Identifying tyrosine kinase drivers in human tumors using 2D Western blotting

*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

Talk Outline

- Overview of receptor tyrosine kinases (RTK) as targets for cancer drugs
- 2D gel phosphoprotein Western Blotting (WB)
- 2D pTyr WB patterns - human lung cancer samples compared to standards
- 2D pSer/pThr WB patterns from tumor samples
- Discussion, Conclusions, Collaborators

Cancer is like a runaway car

- Activated oncogenes act as the gas pedal stuck to the floor (good drug targets)
- Inactivated tumor suppressor proteins (e.g., p53) are defective brakes
- Up-regulated telomerase provides endless gas by maintaining telomeres

Robert Weinberg, Biology of Cancer, 2007, p383

Classes of cancer drivers

- **Receptor tyrosine kinases**
- **Transcription factors like c-Myc**
- **Small G proteins like Ras**
  - Ras typifies a family of 35 proteins with similar cavities that bind and hydrolyze GTP
  - When GTP is in the cavity, an effector loop becomes exposed that binds downstream signaling proteins

The Biology of Cancer by Robert Weinberg, 2007 p175

Receptor tyrosine kinases are known to drive cancer cell division

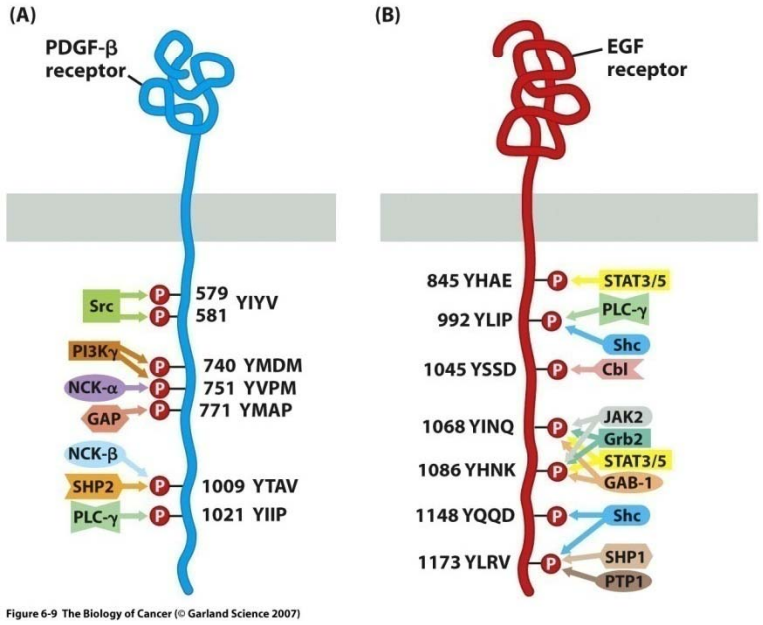


Figure 6-9 The Biology of Cancer (© Garland Science 2007)

Cytoplasmic signaling proteins are brought together by RTKs

Mechanisms of Action

- **Receptor tyrosine kinases** create phosphotyrosine binding sites (pTyr + adjacent aa) on their cytoplasmic chains that attract proteins with matching SH2 domains. **RTKs act *by relocating* signaling proteins that interact.**

- **Serine/threonine kinases act by causing conformation changes** that activate the phosphorylated protein.

Robert Weinberg, BOC, 2007, Ch 6.

Of the 90 known tyrosine kinases, 58 are RTKs in the following families*

1. **EGFR** family (Epidermal growth factor is the ligand)
2. **VEGF** family (Vascular endothelial growth factor)
3. **PDGF** family (Platelet derived growth factor)
4. **FGF** family (Fibroblast growth factor)
5. **Insulin Receptor** family (Insulin)
6. **HGF** receptor family (Hepatocyte growth factor)
7. **Trk** receptor family (Neurotropin)
8. **Eph** receptor family (Ephrin)
9. **AXL** receptor family (e.g., Vitamin K-dependent protein growth-arrest-specific gene 6)
10. **TIE** receptor family (Angiopoietin growth factor P)
11. **RET** receptor family (Glial cell line-derived neurotrophic factor)
12. **ROR** receptor family (Unknown ligand? activates Wnt pathway)
13. **Discoidin domain** receptor family (Unknown ligand)
14. **Leukocyte TK** receptor family (Unknown ligand)
15. **KLG** receptor family (Unknown ligand)

