Abstract

Despite decades of cancer research, mortality rates remain high largely due to the failure of early detection, poor understanding of the epidemiology of rational drug targets, and molecular etiology of human cancers. The discovery of disease markers promises to deliver some solutions to these formidable challenges. Gene and protein expression profiling through DNA microarray and proteomics have already made a tremendous effect in this area. However, protein/gene expression does not necessarily reflect protein activity, which is often regulated via post-translation modifications, of which phosphorylation is one of the most prominent. This is an important consideration because the activity of protein is a more relevant phenotype than its expression during pathogenesis. Tyrosine kinases represent a very important class of enzymes that are critical regulators of mitogenic and angiogenic signaling, hence attractive targets for anticancer drugs as exemplified by BCR-ABL and ErbB2. More than 50% of them are overexpressed or mutated resulting in a gain of function in various human cancers. In this review, we discuss the potential effect of phosphoproteins as cancer markers in cancer diagnosis and therapeutics. Phosphoproteomics strategies that might pave the way to high-throughput analysis for routine clinical applications are also described.

The quest for cancer markers has led to the discovery of existing markers such as prostate-specific antigen, carcinoembryonic antigen, α-fetoprotein, and CA12.5 in the 20th century. However, current biomarkers often lack specificity and sensitivity, leading to false results and late detection of malignancies. With the completion of the human genome project and the advent of DNA microarray and proteomics technologies, expression profiling of multitudes of genes and proteins has begun to affect the field of cancer markers with the goal of achieving better markers (single or in combination). In the 21st century, we expect to see increasing research efforts on the global study of protein post-translational modifications like phosphorylation, degradation, and glycosylation and their relationship to cancer. Phosphorylation, in particular tyrosine phosphorylation, features prominently in the biology of cancer. Apart from being attractive drug targets, tyrosine kinases and their substrates may be useful molecular cancer markers that are invaluable for their potential discriminatory power in molecular classification of cancers (diagnosis), in predicting clinical outcome (prognosis) and response to drugs (therapy). Whereas the serine/threonine kinases represent another group of proteins important to cancer and response to drugs (therapy). The serine/threonine kinases represent another group of proteins important to cancer and response to drugs (therapy). The serine/threonine kinases represent another group of proteins important to cancer and response to drugs (therapy). The serine/threonine kinases represent another group of proteins important to cancer and response to drugs (therapy). The serine/threonine kinases represent another group of proteins important to cancer and response to drugs (therapy).

Growth factors, tyrosine kinases, and cancer biology. The process of tumor initiation, progression, and metastasis are multifactorial and highly complex. Growth factors are critical components in cancer biology. Epidermal growth factor (EGF) and platelet-derived growth factor are potent mitogens, whereas vascular endothelial growth factor and fibroblast growth factor have angiogenic properties. Growth factors stimulate a family of cell surface receptors called receptor tyrosine kinases with intrinsic tyrosine kinase activity. Tyrosine phosphorylation is a key regulatory switch that controls biochemical cascades leading to cellular events such as growth and proliferation. It does so by controlling enzymatic activities, protein stability, protein-protein interactions, subcellular translocation, and transcriptional activities of proteins.

There are about 518 putative protein kinase genes identified in the human genome and about 100 of these are tyrosine kinases. What is remarkable is that >50% of all tyrosine kinases have been implicated in human cancers as a result of aberrant activities arising from gain-of-function mutations such as deletion, translocation and viral insertion and/or overexpression. Examples include BCR-ABL tyrosine kinase in chronic myelogenous leukemia (CML), ErbB2 receptor tyrosine kinase in breast cancers, platelet-derived growth factor receptor tyrosine kinase in gliomas, Src nonreceptor tyrosine kinase in colorectal cancers, c-Met receptor tyrosine kinase in gastric cancers, and focal adhesion kinases in invasive human tumors.

