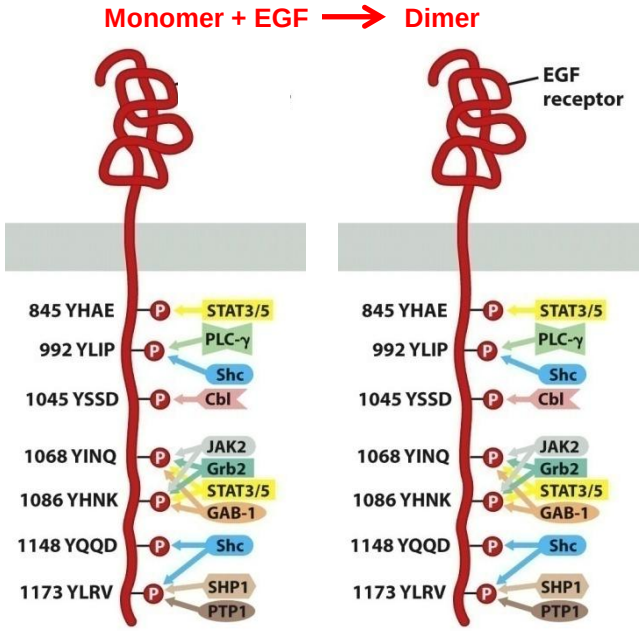
Deglycosylation and Antibody Screening to Identify Receptor Tyrosine Kinases by 2DE

*Jon Johansen[1], Matt Hoelter[1], Mary Ann Gawinowicz[2] & Nancy Kendrick[1]**

[1]Kendrick Labs Inc, Madison, WI, www.kendricklabs.com
[2]Columbia University Protein Core Facility, New York, NY

