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Pathway oriented phospho-proteomics analysis with reverse phase protein array (RPPA) technology in cell growth and cancer therapy

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Abstract

Reverse Phase Protein Array (RPPA) is a powerful technology that facilitates simultaneously measuring the level of a single protein in multiple samples in a high-throughput format. We have developed a signal transduction oriented RPPA system that focuses on phospho-protein profiles in samples. Thus far, our system utilizes 180 phospho-protein specific antibodies and can profile the phosphorylation status of more than 100 Gene Ontology (GO) processes covering more than 10 components in each process. In this study, we show the phospho-protein profiles of two cancer cell lines, A431 and A549 treated with Erlotinib and Gefitinib. Our RPPA system could detect dynamic changes in phosphorylation profiles of both cell lines as well as several differences between them with high sensitivity and reproducibility.

On the other hand, the results of our RPPA experiments were in accordance with those of the *in vitro* kinase assays we have obtained. We are now expanding the panel of cell lines and drugs to devise a method to guide strategies for effective targeting in drug discovery by integrating kinase-inhibition-potency data and cellular-signaling-profile data.

Methods

Cell Culture: A431 (human epidermoid carcinoma) and A549 (human non-small cell lung carcinoma) cells were incubated in serum-free DMEM or F-12 medium, respectively overnight prior to treatment with TKIs. Cells were pre-incubated with 1μM Erlotinib or Gefitinib for 2 hours before stimulating with 500 ng/ml rhEGF. Treated cells were collected at 0 (immediately after adding EGF), 5, 15, and 30 min (Figure 1).

Figure 1. Scheme of Cell Culture Experiment

RPPA: 1-4 x 10^6 cells were homogenized in our proprietary lysis buffer. Lysates were printed onto glass slides with an arrayer equipped with 32 pins. Serially diluted lysates were also spotted in order to place the expression level of samples in a dynamic range for signal detection (Figure 2). Each slide was probed with an anti-phospho antibody and signals were generated by employing a catalyzed signal amplification (CSA) system (Dako) and Alexa-647-conjugated streptavidin. The targets of anti-phospho antibodies utilized in this study span a wide variety of Gene Ontology categories and pathways (Table1 & 2).

Data Analysis: Stained slides were scanned on a desktop scanner (InnoScan 710AL, INNOPSYS, Carbonne, France). SuperCurve (http://bioinformatics.mdanderson.org/main /OOMPA:Overview) algorithms were used to estimate a single value of logarithmic concentration from the four serial dilutions for each sample. Two sample t-test was used to compare values between two groups. All analyses were performed using the R statistical Programming Environment (v.2.15.1).

Pathway Mapping: UniProt ID was ascribed to each target of an anti-phospho antibody based on the antibody information, while the records in UniProt were linked to the information in KEGG pathway and Gene Ontology. This combination (Figure 3) resulted in classification of our targets into GO groups (Table 1) and mapping onto KEGG pathways (Table 2, Figure 4).

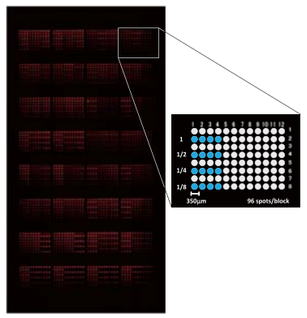

Figure 2. Stained Slide Image and Array Design

A representative stained glass slide image and array design (inset) are shown. Four two-fold serial dilutions of lysates were printed in quadruplicate. Neighboring blocks have the same layout to reduce the effects of local background variation. Each glass slide can accommodate at most 96 samples that are arrayed as two blocks of 192 lysate spots that each contains 6 samples.

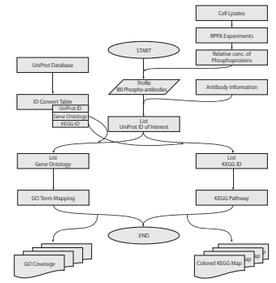

Figure 3. Example of Analysis RPPA Results

RPPA will list antibodies with interesting profile. Usually the antibody targets can be converted to accessions of proteomic database by supplier's information or databases such as PhosphoSite Plus.
On the otherhand, proteomic database such as UniProt provide useful links from accessions to protein functions such as Gene Ontology or KEGG pathway.
By using simple conversion with this accession-function table, Phospho-protein quantities from RPPA experiments can be easily converted to function/pathway profiles.

