Proteomic Analysis of Hepatocellular Carcinoma (HCC)

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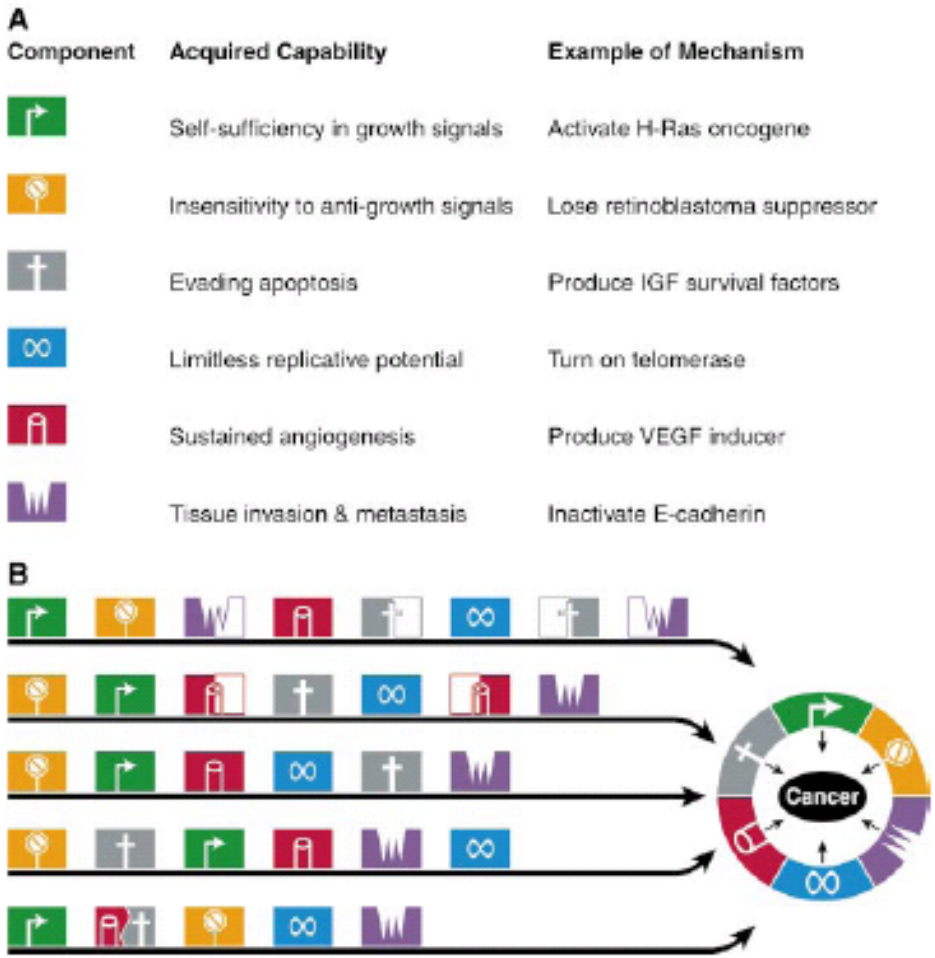
Hepatocellular Carcinoma (HCC)

- Fourth leading cause of cancer death worldwide
- 0.25 – 1 million new cases each year
- Highly metastatic → 5 year recurrence rate of 40 – 70%
- Risk factors
  - Hepatitis B or C virus
  - Aflatoxin B1
  - Cirrhosis
- Tumor markers: α-fetoprotein and imaging
  - Limited sensitivity
  - Non-specific
- Need for improved tumor markers and increased understanding of pathogenesis