Receptor tyrosine kinases are known cancer drivers



Robert Weinberg, BOC, 2007

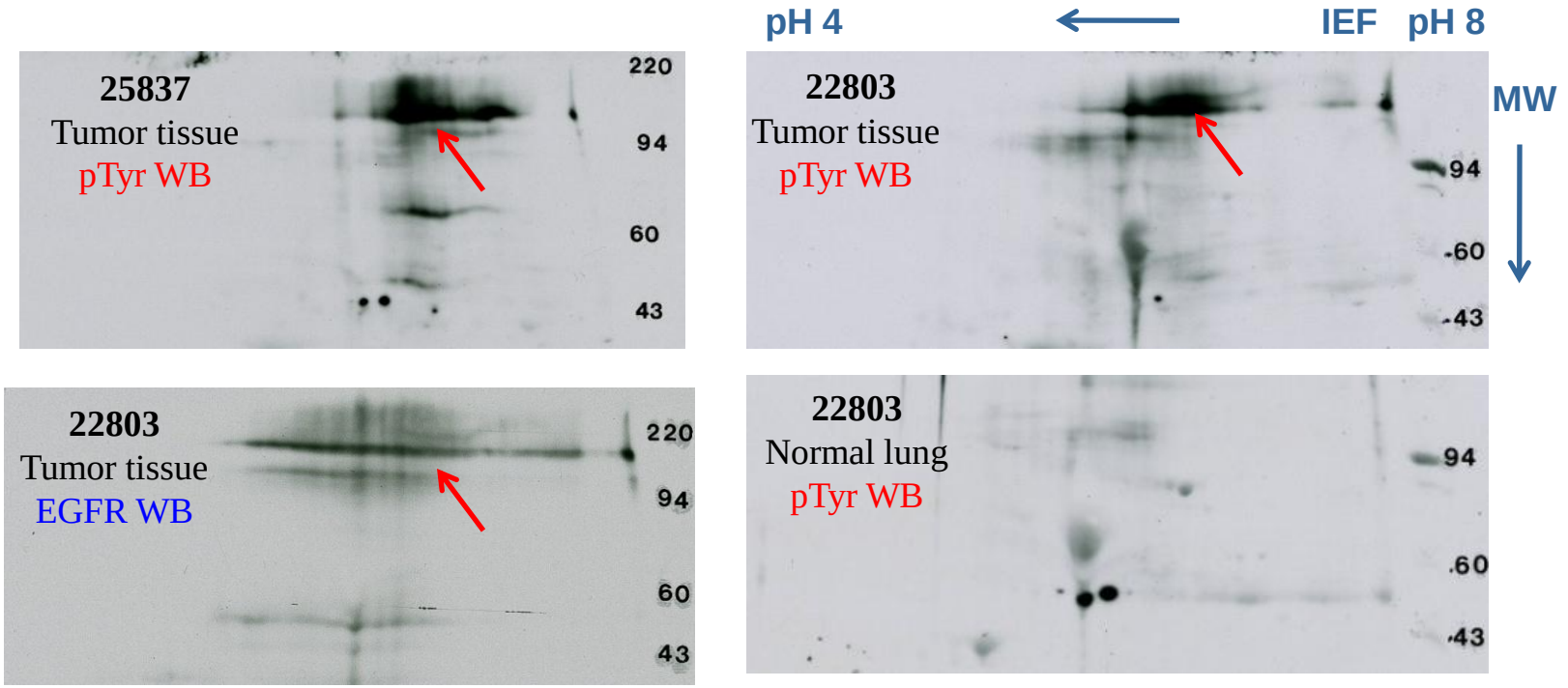
Mechanism of Action

RTK monomers float in the cell membrane until ligand binding triggers dimerization and tyrosine phosphorylation of cytoplasmic chains.

The **phosphotyrosines** (**pTyr**) become binding sites for cytoplasmic proteins that in turn interact. Cascades of reactions are initiated that **drive cell division.**

Active (phosphorylated) RTKs are rare and important

Previously: High MW pTyr proteins were observed in 2/6 lung cancer tumors using carrier ampholine 2D gel Western blotting.



Western Blot (WB) method: 2D gel proteins were transferred to PVDF membrane and incubated overnight with anti-pTyr antibody. The pattern revealed with ECL reagent and exposure to x-ray film .

Results: The pTyr-proteins in the tumor samples migrate to roughly the same area as Epidermal Growth Factor Receptor (EGFR), a Receptor Tyrosine Kinase known to be a powerful lung cancer driver.

Several *EGFR Inhibitors* have been approved for lung cancer treatment by the FDA. Better tests are needed to match patient to inhibitor (1-3).

Our results suggest proteomic tests for activated (phosphorylated) Receptor Tyrosine Kinases (RTK) would be useful. But we have to be sure about the protein's identity.

First two tries at identification by LC/MS/MS didn't work.

References

1. Cappuzzo et. al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst, 2005. 97: 643-55.

2. Eberhard, D.A., G. Giaccone, and B.E. Johnson, Biomarkers of response to epidermal growth factor receptor inhibitors in Non-Small-Cell Lung Cancer Working Group: standardization for use in the clinical trial setting. J Clin Oncol, 2008. 26(6): p. 983-94.

3. Lee, S.M., I. Khan, S. Upadhyay, C. Lewanski, S. Falk, G. Skailes, E. Marshall, P.J. Woll, M. Hatton, R. Lal, R. Jones, E. Toy, D. Chao, G. Middleton, S. Bulley, Y. Ngai, R. Rudd, A. Hackshaw, and C. Boshoff, First-line erlotinib in patients with advanced non-small-cell lung cancer unsuitable for chemotherapy (TOPICAL): a double-blind, placebo-controlled, phase 3 trial. Lancet Oncol, 2012.

The EGF Receptor is known to be heavily *N*-glycosylated (1-2).

