Using reverse-phase protein arrays as pharmacodynamic assays for functional proteomics, biomarker discovery, and drug development in cancer

Yiling Lu[a], Shiyun Ling[b], Apurva M. Hegde[b], Lauren A. Byers[c], Kevin Coombes[d], Gordon B. Mills[a], Rehan Akbani[b],*

[a] Department of Systems Biology, The University of Texas MD Anderson Cancer Center, Houston, TX
[b] Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX
[c] Department of Thoracic/Head & Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX
[d] Department of Biomedical Informatics, The Ohio State University, Columbus, OH

ARTICLE INFO

ABSTRACT

The majority of the targeted therapeutic agents in clinical use target proteins and protein function. Although DNA and RNA analyses have been used extensively to identify novel targets and patients likely to benefit from targeted therapies, these are indirect measures of the levels and functions of most therapeutic targets. More importantly, DNA and RNA analysis is ill-suited for determining the pharmacodynamic effects of target inhibition. Assessing changes in protein levels and function is the most efficient way to evaluate the mechanisms underlying sensitivity and resistance to targeted agents. Understanding these mechanisms is necessary to identify patients likely to benefit from treatment and to develop rational drug combinations to prevent or bypass therapeutic resistance. There is an urgent need for a robust approach to assess protein levels and protein function in model systems and across patient samples. While "shot gun" mass spectrometry can provide in-depth analysis of proteins across a limited number of samples, and emerging approaches such as multiple reaction monitoring have the potential to analyze candidate markers, mass spectrometry has not entered into general use because of the high cost, requirement of extensive analysis and support, and relatively large amount of material needed for analysis. Rather, antibody-based technologies, including immunohistochemistry, radioimmunoassays, enzyme-linked immunosorbent assays (ELISAs), and more recently protein arrays, remain the most common approaches for multiplexed protein analysis. Reverse-phase protein array (RPPA) technology has emerged as a robust, sensitive, cost-effective approach to the analysis of large numbers of samples for quantitative assessment of key members of functional pathways that are affected by tumor-targeting therapeutics. The RPPA platform is a powerful approach for identifying and validating targets, classifying tumor subsets, assessing pharmacodynamics, and identifying prognostic and predictive markers, adaptive responses and rational drug combinations in model systems and patient samples. Its greatest utility has been realized through integration with other analytic platforms such as DNA sequencing, transcriptional profiling, epigenomics, mass spectrometry, and metabolomics. The power of the technology is becoming apparent through its use in pathology laboratories and integration into trial design and implementation.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Targeted therapy has demonstrated marked activity in a number of diseases. However, for most diseases and most agents, targeted therapy has not delivered on its initial promise: favorable treatment responses have been limited to subsets of patients who have the predicted biomarkers, and often have been of short duration. Some of the apparently limited efficacy of targeted therapy likely arises from an unrealistic expectation that monotherapy would be broadly active in complex and heterogeneous diseases such as solid tumors.

The basic precepts of pharmacokinetics and pharmacodynamics in drug development have too often been ignored in the implementation of targeted therapy. The role of pharmacodynamic analysis in oncology is to determine both the early effects of drug

* Corresponding author. Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, 1400 Pressler St, Unit 1410, Houston, TX, 77030. Tel.: +1 (713) 794-5043; fax: +1 (713) 563-4242.
E-mail address: RAkbani@mdanderson.org (R. Akbani).

inhibition on the target and downstream signaling, and the longer-term adaptation of the tumor to the effects of the drug. This is limited by the challenges of obtaining and assessing tumor tissue at the appropriate time points after the delivery of a therapeutic agent. Furthermore, biopsy tissues are often small and of diverse tumor and stromal composition; thus, applicable proteomic approaches to effectively analyze the samples are elusive. The objective of such approaches is to broadly determine the effects of the targeted agent (expected and unexpected effects) on the target and on downstream signaling events, cross-talk, and feedback loops. Delayed adaptive responses to the therapeutic agent can inform analytic approaches that can then be used to determine resistance mechanisms and to facilitate the choice of rational combination therapies to prevent resistance and convert what are often cytostatic effects of single agents into cytotoxic effects.

The failure to identify methods to effectively assess early pharmacodynamic responses (whether to use peak inhibition, the area under the curve, or the trough levels of target inhibition as the key determinants of patient response) obviously contributes to the low success rate of current targeted therapy trials. Indeed, for most agents, we do not know which of these criteria indicate an effective response. Perhaps a "hit and run" approach of maximal target inhibition that induces cell death or, conversely, chronic inhibition, will provide the optimal patient benefit. This remains unknown for most agents. Although a systems biology approach allows us to generate predictions through in vitro and animal model studies combined with mathematical modeling, the implementation of these approaches in humans is limited by several challenges. These include accurately measuring the pharmacodynamics of target inhibition, understanding the pharmacokinetics and off-target activity of current targeted agents, and working with a narrow therapeutic index of target inhibition between tumor and normal tissue for many drugs. A careful evaluation of the mechanisms of drug resistance (pre-existing, acquired and adaptive resistance) will be necessary to design rational combination therapies that can prevent the emergence of resistance or overcome established resistance. Indeed, adaptive resistance, the ability of the tumor to rewire signaling networks to bypass the effects of the targeted therapy, may represent the major mechanism of targetable resistance.