Complex Tumorigenesis



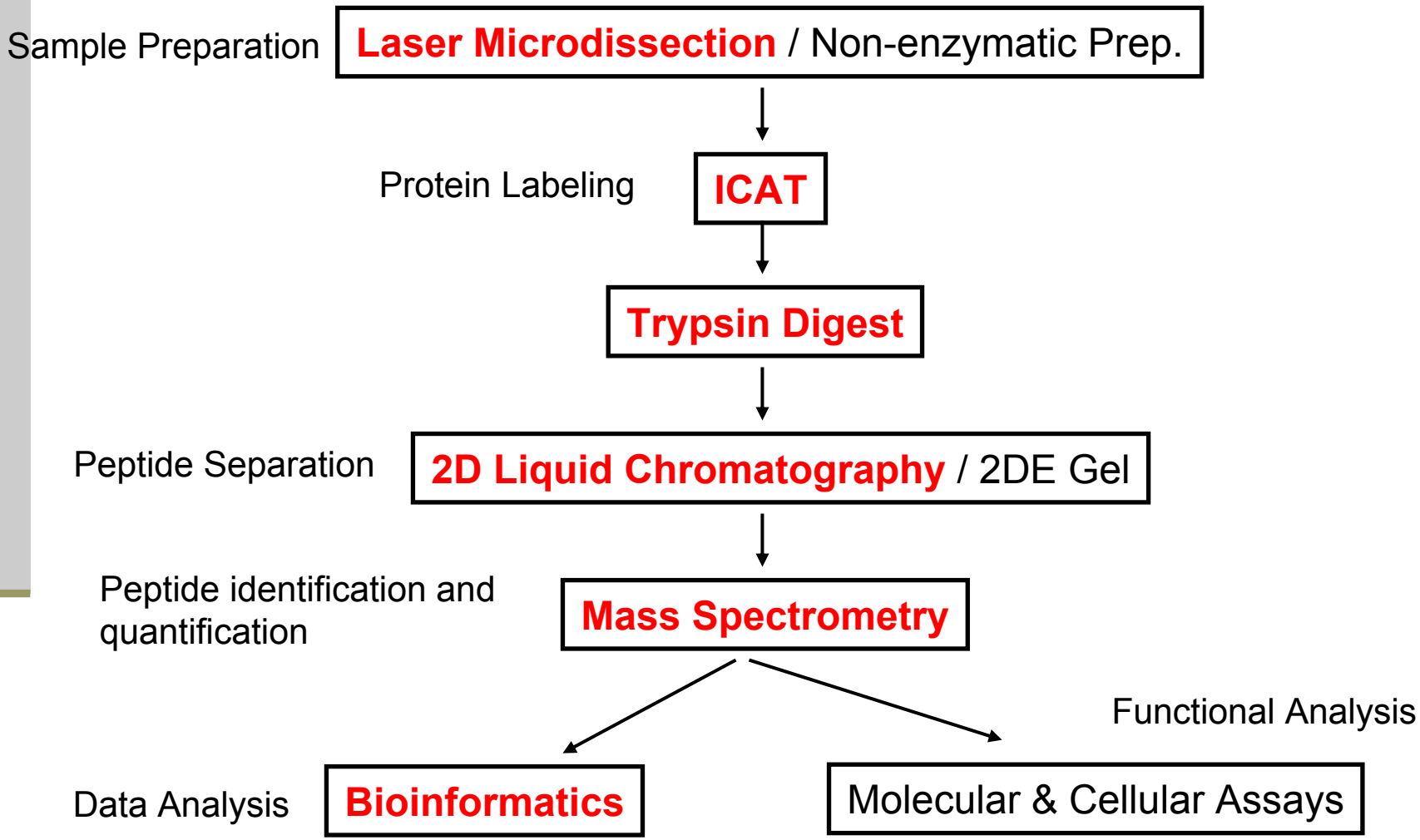
Hanahan and Weinberg, 2000

- Deletions
- Translocations
- Proviral insertions
- Amplifications
- Base pair mutations
- De-differentiation
- Transcription factor changes

Proteomics

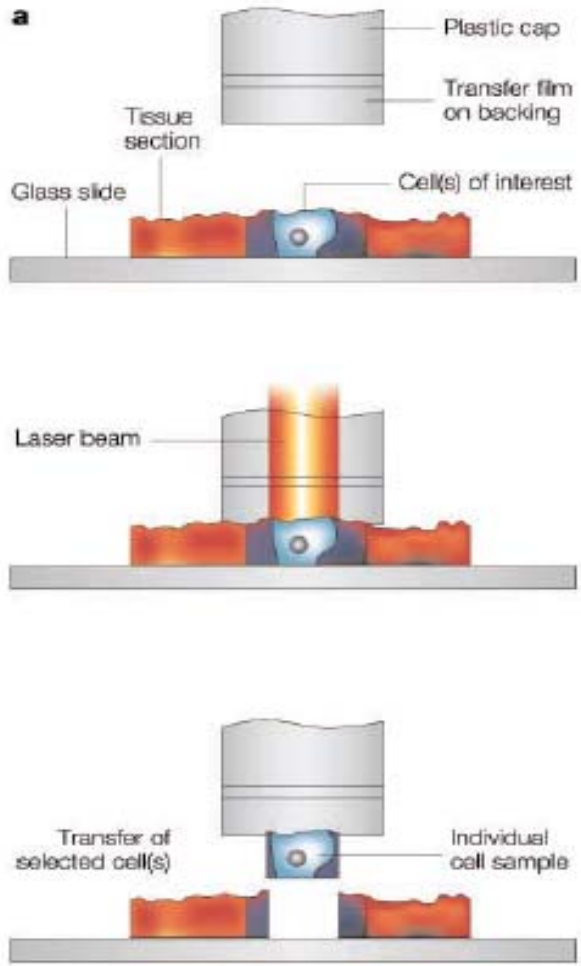
- Proteomics: Attempt to identify and quantify the spectrum of proteins in a cell or tissue at any given time
- Advantages over gene profiling
  - More direct measure of the actual functional unit (proteins)
  - Allows observation of post-translational modifications
- Applications
  - Characterization of cellular responses to stimuli
  - Mapping of signaling pathways
  - Discovery and characterization of novel proteins
  - Identification of disease markers
  - Identification of proteins with pathological function
    - Individual therapies
    - Combinatorial therapies

