Identification of tyrosine kinase protein drivers in human tumors using 2D western blot overlays

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Abstract

Tyrosine kinases (TK) are well known to drive cancer growth. A plethora of tyrosine kinase inhibitors are now available as targeted cancer therapies against driver proteins like EGFR, PDGFR and Src. The problem for physicians is deciding which TK, if any, is driving each patient's cancer. Genomic testing can indirectly detect active mutated TKs but not wild type proteins turned on, for example, by excess growth factor. It would be useful to have a companion diagnostic test that *directly* identifies active TK proteins, both mutant and wild type, in tumor tissue.

Our goal has been to develop such a direct test that could serve as a gold standard showing correct results. Other high-throughput proteomic tests (ELISA, protein microarrays) could then be optimized against ours. This presentation provides evidence that a specialized version of 2D gel electrophoresis (CA-2D) is capable of detecting and identifying at least three active TK proteins when used in combination with western blotting.

CA-2D was used to resolve proteins in 6 tumor and 3 control samples followed by western blotting with high affinity antibodies against phosphotyrosine (pTyr), and the TK of interest. Blot image overlays showed TK activation by phosphorylation. One patient's tumor showed pTyr-EGFR only; another's showed both pTyr-EGFR *and* pTyr-PDGFR; a third showed pTyr-Src only. Three other lung cancer tumors and three control lung samples showed low TK signal. Thus, the framework for a gold standard assay has been established.

Outline

- Review of receptor tyrosine kinase mechanism (4-5)
- Previous work (slide 6-7)
- New approach - 2D western blot overlays (slide 8-14)
- Discussion, Conclusions (slide 15-16)
- Acknowledgments, Methods (slide 17-20)

Abbreviations:

Ab: antibody; **blot:** rigid membrane containing proteins transferred from a 1D or 2D gel. **pTyr**: phosphotyrosine; **TK:** tyrosine kinase; **RTK**: receptor tyrosine kinase; Examples of RTKs are **EGFR**: epidermal growth factor receptor; **PDGFR**: platelet derived growth factor receptor; and **VEGFR**: vascular endothelial growth factor receptor. There are 58 RTKs.

CA-2DE: carrier ampholine 2-dimensional electrophoresis. A method for resolving protein mixtures where individual proteins are first separated by charge (isoelectric point), then by size (molecular weight). Proteins separated on a 2D gel may be visualized by staining, or transferred to a rigid membrane for western blotting.

Western blotting: An immunostain method for detecting individual proteins resolved by 1D or 2DE by reaction with an antibody. This older method has been revitalized by the commercial availability of high-affinity, specific antibodies for virtually all proteins of interest.

