways that are made active during successful clinical treatment of the disease: the main challenge now is the generation of large-scale data sets that will allow us to define kinome profiles with predictive properties on the outcome-of-disease and to obtain insight into tissue-specific analysis of kinase activity. This review describes the current techniques available to generate kinome profiles of clinical tissue samples and discusses the future strategies necessary to achieve new insights into disease mechanisms and treatment targets. *Cancer Res; 70(7); 2575–8. ©2010 AACR.*

Background

The past five years have seen an exponential increase in technological development to explore the genome and the proteome to understand the molecular basis of disease. However, identification of the genes and proteins alone is not enough, and the next step is to develop assays that will help understand the function of these proteins.

A protein, once expressed, undergoes numerous post-translational modifications that are vital for its adequate functioning. Phosphorylation by protein kinases is the most widespread and well-studied post-translational modification in eukaryotic cells. It can regulate almost every property of a protein and is involved in all fundamental cellular processes. Understanding protein phosphorylation is very complicated, as approximately one third of all intracellular proteins may be phosphorylated (1). Identification of the kinome, the complement of all kinases in the human genome, by Manning and colleagues has provided an excellent starting point for understanding the scale of the problem (2). Multiple levels of cellular switches intricately regulate kinase activity, and deregulation of its activity usually arises owing to mutations leading to gain-of-function mutant alleles that are usually pathogenic and in some cases tumorigenic. Hence, it is imperative to delineate the complex network of phosphorylation-based signaling for a thorough and therapeutically applicable understanding of the functioning of the cell in a tumorigenic state. Profiling of the kinome will provide a "'snapshot'" of the current state of the cell or tissue as characterized by proteomic and metabolic measurements (3).

In this review we describe and evaluate the various techniques that have recently become available to study the kinomes of clinical tissue samples.

Development of New Technologies

Classically, protein phosphorylation was studied using in-gel kinase assays or Western blot-based gel-shift techniques exploiting the size difference between the phosphorylated and unphosphorylated forms of proteins. These techniques are fairly cumbersome and do not allow the study of large numbers of samples. The situation improved with the arrival of phospho-specific antibodies in the late 1990s, which recognize the phosphorylated forms of proteins but not their unphosphorylated counterparts. By employing these antibodies, phosphorylation events can be detected using classical Western blotting, or enzyme-linked immunosorbent assay (ELISA) formats to allow high-throughput screening for kinase activity-modifying compounds (4), or tissue arrays enabling histological analysis of protein phosphorylation in evaluating hundreds to thousands of relevant tissue samples simultaneously (5). The main drawback remains that only one type of phosphorylation is studied per experiment.

An improvement on using phospho-specific antibodies is the use of multicolor fluorescence-activated cell sorting (FACS) technology to study phosphorylation as applied by Irish and colleagues to study acute myelogenous leukemia (AML). Briefly, peripheral blood samples of patients suffering from AML were stimulated with a range of relevant stimuli, permeabilized, and subjected to intracellular phospho-specific flow cytometry using a panel of phospho-specific antibodies to various phosphorylated proteins (6). Significantly, response-to-treatment-specific network profiles were obtained, and the observation that individual cancers show multiple cell subsets with unique network profiles was a

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dramatic demonstration of the inherent advantages of the single cell approach (Fig. 1). However, the maximum number of antibodies that can be used in one experiment is limited to 10; this has prompted investigators to explore techniques for studying cellular phosphorylation with few *a priori* assumptions about the phosphorylation events involved.

Proteomic approaches in which phospho-proteins are separated on 2-D gels or by chromatography (7), followed by mass spectrometry (MS), are particularly useful, as such approaches provide a powerful way to monitor the expression and regulation of potentially hundreds of proteins simultaneously. But in practice, these approaches are hampered by the fact that the positions of scarcely more than two dozen protein kinases are available on 2-D proteomic maps. This result reflects that, like most signal transduction proteins, protein kinases are present at very minute levels in cells, and are often undetectable by the most sensitive protein stains, whereas procedures based on the purification of phospho-peptides and determination of peptide structure by matrix-assisted laser desorption ionization (MALDI) MS are time consuming but are completely unbiased (8). However, recent advances in instrumentation, algorithms, and preparation techniques have allowed in-depth analysis of the phospho-proteome in solid tumors. For instance, enrichment of peptides containing phosphotyrosine directly from protease digested cellular protein extracts with a phosphotyrosine-specific antibody and are identified by tandem MS. Applying this approach to various tumors can identify activated protein kinases and their phosphorylated substrates (9). Furthermore, Zanivan and colleagues analyzed the phospho-proteome of tumor tissue from a mouse model of melanoma by enrichment of phospho-peptides from solid tumor samples either by a two-step procedure consisting of strong cation exchange chromatography followed by titansphere enrichment (SCX-TiO2) or with TiO2 alone, and identified more than 5,600 phosphorylation sites (Fig. 1; ref. 10). One of the major drawbacks of these techniques is the requirement for large amounts of tissue. Additionally, different numbers of phosphorylation sites can be identified depending upon how the liquid chromatography-MS and/or MS data are analyzed and filtered. Low stringency filters can result in the identification of large numbers of phosphorylation sites, but will also include high numbers of false positives, and hence the choice of filter stringency is very crucial.