Technique Overview



Sample Preparation: Laser Microdissection (LCM)

- Purpose: Reduce sample heterogeneity by selectively isolating a desired subpopulation of cells in tissue
- Infrared laser expands a thermoplastic polymer that captures cells beneath the laser pulse
- Laser does not damage cellular proteins, DNA, or RNA
- Limitation: Requires fixing and staining of tissue



Petricoin et al., 2002

Sample Preparation: Laser Microdissection (LCM)

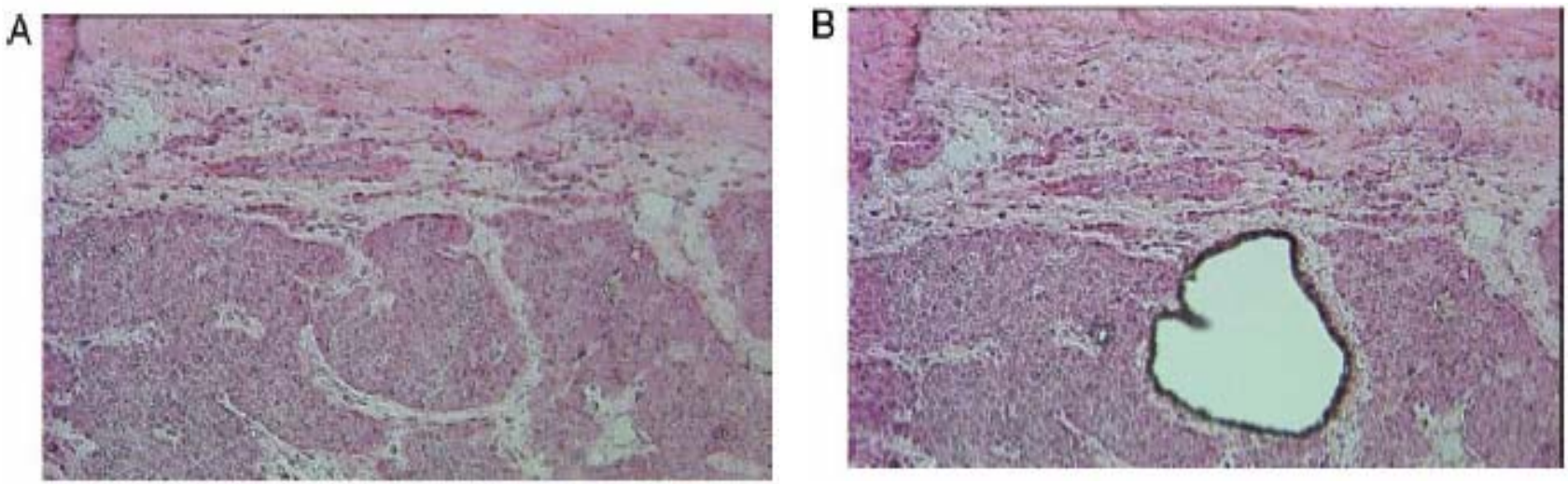


FIG. 2. **HCC tissues before (*A*) and after (*B*) LCM.**

TABLE I
Hemoglobin found with and without LCM

	Contamination	Rank (hits)[a]	AN[b]	Protein name
HCC-NESP-2D-LC-MS/MS	Hemoglobin	4 (214)	AAK15770	Hemoglobin α1 globin chain
		394 (3)	AAB20440	Hemoglobin β chain; β globin
HCC-LCM-2D-LC-MS/MS	Hemoglobin	52 (27)	AAK15770	Hemoglobin α1 globin chain