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

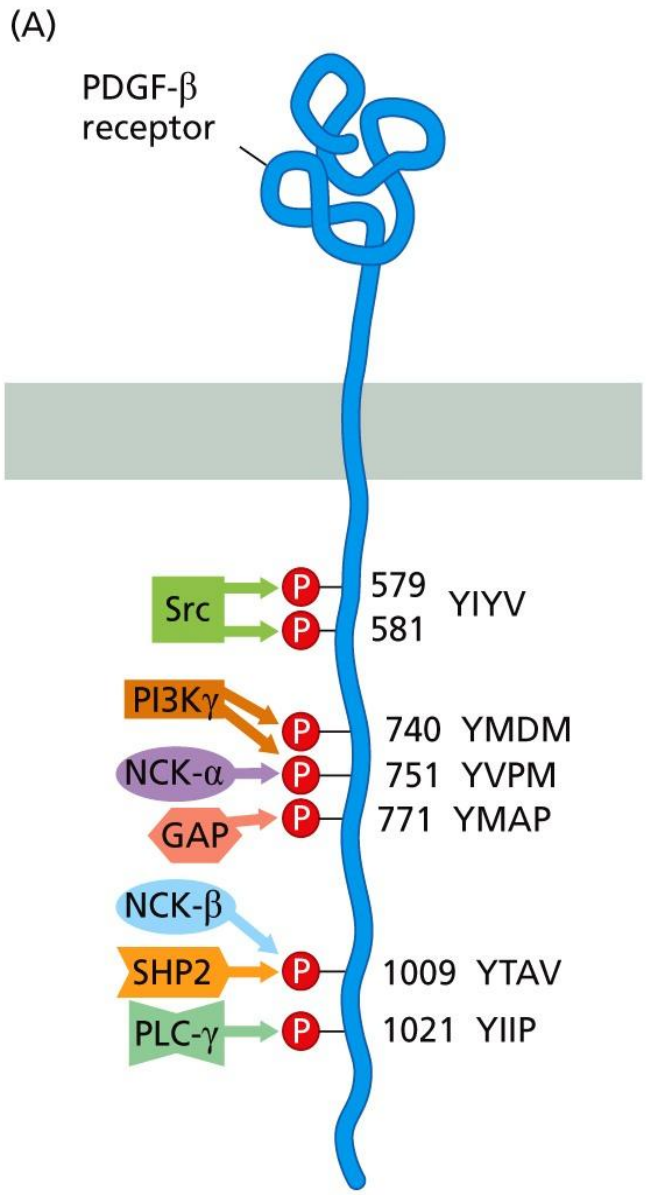
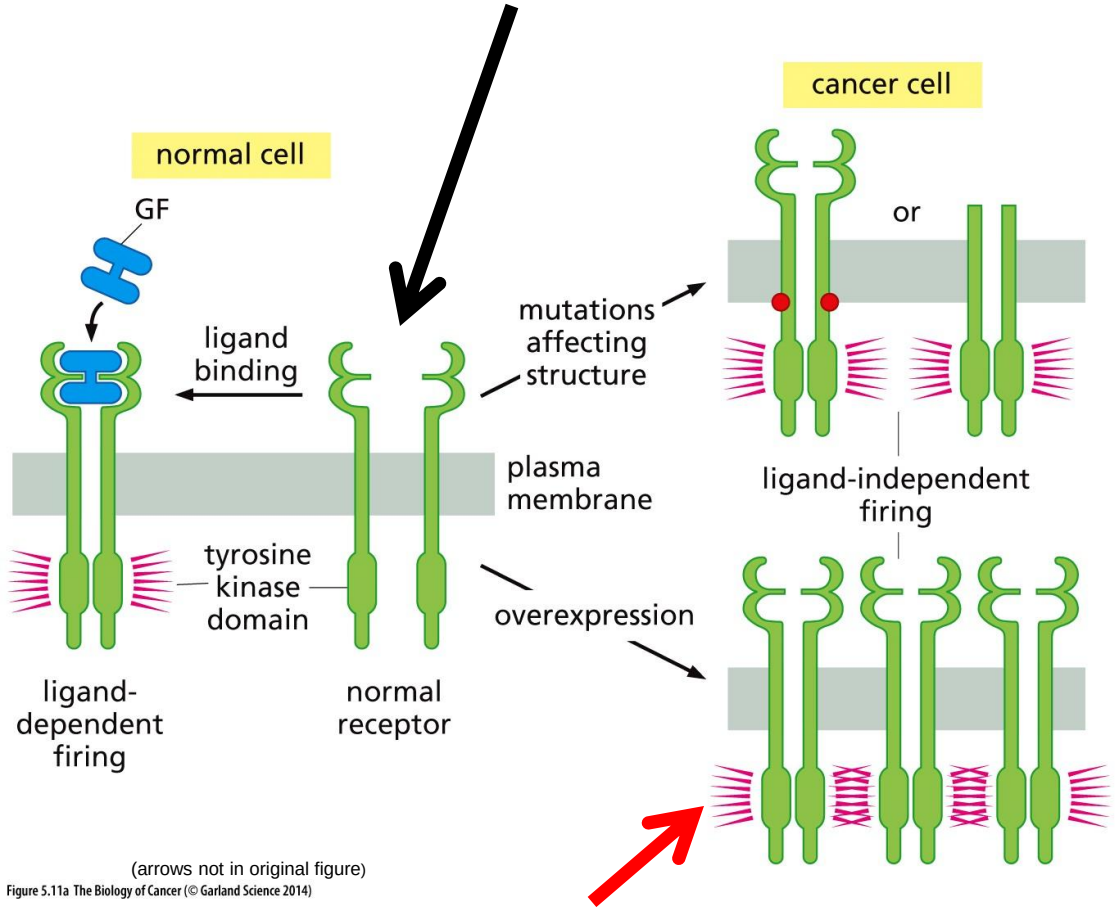


Figure 6.9 The Biology of Cancer (© Garland Science 2014)

Receptor tyrosine kinases are not always phosphorylated

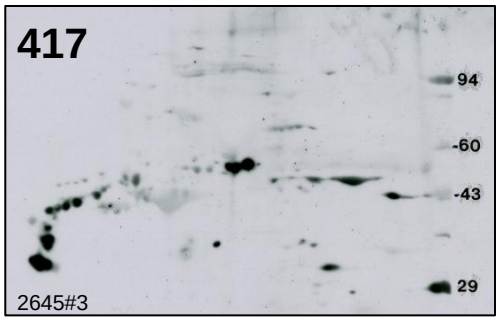


Western blotting can detect protein-bound phosphoTyrosine (pTyr) in tumor tissue lysates.

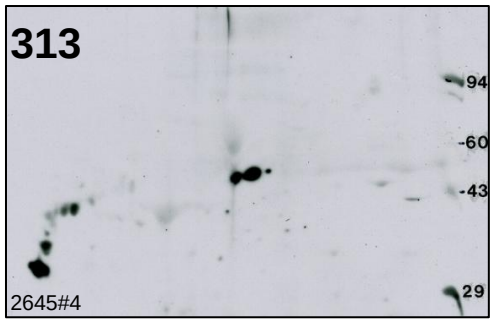
Although much can be learned from model systems like cultured cancer cells and knockout mice, in the end, human tumor tissue must be studied to understand what works. To that end, human lung cancer tumor and control samples were utilized. Initial results using an anti-pTyr antibody are shown in the next slide.

The Methods section (slides 18-20) describes sample preparation and provides more information on carrier ampholine 2D gel electrophoresis. Presentations of earlier work can be found on the Support link of our website.