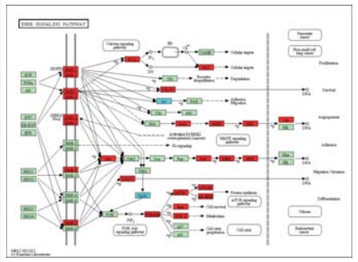

Figure 4. Our Targets Mapped in the ErbB Signaling Pathway

The targets of our anti-phospho antibodies were mapped in the KEGG ErbB signaling pathway (hsa04012). Phosphorylation level of the targets in red boxes are decreased by Erlotinib treatment at 5min in A431 cells. Phosphorylation level of the targets in cyan boxes are not varied significantly(two sample t-test).

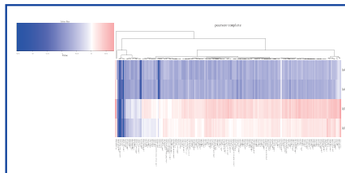

Figure 5. Changes in Phosphorylation of 180 Targets in A431 and A549 Cells Treated with TKIs at 5 min After Addition of EGF Revealed by RPPA

Each column represents a phosphorylated target. Blue color indicates decreased phosphorylation and red indicates increased phosphorylation, compared with control. Following 5min EGF stimulation, almost all targets showed decreases in phosphorylation for A431 cells treated with TKIs relative to a control. On the other hand, in A549 cells, phosphorylation level of most proteins were unchanged or rather increased relative to a control, except for a few proteins which exhibit decreased phosphorylation. Erlotinib- or Gefitinib-treatment showed a similar change in phosphorylation of almost all targets in both A431 and A549. The heat map was derived by calculating the average of the difference in logarithmic concentrations between a TKI-treated and a control group over at least three RPPA experiments.

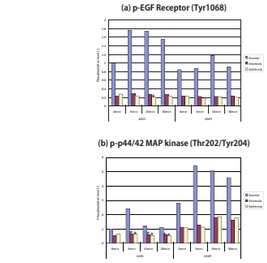

Figure 6. Time-dependent Changes in Phosphorylation Levels of Proteins in the ErbB Signaling Pathway

Erlotinib or Gefitinib effectively reduced phosphorylation levels of (a) EGFR (Tyr1068) and (b) p44/42 MAPK (Thr202/Tyr204) at all time points investigated. A431 or A549 was stimulated with EGF at time 0 in the presence of Erlotinib or Gefitinib. *p<0.05 vs control using two sample t-test.

RPPA Target with this GO	Human protein with this GO	Coverage(%)	GO ID	GO description	GO Type
10	14	71.429	GO:0004697	protein kinase C activity	F
15	33	45.455	**GO:0051403**	**stress-activated MAPK cascade**	P
10	23	43.478	**GO:0046627**	**negative regulation of insulin receptor signaling pathway**	P
6	15	40.000	**GO:0043536**	**positive regulation of blood vessel endothelial cell migration**	P
6	15	40.000	GO:0004708	MAP kinase kinase activity	F
9	24	37.500	**GO:0060397**	**JAK-STAT cascade involved in growth hormone signaling pathway**	P
9	24	37.500	**GO:0031663**	**lipopolysaccharide-mediated signaling pathway**	P
5	15	33.333	**GO:0032007**	**negative regulation of TOR signaling cascade**	P
22	67	32.836	**GO:0035666**	**TRIF-dependent toll-like receptor signaling pathway**	P
22	69	31.884	**GO:0034138**	**toll-like receptor 3 signaling pathway**	P
21	68	30.882	**GO:0034139**	**toll-like receptor 1 signaling pathway**	P
21	69	30.435	**GO:0034134**	**toll-like receptor 2 signaling pathway**	P
6	20	30.000	**GO:0030010**	**establishment of cell polarity**	P
21	73	28.767	**GO:0002755**	**MyD88-dependent toll-like receptor signaling pathway**	P
14	49	28.571	**GO:0018108**	**peptidyl-tyrosine phosphorylation**	P
22	80	27.500	**GO:0008063**	**Toll signaling pathway**	P
22	80	27.500	**GO:0034142**	**toll-like receptor 4 signaling pathway**	P
12	45	26.667	GO:0004725	non-membrane spanning protein tyrosine kinase activity	F
13	49	26.531	**GO:0033138**	**positive regulation of peptidyl-serine phosphorylation**	P
5	19	26.316	**GO:0046320**	**regulation of fatty acid oxidation**	P
16	61	26.230	**GO:0018105**	**peptidyl-serine phosphorylation**	P
7	27	25.926	**GO:0050853**	**B cell receptor signaling pathway**	P
17	68	25.000	**GO:0048015**	**phosphatidylinositol-mediated signaling**	P
6	25	24.000	**GO:0043352**	**positive regulation of phosphatidylinositol 3-kinase activity**	P
6	25	24.000	**GO:0045931**	**positive regulation of mitotic cell cycle**	P
27	113	23.894	**GO:0008543**	**fibroblast growth factor receptor signaling pathway**	P
31	142	21.831	**GO:0008286**	**insulin receptor signaling pathway**	P
5	23	21.739	GO:0000979	RNA polymerase II core promoter sequence-specific DNA binding	F
27	128	21.094	**GO:0007173**	**epidermal growth factor receptor signaling pathway**	P
6	29	20.690	**GO:0043491**	**protein kinase B signaling cascade**	P
9	44	20.455	**GO:0000186**	**activation of MAPKK activity**	P
31	153	20.261	**GO:0046777**	**protein autophosphorylation**	P

