Protein Pathway Activation Mapping for Multi-Omic-Based Precision Medicine

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Abstract

The development of precision medicine in oncology is entirely dependent on the ability to obtain accurate molecular information that can assist physicians in selecting targeted treatments for cancer patients. To date, most clinical studies and companion diagnostic efforts utilizing this approach have relied heavily upon genomic information as the sole determinant for patient selection. Unfortunately, many of these genomic alterations are infrequent, occurring in 1 to 5% of cancers, which generates a frustrating dynamic in patient accrual and the powering of clinical outcome correlations. Moreover, while these rare events can be readily identified by genomic profiling, it is impossible to know ahead of time which genomic alterations are true driving events for any individual patient's tumor. Analysis of the functional proteome may provide a synergistic solution, since these genetic derangements ultimately lead to aberrant protein synthesis and activation of signaling networks responsible for sustaining tumor growth and progression. Because changes within the signaling architecture—and, particularly in kinase activity, are the ultimate causal event of cancer, the signaling network, itself, has become the direct target of the new generation of therapeutic agents. For these reasons, high-throughput proteomic platforms able to capture changes within these signaling networks have received increasing attention in recent years. Among others, the Reverse Phase Protein Microarray (RPPA), a widely used proteomic platform for signaling network mapping of biological samples, is currently used as a companion diagnostic for patient stratification to personalized treatment. This platform generates high-throughput, multiplex, quantitative information starting from a relatively low amount of biological material such as a fine needle biopsy. In this article, two clinical case studies are presented that illustrate the clinical potential for utilizing functional proteomics data into the precision medicine workflow.

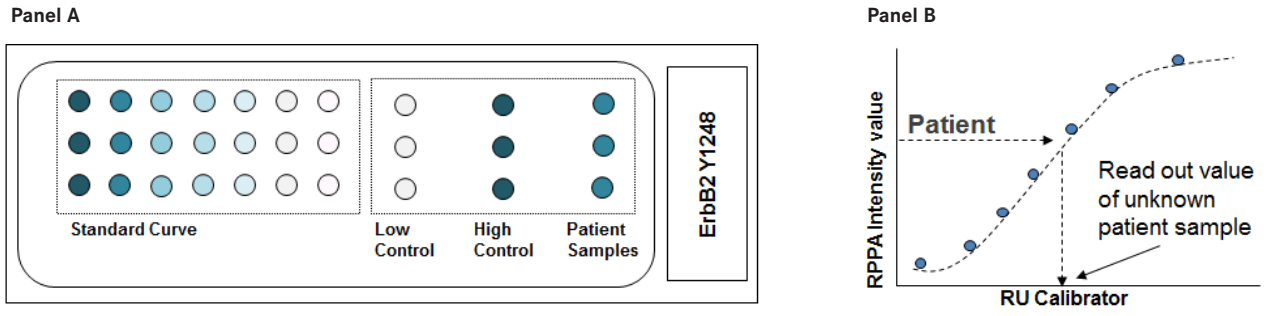
Key words: molecular profiling, heterogeneity, trastuzumab, HER2 mutations, Reverse Phase Protein Microarray

Introduction

Molecular studies mapping malignant lesions of similar anatomical origin have led to the understanding that tumors originating from the same organ can be extremely heterogeneous at the molecular level.[1] This underpinning molecular heterogeneity is considered one of the underlying causes of high discrepancy in treatment success rates across patients with lesions that have similar histopathologic characteristics.[2,3] At the same time, broad-scale molecular profiling efforts such as The Cancer Genome Atlas (TCGA) have revealed that tumors originating from different organs also share common molecular alterations.[4] For these reasons, drugs such as trastuzumab (Herceptin®), a monoclonal antibody targeting the HER2 protein, is FDA-approved for treating patients with breast or gastric cancer overexpressing the HER2 protein. This approval may be broadened in the near future to other HER2 overexpressing solid tumors and to a subpopulation of patients presenting with HER2 mutations.[5-7] Because these molecular characteristics play such a central role in determining the most appropriate therapeutic approach for patients with cancer, upfront molecular profiling is becoming a de facto part of the standard of care work-up for classifying and describing malignant lesions, and true oncogenic drivers are being used as key targets for effective therapy.