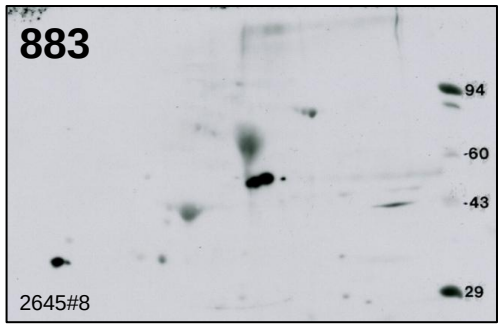
Phosphotyrosine 2D western blots from 6 lung cancer + 3 control samples



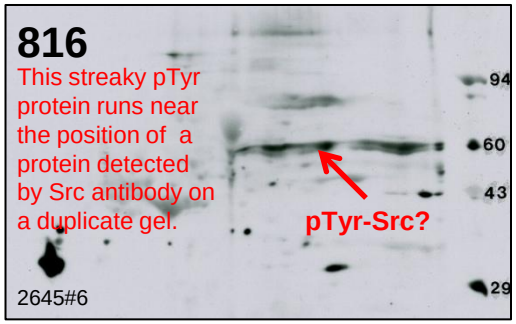
Squamous cell carcinoma



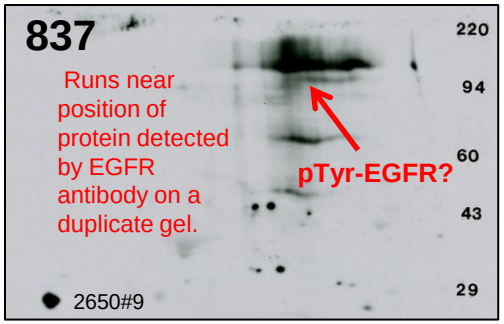
Squamous cell carcinoma



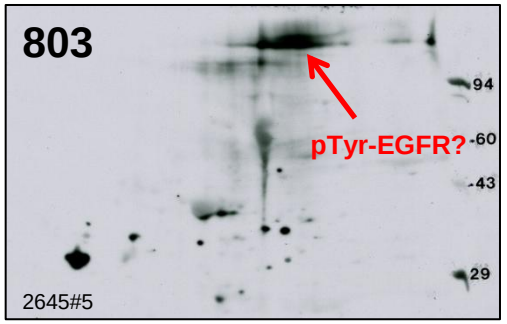
Adenocarcinoma



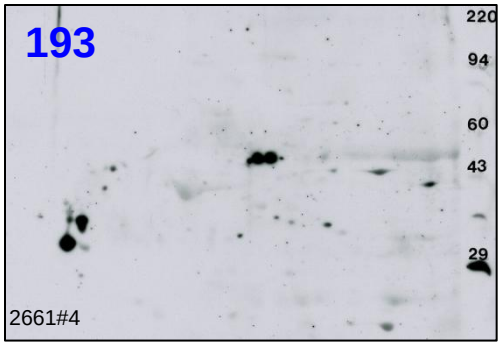
Squamous cell carcinoma



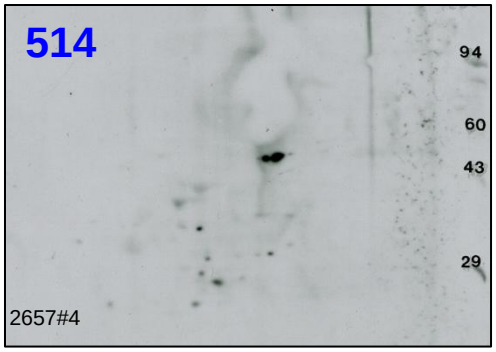
Squamous cell carcinoma



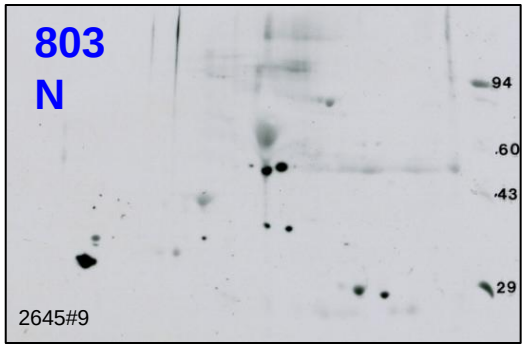
Squamous cell carcinoma



Control: Tuberculosis – lung



Control: Asthma – lung



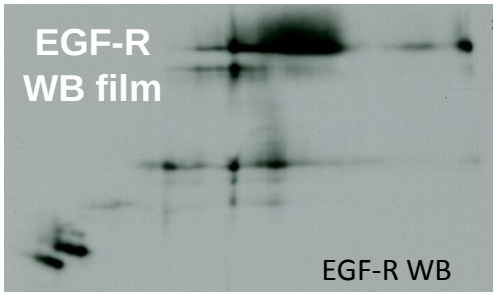
Control: Normal lung tissue

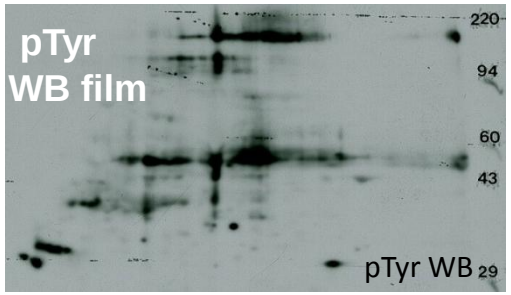
We needed to identify the proteins with pTyr residues unequivocally.