***** From Wikipedia
http://en.wikipedia.org/wiki/Receptor_tyrosine_kinase#Receptor_tyrosine_kinase_classes

TK inhibitors are rapidly being developed as cancer treatments

- Eleven TK Inhibitors are approved by the FDA, including:
  - Herceptin - **EGFR**
  - Avastin - **VEGFR**
  - Imatinib - **PDGFR**
  - Dasatinib - **SRC – nonreceptor TK**

- Entire companies are devoted to screening new TK inhibitors, for example Proqinase (www.proqinase.com)

- Searching http://ClinicalTrials.gov for keyword “tyrosine kinase inhibitor” found 258 studies as of Oct, 2011.

Major problem: How to pair the RTK driver(s) in individual cancer patients with the appropriate RTK inhibitor(s)?

Problem because RTK activity may be increased by different mechanisms

- **Gene amplification** (FISH assay)
- **Point mutations** may activate the receptors (PCR)
- **Increased transcription or translation**
- **Receptor exposure time at the cell surface may be increased**
  - Because of disruption of endocytosis (overexpressed HIP1 or underexpressed GAK)
- **Growth factor secretion by the tumor** triggers constant firing of receptor (autocrine signaling loop)

R. Weinberg, BOC 2007, especially p130-134

An assay for activated RTK protein is needed

- Genomic assays (FISH, microarrays, PCR) are often used. However, the *correlation between mRNA and protein levels is known to be less than 40%.* See review: www.kendricklabs.com/WP1_mRNAvsProtein.pdf

- Immunohistochemistry is often used for tumor biopsies but has problems: subjective scoring and low antibody dilutions (~1:100).

- Phosphotyrosine 2D Western blotting would detect *activated* RTK; it's very sensitive - might work.

Purchased 13 tumor samples from ILSBio

Patient's Barcode	Organ	Sex	Age	Clinical Diagnosis/ Cause of Death	Pathological Diagnosis	Stage	Grade
ILS28883	Lung	M	74	Lung Cancer	Adenocarcinoma	III B	4
ILS21417	Lung	F	66	Lung Cancer	Squamous Cell Carcinoma	n/a	3
ILS23313	Lung	M	63	Lung Cancer	Squamous Cell Carcinoma	IIIB	2
ILS24816	Lung	M	n/a	Lung Cancer	Squamous Cell Carcinoma	III a	3
ILS25837	Lung	M	48	Lung Cancer	Squamous Cell Carcinoma	III	3
ILS22803	Lung	F	68	Lung Cancer	Squamous Cell Carcinoma	IIIB	1
ILS22803	Lung	F	68	Adj. normal lung	normal tissue	IIIB	1
AGR-00193	Lung	M	44	Lung Diseased	Tuberculosis	n/a	n/a
ILS-1514	Lung	F	74	PM/Asthma attack	Asthma	n/a	n/a
ILS22017	Liver	M	78	Liver Cancer	Hepatocellular Carcinoma	n/a	2
ILS26801	Liver	M	54	Liver Cancer	Hepatocellular Carcinoma	III A	3
ILS26801	Liver	M	54	Adj normal liver	normal tissue	III A	3
ILS26852	Kidney	M	75	Kidney Cancer*	Renal Clear Cell Carcinoma	I	2
ILS26852	Kidney	M	75	Adj. normal kidney	normal tissue	I	2
USA-00329	Breast	F	58	Breast Cancer	Ductal Carcinoma	II B	3
ILS26870	Pancreas	M	56	Pancreatic Cancer*	Undifferentiated Sarcoma	n/a	3