Deglycosylation might be an easy way to free up peptides for identification by LC/MS/MS

PNGase F cleaves N-glycans

References

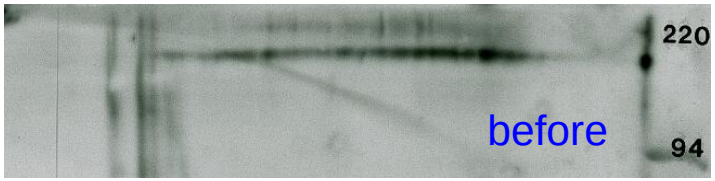
1. Soderquist, A.M. and G. Carpenter, Glycosylation of the epidermal growth factor receptor in A-431 cells. The contribution of carbohydrate to receptor function. The Journal of biological chemistry, 1984. 259(20): p. 12586-94.
2. Zhen, Y., R.M. Caprioli, and J.V. Staros, Characterization of glycosylation sites of the epidermal growth factor receptor. Biochemistry, 2003. 42(18): p. 5478-92.

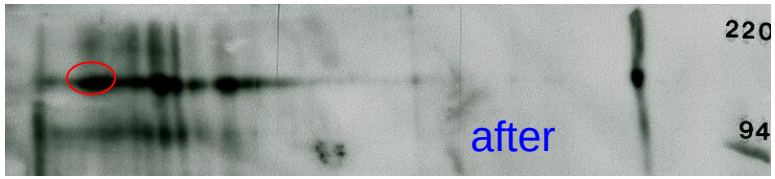
Deglycosylation with PNGase F

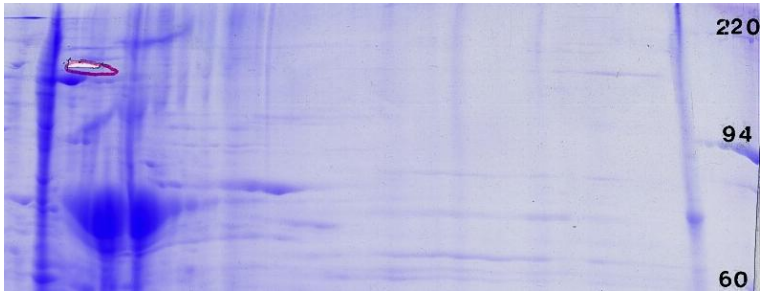
I. A 500 µg aliquot of lung cancer homogenate 22803 was treated with PNGase F from Q-Bio according to their directions.

II. 2DE with pTyr WB showed the putative RTK protein shifted from basic to acid position consistent with conversion of N-blocked asparagines to aspartic acids. The protein also shifted from high to lower MW, consistent with loss of glycan chains.

III. The ECL film pattern was matched to that of the stained blot image, and from there to a duplicate 2D gel stained with Coomassie blue. A small area was cut for identification by LC/MS/MS, then a second cut.

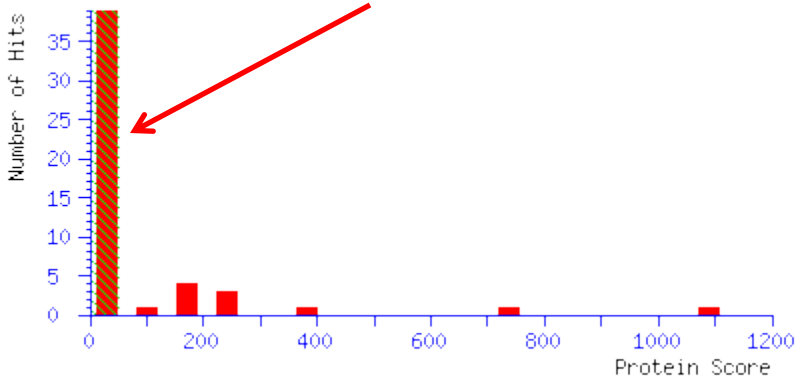






MS results provided by Columbia University protein core

Laminin gamma-2 subunit

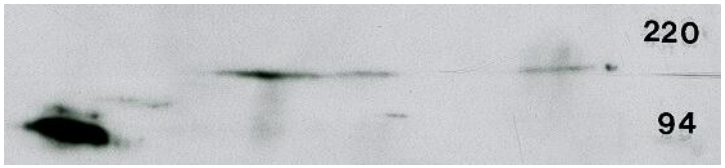


Laminins are major proteins in the basement membrane underlying epithelial cells. Sometimes they are phosphorylated.