- Comparison between pTyr and TK western blots run on duplicate gels were suggestive but not certain.
- Mass spectrometry didn't work.
- Western blot overlays – Bingo!
  - Method to overlay one 2D image showing proteins detected by a specific TK antibody over a second image of proteins detected by a pTyr antibody. Both images are obtained from the same 2D blot (one sample) and are superimposable.

2D WB image overlay summary slide

A single 2D blot was probed with TK ab, stripped and reprobed with pTyr ab.

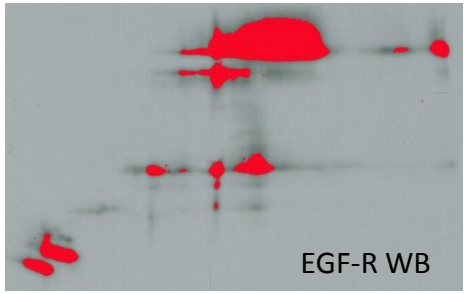




desktop scanned

desktop scanned

Image colorized in Adobe Elements



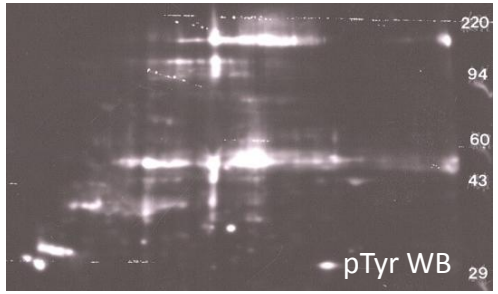
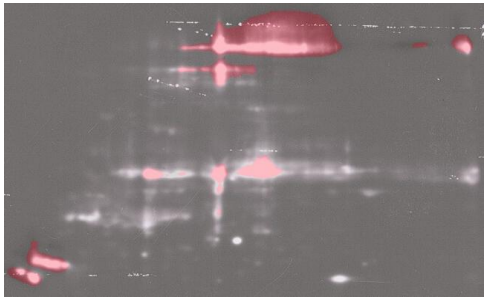


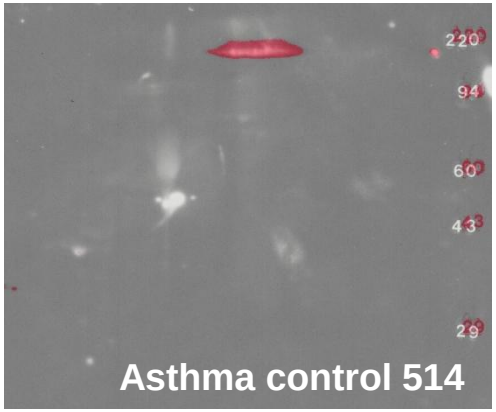
Image inverted in Adobe Elements

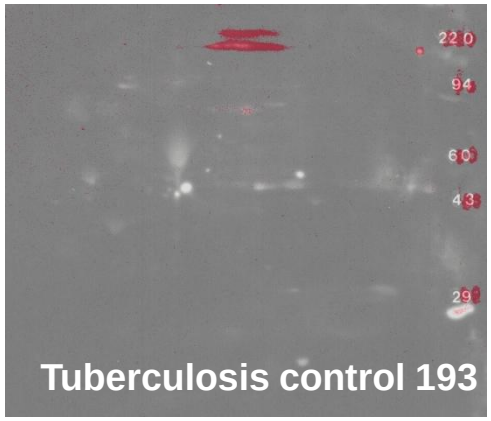


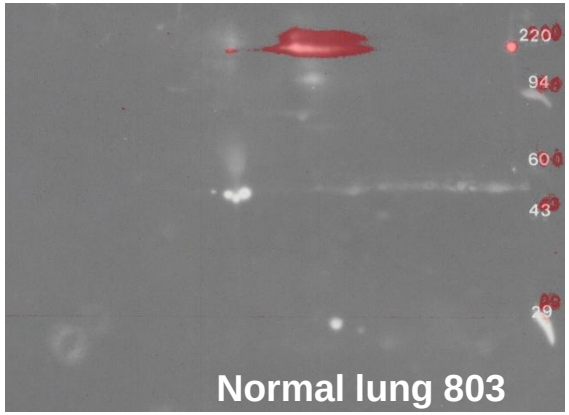
Images are overlaid and aligned. Colorized image opacity is set to 45% to show both patterns.

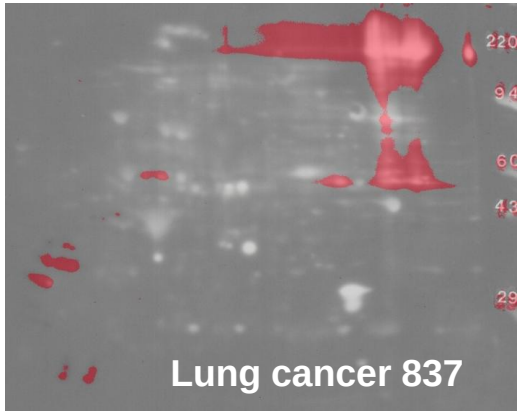
Result: EGFR proteins that contain phosphotyrosine are revealed (pink).

