RPPA Description

Rationale

Molecular therapeutics is designed to capitalize on tumor cells that arise from the rewiring of functional networks due to genomic and epigenetic changes in tumor or their effects on the tumor environment. Although DNA and RNA analysis have been used extensively to identify novel targets and to define patients likely to benefit from targeted therapies, they provide only indirect measurements of the functions of most therapeutic targets. Therefore, assessing changes on the levels of protein expression and function is the most efficient way to evaluate the mechanisms underlying sensitivity and resistance to targeted therapy.

Reverse phase protein array (RPPA), an antibody-based assay, has emerged as a robust, sensitive, and cost-effective approach to analyze large numbers of samples for quantitative assessment of key protein molecules in functional pathways. The RPPA platform is a powerful measurement to identify and validate targets, classify tumor subsets, assess pharmacodynamics, and define prognostic and predictive markers, adaptive responses and rational drug combinations in model systems as well as in patient samples. Its greatest utility is through integration with other analytic platforms such as DNA sequencing, translational profiling, epigenomics and metabolomics.

RPPA determines levels of protein expression and modifications such as phosphorylation, cleavage, and fatty acid alteration. RPPA allows concordant interrogation of multiple signaling molecules along their functional status. We utilized RPPA to profile and validate signaling networks in human cancer cell lines and tumor tissue.

Each sample is analyzed for cell cycle progression, apoptosis, functional proteomics, and signaling network activity. The results will be classified and compared with disease patterns to generate a "molecular signature." The integrated information will display potential therapeutic targets or biomarkers to accurately predict or rapidly define intracellular signaling networks and functional outcomes affected by therapeutics, providing an expanding repertoire for clinical evaluation.

Availability of key reagents required for execution of the high throughput RPPA project

We have extensively validated about 500 different monospecific antibodies to signaling molecules that are useful for the RPPA approach. These antibodies are assessed for specificity, quantification and sensitivity (dynamic range) using protein extracts from cultured cells or tumor tissue. These antibodies specifically recognize proteins acting on multiple signaling pathways, including receptor tyrosine kinases, PI3K-AKT and MAPK cascades, LKB1-AMPK and TGFβ cascades, as well as DNA repair, cell cycle and apoptosis/autophagy regulators. We are currently validating a group of antibodies for monitoring immune responses to cancer development and to cancer therapy. We update our antibody list routinely and post it publicly on our website to the proteomics community around the world. The list can be found on our RPPA website under "Resources and Protocols."

We have also established QC processes to improve the quality and accuracy of RPPA data sets. A set of cell lysates has been defined, prepared in large quantities and designated as "Control Lysates." Technical replicates of these "Control Lysates" are placed on each RPPA slide at different locations to assess assay sensitivity, stability, and reproducibility. These "Control Lysates" also serve as a standard for batch variation adjustment. Additionally, a large quantity of "Mixed Lysates" has been prepared

from 32 different cell lines. Serial dilutions of "Mixed Lysates" are printed for 96 technical replicates on each slide at different locations as a standard for spatial correction and quality control in data analysis to determine relative protein concentration. The QC score from quality control samples indicate good (above 0.8) or poor (below 0.8) antibody staining. Poor QC slides are excluded from further data analysis and in most cases, are repeated for staining with different antibody concentrations.

We have full access to an Integra Assist Plus pipetting robot for serial dilution of cell lysates and sample transfer, two Quanterix 2470 arrayers for printing up to 100 slides per run with several automated runs continuing for several days, and three Agilent Link 48 autostainers that probe each slide with a different antibody. Each autostainer is capable of staining up to 48 slides per day under conditions that are specific for each individual antibody.

Approach

Currently, we perform RPPA on samples prepared from frozen tissue or from cultured cell lines.

For tumor tissue, we extract proteins from about 15 mg of snap frozen tissue by homogenizer or ceramic beads. Protein concentration will be determined and adjusted to 2μg/μl.

For cell lines, we prefer a 6-well format to obtain enough protein for the entire procedure. Briefly, we select cell lines based on a specific disease model. Cells are seeded in 6-well plates and treated according to experimental design. Cells are lysed in 6-well plates and protein concentration adjusted to 1.5μg/μl.

Proteins extracted from frozen tissue or cultured cells are denatured by 1% SDS + B-Me followed by serial dilution (to detect the antigen-antibody reaction in a linear range for accurate quantification). Serially diluted cellular proteins are arrayed on nitrocellulose-coated slides and probed with validated antibodies that recognize signaling molecules in their functional state. Signals are captured by tyramide dye deposition and a DAB colorimetric reaction. Data is collected and quantitative analysis is performed using custom spot finding and curve fitting software developed for this purpose. Features include automated spot identification, background correction, controlling for location, serial dilution-signal intensity curve construction, and concentration determination. The values derived from the slope and intercept of the "supercurve" construction are expressed relative to standard control cell lysates or control peptides on the array. These values indicate the levels of protein expression and modification (phosphorylation or cleavage based on antibody specificity).

We analyze the data for the presence of clusters, based on differential protein expression by using available methods with the R statistical software package (http://cran.r-project.org). We will use a variety of unsupervised clustering methods (including hierarchical clustering, K-means, independent component analysis, mutual information, and gene shaving) to classify the samples into statistically similar groups. We will evaluate the robustness and statistical significance of these groups using bootstrap resampling of the data. By plotting the data under each condition or each disease pattern independently, we will be able to evaluate linked events and create a database for pathways and networks. Alterations in important signaling molecules in multiple pathways will be correlated to the data from cell survival assay or patient outcomes and integrated to allow rapid assessment of functional proteomics studies.