Identification of Tyrosine Kinase Protein Drivers in Human Tumors by 2DE Duplex Western Blotting

Nancy Kendrick, Matt Hoelter and Jon Johansen

Kendrick Labs, Inc, Madison, WI
www.kendricklabs.com

Outline

- Review RTK mechanism
- Previous work (6)
- New approach - 2D WB duplexing (11)
- Summary, Conclusions (18)

RTK Mechanism of Action

Receptor tyrosine kinases are transmembrane proteins. They act by relocating cytoplasmic proteins with SH2 domains (affinity for pTyr) to be near the plasma membrane and each other. This triggers cascades of cell growth reactions.

Examples:

- Src – drives cell division
- GAP – stimulates Ras GTPase activity. Ras drives cell division
- SHP2 – tyrosine phosphatase negative feedback control

If no tyrosine phosphorylation, then no RTK activity

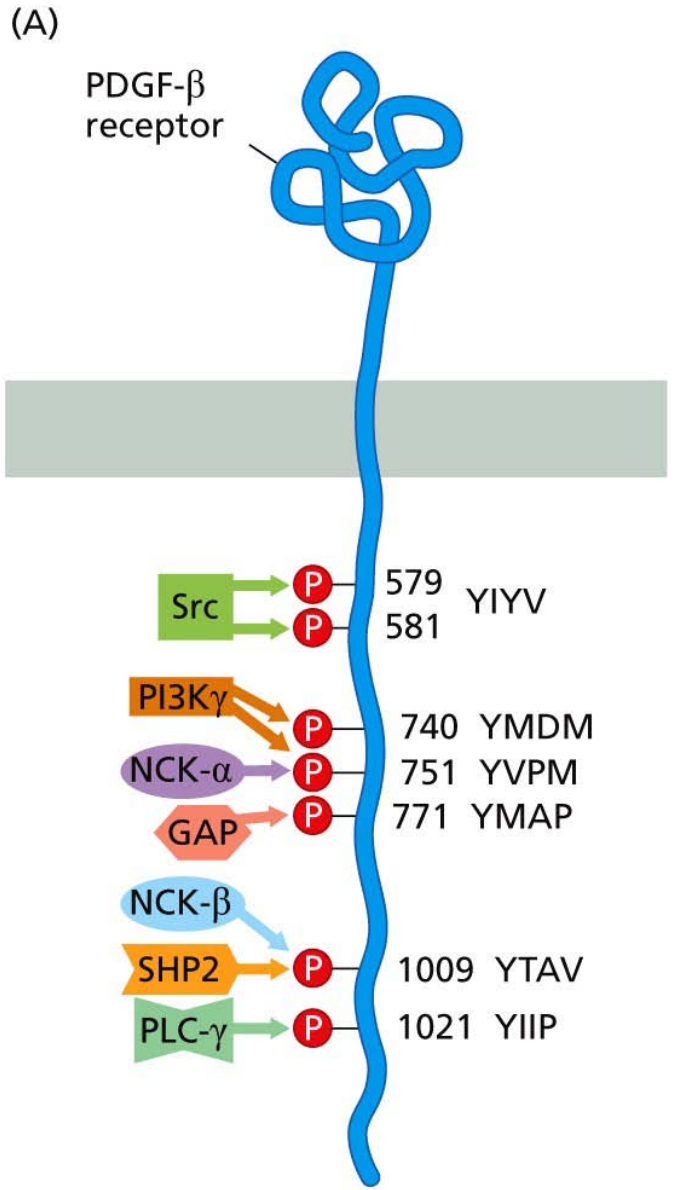
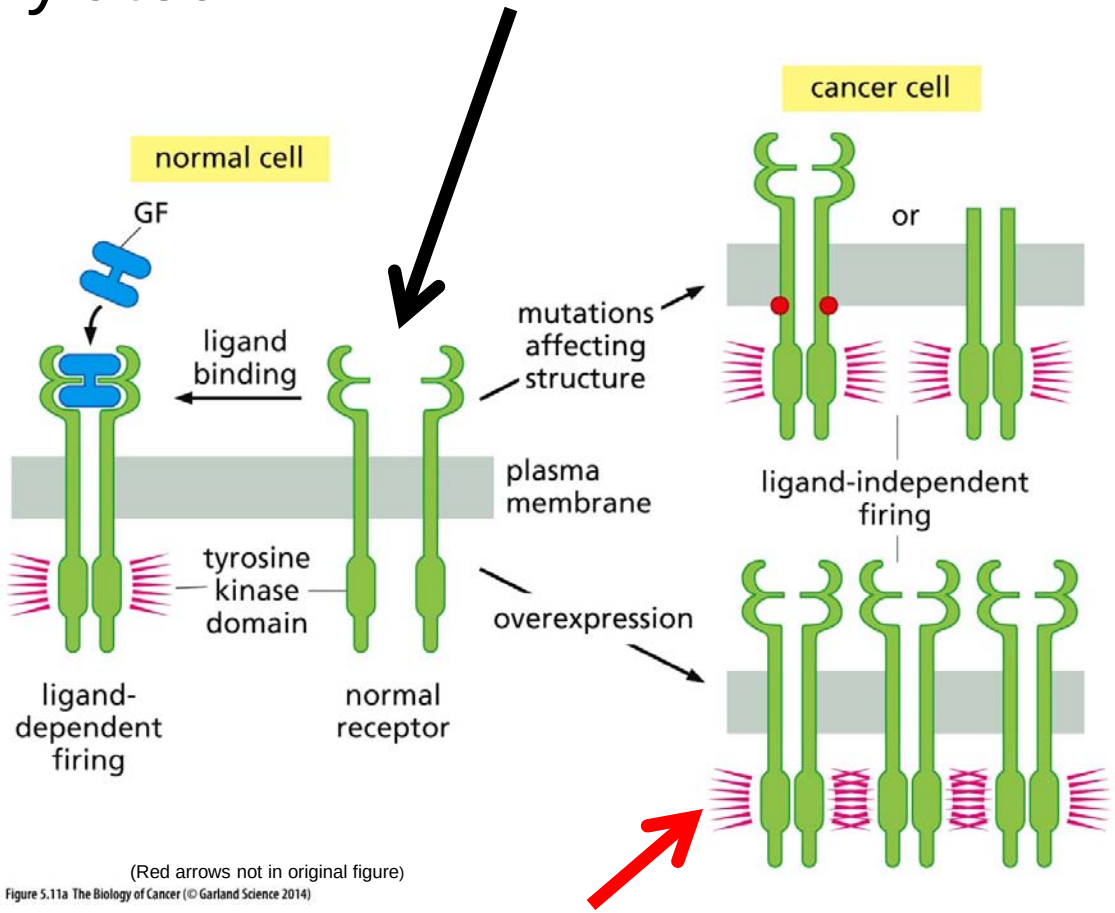