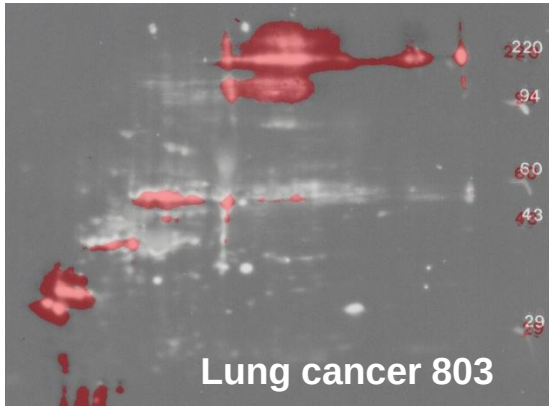
2D western blot overlays can differentially detect tyrosine-phosphorylated EGFR in tumor samples: Lung cancer >> control lung.











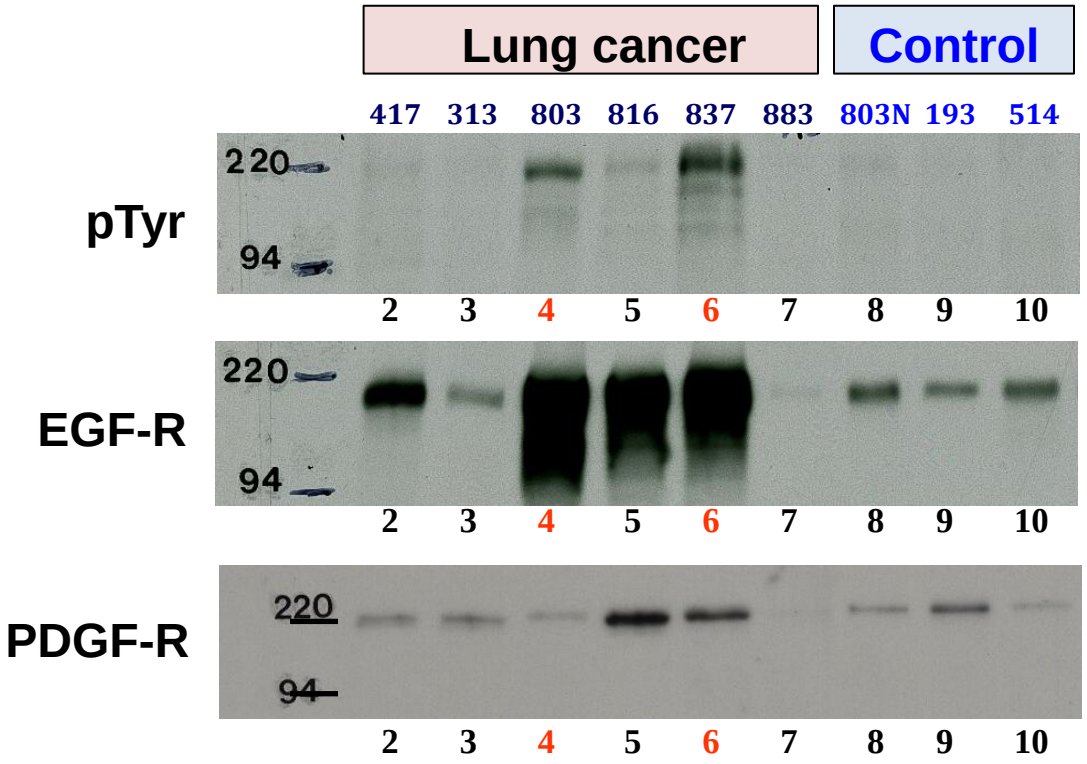
Western blot overlays: EGFR (red) over pTyr (white). Standardized WB conditions
(EGFR ab 1:10,000 3min exp; pTyr ab 1:5000 10 min exp)

1D screen for presence of PDGFR and VEGFR

EGFR, PDGFR and VEGFR are known cancer drivers that run at ~170 kDa. Are they present?

Method:

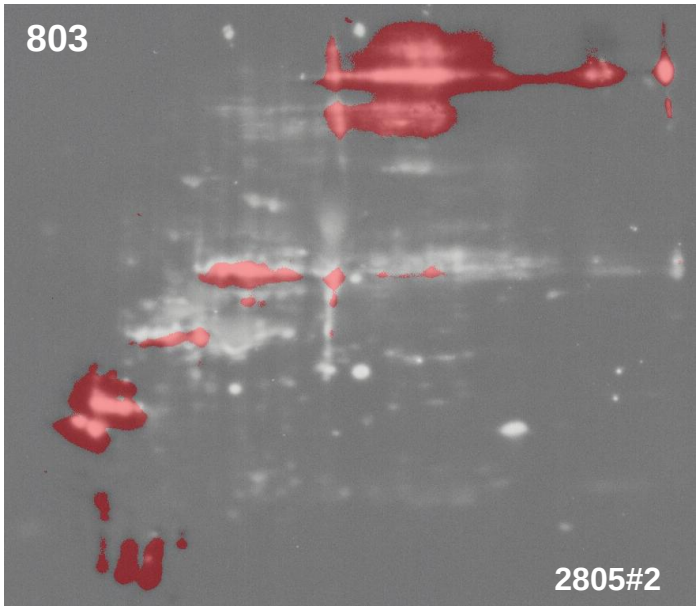
- The 6 lung cancer and 3 controls were identically loaded on four 1D gels.
- Each gel was western blotted with a different antibody.