Tyrosine kinases as rational drug targets. Given the critical roles of tyrosine kinases in cancers, researchers and drug companies have for a long time envisaged the arrival of target specific drugs or magical bullets against these signaling molecules. In recent years, Herceptin (trastuzumab) against ErbB2 receptor tyrosine kinase for the treatment of metastatic breast cancer and Gleevec (imatinib mesylate) against BCR-ABL for the therapy of patients with CML and gastrointestinal...
stroma tumors emerged as early examples of protein-based cancer drugs against signal transduction molecules (9). More recently, Iressa (gefitinib, AstraZeneca) has been approved by for chemotherapy-recalcitrant non–small cell lung cancer (NSCLC) by the Food and Drug Administration (10). These agents not only prolong and improve the quality of lives they also provided clinical validation of rational drug design.

Gleevec, in particular, is a star performer. Its impressive results during phase II trials with 532 patients with CML, achieving a 95% response, all of whom had previously failed standard IFN therapy, has led to its fast-track approval by Food and Drug Administration (11). A new wave of “smart drugs” are currently making its way to the market and they include Avastin (Genentech, South San Francisco, CA) and vascular endothelial growth factor trap (Regeneron, Tarrytown, NY), both with antiangiogenic properties against vascular endothelial growth factor for colorectal cancer and non-Hodgkins lymphoma, respectively; ABX-EGF (Abgenix, Fremont, CA) and Tarceva (Genentech) against EGF receptor (EGFR) for NSCLC (12). The mode of action of these drugs includes inhibition of ATP binding to the kinase domain, ligand-mediated receptor modulation (endocytosis and/or degradation) and trapping of receptor or ligand (9).

Although some difficulties have been encountered by some of these drugs (e.g., the relapse of patients seen in late-stage CML as a result of mutations in BCR-ABL resulting in drug resistance; refs. 13–18), the stage is set for more demonstrations of the power of target-directed therapy development, as is resistance; refs. 13–18), the stage is set for more demonstra-

tions in early detection and predicting drug response are results of poor understanding of the molecular etiology of the disease and the epidemiology of drug targets. This is partially attributed to a prominent characteristic of biomedical research in the 20th century: the “reductionist” approach to complex biology such as the etiology of cancer, which is a result of interactions between the cellular and biochemical environments. Consequently, despite identification of many oncogenes and tumor suppressors from in vitro cell models or animal models, their roles in human tumors in vivo remain unclear. However, it must be highlighted that the classic reductionist approach has laid a solid foundation in our understanding of the molecular mechanisms of oncogenes and tumor suppressors and will continue to be extremely important in the 21st century. But as Philip Cohen stated in an elegant review, “research emphasis would shift increasingly from the use of transformed cell lines, which have been invaluable in dissecting signaling pathways, to the analysis of tissues” (24). The natural state of cells in a physiologic “soup” provided by the tissues compared with the “artificial” state created by the addition of exogenous factors during in vitro cell culture is a great advantage to obtain physiologically relevant data. One of the commonest hurdles associated with tissue analysis is the heterogeneity of cell types such as epithelial cells, stroma fibroblasts, and endothelial cells. Thus, laser-captured microdissection technique to tissue analysis may become as common as cell culture is to in vitro studies. Other potential issues that remain to be resolved include a variety of factors such as nonstandardized tissue handling procedures between different centers and different genetic and environmental backgrounds of patients.

A further level of complexity exists at the cellular level where cell fate is regulated by a myriad of oncogenes, tumor suppressor genes, and other regulatory molecules that are intricately wired. How does one capture all these interactions and get a complete picture of what is really happening during pathogenesis and disease progression? As a result of these challenges, conventional tools to study single protein or pathway are proving inadequate and require complementation by approaches that provide information on a global scale. Alterations in protein/gene expression as measured by DNA microarray and proteomics technologies have been well established to participate in tumorigenesis and the expression profiles useful for molecular classification of subtypes, predicting clinical outcome and response to chemotherapy (25–39). Here, we propose that the phosphoproteome changes during cancer development and phosphoproteins could constitute cancer markers useful to cancer diagnostics and therapeutics.

