Pacific Northwest National Laboratory
Operated by Battelle for the U.S. Department of Energy

Analysis of Receptor Tyrosine Signaling in Stimulated Human Mammary Epithelial Cells

Tyler Heibeck[1], Shi-Jian Ding[2], Lee Opresko[1], Rui Zhao[1], Feng Yang[1], Athena Schepmoes[1], Matthew Monroe[1], David G. Camp[1], Richard D. Smith[1], H. Steven Wiley[1], and Wei-Jun Qian[1]
[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA; [2]University of Nebraska Medical Center, Omaha, NE

Stimulation under multiple conditions with replicates increases both the confidence for relative quantitation and proteome coverage

Overview

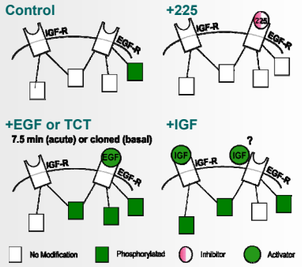
- Our intent was to semi-quantitatively examine tyrosine signaling pathways in human mammary epithelial cells (HMECs) based on spectral counting.
- HMECs were treated with epidermal growth factor (EGF), 225 (a monoclonal antibody inhibitor for the EGF receptor), TCT (a constitutively expressed EGFR ligand), or insulin-like growth factor-1 (IGF).
- Cell lysates were digested, enriched for phosphotyrosine (pTyr) with a peptide immunoprecipitation (IP) step, and analyzed by LC-MS on a metal-free, nano-flow 30 μm HPLC system.
- 531 unique tyrosine phosphorylation sites were identified with their phosphorylation levels characterized across all conditions (consisting of 2–4 biological replicates for each) with an FDR of < 1%. Of these, 48 sites have not been previously reported.

Introduction

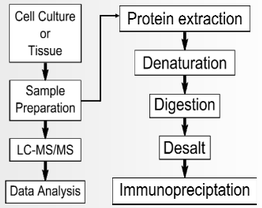
- Phosphorylation is a well characterized cell signaling mechanism that is critical to normal and disease state functions.
- Phosphotyrosine (pTyr) presents at extremely low abundances compared to pSer and pThr (pSer: pThr: pTyr = ~95:5:0.05) and requires an effective enrichment method such as peptide-IP in addition to the IMAC and TiO_2 enrichment techniques, which are commonly used to isolate low-abundant Ser and Thr phosphopeptides.
- Phosphopeptides are known to bind to metal surfaces. We use a metal-free, nano-flow HPLC system to decrease loss of peptide due to retention.
- Semi-quantitative measurements for phosphorylation levels can be achieved based on spectral counting for the identified sites and by performing multiple biological replicates to increase the confidence of quantitation.

Methods

HMECs were grown in cell culture to 40% confluence and treated with several growth factors and an EGFR antibody. Each condition replicate used about 2 x 10^8 cells (10–20 mg total protein).



Conceptual scheme of the different treatment conditions and its effect on cell signaling



Sample Preparation

Cells were scraped and lysed in 20 mM NH_4HCO_3 with 8 M urea and phosphatase inhibitors. Proteins were reduced and alkylated with dithiothreitol and iodoacetamide. Digested peptides were desalted on SepPak Plus cartridges (Waters) and lyophilized.

Peptide Immunoprecipitation[1]

~4 mg peptides were re-dissolved in binding buffer (50 mM MOPS, pH 7.2) and incubated with the pre-bound antibody (PY-100, Cell Signaling) overnight at 4 °C followed by washing. Peptides were eluted with 0.15% trifluoroacetic acid.

LC-MS Analysis

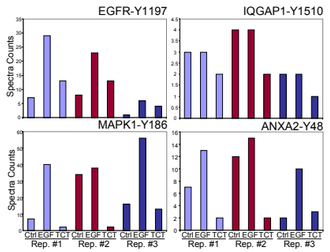
Samples were injected on a nano-flow, metal-free manual HPLC system with a 40 cm long 30 μm I.D. C18 silica capillary. A gradient was run from 0% - 70% acetonitrile with constant 0.1 M acetic acid. Mass spectra were acquired on an LTQ-Orbitrap.

Data Analysis

All MS/MS spectra were searched using X!Tandem against the human IPI database. FDR were calculated with a scrambled database search to be < 1%. Spectral counts were summed between technical replicates and the sum was adjusted for the number of technical replicates among conditions.