Figure 6.9 The Biology of Cancer (© Garland Science 2014)

Receptor tyrosine kinases are not always phosphorylated



(Red arrows not in original figure)

Figure 5.11a The Biology of Cancer (© Garland Science 2014)

Western blotting can detect protein-bound phosphoTyrosine (pTyr) in tumor tissue lysates.

Human lung cancer tumor and control samples were purchased from a tissue bank, ILSbio

Sample Preparation was straightforward:

- The samples were homogenized on ice with protease and phosphatase inhibitors in SDS buffer, and heated in a boiling water bath until the solution clarified. (No centrifugation step)
- After protein determination and final dilution, the ready-to-load samples were divided into small aliquots, and stored frozen at -80º C.

Previously:

2011 AES Talk: (PP posted on web page)

- 2D WB against pTyr was performed on 6 human lung tumor and 3 control lung samples
- Results: A strong pTyr–protein lit up in 2/6 tumor samples at ~170 kDa (near EGF-R)
  Another strong pTyr protein was seen in 1 tumors at 60 kDa (near Src)
  3/6 tumors and 3/3 controls showed only minor pTyr spots
- The pTyr and EGF-R WBs were run on different gels, on on different days - didn't exactly match. Protein identification by mass spectrometry (MS) failed twice. The human genome shows many possibilities: 90 TKs and 58 RTKs. **We couldn't be sure of what we'd found.**

2012 AES Talk: (posted)

- All attempts to identify the pTyr-proteins using deglycosylation prior to MS at Columbia University Protein Core **failed totally.** Mass spectrometry is not nearly as sensitive as western blotting in complex protein mixtures. Antibody binding K_ds = 10^{-8} to 10^{-12}, very high affinity! **We gave up on MS.**

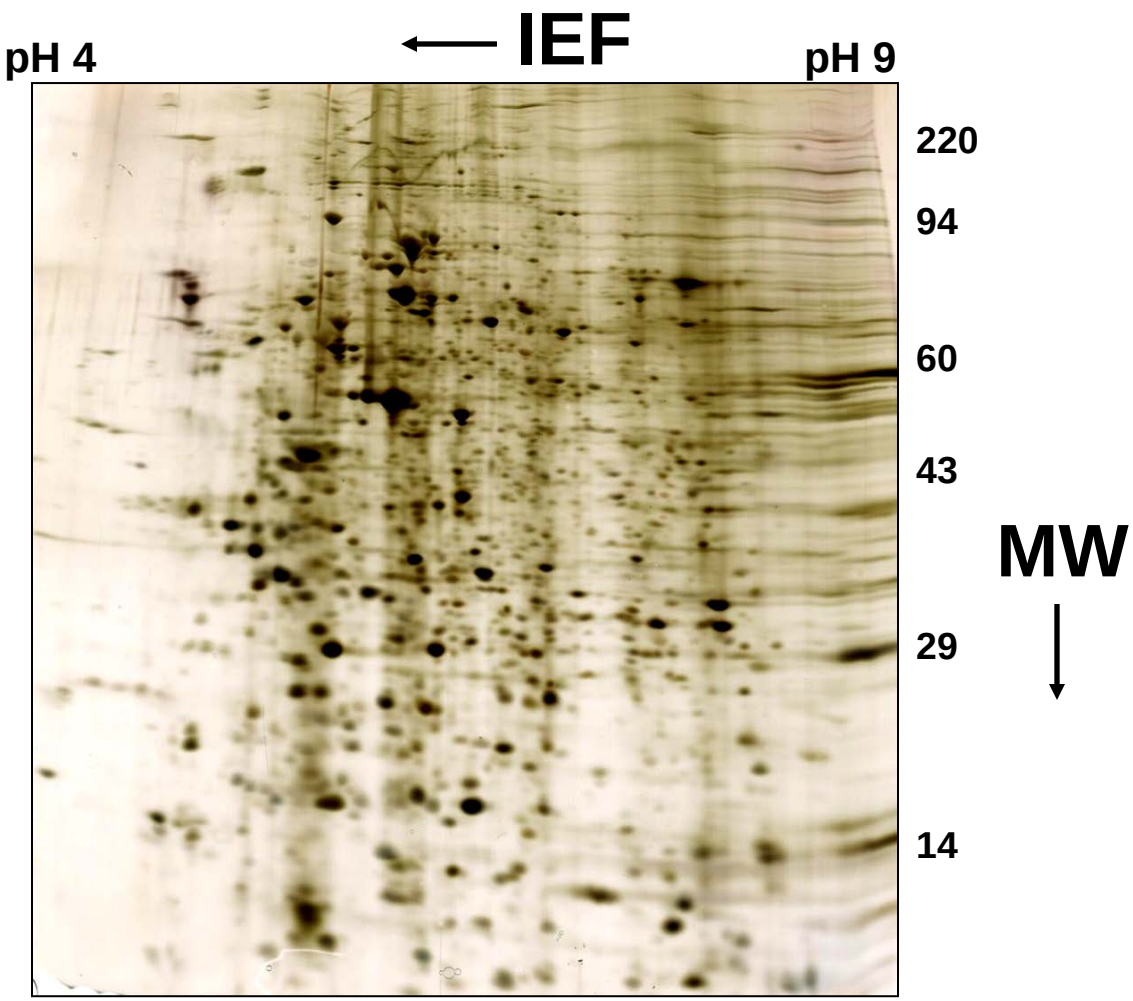
The TK phosphotyrosine signals are robust. Observations made in 2011 are still detectable in newly thawed aliquots in 2013.