* 90% cancer cells

Metastatic tissue is too expensive

2D Phosphoprotein Western blotting was performed using standardized methods

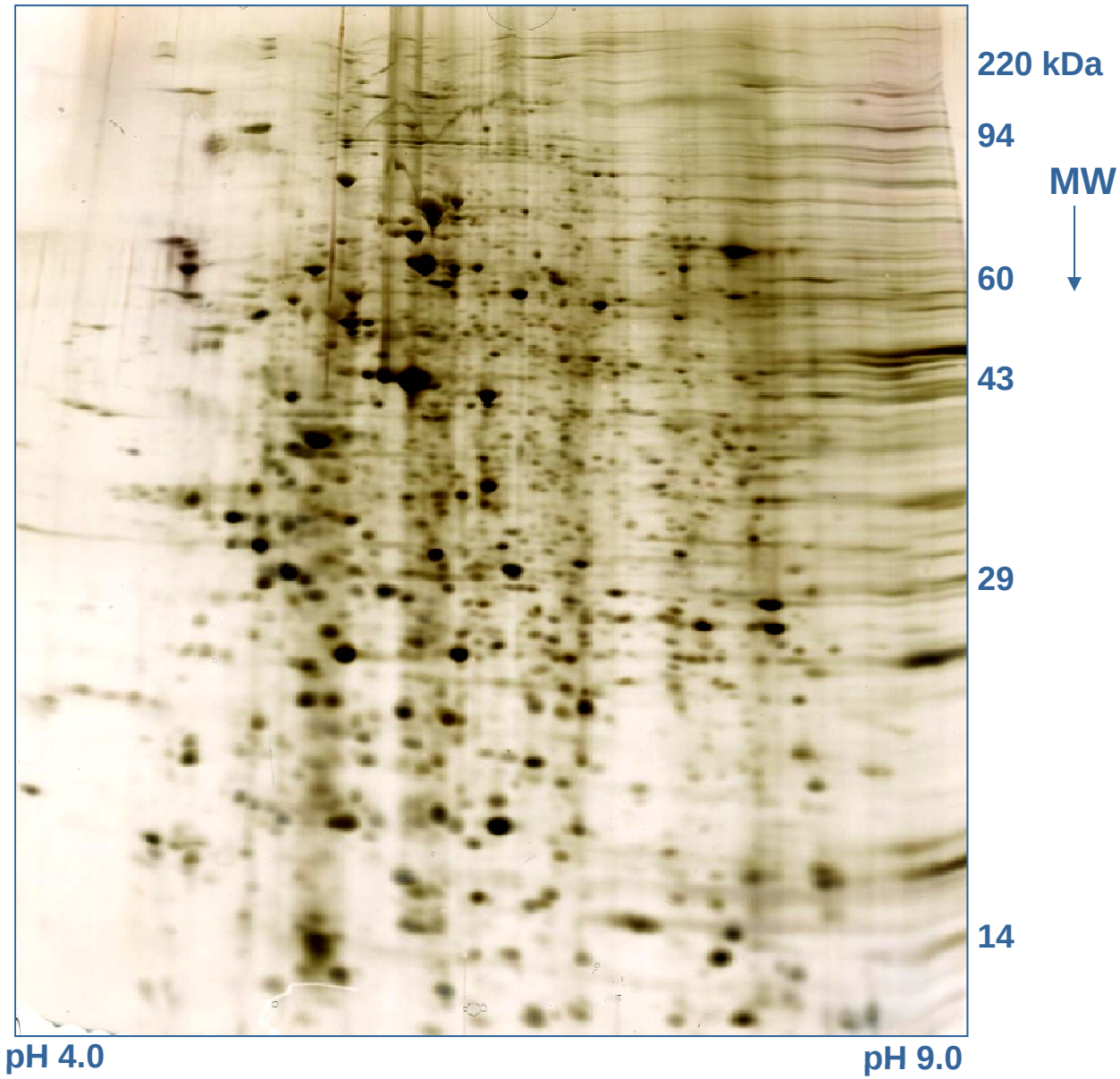
- The tumor tissue was homogenized *in SDS buffer* and the solution clarified by heating in a boiling water bath (no discarded pellet).
- A carrier ampholine 2D gel (not IPG strip) was run, the proteins transferred to PVDF, the membrane shaken overnight with an antibody, and then treated with ECL or ECL Plus before exposure to x-ray film.
- The antibody was either a generic antibody,PY20 (pTyr) or a mixture of Qiagen antibodies, Q5 (pSer) & Q7 (pThr).