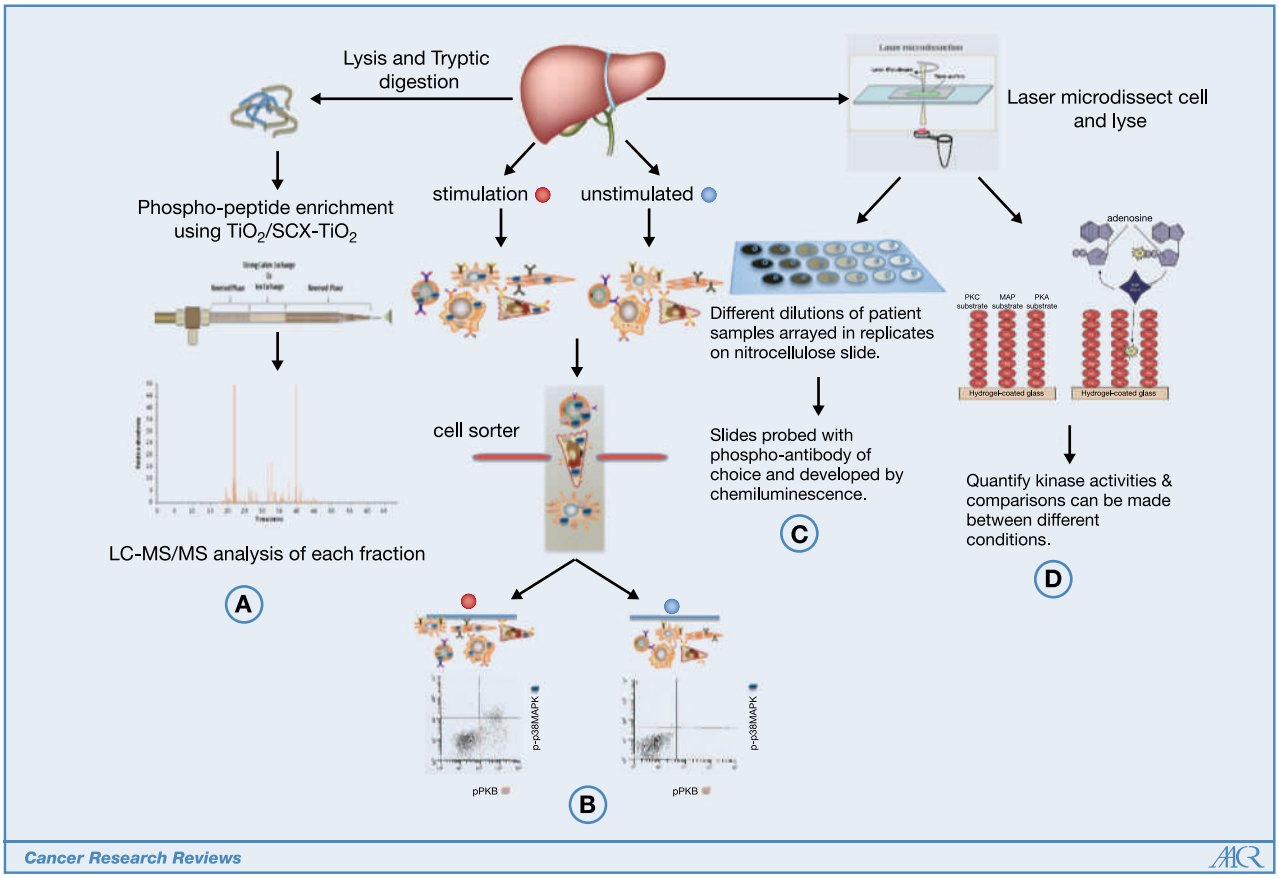

Figure 1. Kinome profiling of clinical tissue samples. A, strategy used for the large-scale identification and characterization of phosphorylation sites from solid tissue and/or tumor. B, strategy used for intracellular flow cytometry technique. C, principle of RPPA. D, principle of the Pepchip technology: Kinase arrays are spotted with peptides that are consensus motifs for the different kinases in the human genome. These arrays allow the direct measurement of the kinase activities.

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In contrast, Felsher and colleagues have developed a nanofluidic proteomic assay (NIA), which separates proteins according to their individual isoelectric points (which also change according to phosphorylation), and the separated proteins are detected with antibodies, which generates a quantifiable chemiluminescent signal. The authors used NIA to assess signaling pathway activation in Burkitt lymphoma samples through the quantitative detection of phosphorylated forms of the extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) signaling pathway (11). The ability to take high-resolution snapshots of the kinome, while at the same time consuming extremely minute amounts of patient sample, is ideal for the analysis of clinical biopsy specimens and micro-dissected tissue sections. It seems that this method is largely limited by the availability of selective monoclonal antibodies and the need to optimize the isoelectric focusing conditions for maximum sensitivity.