Method review: 1st dimension of CA-2DE is isoelectric focusing of samples (dissolved in SDS buffer) in tube gels, not IPG strips

Keith Oberle, Senior Biochemist, loading IEF tube gels

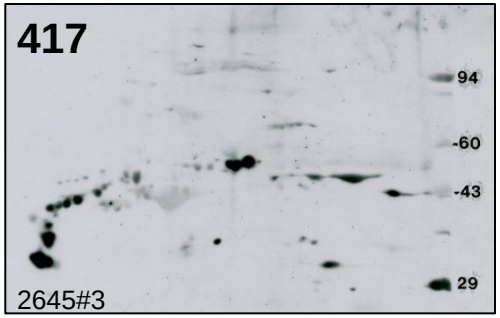
2nd dimension is SDS slab gel electrophoresis

- Whole cell lysates contain many proteins but only a few show up in WBs.
- Duplex WBs were run on smaller gels to conserve antibody.
- This method is highly standardized at Klabs.

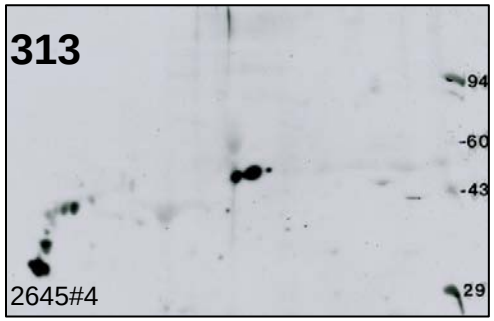


Acute Lymphoblastic Leukemia cells lysates run on large format 2D gels (20x20cm). Shown with permission of Dr. Terzah Horton, Baylor College Med.

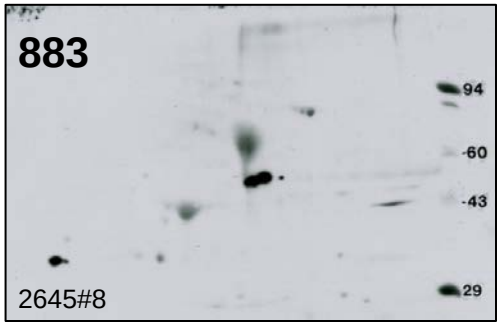
2011 results: anti-pTyr western blots for 6 lung cancer & 3 controls