Table 1. Classification of Our Antibody Targets According to Gene Ontology

Gene Ontology IDs were listed in the order of higher proportion of our targets to all proteins (UniProt IDs) belonging to the same GO. Multiple GO IDs were sometimes assigned to a target because some anti-phospho antibodies can recognize epitopes of homologous proteins (ex. AKT1 & AKT2). The proportion of our targets in a GO group was calculated by counting the GO IDs assigned to the targets non-redundantly.

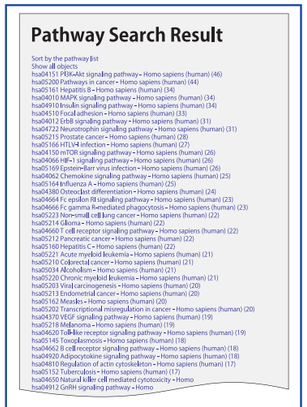

Table 2. Classification of Our Antibody Targets According to Pathways

KEGG pathways were listed in the order of higher count of our targets in a pathway. The number of our RPPA targets in each pathway is indicated in parenthesis.

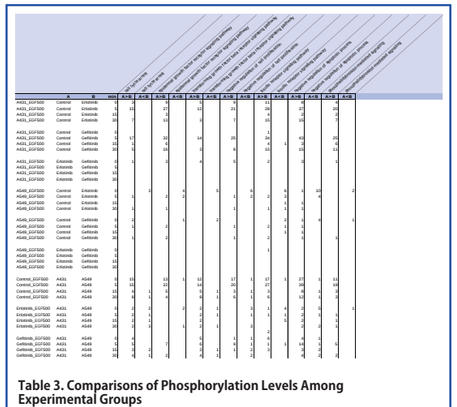

Table 3. Comparisons of Phosphorylation Levels Among Experimental Groups

Comparisons in phosphorylation level of the targets are made in this table. The number of targets that showed a significant difference between two groups (two sample t-test, $p<0.05$, $n>3$ for each group) were counted in each Gene Ontology category. In A431 cells, numerous proteins showed reduced phosphorylation in the presence of TKIs, especially at 5 min after EGF stimulation, while a few proteins exhibited significant differences in phosphorylation for A549 cells treated with TKIs, compared with control.

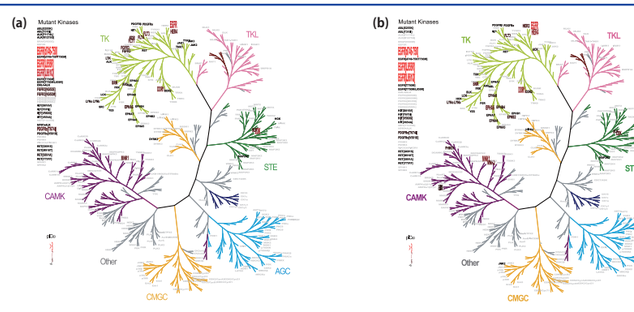

Figure 7. *In vitro* Kinome Profile of (a) Erlotinib and (b) Gefitinib

IC_{50} values of drugs against the kinome (311 kinases including mutants) are determined in the presence of 1mM ATP (Kitagawa D. et al., Genes to Cells 18(2), 110-22(2013)) and are indicated with size and color of kinase names on the kinome trees.

Conclusions

- We have developed a pathway oriented RPPA on glass slides with extremely small amount of samples.
- The outputs of the RPPA phosphoprotein profiles can be converted to protein functions/pathways by using publicly available proteomic data bases.
- Phosphoprotein profiles from two distinct cancer cell lines could be clearly distinguished using our highly sensitive RPPA.
- Two EGFR-TKIs demonstrate similar effects on A431 and A549 cancer cell lines that are consistent with *in virto* kinase inhibition profiles.