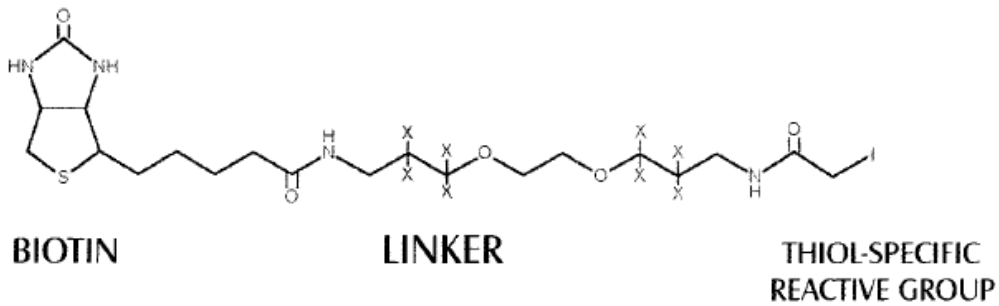
[a] The hit numbers of peptides.
[b] NCBI accession number.

Li et al., 2004

Protein Labeling: ICAT

- **Purpose:** Allows for simultaneous identification and quantification of proteins from two samples in a complex mixture
- **Examples:** Compare protein levels in cancer vs. non-cancer cell, metastatic vs. non-metastatic, stressed vs. non-stressed…
- **Steps:**
  1. Covalent attachment of ICAT reagents to reduced cysteines (Different isotope for each sample)
  2. Trypsin digestion
  3. Avidin column purification
  4. Peptide separation and mass spectrometry
- **Limitations:** Peptides that do not contain a cysteine will be lost

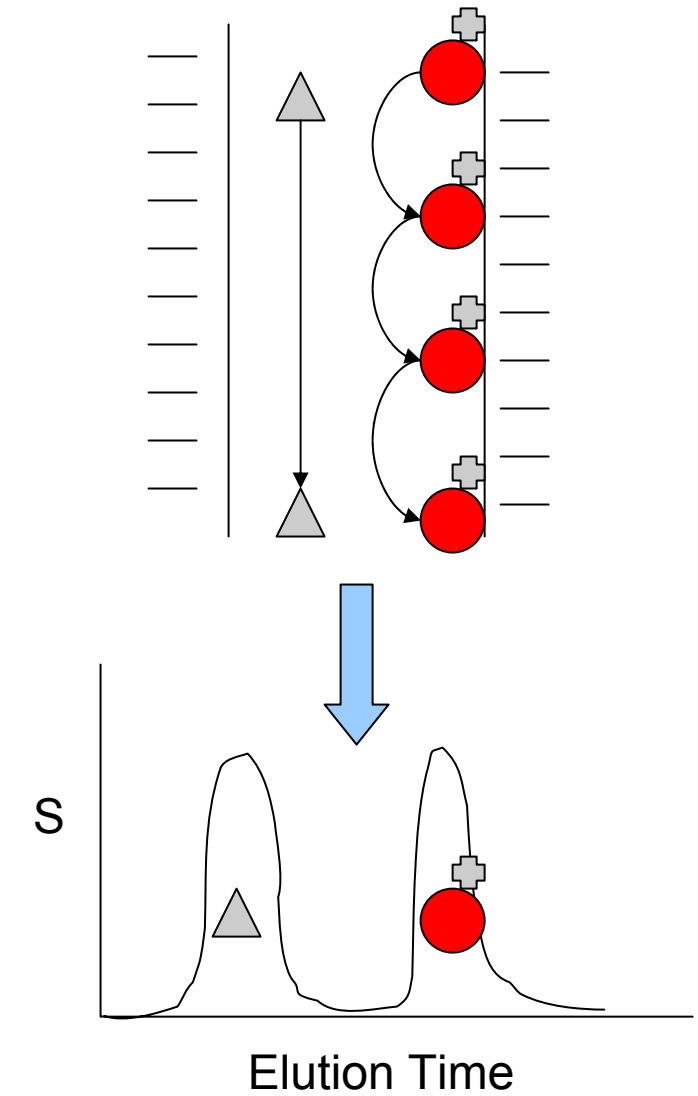
Smolka et al, 2001



X = carbon isotope (^{12}C or ^{13}C)