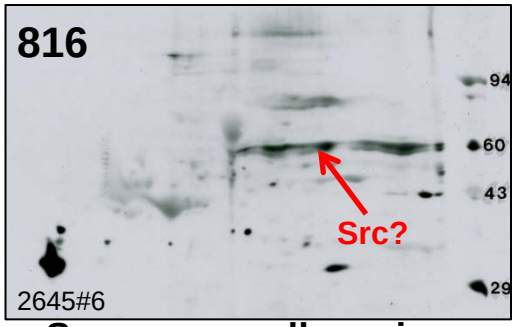
Squamous cell carcinoma



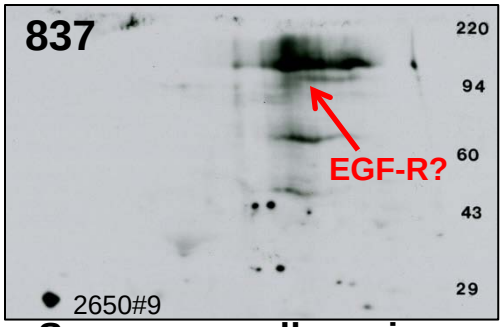
Squamous cell carcinoma



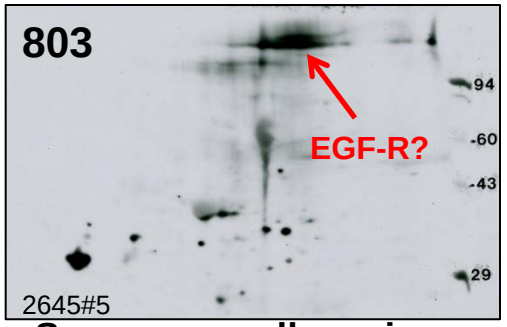
Adenocarcinoma



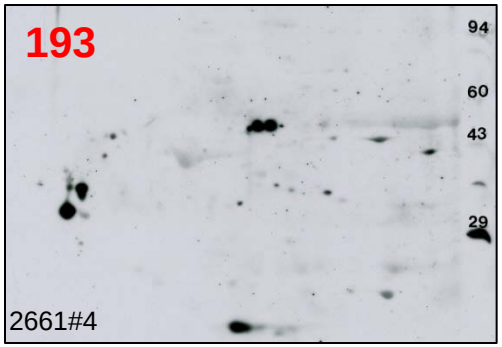
Squamous cell carcinoma



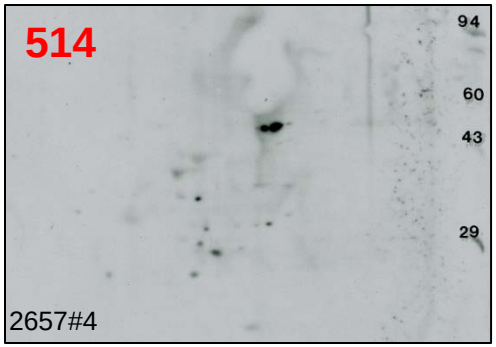
Squamous cell carcinoma



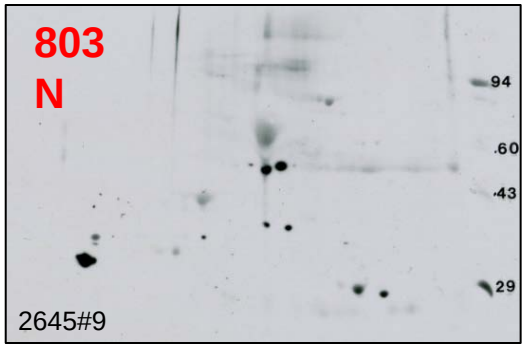
Squamous cell carcinoma



Control: Tuberculosis – lung



Control: Asthma – lung



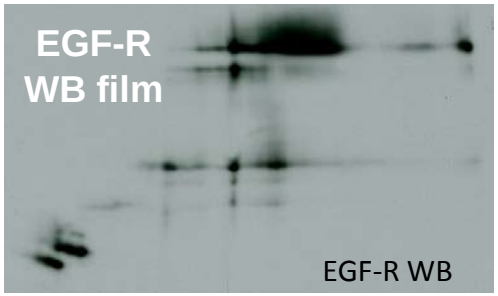
Control: Normal lung tissue

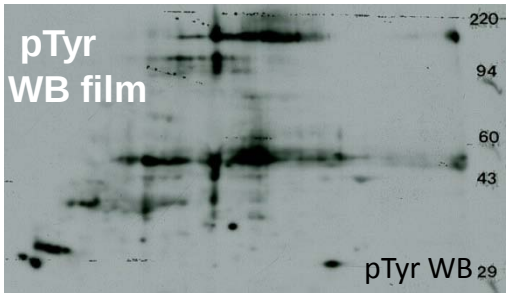
We needed to identify the pTyr proteins unequivocally

- Comparison between gels didn’t work
- Mass spectrometry didn’t work
- WB duplexing – Bingo!
  - Method to optically overlay protein TK and pTyr WB images obtained from the same blot

2D WB duplexing summary slide

One 2D PVDF membrane was probed with RTK ab, stripped, reprobed with pTyr ab.