**Tyrosine kinases and substrates in cancer diagnostics.** The Vogelstein’s model for colorectal cancer states that the progression from small adenoma to large adenoma and carcinoma, representing tumor initiation, promotion, and progression, correlates with the accumulation of genetic alterations involving the tumor promoter K-ras, and tumor suppressors APC, SMAD, and p53 (40, 41). Activating mutations in K-ras and loss-of-function mutations in APC, SMAD, and p53 confer growth advantage to tumor cells. Given the predominance of tyrosine kinases in cancer biology, it is conceivable that these genetic alterations could also involve tyrosine kinases and their substrates (Fig. 1). The hypothesis is supported by the observation that ErbB2/Her-2 gene amplification can be acquired as breast cancer progresses (42). The evidence for the existence of tumor-specific phosphoproteome came from our novel finding that the phosphotyrosine proteome of the breast and liver tumors were indeed distinct (43). This provided a proof of principle that it is possible to mine the tumor phosphoproteome for potential biomarkers. In the same report, three proteins (i.e., vimentin, hsp70, and actin) were observed to be consistently hyperphosphorylated at tyrosine residues in breast tumors but not in normal tissues. Overexpression of these proteins have been implicated in metastasis or drug resistance of breast cancers (44, 45). It would be interesting to understand the role of tyrosine phosphorylation on the function of these proteins in tumor invasion and metastasis. Similarly, constitutive tyrosine phosphorylation of p62dok, a substrate of BCR-ABL fusion protein tyrosine kinase, has been observed in the haemopoietic progenitor cell population of CML patients (46). These data provided a proof of principle that distinctive phosphoproteins exist in various tumors and that tyrosine phosphorylation profiling could be used to fingerprint cancers from different origins. By correlating these experimental data to clinical data such as disease outcome and drug response, potential cancer markers could be generated and subsequently validated for applications in cancer diagnosis,

**Tumor Phosphoproteome and Cancer Markers**

Early detection and therapeutics are two important pillars in the management of cancers. Despite decades of cancer research, mortality rates remains high largely because many cases are not being diagnosed before disease progression (23). The deficiencies in early detection and predicting drug response are results of poor understanding of the molecular etiology of the disease and the epidemiology of drug targets. This is partially attributed to a prominent characteristic of biomedical research in the 20th century: the “reductionist” approach to complex biology such as the etiology of cancer, which is a result of interactions between the cellular and biochemical environments. Consequently, despite identification of many oncogenes and tumor suppressors from in vitro cell models or animal models, their roles in human tumors in vivo remain unclear. However, it must be highlighted that the classic reductionist approach has laid a solid foundation in our understanding of the molecular mechanisms of oncogenes and tumor suppressors and will continue to be extremely important in the 21st century. But as Philip Cohen stated in an elegant review, “research emphasis would shift increasingly from the use of transformed cell lines, which have been invaluable in dissecting signaling pathways, to the analysis of tissues” (24). The natural state of cells in a physiologic “soup” provided by the tissues compared with the “artificial” state created by the addition of exogenous factors during in vitro cell culture is a great advantage to obtain physiologically relevant data. One of the commonest hurdles associated with tissue analysis is the heterogeneity of cell types such as epithelial cells, stroma fibroblasts, and endothelial cells. Thus, laser-captured microdissection technique to tissue analysis may become as common as cell culture is to in vitro studies. Other potential issues that remain to be resolved include a variety of factors such as nonstandardized tissue handling procedures between different centers and different genetic and environmental backgrounds of patients.

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progression, and prediction of response to drugs. In addition, a systematic cataloguing of tumor-specific phosphoproteins in individual patient would also reveal multiple causative players during cancer formation. This allows formulation of polytherapy regime, which presumably would be more effective than monotherapy because it is uncommon for cancer to progress as a result of aberration in a single protein.  