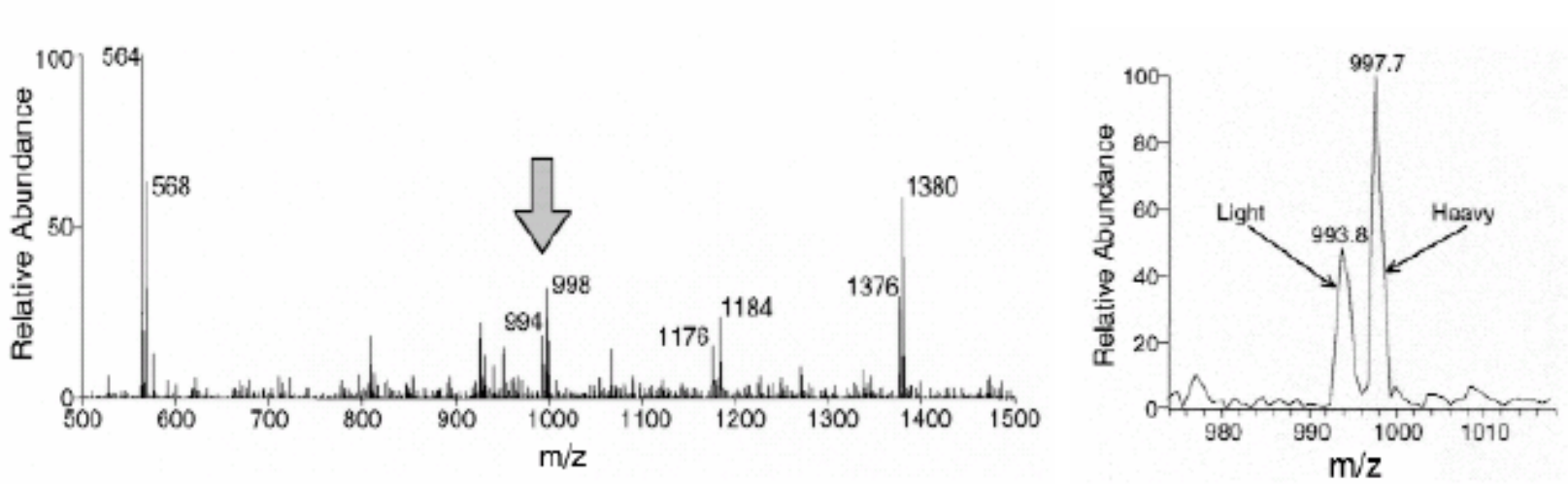
Peptide Separation :2D Liquid Chromatography (LC)

- Purpose: Separate peptides in the proteome based on physical properties (Most commonly size and charge)
- Molecules separate based on differential interactions with mobile and stationary phases
  - Reversed-phase: Polar mobile phase, non-polar stationary phase
  - Ion-exchange: Uncharged mobile phase, charged stationary phase
- Generally increased resolution over 2D gels



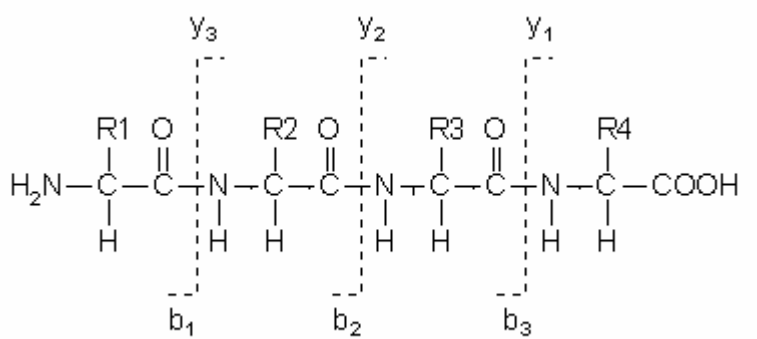
Peptide Quantification: Mass Spectrometry

- Ionization: Maldi, Electrospray
- Acceleration and Detection: Time-of-flight, quadrupole, ion trap