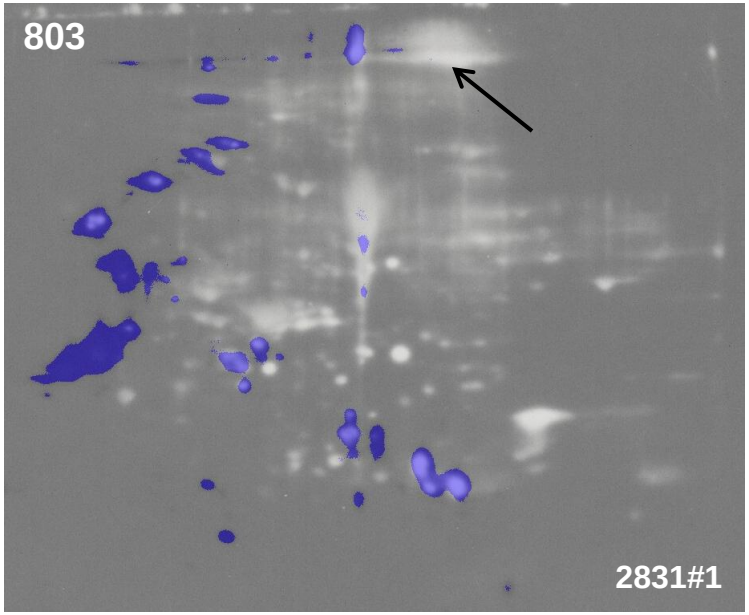
Conclusions

- EGFR is in all the samples but is only active in patients 803 and 837 (see pTyr - slide 9)
- PDGFR is in all the samples, perhaps at lower levels; it may be active in 837
- VEGFR does not drive any of these tumors as it did not react, even at low dilution and long exposure (not shown)
- Thus, a 1D check can reveal whether a tumor might contains >1 driver.

Lung tumor 803: EGFR/pTyr and PDGFR/pTyr image overlays



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



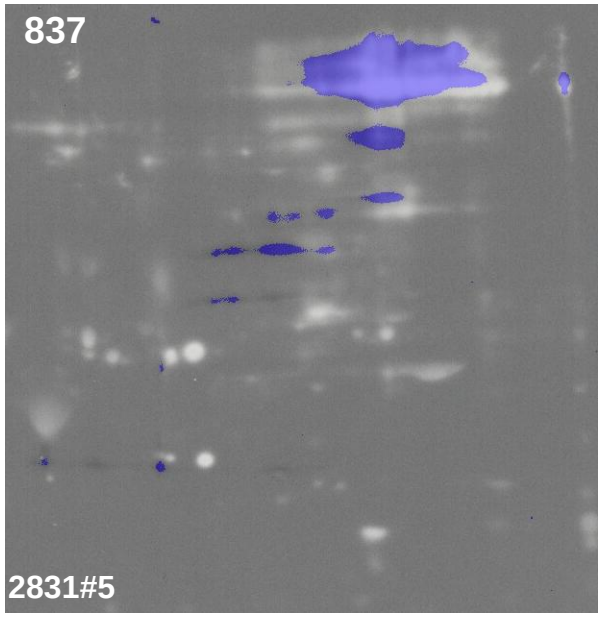
PDGFR (blue) over pTyr (white)
2831#1 PDGFR-3minOver_pTyr1:5000-10min

Conclusions:

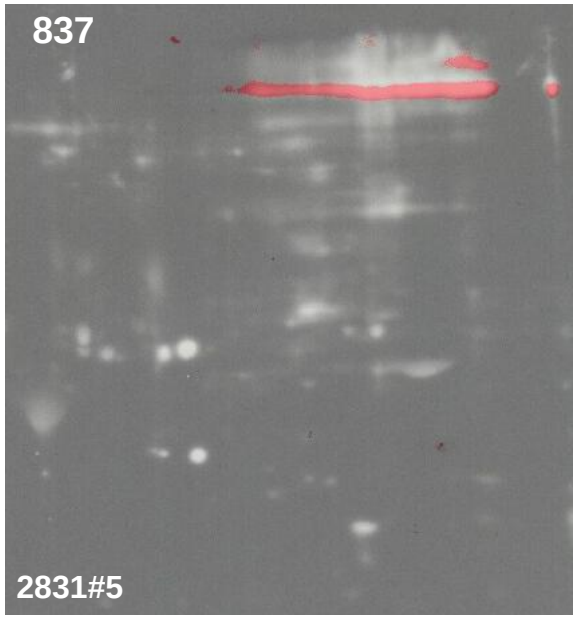
- The diffuse 170 kDa pTyr signal (arrow) does not co-migrate with the PDGF-R signal. Mostly lower MW PDGF breakdown products are seen. *PDGF-R is probably not active in this tumor.*
- EGFR is active in 803, i.e. the diffuse 170 kDa EGFR signal co-migrates with that from the pTyr signal. *Patient 803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