Tyrosine kinases and substrates in cancer therapeutics. Chemotherapy and clinical trial would benefit from the discovery of markers that could help to predict response or resistance to drugs. Two independent groups screened the EGFR kinase domain for somatic mutations and reported that mutations in EGFR (L858R) correlated with response to Iressa (gefitinib) in NSCLC patients (47, 48). This is likely due to increased sensitivity of the mutant EGFR to inhibition by gefitinib as suggested by experiments in vitro (47, 48). Recently, PTEN deficiency was reported to be a powerful predictor for resistance to Herceptin (trastuzumab) treatment (49). It was found that Herceptin (trastuzumab), which targets ErbB2 in breast cancers, activated PTEN and the therapeutic agent was efficacious in patients with ErbB2-overexpressing tumors only when PTEN was present. Thus, the availability of molecular markers such as mutant EGFR and PTEN helps to identify patients who would respond to a specific therapeutic regime or those who would make better subjects during the design of clinical trials.

In the case of NSCLC and CML, genotyping patients for specific EGFR or BCR-ABL mutations could well become a standard practice before administration of target-directed drugs, resulting in a cost-effective cancer management. However, several potential hurdles lie ahead. First, is screening for EGFR mutation alone sensitive enough to predict response to Iressa? As a result of the heterogeneity of cancers, the answer is likely to be yes for a specific subset of patients and no for others. The challenge is therefore to continue to search for additional markers that would complement EGFR mutation in predicting response to the drug. Cross-talking of other signaling systems with that of EGFR is increasingly evident. The convergence of intracellular signals from G-protein-coupled receptor and cytokine receptors into EGFR signaling are mediated by intracellular tyrosine kinases, c-Src and Janus-activated kinase, respectively, and have a potential regulatory influence on EGFR activity (50–56). It is therefore likely that secondary tyrosine kinases could be involved in determining sensitivity of EGFR to Iressa (Fig. 2A–D). The crucial role of secondary kinases in the etiology of cancer is supported by a recent observation that Lyn, Hck, and Fgr, members of the Src family of tyrosine kinases, play a role in B-cell acute lymphoblastic leukemia in addition to BCR-ABL in mouse model (57). In the same study, whereas Gleevec alone was efficacious in CML, a combination of Src tyrosine kinase inhibitors together with Gleevec was required for suppressing B-cell acute lymphoblastic leukemia.

Second, it is logical to preempt the development of clinical resistance to Iressa. Because Iressa targets the EGFR, any other resistance to the drug, apart from that contributed by the target itself, would be expected to arise from downstream signal transduction components that are either regulators or effectors of the cellular phosphorylation/dephosphorylation circuitry (Fig. 3). If both cases were true, phosphoproteomics may prove to be valuable in unraveling phosphoproteins as markers that are potential drug targets or of predictive value in cancer therapeutics. The latter is especially relevant because reliable markers that could aid in predicting drug response are scarce for most cancers and that treatment of cancer patients with chemotherapeutic agents is frequently a process of elimination, with the exception of hormonal therapy where steroid receptor status could help to select potential responders from non-responders. In addition to clinically approved drugs, phosphoproteomics could also be extended to novel target-directed anticancer compounds such as new protein tyrosine kinase inhibitors in preclinical studies to characterize and understand the molecular effects on cell signaling thus facilitating the progress of anticancer drugs into clinical trials.