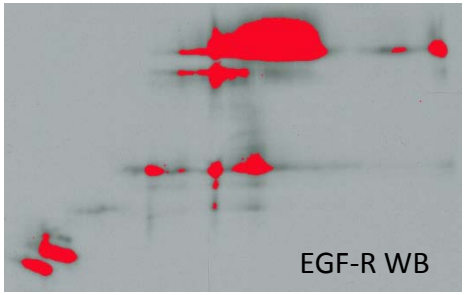


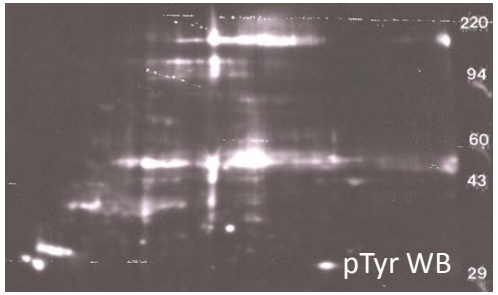


desktop scanned

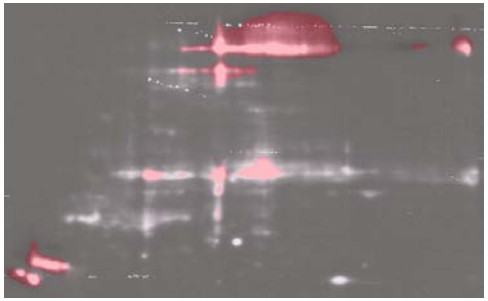
desktop scanned

Tif image colorized in Adobe Elements





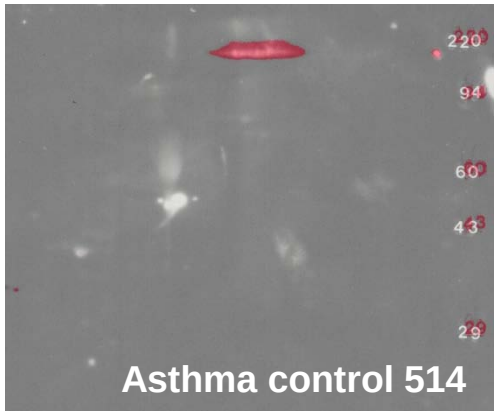
Tif image inverted in Adobe Elements

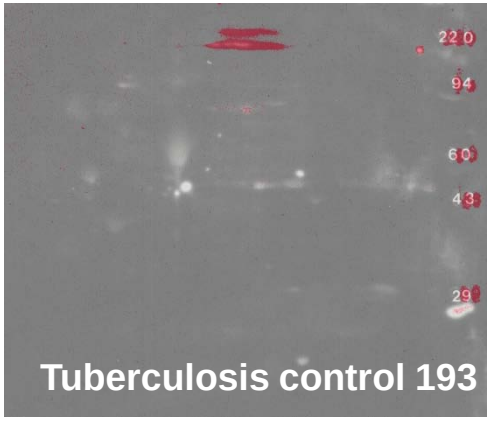


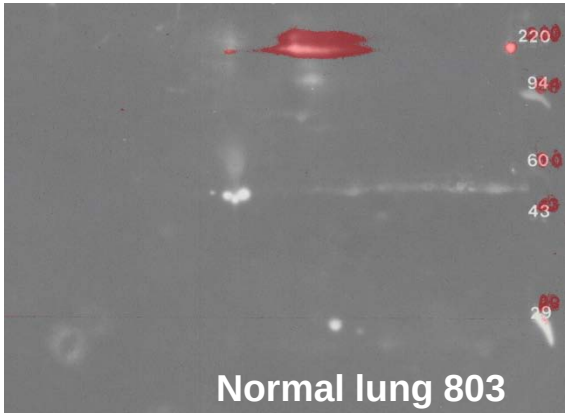
Images are overlaid and aligned. Colorized image opacity is set to 45% to show both patterns. The overlay image is called a duplex.

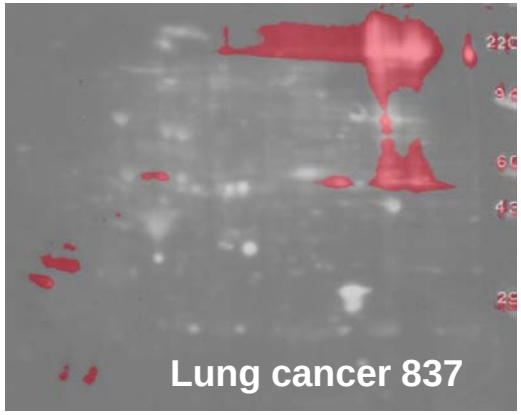
Result: Pattern matching shows EGF-R proteins that contain phosphotyrosine (pink).

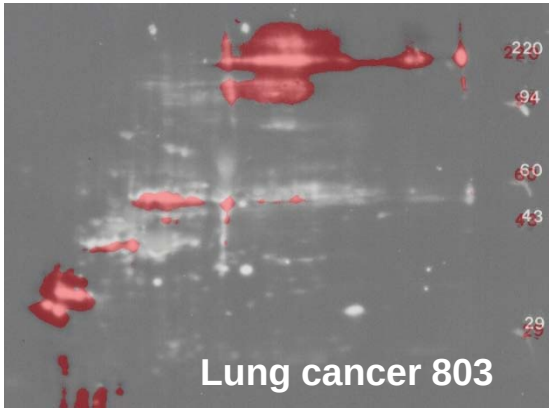
2D Duplexing WB can differentially detect active EGF-R in tumor samples: Lung cancer >> controls











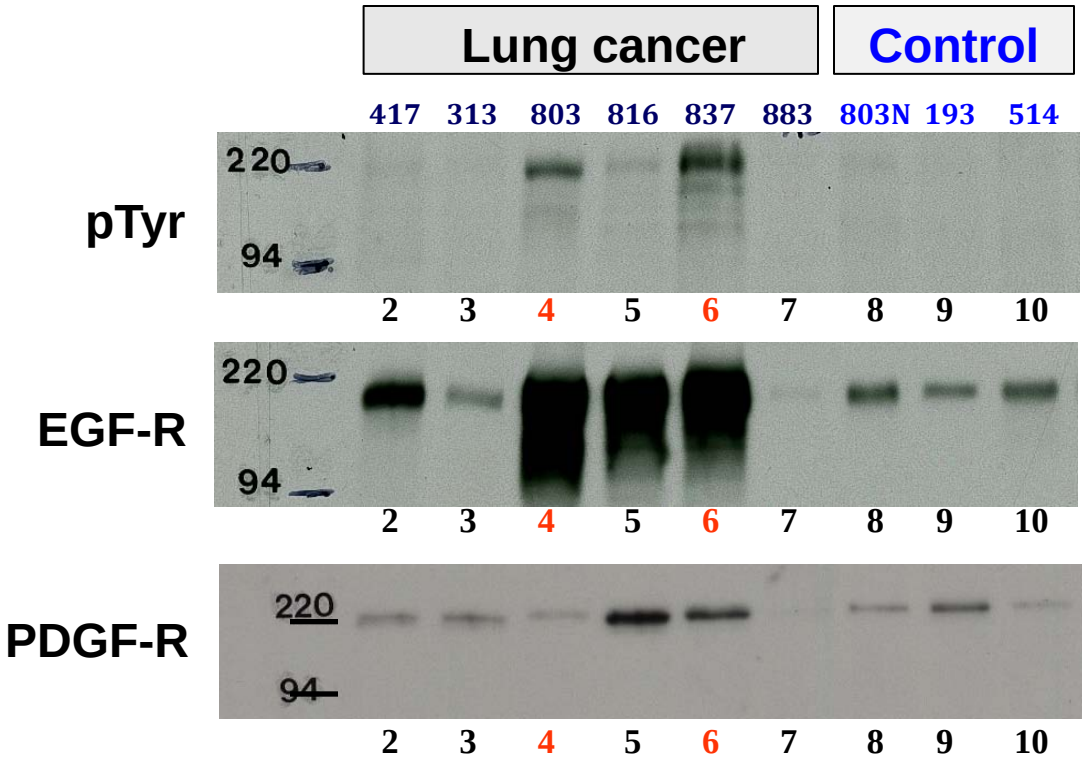
Duplex overlays: EGF-R (red) over pTyr (white).
Standardized WB conditions: EGF-R ab 1:10,000 3min exp; pTyr ab 1:5000 10min exp