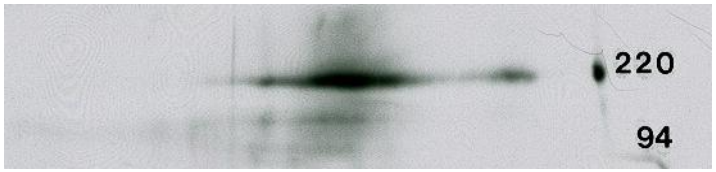
(Trachana, V. et al. Int J Biochem Cell Biol., 2005. 37: 478-92).

2D WB Overlay Image

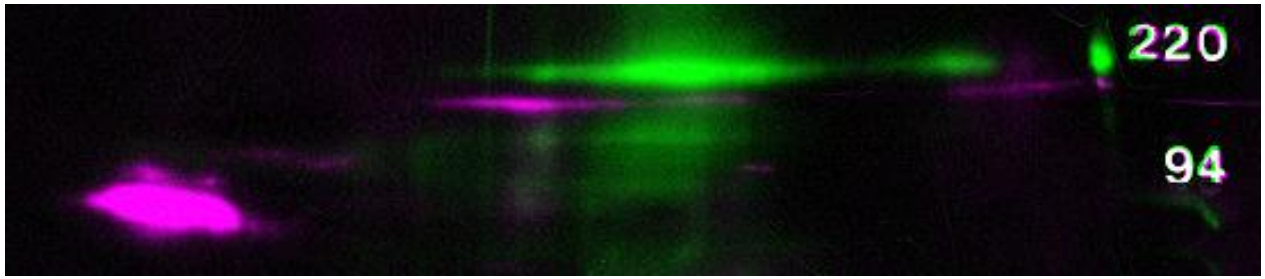
Method: An antibody against Laminin γ-2 was purchased. Laminin and pTyr 2D Western blots were obtained for lung cancer tumor 22803. The images were aligned with Progenesis SameSpots Software and an Overlay Image created.



Laminin γ-2 WB



pTyr WB



Overlay Image: The pTyr WB proteins are green; laminin WB proteins are magenta. The patterns clearly don't match. The putative RTK is not Laminin.

Glycosylation is thought to dramatically change the MW of EGFR

- We saw only a small change. Maybe our deglycosylation didn't fully work.
- Tried deglycosylating using 5 enzymes from the Prozyme kit (PNGase F, O-glycanase, galactosidase, glucosaminidase, and sialidase).
- This time the deglycosylation didn't work well. Probably too much SDS.

References

1. **http://www.phosphosite.org**

2. Soderquist, A.M. and G. Carpenter, Glycosylation of the epidermal growth factor receptor in A-431 cells. The contribution of carbohydrate to receptor function. The Journal of biological chemistry, 1984. 259(20): p. 12586-94.

Deglycosylation didn’t work, what to do next?

We decided to try 1D and 2D WB screening using anti-RTK antibodies with overlay images.