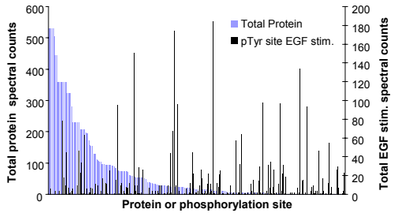
Results

Replicate reproducibility



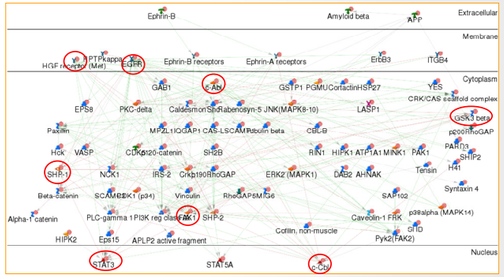
Representative spectral count data for different tyrosine phosphorylation sites. The same relative abundance patterns are observed across 3 treatment conditions for 3 biological replicates.

Protein abundance and phosphorylation level



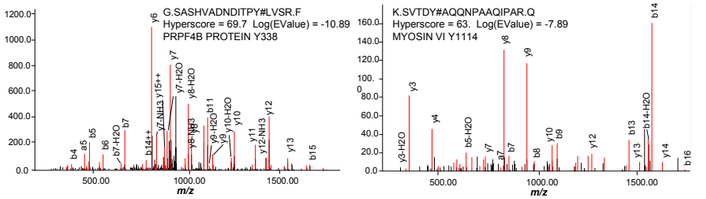
Relationship between the total protein abundance (as previously measured[2]) and the amount of site-specific phosphorylation. Each unique phosphorylation site (273 total) is matched to its respective protein (155 total unique). At the site level the observed amount of phosphorylation does not correlate to total amount of protein. The majority of phosphorylation events were observed from low-abundant proteins.

Interaction network of detected and quantified phosphotyrosine proteins

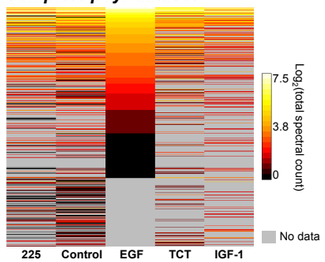


All proteins in the network show increased tyrosine phosphorylation following EGF or TCT stimulation as analyzed by GeneGo MetaCore. The network clearly illustrates some key protein interaction nodes such as EGFR, HGFR, FAK1, GSK3 beta, SHP-1, STAT3, c-abl, c-Cbl that involved in the EGFR pathways. The symbols of proteins indicate different functional classes.

Representative MS/MS spectra of novel pTyr sites

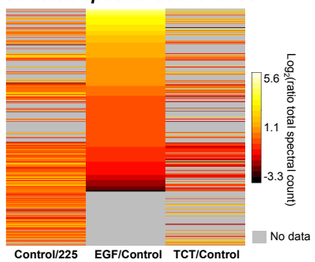


Complete pTyr dataset



Phosphorylation abundances for 531 different sites over five different treatment conditions. 48 of these sites have not previously been identified (compared to the PhosphoSite[3] database). Each column represents the spectral count sum over 2–4 biological replicates. Data was sorted on the EGF dataset. Stimulation over multiple conditions yields broader site coverage, providing a more complete characterization of the system.

Selected spectral count ratios



Ratio of spectral counts for three conditions. As before, numbers are sorted by the EGF results. 185 Tyr residues were affected by EGF stimulation. All 531 found sites are displayed here. As expected EGF stimulation gives a large response. Note that not all TCT activated sites overlap with EGF.