More method details including linearity of load versus spot density plot at: www.kendricklabs.com

Example of our most sensitive silver stain used to show > 1000 proteins in cultured cell lysate run on a large format 2D gel. 2D *Western blotting is highly specific and much more sensitive than silver staining.*

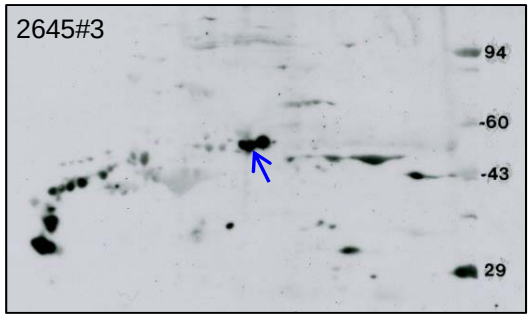
Cultured cells contain 5,000-8,000 proteins. Differentiated cells in mammalian tissue probably have fewer expressed proteins, ~3,000 – 5,000.

The lung cancer samples were run on smaller 2D gels (13x15cm) to conserve antibody.

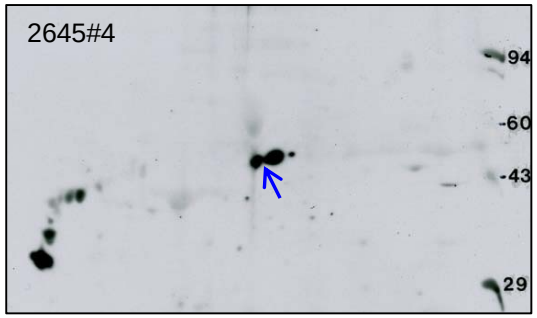


Acute Lymphoblastic Leukemia (ALL) cell line. Shown with permission of Dr. Terzah Horton, Baylor College Medicine.

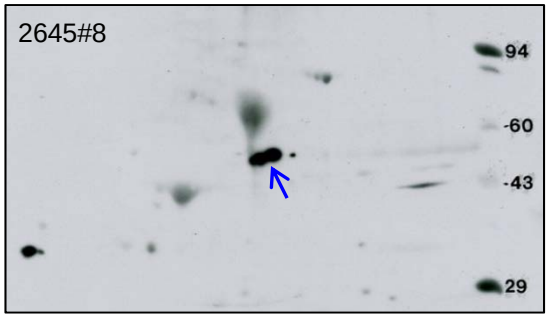
Six lung cancer phosphotyrosine Western blots vs 3 controls