Phosphoproteomics potentially provides two additional layers of information compared with conventional expression profiling studies. First, it provides clues on what protein or pathway might be activated because a change in the phosphorylation status of proteins almost always reflects a change in protein activity. Second, it indicates what proteins might be potential drug targets because phosphoproteomics focuses on proteins that have kinase activities or are substrates of kinases. Thus, there is increasing interests in phosphoproteomics as reflected by a considerable number of works describing various strategies including the use of different isotopic technologies (e.g., isotope-coded affinity tag, stable isotope labeling by amino acids in cell culture), protein separation techniques (e.g., gel-based versus liquid chromatography-based methods), and chemical modifications (e.g., β-elimination and esterification) to identify phosphoproteins and/or phosphorylation sites in a global fashion (58–67). Recently, our group developed a method that combines the capability of anti-phosphotyrosine 4G10 antibodies (68) to enrich for tyrosine-phosphorylated proteins, the high-resolution power of two-dimensional PAGE to separate proteins and the high sensitivity of mass spectrometry to identify proteins of interest. The tool enabled the concurrent visualization of >50 protein spots in a single phosphoproteome.
map belonging to about 20 proteins which can be broadly categorized into five functional classes of signaling proteins activated by EGF in the A431 human cancer cell line (69). They were the receptor tyrosine kinases (e.g., EGFR), SH2-containing adaptor proteins [e.g., growth factor receptor binding protein 2, p85 PI3-K, Src homology and collagen protein], SH2-containing signaling enzymes (e.g., PLC-γ), heat shock proteins (e.g., Hsc71), and cytoskeletal proteins (e.g., tubulin and γ-catenin), all of which have pro-mitogenic or anti-apoptotic properties. The potential application of this valuable tool is tremendous, ranging from the fingerprinting of growth factor signaling pathways to drug screening and to comparative studies between normal and tumor tissues. However, the current sensitivity of this gel-based tool limits its success to only in vitro studies and we are attempting to achieve greater sensitivity for tissue analysis by adapting liquid chromatography–based mass spectrometry into this approach.

Whereas general profiling of phosphorylation status in proteins offers useful information, it is possible that proteins are tyrosine phosphorylated in dissimilar fashions in terms of the sites and degree of phosphorylation in different states of disease and between subjects. Our recent study reveals that of the 20 or so proteins mapped on two-dimensional gels, about nine or 45% of them have varying numbers of tyrosine-phosphorylated isoforms within the same signaling system (EGF) and between two different signaling systems (EGF versus platelet-derived growth factor; Table 1; ref. 69). These differences reflected the predominant functional effects of the individual signaling system. Platelet-derived growth factor induced a more potent PI3-K/actin–mediated chemotactic pathway relevant to tumor metastasis, whereas EGF induced a more prominent focal adhesion pathway involving γ-catenin. Similarly, phosphorylation isoforms exist for proteins that are phosphorylated at serine and threonine (70).

Will phosphorylation isoforms have important bearing on the causative nature of oncoproteins? The EGF receptor is known to be phosphorylated at multiple sites upon activation. Each phosphorylated tyrosine residue on the receptor is a potential docking site for downstream signaling proteins, thereby triggering different effector pathways. It has been shown that c-Src phosphorylates Y845 in the activation loop of the EGFR and maintains the enzyme in an active state (56). Phosphorylated Y992 and Y1068 of EGFR are direct binding sites for the SH2 domain of PLC-γ and growth factor receptor binding protein 2, respectively, and these interactions stimulate the mitogen-activated protein kinase cascade critical for growth and proliferation in the cells (71). On the other hand, phosphorylation of Y1045 creates a major docking site for c-Cbl, the binding of which leads to receptor ubiquitinylation and degradation (71). Examples of the interaction of other cytosolic signaling proteins with various receptor tyrosine kinases have been reviewed elsewhere (72). Some questions

