Rationale

Molecular therapeutics is designed to capitalize on tumor cells that arise from the rewiring of functional networks as a consequence of the 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 analysis, has emerged as a robust, sensitive, cost-effective approach to analyze large number 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 has been realized 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 at 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 result will be classified and compared with disease patterns to generate a “molecular signature”. The integrated information will display the 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 over 300 different monospecific antibodies to signaling molecules as 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 in the process of 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 publically on our website to the proteomics community around the world. The list can be found on our RPPA website under "Antibody Information and Protocols".

In addition, we have established QC processes to improve the quality and accuracy of RPPA data sets. A set of cell lysates has been defined, prepared in large quantity 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" serve as a standard for batch variation adjustment. In addition, a large quantity of "Mixed Lysate" is prepared from 32 cell lines. Serial dilutions of this "Mixed Lysate" are printed for 96 technical replicates on each slide at different locations, as a standard for spatial correction and quality control in the program of 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 a Tecan Robotic Liquid Handling System for serial dilution of cell lysates and sample transfer; two Aushon 2470 Arrayers for printing of up to 100 slides per run with several automated runs feasible in a day; three DAKO Universal Staining Systems that probe each slide with a different antibody. Each autostainer is capable of staining approximately 60 slides per day under conditions that are specific for each individual antibody.

Approach

At this current stage, we perform RPPA on samples prepared from frozen tissue or from cultured cell lines.

For tumor tissue, we extract proteins from 8-10 mg of snap frozen tissue by homogenizer or ceramic beads. Protein concentration will be determined and adjusted to 2ug/ul.

For cell lines, we prefer a 6-well format to get enough protein for the entire procedure. Briefly, we will select cell lines based on a specific disease model. Cells will be seeded in 6-well plates and treated with different manipulations according to experimental design. Cells will be lysed in 6-well plates and protein concentration will be adjusted to 1.5ug/ul.

Proteins extracted from frozen tissue or cultured cells are denatured by 1% SDS + B-Me, followed by serial dilution (in order to define 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 "Supercurve" 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 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 will 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 conditions or each disease pattern independently, we will be able to evaluate linked events and to 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.