Recently, important progress has been made to adapt array technology to measure enzymatic activity in whole cell lysates with the preparation of protein and/or peptide chips for the assessment of protein substrate interactions (12, 13). Another variation on the theme of microarrays is the high-density reverse-phase protein array (RPPA). Herein the arrays are spotted with cellular lysates, and the analytes are quantified by exposing the array with a specific primary antibody. Petricoin and coworkers have successfully applied RPPA analysis of laser capture micro-dissected colorectal cancer tumor specimens to profile broad cell-signaling pathways from patients who presented with liver metastasis versus patients who remained recurrence free after follow-up (Fig. 1; ref. 14). A limiting factor of this technique is the requirement for highly specific antibodies, which are currently available only for a small percentage of the proteins and only a limited number of proteins can be analyzed simultaneously. Additionally, because the technique requires spotting of the patient material, a critical step is the specimen acquisition, which would require optimization to ensure that the phosphorylated isoforms of proteins are kept intact and can be correctly identified. An exhaustive review of this methodology can be found elsewhere (15). Golub and coworkers have developed a bead-based kinase profiling approach that involves coupling of tyrosine kinase antibodies to color-coded polystyrene beads, with one kinase per bead. The beads are subsequently exposed to tissue lysates to isolate the respective tyrosine kinases, following which a second labeled antibody that binds specifically to phosphotyrosine residues is added and detected using flow cytometry. Using this technique, the authors analyzed primary glioblastoma multiforme (GBM) tumor samples from human patients and identified Src as an important target for therapy in GBM (16). The limitation, however, is the requirement for highly specific anti-tyrosine kinase antibodies, and would ideally need to be extended to serine and threonine kinases as well.

Houseman and colleagues showed that employing peptide chips, prepared by the Diels-Alder–mediated immobilization of one kinase substrate (for the tyrosine kinase c-Src) on a monolayer of alkanethiolates on gold, allows quantitative evaluation of kinase activity (17). Another such technique is the solid-phase Bcr-Abl kinase assay in 96-well hydrogel plates developed by Kron and coworkers (18). Hence in principle, an array exhibiting specific consensus sequences for protein kinases across the entire kinome allows a comprehensive detection of signal transduction events in whole cell lysates. Van Baal and colleagues have successfully used this technique to identify several signaling pathways that are specifically active in Barrett's esophagus when compared with adjacent normal epithelia, thereby providing novel insight in the pathogenesis of Barrett's esophagus (19). The advantage of this technique is that it allows a broad view on kinase changes taking place, because there are more than 1,000 peptide substrates spotted on a single microarray. This allows the use of minute amounts of clinical samples, and because each array contains the peptides spotted in triplicate, allows for a more robust experimental setup. This method has also been successfully applied to study the usefulness of c-Met inhibitors in colon cancer (20). An adaptation of this technique makes use of a porous microarray platform that differs from conventional microarray formats in that it allows exploitation of features such as speed and the ability for kinetic measurements. In the PamChip tyrosine kinase profiling system, a sample is constantly pumped up and down through the porous microarray of 144 kinase substrates. The phosphorylation is determined by measuring the association of a fluorescent labeled anti-phosphotyrosine antibody to the substrates phosphorylated by the kinase activities in the sample. Sikkema and colleagues have employed this technique to detect aberrant kinase activity in pediatric brain tumors (21). They identified Src activity as a potential target in pediatric brain tumors for therapeutic intervention.

A potential drawback of all array technology is the variation in the number of potential upstream kinases for the various substrates. Until the issue of specificity is resolved, all substrates potentially sensitive to a kinase of interest should be considered in the analysis, allowing a better interpretation of the results. This analysis, in turn, leads to the requirement for better bioinformatic tools and more reliable annotation of phosphorylation databases. Thus, determining the sensitivity of a substrate for specific kinases will prove to be of tremendous value in future application of the peptide array. Most likely, future application of recombinant and/or purified kinases will tell us more about the specificity of individual peptides, thus making it easier to pinpoint specific kinases on the basis of the obtained peptide array results. Therefore, generating hypotheses is the limit of what is possible at the current developmental stage of peptide microarray technology.

Conclusions

Applications of the technologies described in this review provide a powerful toolkit to enhance our understanding of cell signaling in clinical tissue samples. This information does not mean that traditional hypothesis-driven research should be completely abandoned, because most omics-based studies have intrinsic limitations. The results obtained from such

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technology do not always provide a very clear picture of the mechanism and only provide a rather "blurred image" of the system under investigation. Only classical hypothesis-driven research can help dissect the mechanisms involved. The advantage though is that integrating the data obtained from such kinomic technologies can feed into hypothesis-driven research, and furthermore, kinomic approaches can sometimes lead to completely unexpected results that could not have been anticipated by hypothesis-driven research. In short, traditional hypothesis-driven research and omics-based research complement each other effectively. However, the future lies in moving beyond the idea of an individual "omics" approach and studying a biological system in its entirety by combining data from all different omics technologies, so that disease can be better modeled and thoroughly understood compared with a single pathway approach. This approach combined with phenotypic measurements, mathematical modeling, and biochemical manipulations will lead to a comprehensive understanding of signaling pathways and, ultimately, identification of potential drug targets for treatment of disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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