Fig. 2. Role of secondary kinases and phosphoproteins in predicting sensitivity of target to drugs. A, convergence of G-protein–coupled receptor (GPCR) and cytokine signaling into EGFR signaling. G-protein–coupled receptor and cytokine–induced, EGF–independent activation of EGFR is mediated by c-Src and Janus-activated kinase (JAK) tyrosine kinases, respectively. B, in NSCLC, wild-type, inactive EGFR is not sensitive to Iressa, whereas L858R mutant EGFR is active and sensitive to Iressa. C, generation of a COOH terminus–truncated or Y527F Src during cancer formation may render the wild-type EGFR sensitive to Iressa as a result of the phosphorylation and stabilization of the activation loop by constitutively active Src. D, distinct phosphoproteomes (PP1–PP8) are likely in normal tissues, tumors with wild-type EGFR, L858R mutant EGFR, and wild-type EGFR with mutant Src backgrounds in the presence or absence of Iressa. The identification of differentially phosphorylated proteins between these backgrounds is likely to provide clues on ancillary factors that might predict drug sensitivity. Due to space constraint, activated EGFR is depicted as a monomer instead of a dimer.
that beg to be answered are whether all the sites are phosphorylated in an all-or-none fashion or are these phosphorylations hierarchical and temporal in nature and whether subpopulations of receptor with distinct phosphorylation sites exist. For the non-receptor tyrosine kinase, c-Src is reciprocally phosphorylated at Y527 and Y416 when it is activated and inactivated, respectively (73). In the case of receptor tyrosine kinases where multiple phosphorylation sites are involved, the temporal/spatial stoichiometry of phosphorylation events may be more complicated. Nevertheless, it is obvious from the above examples that site-specific phosphorylation has important functional implication in terms of the molecular pathways activated and needs to be more thoroughly understood with respect to tumor biology and cancer therapeutics.

Technical Challenges in Clinical Applications of Phosphoproteomics

The prerequisite features for the translation of protein-based techniques into routine clinical use include high throughput and high sensitivity because critical decisions have to be made within the shortest possible time from minute quantities of patients' samples. Whereas liquid chromatography–based mass spectrometry is instrumental in mapping of the phosphorylation sites on different cancer-causing tyrosine kinases and/or their substrates, it is tedious and is not amenable for high-throughput analysis of tissues. A viable strategy towards the fine scale mapping of the phosphorylation status of tyrosine sites in biomarkers is to exploit the experimental data from liquid chromatography–based mass spectrometry analysis to generate phosphorylation site-specific antibodies, which could be used as reagents in the development of antibody array/chip or for immunohistochemistry on tissue microarrays (TMA) in molecular pathology. The establishment of a methodology for raising phosphorylation site-specific antibodies (74) and the availability of a vast arsenal of phosphorylation site-specific antibodies in the marker (70) have kick-started pilot-scale studies employing

<table>
<thead>
<tr>
<th>Class of proteins</th>
<th>No. isoforms</th>
<th>Experimental MW (kDa)</th>
<th>Experimental pl</th>
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<tbody>
<tr>
<td>Signal enzymes/adaptor</td>
<td></td>
<td></td>
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<tr>
<td>PLC-γ</td>
<td>3</td>
<td>145</td>
<td>5.9, 6.0, 6.1</td>
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<tr>
<td>p85/p13-K (EGF)</td>
<td>2</td>
<td>90</td>
<td>6.3, 6.4</td>
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<tr>
<td>p85/p13-K (PDGF)</td>
<td>4</td>
<td>90</td>
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<tr>
<td>Plakoglobin/γ-catenin (EGF)</td>
<td>3</td>
<td>85</td>
<td>5.9, 6.0, 6.1</td>
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<td>Plakoglobin/γ-catenin (PDGF)</td>
<td>1</td>
<td>85</td>
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<td>Focal adhesion/cytoskeletal</td>
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<tr>
<td>Cortactin</td>
<td>2</td>
<td>78</td>
<td>5.2, 5.3</td>
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<td>α-Tubulin</td>
<td>2</td>
<td>50</td>
<td>5.1, 5.2</td>
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<td>Heat shock proteins/chaperones</td>
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<tr>
<td>HSP70</td>
<td>2</td>
<td>70</td>
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<tr>
<td>HSC71</td>
<td>3</td>
<td>75</td>
<td>5.4, 5.5, 5.6</td>
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<tr>
<td>GRP78/BIP</td>
<td>2</td>
<td>80</td>
<td>5.1, 5.2</td>
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NOTE: Compilation of raw data from the reported study (69) reveals that there were eight signaling proteins with varying number of isoforms. Not included in the table is EGFR that had an undetermined number of isoforms because of the smeary nature of the protein in two-dimensional gels. Hence, a total of 9 of 20 proteins or 45% of the proteins identified possessed isoforms. Because all the spots representing isoforms of the same protein were immunoreactive to anti-phosphotyrosine antibodies and they differed only in isoelectric points but not molecular weights, they were implied to be phosphorylation isoforms. It is possible that additional isoforms exist for each protein as a result of a change in molecular weight due to phosphorylation and/or other post-translational modifications (e.g., glycosylation and proteolytic cleavage). However, such events are difficult to ascertain from two-dimensional gels directly.