Lung cancer 837: Triple western blot overlay

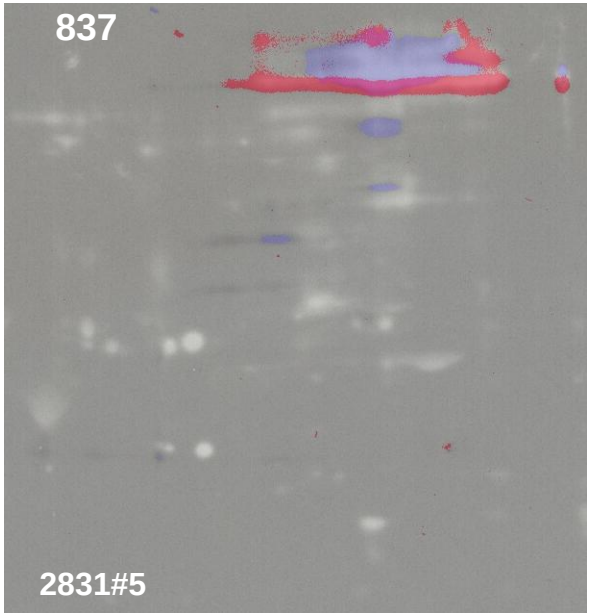
Blotting Order: PDGFR, then pTyr, then EGFR



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



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



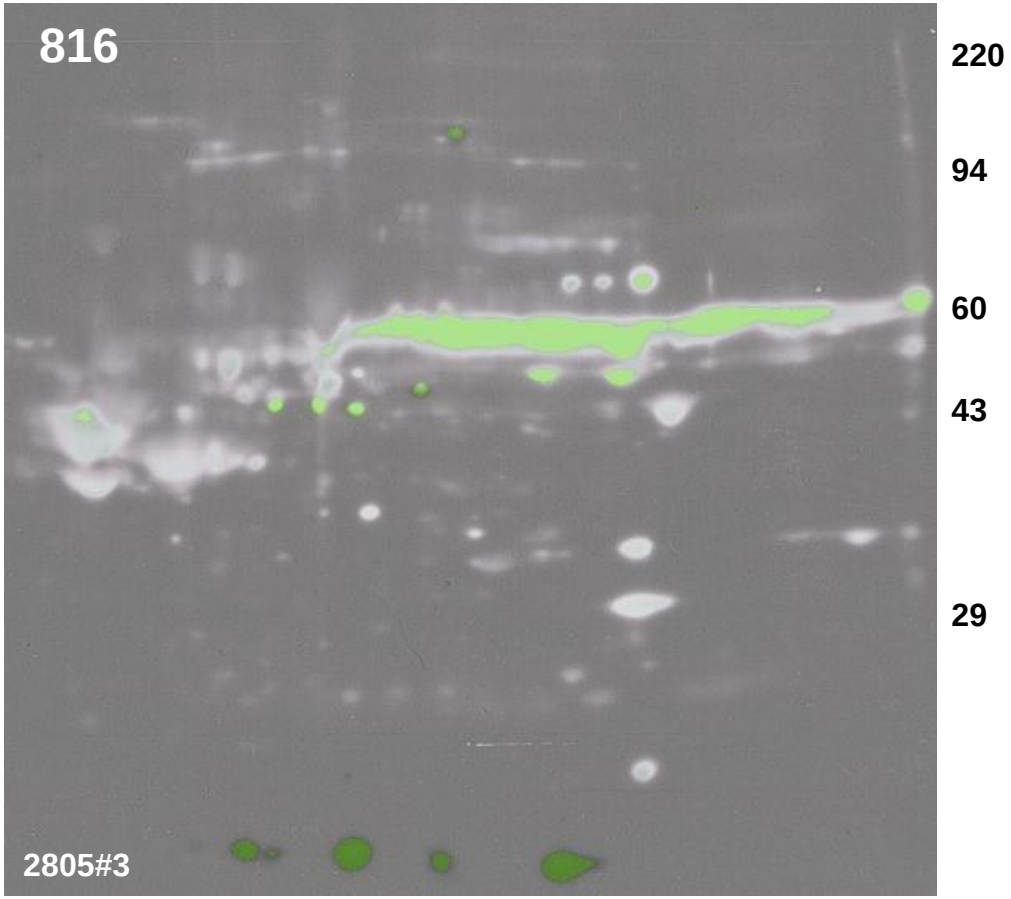
TriPlex – all 3 images aligned

Conclusions:

• The EGFR and PDGFR signal co migrate 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 might be too much; the third WB (EGFR) seemed washed out compared to an earlier result.

Lung Cancer 816 contains active Src, for sure.

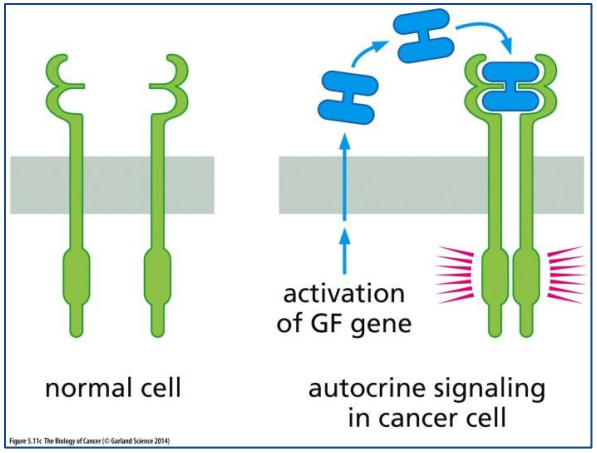


WB Conditions: Src (10,000, 3 min, **green**) over pTyr (1:1000, 3 min, white).