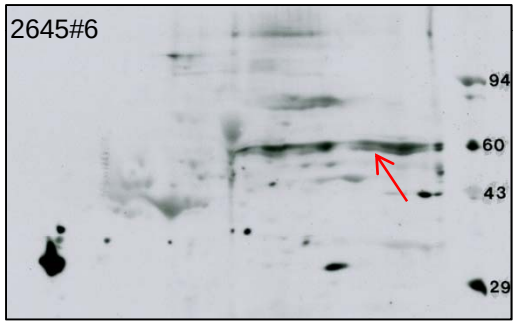
Sqamous cell carcinoma ILS21417



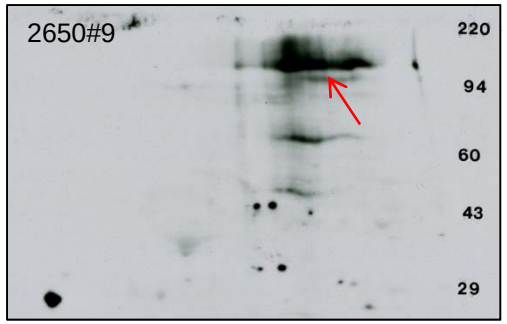
Sqamous cell, ILS23313



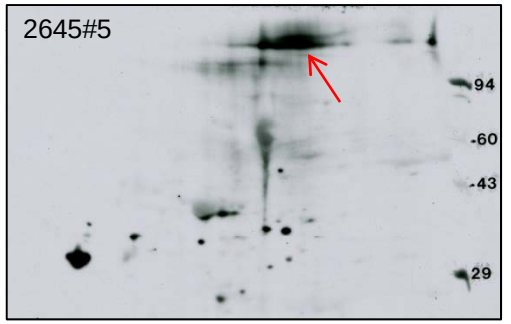
Adenocarcinoma, ILS28883



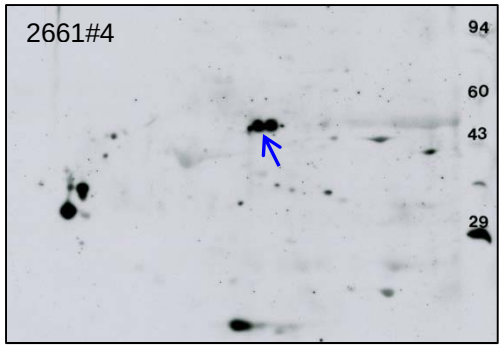
Squamous cell, ILS24816



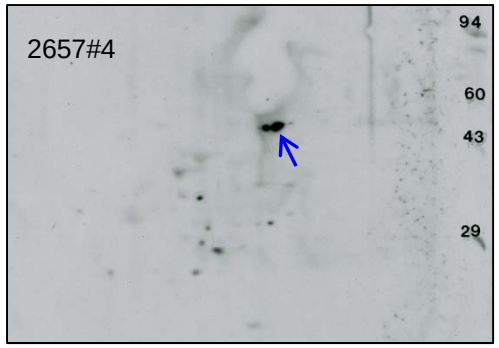
Squamous cell, ILS25837



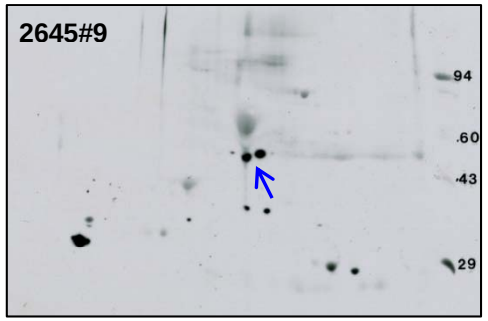
Squamous cell, ILS22803



Control: Tuberculosis – lung

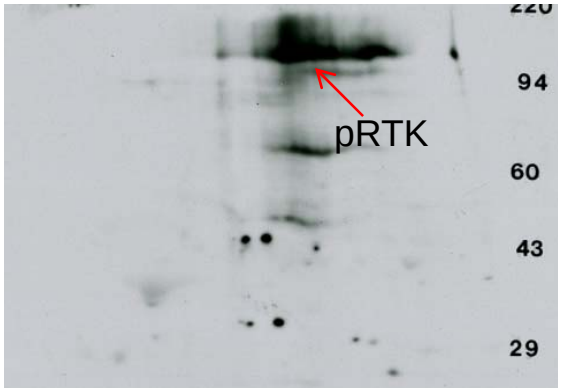


Control: Asthma - lung

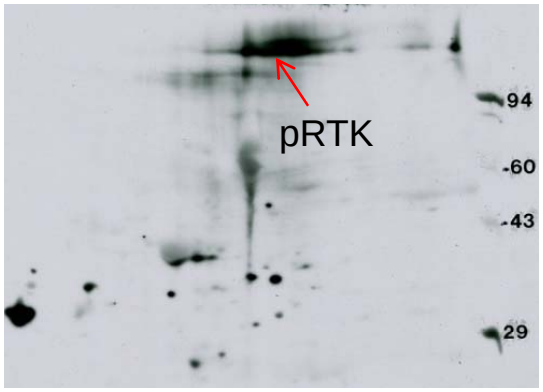


Control: Normal lung tissue from ILS22803N

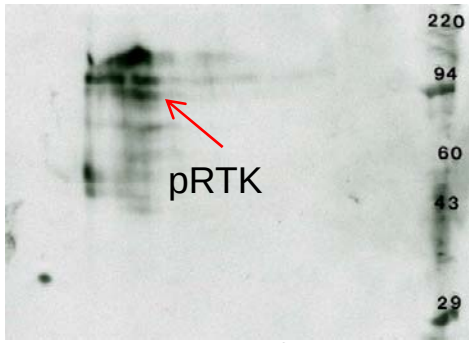
Lung cancer versus recombinant standards