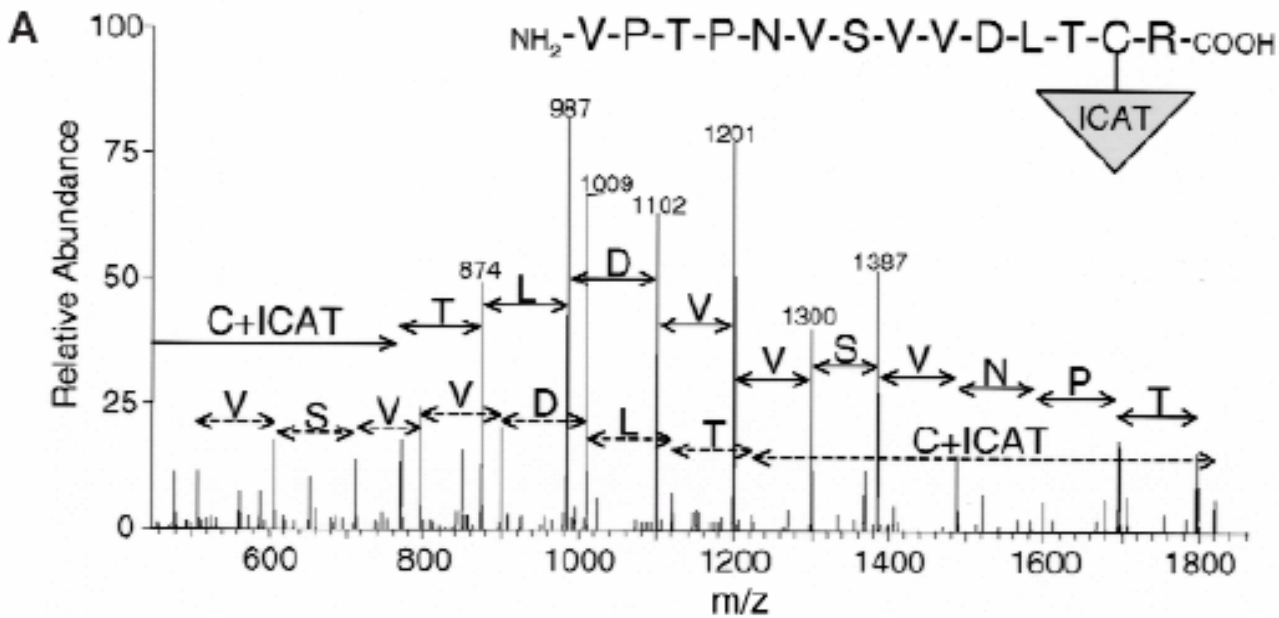
Gygi et al., 1999

Peptide Identification: Tandem Mass-Spectrometry



http://www.matrixscience.com/help/fragmentation_help.html

(C+ICAT)R
T(C+ICAT)R
LT(C+ICAT)R
…
NVSVVDLT(C+ICAT)R
PNVSVVDLT(C+ICAT)R
TPNVSVVDLT(C+ICAT)R



Gygi et al., 1999

2 Papers- Essentially same goal

- Chen Li, et al. Accurate qualitative and quantitative proteomic analysis of clinical hepatocellular carcinoma using laser capture microdissection coupled with isotope-coded affinity tag and two-dimensional liquid chromatography mass spectrometry. *Mol Cell Proteomics.* 3:399-409 (2004)

- Shi-Jian Ding, et al. From Proteomic Analysis to Clinical Significance: Overexpression of cytokeratin 19 correlates with hepatocellular carcinoma metastasis. *Mol Cell Proteomics.* 3:73-81 (2004)

Chen Li, et al. *Mol Cell Proteomics.* 3:399-409 (2004)

- Advanced proteomic techniques
  - Laser capture Microdissection (LCM)
  - Isotope-coded affinity tag technology (ICAT)
  - Two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS)
- Compared proteins from HCC and non-HCC tissue
  - ONE male, HBV+, HCC patient
  - Identified 261 proteins with quantitative differences