In general, targeted therapy is designed to capitalize on the vulnerabilities of tumor cells that arise from the rewiring of functional networks as a consequence of the genomic and epigenetic changes in the tumor or their effects on the tumor microenvironment. Targeted agents typically inhibit, or in rare instances stimulate, protein function. Thus, in order to determine the consequences of target engagement, we need to develop technology that can assess target inhibition as well as the resulting functional changes to the signaling networks. The ability to quantitate RNA levels has rapidly matured; however, the correlations between RNA and protein levels and protein function vary markedly for different proteins, ranging from very high to very limited correlations and thus very limited predictive ability [1,2]. Furthermore, transcriptional analysis, RNA-Seq in particular, is sufficiently complex that it is challenging even under the best circumstances to impute the treatment effects on protein networks and signaling functions. Thus, there is a need to directly assess the effects of the targeted agents on hundreds of different proteins, both predicted and unexpected.

2. Pharmacodynamic assays for large-scale protein level determination

Two technologies have emerged to fulfill these criteria, each with different strengths and weaknesses. The first technology is mass spectrometry, which can assess thousands of proteins and post-translational events (such as phosphorylation or methylation) that can change function in a single assay [2,3]. Mass spectrometry can unambiguously identify and quantify both wild-type and mutant proteins, identify expected and unexpected proteins and post-translational modifications, and determine the presence of splice variants. However, mass spectrometry is limited in its ability to detect rare events, such as proteins or post-translational modifications that are present at low levels, due to a bias toward common molecules such as actin or albumin. This challenge can be partially overcome by new mass spectrometry technologies such as single reaction monitoring (SRM), multiple reaction monitoring (MRM), sequential window acquisition of all theoretical mass spectra (SWATH); however, even at their most effective implementation, these technologies lack the sensitivity of a high affinity antibody. The necessity to generate protein fragments that will "fly" in the mass spectrometer also limits the ability to identify post-translational modifications to those with convenient proteolytic cleavage sites and an appropriate charge. Indeed, in a recent analysis of human ovarian and breast cancer xenograft tissue [3], only about 60% of phosphorylation sites identified by parallel antibody-based approaches could be detected, and fewer sites could be quantitated by mass spectrometry. Furthermore, for deep analysis, mass spectrometry requires significant amounts of starting material, expensive equipment, and specialized operators and analytical approaches, all of which limit its utility to a few centers. Nevertheless, mass spectrometry assays designed to assess patient samples have become commercially available and have been implemented in Clinical Laboratory Improvement Amendments (CLIA) laboratories.

The second technology is antibody-based analysis, including flow cytometry and its mass spectrometry-based cytometry by time of flight (CyTOF) variant, multiplexed immunohistochemistry, and forward- and reverse-phase protein arrays. Bar coding of antibodies can allow for concurrent detection of nucleotides and proteins, which facilitates the analysis of DNA, RNA, and proteins in a single assay [4]. In terms of the analysis of signaling networks, reverse-phase protein arrays (RPPAs) have emerged as a cost-effective, robust, sensitive, and tissue-sparing technology that can assess hundreds of different signaling molecules in a single assay [5–8]. This technology is limited by the need for high-quality monospecific antibodies, which is being met by the development of antibodies in commercial and academic laboratories. In addition, large-scale efforts to validate the utility of antibodies to a broad spectrum of targets are being conducted through the Human Proteome Atlas and the National Cancer Institute and research centers with RPPA platforms [9]. However, even antibodies predicted to be highly specific can be plagued by unexpected off-target activity, resulting in spurious results. Indeed, the demonstration of a single dominant band of the correct size on Western blotting that correlates in expression with RPPA is a minimal requirement for antibody utility. Multiple antibodies that perform well on Western blotting do not perform well in RPPA because of the essential dot-blot characteristics of the RPPA assay, in which materials that do not enter or run through the Western blot gel are present in the "dot" and can interact with the antibodies. Additional information can increase the confidence that the antibody is indeed faithfully reporting the protein or phosphoprotein levels on RPPAs. Correlations with mRNA levels in the same samples provide "one-way" confidence as translational and post-translational controls can result in markedly different mRNA and protein levels. That is, if RNA and protein levels are highly correlated, this adds to the confidence; if they are not, they are non-informative. For phosphoproteins, the demonstration of increased phosphorylation of the specific site in the presence of growth factors and decreased phosphorylation by phosphatases in