1D Check for presence of PDGF-R and VEGF-R

EGF-R, PDGF-R and VEGF-R are known cancer drivers that run at ~170 kDa. Are they present?

Method:

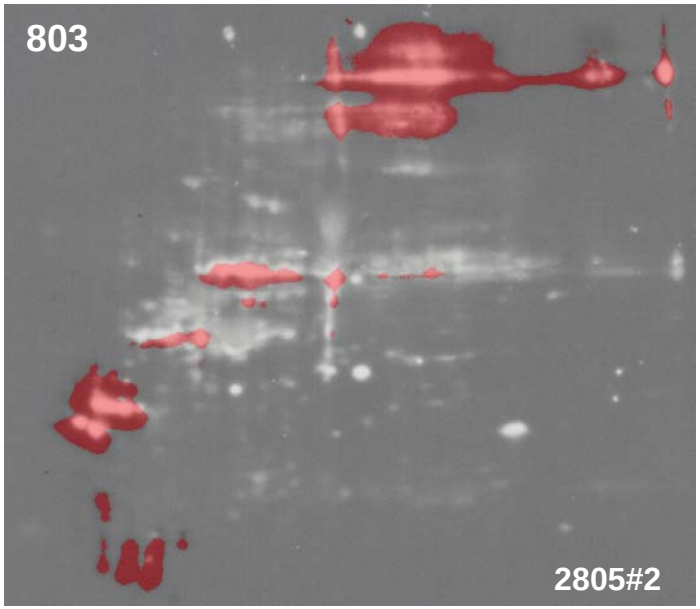
- The 6 lung cancer and 3 controls were identically loaded on four 1D gels.
- Each gel was western blotted with a different antibody.



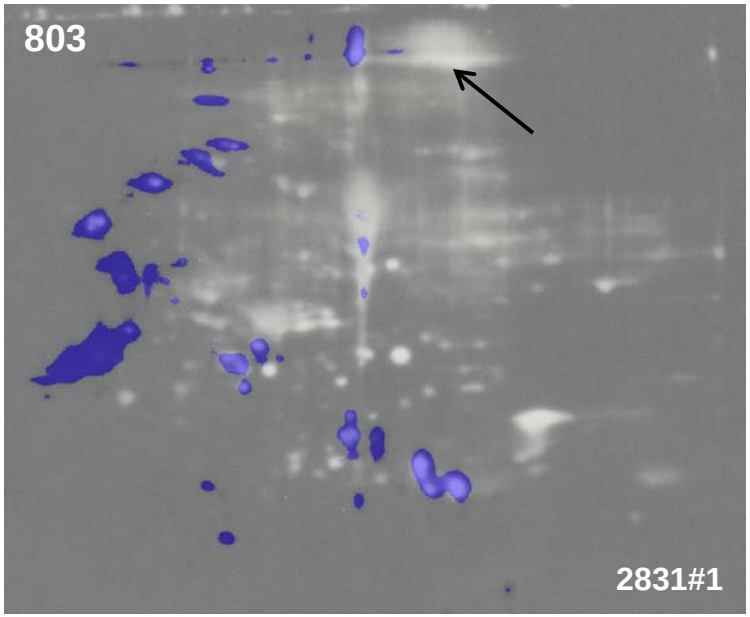
Conclusions

- EGF-R is in all the samples but is only active in patients 803 and 837 (see pTyr - slide 9)
- PDGF-R is in all the samples, perhaps at lower levels; it may be active in 837
- VEGF-R does not drive any of these tumors as it did not react, even at low dilution and long exposure (not shown)

Lung tumor 803: EGF-R vs PDGF-R Duplex WB



EGF-R (red) over pTyr (white)
2805#2 EGFR-3minOver_pTyr1:5000-10min



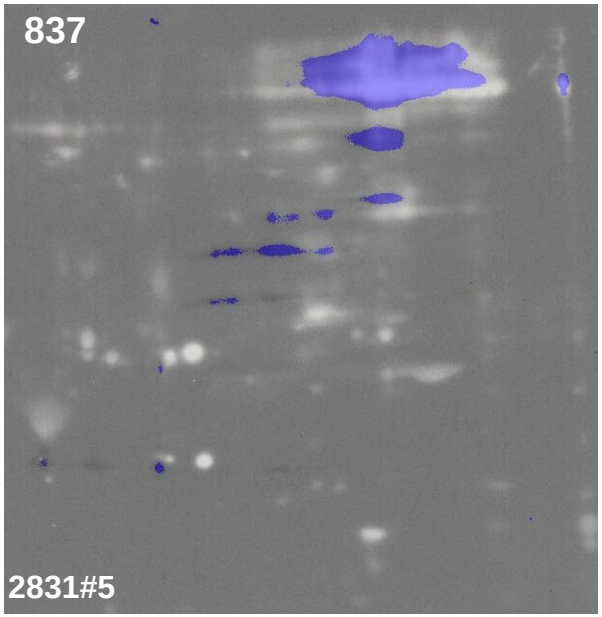
PDGF-R (blue) over pTyr (white)
2831#1 PDGFR-3minOver_pTyr1:5000-10min