Table III: 55 proteins upregulated in HCC detected by ICAT-2D-LC-MS/MS

Ratio: HCC/non-HCC

AAK37554	Hemoglobin α1 globin chain	3.42	36
BAA25845	Ribosomal protein P0	3.52	
P00751	Complement factor B precursor	3.54	
BAC11648	Unnamed protein product	3.56	
NP_001345	Aldo-keto reductase family 1, member C2	3.61	
NP_003730	Aldo-keto reductase family 1, member C3	3.61	
XP_295998	Hypothetical protein XP_295998	3.96	
XP_218909	Similar to hypothetical protein FLJ12587	4.14	
XP_209360	Similar to ribosomal protein S4	4.2	
NP_000509	β globin	4.35	27
NP_001449	γ filamin; filamin 2	4.39	
NP_001822	Clusterin; complement-associated protein SP-40	4.4	
Q9UK39	Nocturnin (CCR4 protein homolog)	4.58	
AAD03059	Eph-family protein	5.23	
CAA36901	Acid sphingomyelinase (502 AA)	5.81	
XP_204690	Similar to hypothetical protein	5.87	
NP_004292	BAF53a isoform 1	6.1	
AAF32274	Transcription factor LBP-1b	6.28	
NP_149973	Similar to zinc finger protein 85	6.41	
NP_000510	δ globin	7.27	17
NP_001632	APEX nuclease	7.72	
NP_006793	Splicing factor 3a, subunit 3, 60 kDa	7.83	
AAG29811	ORF S/L	7.95	
AAB32928	Alanine-glyoxylate transaminase 1, AGT1 (EC 2.6.1.44)	8.03	
AAG33618	ATP-binding cassette half-transporter	8.68	
XP_299384	Hypothetical protein XP_299384	9.55	
BAC05016	Unnamed protein product	12	
AAK18723	Immunoglobulin α heavy chain variable region	13.86	
XP_102109	Similar to RIKEN cDNA C230081A13	13.88	
XP_123864	Similar to hypothetical protein FLJ14007	16.75	
AAH06751	Calpain 1, large subunit	17.72	
AAC17474	Neuralized homolog	23.28	
XP_129872	Similar to hypothetical protein FLJ23861	24.43	
AAF78677	Immunoglobulin A heavy chain	28.6	
NP_071368	Thioredoxin domain containing 5	35	
NP_003085	Small nuclear ribonucleoprotein polypeptide E	104.57	