Although a number of DNA mutations and chromosomal amplifications/translocations are recognized molecular 'drivers' of many cancers, it is impossible to distinguish in any given tumor which alterations are drivers of the disease and which adjustments were necessary in the early stage of the malignancy, but are not any more necessary or sufficient to sustain tumor progression. Despite the development of high-throughput genomic technologies that have opened new opportunities for precision medicine, the results of these analyses can generate unclear data for the treating physician because multiple genomic abnormalities are often identified within the same lesion.[8] Moreover, different organ sites, with ostensibly the same driving genomic event, often show diverse response rates to the same targeted compound.[9] Therefore, the identification of genomic changes in isolation may be only partially sufficient for stratifying patients to the most appropriate line of treatment.

FIGURE 1. Graphic representation of the RPPA platform



Panel A illustrates the RPPA array format, including standard curve, internal control for quality assurance, and patient sample. Panel B exemplifies how standard curve can be used to convert experimental values into standardized relative unit of the reference standard.

Finally, while genomic alterations play a central role in cancer progression, the overall cellular signaling architecture is comprised of proteins, and genetic aberrations ultimately exhibit their phenotypic consequences through the action of activated proteins. Moreover, the activation of specific signaling pathways in tumor cells via feedback mechanisms or the activation of cellular receptor by their ligands are often independent from genomic alterations. For these reasons, kinase-driven activation via phosphorylation represents the most direct means for measuring pathway activation in tumor cells.[10,11] Because derangements in cellular signaling processes are most often the causal aspect of the disease and thus targets for new therapies, molecular analysis of the proteome and phosphoproteome are becoming centrally important for identifying new predictive biomarkers as well as new therapeutic targets.[12] Therefore, there is an urgent need to find more effective tools for exploring the impact of genetic changes on the function of their protein products.