Abbreviation: PDGF, platelet-derived growth factor.
these reagents for tumor analysis (75–78). One very recent and interesting application of this phosphoproteomics site–specific antibodies was in single cell profiling of signaling networks using multivariable flow cytometry (79). However, current literature reporting the use of phosphoproteome site–specific antibodies in tissues is restricted to the concurrent investigation of at most a limited number of target proteins such as ErbB2, p38, mitogen-activated protein kinase, and signal transducers and activators of transcriptions. It is becoming increasing evident that multiple markers is of better utility than single marker in cancer prognosis and diagnosis (80). Hence, arrays of phosphotyrosine site–specific antibodies against multiple targets are likely to emerge in greater visibility in the next decade.

It is obvious that one of the key bottlenecks of such a challenge lies in the cost-effective production of large quantities of a wide range of highly purified and phosphorylation site–specific antibodies. High-density antibody array remains difficult to construct and its current density of about $10^3$ cm$^{-2}$ is considerably lesser than that of $10^5$ cm$^{-2}$ in DNA microarray. This means that a larger amount of samples would be needed for protein analysis than for DNA analysis. Another major hurdle is the concoction of a condition whereby all antibody-antigen interactions could take place optimally. This is apparently impossible and is a major drawback of antibody array. Thus, a considerable amount of technical inertia has to be overcome before a high-throughput antibody/protein chip could be clinically applied in a routine basis. Whereas the Aptamer technology is making inroads to provide some solutions to this problem (81), semi-high throughput TMAs may be the first technology is making inroads to provide some solutions to this problem. Application of the phosphorylation site–specific antibodies on tissue microarrays may comprise of only some 10 to 20 targets that are of interest. Consequently, phosphoproteomics may yield hundreds or even thousands of candidate genes/proteins, the final cancer-specific signature consisting of up to 1,000 tissues can now be constructed and immunohistochemistry on TMAs is now routinely done (82). Application of the phosphoproteome site–specific antibodies on TMA and correlation of the phosphorylation status of proteins at specific tyrosine residues with clinical data is likely to lead to the discovery of potential biomarkers. Current efforts are being made to construct TMAs from frozen tissue blocks, which will be especially useful for protein analysis because better preservation of protein antigens is expected than from paraffin blocks.

## Conclusion

Whereas technology development is a critical component, what is equally important, if not more important, is the definition of the molecular signature of various cancers through biomarker discovery. In this respect, much effort remains necessary to study aberrant signaling pathways in cancer cells from tumors of different origins in a global fashion. The discovery of cancer markers from the tumor phosphoproteomes will undoubtedly contribute to a paradigm shift towards individualized medicine. Along with this is the spinning off of basic research programs to understand the role of phosphorylation in regulating the function of potential phosphoprotein markers in cancer biology. Ironically, whereas phosphoproteomics may yield hundreds or even thousands of candidate genes/proteins, the final cancer-specific signature may comprise of only some 10 to 20 targets that are of significant discriminatory value. This bodes well for the feasibility of creating phosphoprotein-specific antibody chip, which is one of the ultimate goals for routine application of phosphoproteomics in the clinical setting.

## References


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41. Finlay GJ. Genetics, molecular biology and colorectal cancer. Mutat Res 1993;290:3–12.
Mining the Tumor Phosphoproteome for Cancer Markers

Yoon Pin Lim


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