Proteins most affected by EGF treatment

Gene Name	Mod. Pos.	Description	EGF/ Ctrl	TCT/ Ctrl	Ctrl/ 225
AHNAK	Y589	NEUROBLAST DIFF.-ASSO. PROTEIN AHNAK	47±1.8	---	-----
EGFR	Y1092	EPIDERMAL GROWTH FACTOR RECEPTOR PRE.	44±3.3	-----	1
PLCG1	Y905	PLCG1 VARIANT PROTEIN	23±2.2	7±1.9	-----
EPS15	Y849	EPIDERMAL GROWTH FACTOR RECEPTOR SUB.	21±2.7	-----	-----
INPPL1	Y1135	INOSITOL POLYPHOSPHATE PHOSPHATASE-LIKE	19±0.4	5±2.0	1
CBL	Y774	E3 UBIQUITIN-PROTEIN LIGASE CBL.	19±2.8	-----	-----
TRIB3	Y330	UBIQUITIN CONJ. ENZYME 7 INTERACTING PRO.	18±1.7	-----	-----
EGFR	Y1138	EPIDERMAL GROWTH FACTOR RECEPTOR PRE.	15±2.0	-----	-----
STAT5B	Y699	SIGNAL TRAND. AND ACT. OF TRANSCRIPTION 5B	15±0.5	5±0.0	-----
STAM2	Y192	SIGNAL TRANSDUCING ADAPTER MOLECULE	15±0.7	-----	-----
CBLB	Y889	E3 UBIQUITIN-PROTEIN LIGASE CBL-B	14±2.1	2±1.0	-----
TNK2	Y914	ACTIVATED CDC42 KINASE 1	14±1.1	-----	-----
TAGLN2	Y213	24 KDA PROTEIN	12±0.9	-----	-----
AHNAK	Y717	NEUROBLAST DIFF.-ASSO. PROTEIN AHNAK	12±2.2	1±0.5	1±0.2
CAV1	Y6	CAVEOLIN 1	11±2.0	-----	2±0.6
EPN3	Y17	EPSIN-3.	11±1.2	-----	-----
PTPN18	Y389	PROTEIN TYROSINE PHOSPHATASE	10±2.2	3±1.3	2±0.6
STAM2	Y371	SIGNAL TRANSDUCING ADAPTER MOLECULE	10±0.9	-----	-----
JUP	Y722	JUNCTION PLAKOGLOBIN	10±1.9	-----	-----
EGFR	Y1110	EPIDERMAL GROWTH FACTOR RECEPTOR PRE.	10±1.1	5±2.1	2±1.1
CBL	Y674	E3 UBIQUITIN-PROTEIN LIGASE CBL	9±2.5	1±0.3	5±1.1
EGFR	Y998	EPIDERMAL GROWTH FACTOR RECEPTOR PRE.	9±1.2	-----	-----
GAB1	Y183	GRB2-ASSOCIATED-BINDING PROTEIN 1	9±1.1	2±1.0	1±0.3
STS-1	Y19	SUPPRESSOR OF T-CELL RECEPTOR	9±1.2	2±1.0	-----
PRKCD	Y313	PROTEIN KINASE C, DELTA	9±1.5	7±1.9	-----
PLEC1	Y4478	PLECTIN 1 ISOFORM 11	9±1.1	-----	-----
TNK2	Y913	ACTIVATED CDC42 KINASE 1	9±1.5	-----	2±0.6
PKP3	Y195	PLAKOPHILIN-3	9±1.7	-----	2±0.6

Conclusions

- Metal-free, nano-flow HPLC gives consistent, reproducible separations. Our LC platform can be modified to incorporate a TiO_2 pre-column, which could further enrich pTyr peptides.
- A consistent and extensive set of pTyr sites were identified and semi-quantitatively measured. The dataset not only reflects the known biology, but also reveals 48 novel pTyr sites.
- Spectral counting yielded relatively reproducible comparisons for a large number of modification sites over several different stimulation conditions based upon use of biological replicates.
- The vast majority of observed pTyr sites were revealed to originate from lower-abundant proteins as compared to a global HMEC proteomic dataset.
- Current work is focusing on increasing immunoprecipitation efficiency and incorporating isotopic labeling for improved quantitation.

Acknowledgements

Portions of this research were supported the Pacific Northwest National Laboratory (PNNL) Laboratory Directed Research Development program (W.-J.Q.) and the NIH National Center for Research Resources RR018522 (R.D.S.). Much of the work was performed at the Environmental Molecular Science Laboratory, a national scientific user facility located at PNNL and sponsored by the U. S. Department of Energy (DOE) Office of Biological and Environmental Research. PNNL is operated by Battelle for the DOE under Contract No. DE-AC05-76RLO-1830.

References

1. Rush, J. *et al.*, Immunoaffinity profiling of tyrosine phosphorylation in cancer cells, *Nat Biotechnol* . **2005**, 23:94-101.
2. Liu, T. *et al.*, Improved proteome coverage by using high efficiency cysteinyl peptide enrichment: The human mammary epithelial cell proteome, *Proteomics* **2005**, 5:1263-1273
3. Hornbeck, P.V. *et al.*, Phosphosite: A bioinformatics resource dedicated to physiological protein phosphorylation, *Proteomics* **2004**, 4: 1551-1561. http://www.phosphosite.org

Contact Information

Tyler Heibeck, PhD
Biological Sciences Division, K8-98
Pacific Northwest National Laboratory
P.O. Box 999, Richland, WA 99352
e-mail: tyler.heibeck@pnl.gov