Conclusions:

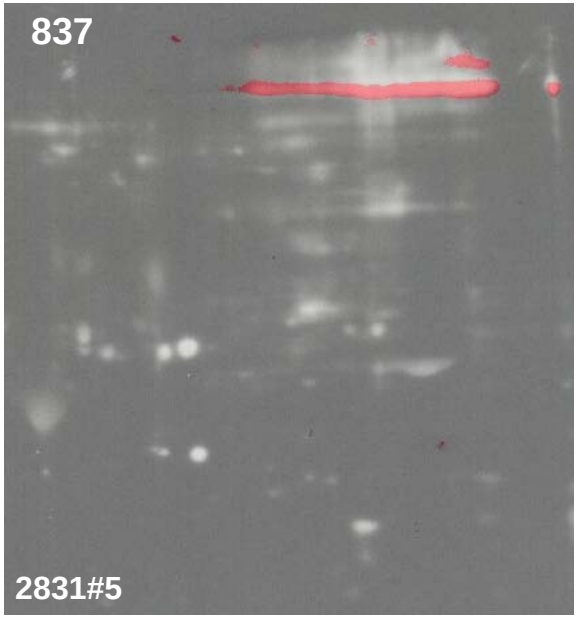
- The diffuse 170 kDa pTyr signal (arrow) does not co-migrate with the PDGF-R signal. Mostly lower MW PDGF breakdown products are seen. PDGF-R is probably not active in this tumor.
- EGF-R is active in 803, i.e. the diffuse 170 kDa EGF-R signal co-migrates with that from the pTyr signal. Patient 803 would probably respond to Gefitinib, an EGF-R inhibitor.

.

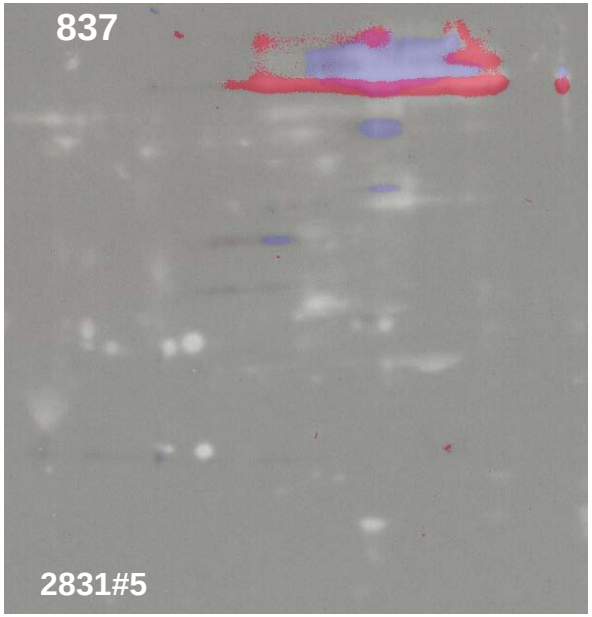
Lung cancer 837: Triplex western blot
Blotting Order: PDGF-R, then pTyr, then EGF-R