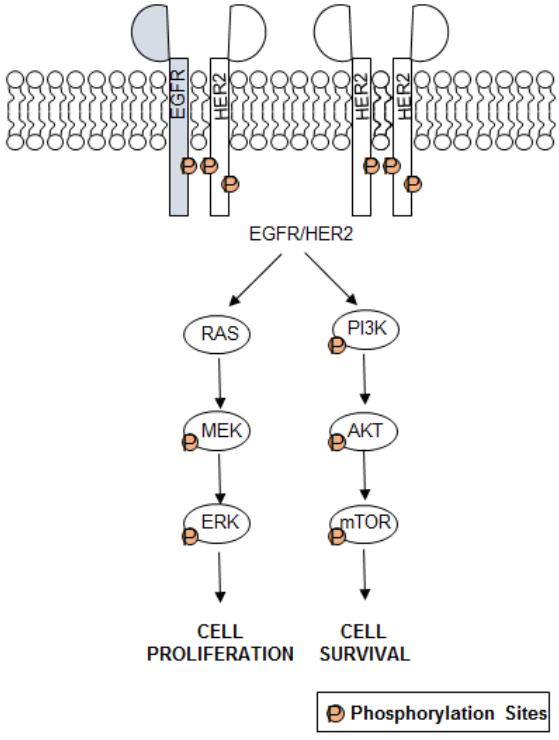
Planar/Suspension Antibody-Based Arrays

So far, planar or suspension antibody-based arrays are the most commonly used platforms for measuring protein kinase signaling in clinical samples.[13] A major advantage of these platforms is their ability to generate high-throughput, and in most cases, multiplexed data allowing for the concomitant analysis of hundreds of analytes across a large number of samples. Although antibody specificity can be challenging from time to time, these methodologies have the advantage of accurately measuring low abundance proteins with detection limit in the range of ng-pg/mL. As recently described by Pierobon and colleagues,[14] when compared to other antibody-based assays suitable for the analysis of clinical samples, the Reverse Phase Protein Microarray (RPPA) has distinguished itself for the ability of reproducibly and sensitively quantifying the activation level of hundreds of kinases starting from a limited amount of biological material (<10,000 cells) while being able to generate quantitative data from hundreds of patients at once.[14,15] As such, this technology captures the linear dynamic range of most targetable kinases and downstream substrates.[16]

Because standard curve and internal controls for quality assurance can be mounted on each array, this platform is been used as a Clinical Laboratory Improvement Amendments (CLIA)/College of American Pathologists (CAP)-based technology for measuring protein signaling activation of individual tumors and for identifying patients who can benefit from specific targeted treatment (**Figure 1**).[8] Upfront tissue processing techniques such as Laser Capture Microdissection (LCM) have been effectively coupled to the RPPA workflow and found to be an essential and necessary component in generating accurate protein activation-based clinical data, especially when used to guide treatment selection.[17,18] The use of core needle biopsies, even under Formaldehyde Fixed-Paraffin Embedded (tissue) (FFPE) fixation, is an appropriate process for the optimal preservation of the phosphoproteome, and has been successfully analyzed using an LCM/RPPA workflow.[16,19,20]

We recently used the RPPA technology to measure the activation of HER2 in patients with inflammatory breast cancer (IBC) and non-small cell lung cancers (NSCLC) harboring HER2 mutations by evaluating the level of phosphorylation at the tyrosine 1248 residue, a well-described site of modification known to control and modulate transmission of downstream signaling (**Figure 2**).[21] In particular, we evaluated whether measuring the activation level of HER2 is clinically relevant and has added value to conventional genomic characterization in terms of outcome prediction. For these case studies, the protein activation level of HER2 in the tested samples was compared to the distribution of activated HER2 in a large cohort of breast cancer samples with known HER2 expression and amplification (data not shown). In brief, activation and dimerization of HER2 with other receptor tyrosine kinases (RTKs), including other members of the HER family, regulate a number of important cellular pro-

FIGURE 2. HER2 signaling pathway



Graphic representation of HER2 protein kinase-driven signaling cascades. Activation and dimerization of HER with other RTKs, including the epidermal growth factor receptor (EGFR), stimulate the activation of the MAPK proliferative pathway, including RAS, the mitogen-activated protein kinase (MEK), the extracellular signal-regulated kinase (ERK), and the AKT/mTOR pathway via activation of phosphoinositide 3-kinase (PI3K), protein kinase B, commonly known as, AKT, and mechanistic target of rapamycin (mTOR).

cesses by modulating different signaling cascades. In particular, HER2 can stimulate cell proliferation by activating the MAP kinase pathway. Activation of the AKT/mTOR kinase pathway, on the other hand, regulates cell survival. Because most of the members of the MAP and AKT/mTOR pathways are kinases, their activity depends on the phosphorylation status of each of the pathway components.

HER2 Mutation and Activating Base Substitutions

Ali and colleagues recently reported the first IBC case with HER2 mutation and activating base substitutions.[22] The patient had extensive local and distant recurrent disease classified by standard pathologic testing as a triple-negative tumor that had progressed despite several cytotoxic treatment regimens. After the molecular test results and the implementation of HER2 targeted therapy, she showed remarkable symptomatic improvement and clinical response. RPPA analysis of a tumor biopsy collected from the same lesion showed an HER2 activation level similar to those seen in breast cancer tumors with overexpression/amplification of HER2, a subgroup of patients who routinely benefit from anti-HER2 targeted treatments (**Figure 3**). These data indicated that a small subgroup of IBC patients with unamplified (by fluorescent in situ hybridization [FISH] and immunohistochemistry [IHC]), but mutated HER2 and high levels of HER2 protein activation, could benefit from anti-HER2 treatment.