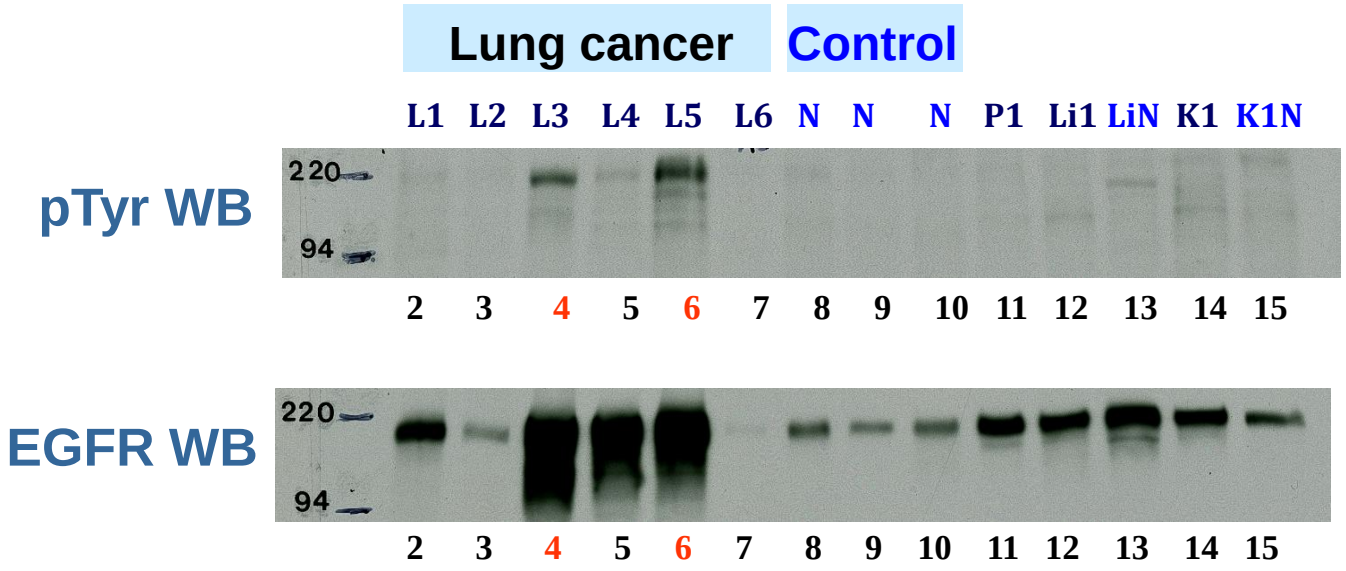
Questions:

- Are the pTyr-proteins in the two lung cancers the same or different?
- Is one or both EGFR, or something else?

1D Gel loading:

Lane	1D Gel Loading	
1	MW Markers	
2	21417	Lung cancer
3	23313	Lung cancer
4	22803	Lung cancer
5	24816	Lung cancer
6	25837	Lung cancer
7	28883	Lung cancer
8	22803N	Normal lung
9	AGR00193	Normal lung - Tub
10	1514N	Normal lung- Asth
11	26870	Pancreatic cancer
12	26801	Liver cancer
13	26801N	Normal liver
14	26852	Kidney cancer
15	26852N	Normal kidney

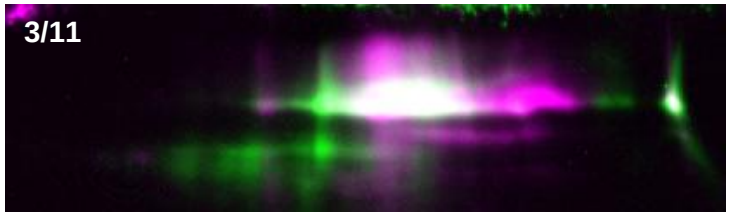
1D WB Results

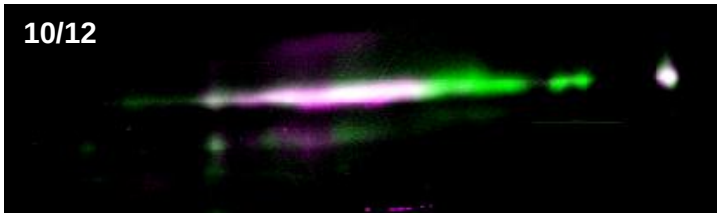


Abbreviations: L = lung cancer, N = Normal lung, P = pancreatic cancer, Li = Liver cancer, LiN = normal liver, K = kidney cancer, K1N = normal kidney. All are from human tumors purchased from a tissue bank prepared by homogenization and heating in SDS buffer.