PDGF-R (blue) over pTyr (white)
2831#5 PDGF-3minOver_pTyr1:5000-10min



EGF-R (red) over pTyr (white)
2831#5 EGFR-3minOver_pTyr1:5000-10min

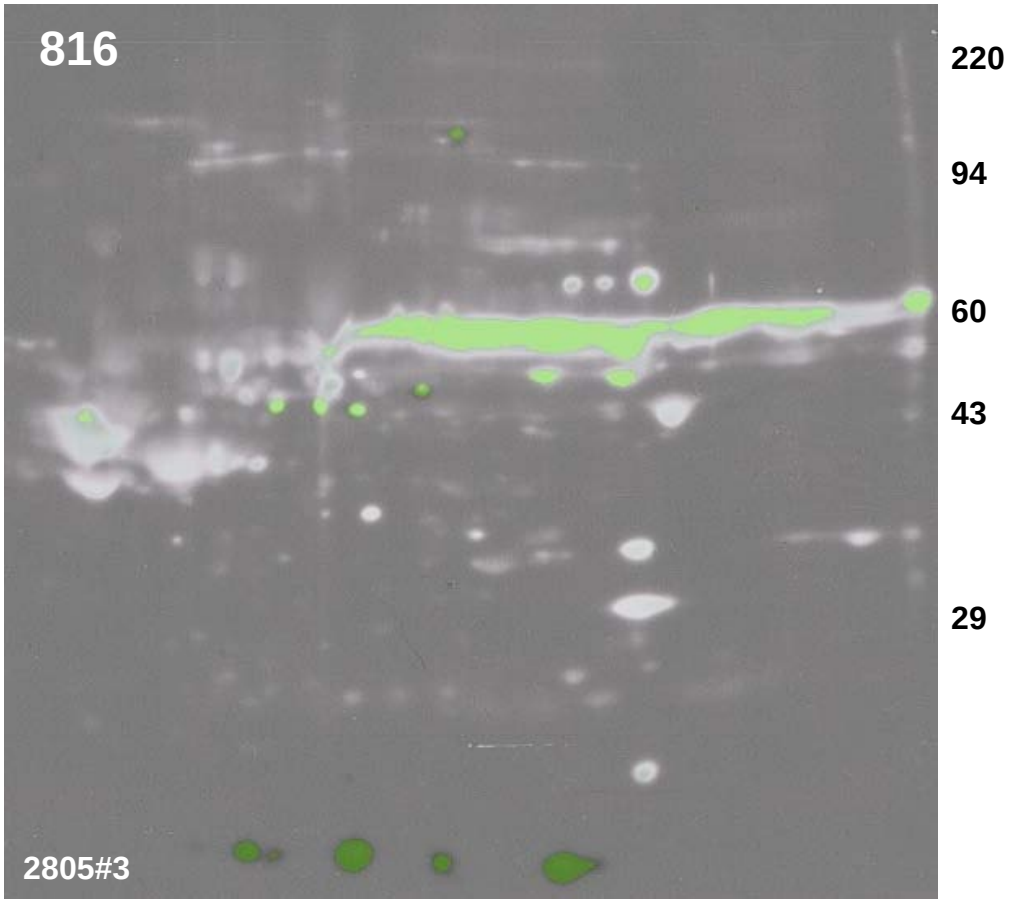


TriPlex – all 3 images aligned

Conclusions:

- The EGF-R and PDGF-R signal comigrate with different parts of the pTyr signal. **Both** are active in this sample. Patient 837 would probably respond best to a cocktail containing inhibitors against both RTKs.
- Stripping twice for triplexing might be too much; the third WB (EGF-R) seemed washed out compared to an earlier result.

Lung Cancer 816 contains active Src

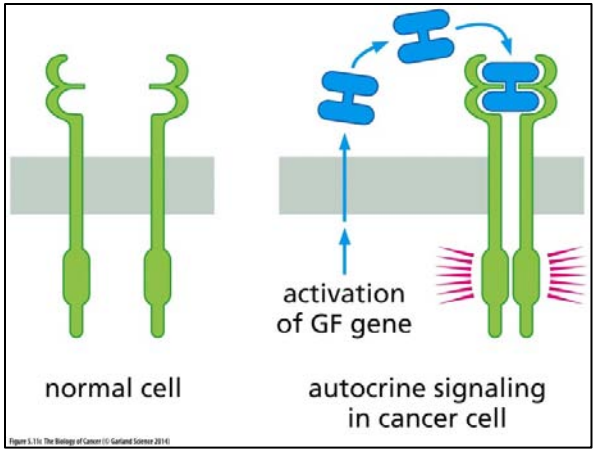


WB Conditions: Src (10,000, 3 min, green) over pTyr (1:1000, 3 min, white).

Conclusion: Src is active in this tumor sample. Patient 816 would probably respond well to Dasatinib, a Src inhibitor, but not to Gefitinib, an EGFR inhibitor.

“Gene Breakthroughs Spark a Revolution in Cancer Treatment” (WSJ Front page article Aug 13, 2013)

Personalized testing for genomic mutations that activate RTKs is the new way to match kinase inhibitor drugs to patients. But genomic testing is not perfect. For example it would miss autocrine signaling that might be detected by 2D WB duplexing.



In malignant autocrine signaling, RTKs are not mutated.

Twelve examples of human tumors making autocrine growth factors are listed in Biology of Cancer 2nd Ed, Table 5.3. 2D WB duplexing is worth pursuing.

Conclusions

- CA-2D WB Duplexing is able to visualize receptor tyrosine kinases (RTKs) in human tissue in a way never before seen. How is this possible?
  - Membrane-bound RTKs are hydrophobic and thus difficult to solubilize. Boiling homogenized tumor tissue in a buffer containing 2% SDS completely solubilizes all proteins including RTKs. CA-2D is compatible with SDS and resolves RTKs. Other proteomic methods like IPG-2D, ELISA, and MS are not compatible with SDS and do not resolve these proteins.
  - Immunostaining is *very* sensitive. Antibody-antigen binding constants range between 10^{-8} and 10^{-12} ;10^{-10} is common. Immunostaining of transblotted membranes from CA-2D gels (western blotting) with sensitive antibodies detects tyrosine kinase proteins on 2D gels.
  - Duplexing (immunostaining a single western blot with two antibodies, anti-pTyr and anti-RTK) proves not only that the RTK in question is present, but that it is present in an active form. This is important; 1D gels show that RTKs like EGF-R are often present but not necessarily active.
- To our knowledge, this is the first clear test that determines which of several intact, activated TK and RTK *proteins* are present in human lung tumor tissue at levels expected to drive cancer growth. This is a direct test, not an indirect test like genomic testing. This information should aid a physician in deciding which inhibitor drug to prescribe.
- CA-2D WB Duplexing is an experimental procedure that needs to be standardized, validated, and given a quantitative endpoint. CA-2D WB Multiplexing with labeled, mixed antibodies detectable with a fluorescent scanner is an obvious next step.
- Kendrick Labs, Inc is looking for collaborators and clients.

Acknowledgements

Kendrick Collaborators:

Jon Johansen
Lab Manager

Matt Hoelter
Western Blot Manager

Andrew Koll, Biochemist

Source of diagrams, inspiration

The Biology of Cancer by Robert Weinberg
Publisher: Garland Science
Second Edition 2013