HER2 activation was then evaluated in 2 patients with NSCLC harboring HER2 mutations. Both patients were treated with the small kinase inhibitor targeting HER2, neratinib, in combination with a downstream mTOR inhibitor, temsirolimus, as part of a phase I study.[23] Retrospective analysis by RPPA showed heterogeneity in terms of HER2 phosphorylation/activation across the 2 clinical specimens. In the first sample, the activation level of HER2 was comparable to that in patients with HER2 FISH/IHC-negative breast cancer (**Figure 3**). As expected, because the drug target was not activated, the patient did not benefit from treatment with the HER2 inhibitor. Indeed, the lack of activation of the receptor indicates that, although a HER2 mutation was present, this genetic event did not result in high levels of activation/phosphorylation of the receptor in this particular lesion. The development of feedback mechanisms in the receptor tyrosine kinases expression and/or turnover as well as the interaction with specific ligands are a few possible explanations for these findings. On the contrary, the activation level of HER2 in the second NSCLC patient analyzed was similar to activation levels seen in patients with breast cancer with overexpressed/amplified HER2 (**Figure 3**). Increased activation of HER2 in this patient was associated with clinical response that lasted close to a year, consistent with the benefit seen with other effective targeted therapies against true oncogenic drivers.

Conclusions

As shown by our data, directly measuring protein signaling activity in human samples has the unique advantage of providing an ex vivo readout of the in vivo cellular signaling network even in patients presenting with similar genomic characteristics, and provides information on actual drug-target activation in the presence or absence of genomic alterations. This approach could greatly assist in identifying responding patients missed by genomic-only means as well as help credential and prioritize which genomic alterations are truly functional drivers of malignant progression. Moreover, pathway activation as well as the activation of proteins like HER2 (regardless of their expression) are much more frequent events than genomic alterations, including the rare frequency of HER2 mutation.[16] The addition of functional proteomic analysis to precision medicine is poised to alter the landscape of clinical trials and routine molecular profiling that rely on genomic analysis alone, because superior

and increased clinical benefit using the addition of proteomics and phosphoproteomics to genomic analysis is beginning to be demonstrated. As shown in the published results from a recent clinical trial where treatment selection was recommended based on integrated proteomic, phosphoproteomic, and genomic data, this 'multi-omic' approach can improve progression-free survival in patients with cancer, providing optimism for the impact of this strategy going forward.[24]

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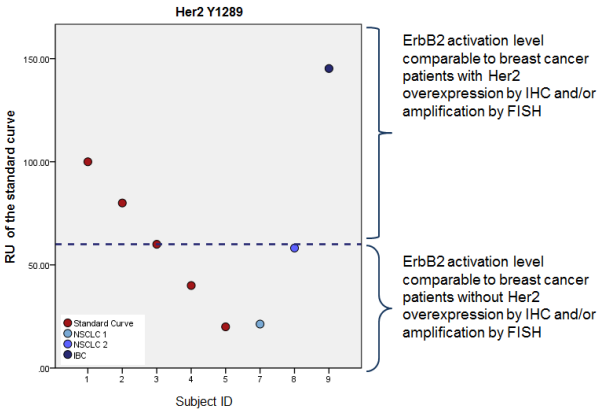
FIGURE 3. Standard curve and interpolated values (1) in inflammatory breast cancer (IBC), and (2) in non-small cell lung cancer (NSCLC).



Intensity values of the clinical samples are shown in relationship to the standard curve. HER2 activation level of a reference population of patients with breast cancer with known HER2 status measured by conventional FISH/IHC (data not shown), along with the presence of standard curves, were used to establish a cut-point that allows to identify patients with high- and low-activation levels of HER2. The blue line indicates the level of the standard curve where HER2-positive and HER2-negative breast cancers are discriminated.

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