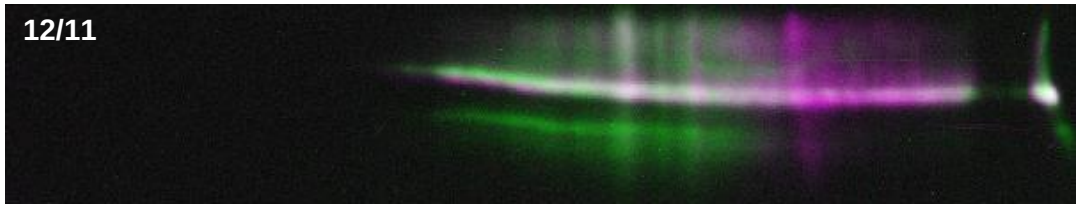
The EGFR signal is strong in the lanes with the pTyr signal, but it's fairly strong in other lanes too.

2D WB film overlays show whether lung tumor pTyr proteins co-migrate

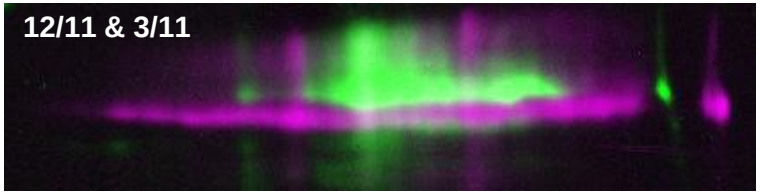




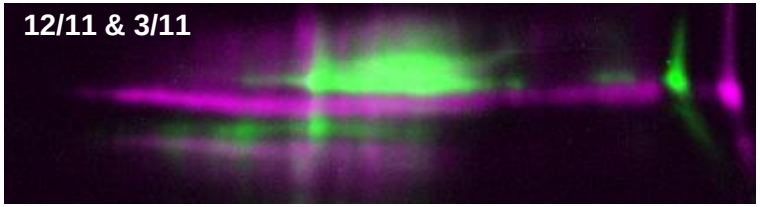
pTyr WBs: **Tumor 25837 (magenta)** over **22803 (green)**. **White indicates a match.**
Both run dates, 3/11 and 10/12, show matches.