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 western blot overlays.



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. Thus, 2D WB duplexing is worth pursuing.

Conclusions

- CA-2D western blot overlays may be used to visualize activated tyrosine kinases 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 EGFR 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.
- Furthermore, in conjunction with 1D gels, CA-2D western blot overlays can identify when >1 driver is present in the same tumor, a key for when to administer an inhibitor cocktail.
- CA-2D western blot overlaying is an experimental procedure that needs to be standardized, validated, and given a quantitative endpoint. Multiplexing with labeled, mixed antibodies detectable with a fluorescent scanner is an obvious next step.
- Kendrick Labs, Inc is looking for collaborators and clients.

Our team at Kendrick Labs

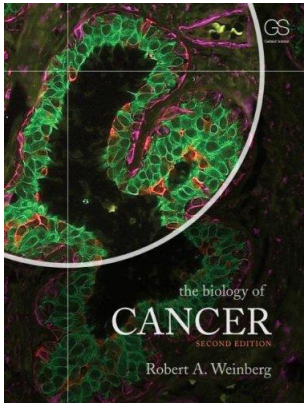
Jon Johansen
Lab Manager

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



Methods

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

www.ilsbio.com

Sample Preparation

- 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 -80^{o} C.

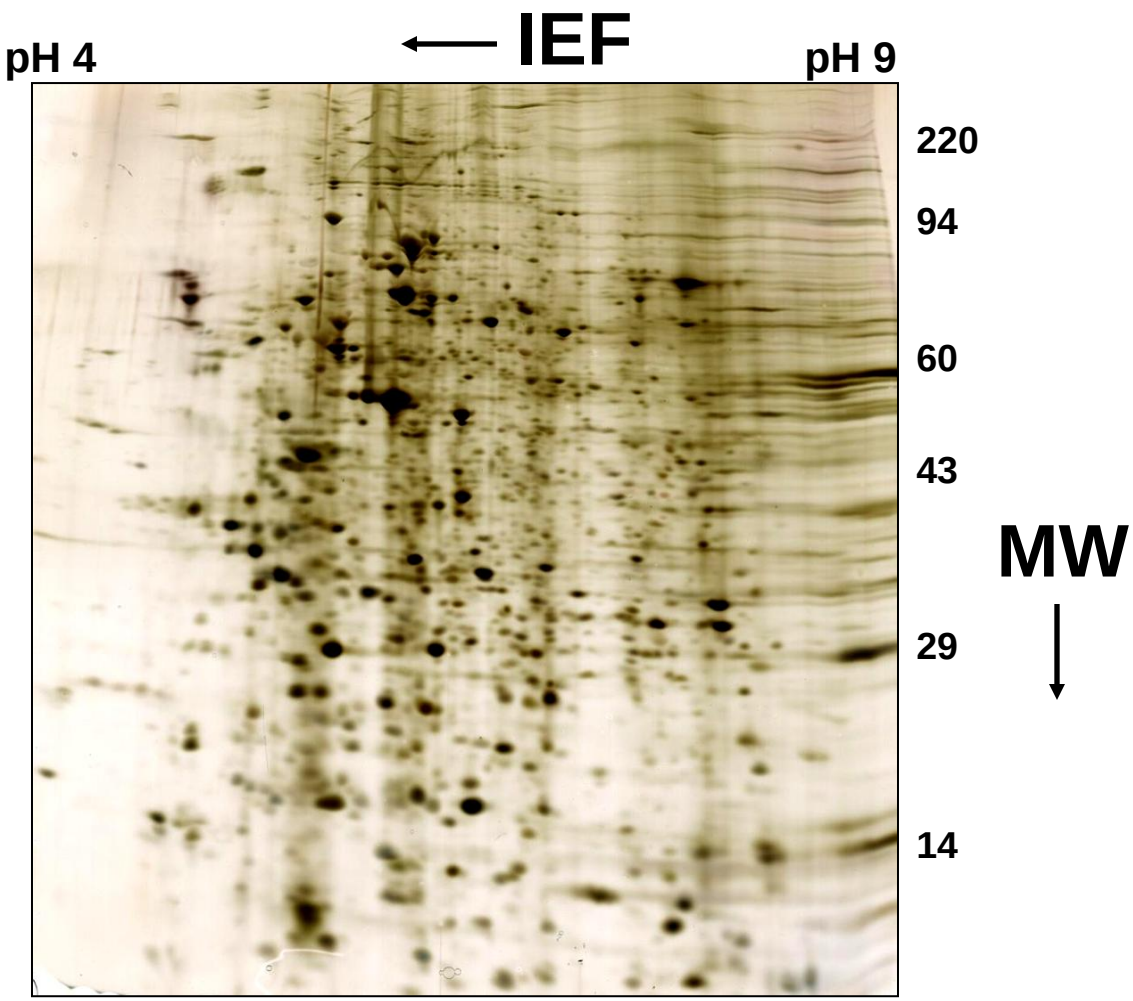
First dimension: Isoelectric focusing

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: 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 Kendrick Labs, Inc.



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