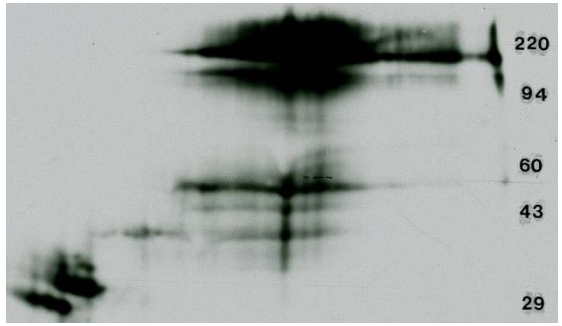
Squamous cell carcinoma ILS25837
Immunostained with anti-PTyr ab



Squamous cell carcinoma ILS22803
immunostained with anti-PTyr ab

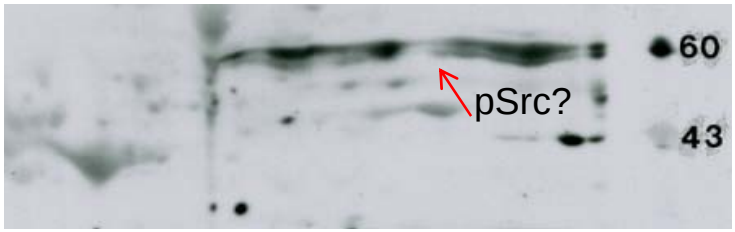


10 ng VEGFR std from ProQinase
Immunostained with anti-PTyr ab

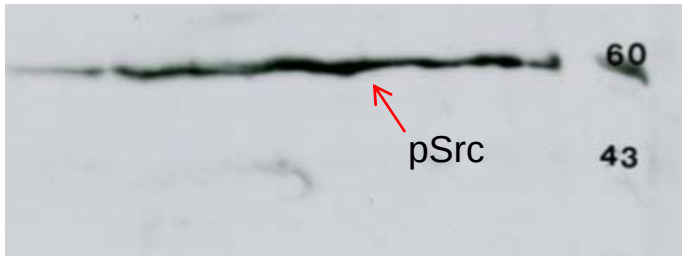


ILS22803 immunostained
with anti-*EGFR* ab

Phospho-Src std versus lung cancer



Squamous cell carcinoma
ILS24816

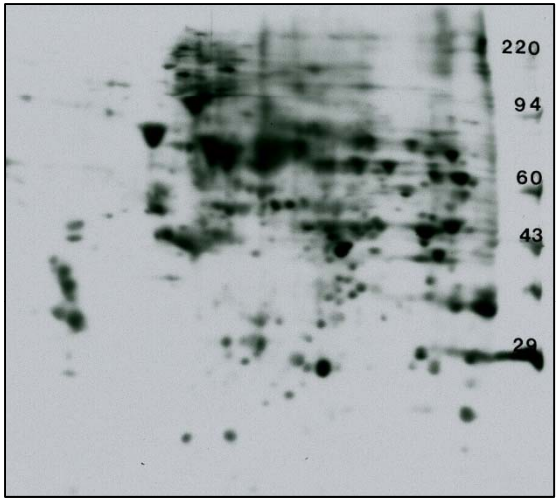


10 ng Src std from ProQinase

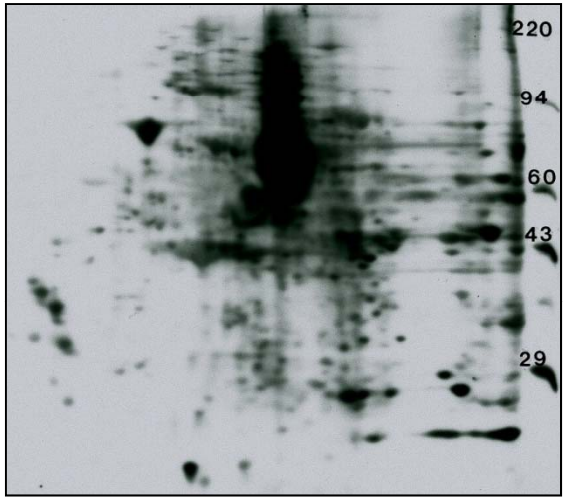
Identification of the diffuse high MW proteins by LC/MS/MS at Columbia University Protein Core Facility hasn't worked yet