EGFR WBs: 25837 (magenta) over 22803 (green). **The patterns match.**

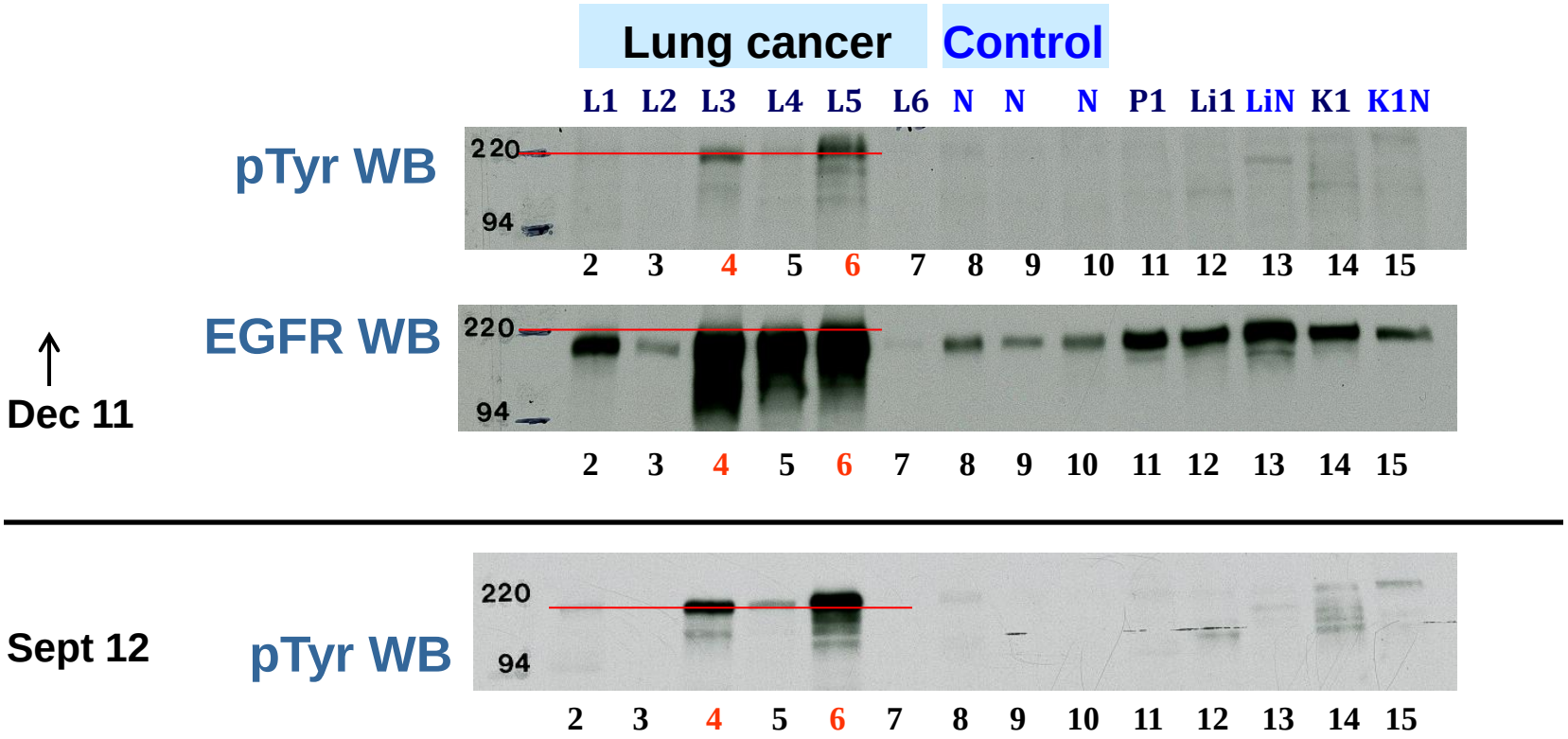


EGFR WB of **22803 (magenta)** over **pTyr WB** of **22803 (green). Not a match**.



EGFR WB of **25837 (magenta)** over **pTyr WB** of **25837 (green). Not a match.**

1D WB Results reconsidered



Carefully measured 1D results confirm the 2D overlays; the pTyr signal runs on or above the 220 kDa marker while the EGFR signal runs below it. They are different.

Other RTK candidates (of about the right MW and implicated in lung cancer)

- ~~**cMET**~~ (Hepatocyte Growth Factor Receptor)
- ~~**Alk**~~ (Anaplastic lymphoma kinase)
- **Other EGFR isomers:** HER2, HER3, HER4
- **PDGFR** (Platelet Derived Growth Factor Receptor)
  - PDGFR-alpha & -beta
- **VEGFR** (Vascular Endothelial Growth Factor Receptor)
  - VEGFR-1, VEGFR-2, VEGFR-3

Crossed out RTKs do not co-migrate.

Conclusions:

- We are able to identify RTKs in lung cancer using 2D WB image overlays
- The putative RTKs in the two tumor samples are the same protein but are not EGFR (or at least not the EGFR isomer with which our antibody reacts)
- Deglycosylation of whole cell lysates is tricky, doesn’t help with protein identification by MS

Collaborators:

Jon Johansen
Lab Manager

Matt Hoelter
Senior Biochemist
AES Executive Director

Mary Ann Gawinowicz
Facility Director
Columbia University
Protein Core Facility