- Ran duplicate LF 2D gels loaded with 600 ug protein from the two interesting tumor samples. Did PTyr WB for one and Coomassie staining of the other for spot cutting. (EGFR is the expected result.)
  - Tried cutting a small spot out in the darkest area → no TKR
  - Tried cutting a nickel-sized spot out → >200 protein IDs, none of them a TKR. Apparently too many peptides have PTMs.
- Lots of things to try:
  - Determine LC/MS/MS sensitivity with recombinant EGFR standard, look for RTK fingerprint
  - Deglycosylate peptides after trysin digestion, then try MS
  - Try specific antibodies for RTK's - 1D SDS PAGE screening
  - Look at TKR Protein arrays (antibodies on chips). They're commercially available but it's not clear they're compatible with SDS.

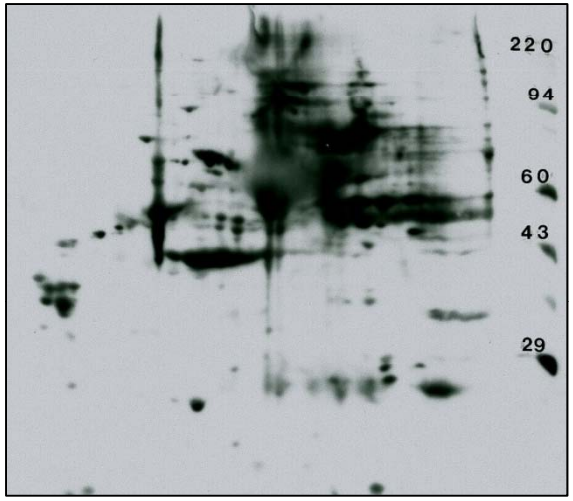
pSerine/pThreonine Western blotting of liver and pancreatic tumor samples



Liver Cancer ILS22017

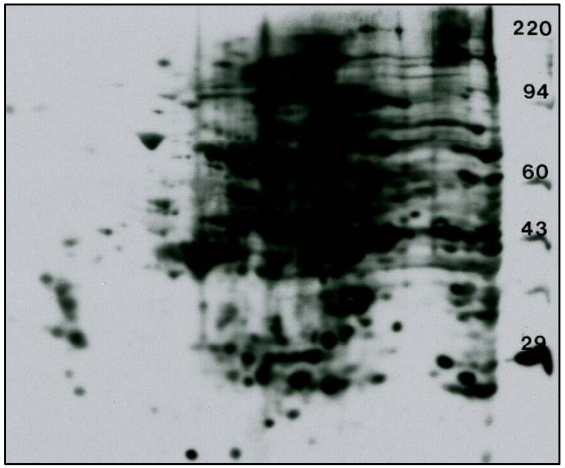


Liver Cancer ILS26801



Pancreatic Cancer ILS26807

Similar



Control Liver ILS26801N

Different

up to 70% of cell proteins are pSer/pThr phosphorylated during the cell cycle.

Discussion and conclusions

Cancer has lots of red herrings

- Tumors differentiate differently than the normal tissue they invade. For example, when lung cancer metastasizes to the brain, it retains some of the characteristics of lung tissue.
- Comparing adjacent normal to tumor tissue using micro-arrays (mRNA) or 2D gels (proteins) shows numerous clear differences that are difficult to interpret and not drug targets.
- Detecting a small set of *activated cancer driver proteins* is the way to proceed, even though it's difficult.

Collaborators:

Jon Johansen
Lab Manager

Matt Hoelter
Senior Biochemist
AES Executive Director

Mary Ann Gawinowicz
Facility Director
Columbia University
Protein Core Facility