Table III: 94 proteins downregulated in HCC detected by ICAT-2D-LC-MS/MS

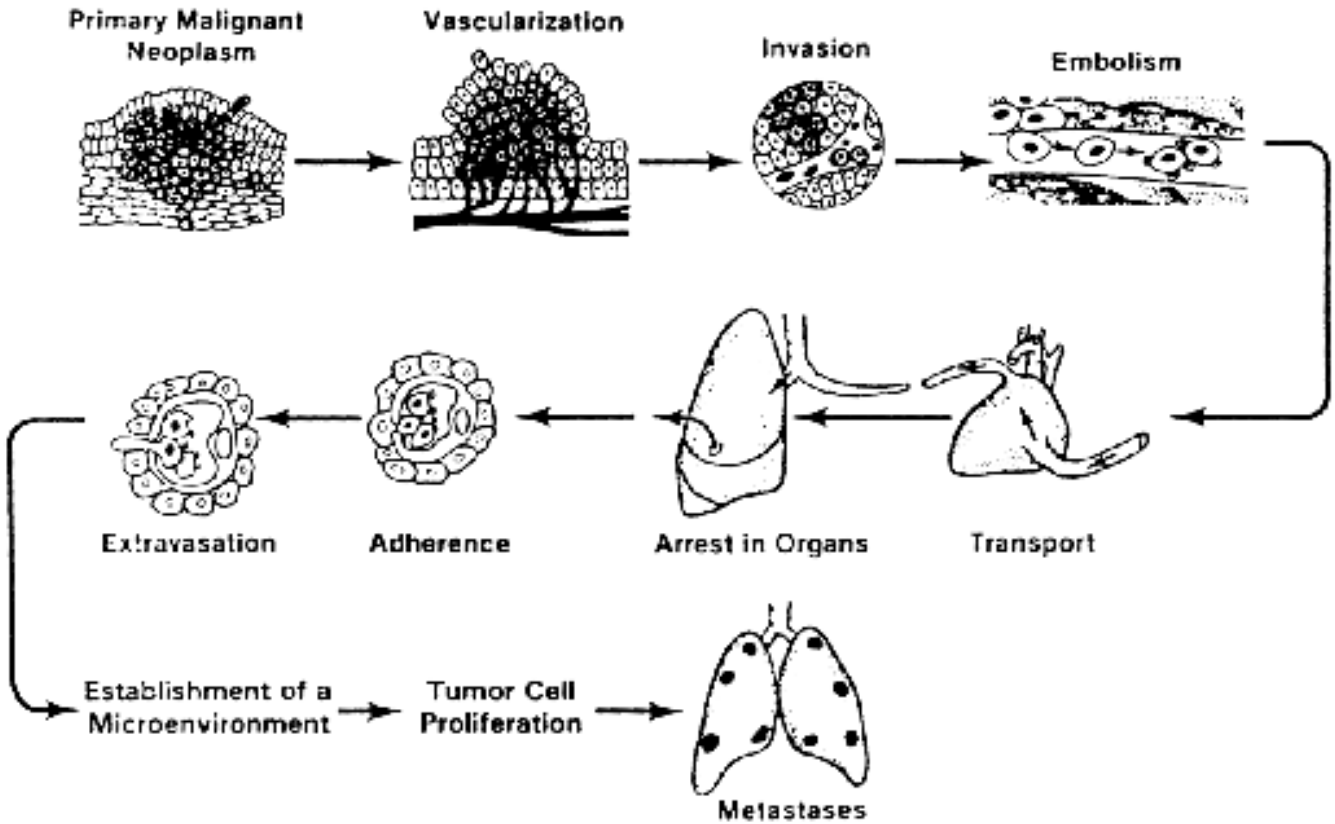
Ratio: HCC/non-HCC

AAC33435	Mitotic checkpoint protein kinase Bub1A	0.01
XP_290472	Similar to hypothetical protein FLJ31846	0.02
NP_067695	Potassium channel, subfamily K, member 9	0.03
AAD02030	Eph-like receptor tyrosine kinase hEphB1	0.04
NP_612641	PWWP domain containing 1; HDGF like	0.04
AAH26290	Similar to fatty-acid-Coenzyme A ligase, long-chain 2	0.05
CAA58470	70-kDa peroxisomal integral membrane protein	0.06
NP_002790	Proteasome α4 subunit	0.07
XP_212354	Hypothetical protein XP_212354	0.07
NP_068657	Fibrinogen, α chain isoform α preproprote	0.07
XP_302020	Similar to C-terminal binding protein 2	0.07
AAC51070	Immunoglobulin heavy chain variable region	0.07
BAA03001	Mitochondrial short-chain enoyl-CoA hydratase	0.08
XP_113986	Hypothetical protein XP_113986	0.08
XP_235716	Similar to ribosomal protein L10a	0.09
CAA39813	Alcohol dehydrogenase	0.1
XP_234923	Similar to KIAA1086 protein	0.1
1OUA	Chain, contribution of hydrophobic residues to the stability of human lysozyme	0.11
P00325	Alcohol dehydrogenase β chain	0.12
NP_004930	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	0.12
AAG24441	Putative prostate cancer susceptibility protein HPC2/ELAC2	0.12
CAA12583	Ig heavy chain variable region	0.13
XP_095725	Similar to 5133401N09Rik protein	0.13
AAD20534	Immunoglobulin heavy chain variable region	0.15
CAC86915	Fibronectin	0.16

Shi-Jian Ding, et al. *Mol Cell Proteomics*. 3:73-81 (2004)

- More aged proteomic approach
  - Two dimensional gel electrophoresis (2-DE)
  - MALDI-TOF-MS
- Compared proteins from cell lines with “high” and “low” metastatic potentials
  - Identified one protein with differential expression
- Expanded analysis of cytokeratin 19
  - Tissues from 102 HCC patients
  - Mouse model of metastasis

Metastasis



< 1 in 1000 cells survive to form metastases (Alberts 4th ed)

http://www.moffitt.usf.edu/pubs/ccj/v2n5/artl3img2.gif

Origin of “high” and “low” metastatic cell lines

MHCC97
“High metastatic human HCC cell line”

↓

Clones derived by limiting dilution in tissue culture

↓

<u>Clonal populations screened for metastatic propensity *in vivo*</u>

1) Injected sc into right flank of male athymic BALB/c nu/nu 4-6 wk old mice
2) 1 to 1.5 cm tumors were harvested cut in 6 pieces and implanted into the liver of 6 nude mice
3) Mice were inspected for metastases

Li et al. World J Gastroenterol. 2001 Oct;7(5):630-6.

Table 1 Lung metastases of 10 clones

Clone number	*n* Tumor implanted	*n* Lung metastases after 5 wk
1, 7, 8, 10, 11, 13	6	6
4	6	5
6, 24	6	4
25	6	2
MHCC97	6	6

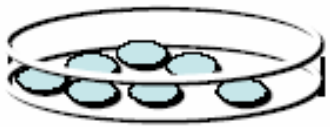
Table 2 Abdominal events and pulmonary metastases after liver implantation of subcutaneous tumor tissue

	Clone number	
	No. 8 (MHCC97-H)	No. 25 (HMCC97-L)
No of mice with tumor implantation	10	10
Tumor size by d 35/cm	1.42±0.11	0.90 ±0.26[a]
Abdominal events		
Abdominal wall invasion	40% (4/10)	20% (2/10)
Bloody ascites	10% (1/10)	0% (0/10)
Intrahepatic metastases	80% (8/10)	0% (0/10)
Diaphragm invasion	10% (1/10)	0% (0/10)
Hepato-splenic/hepato-gastric ligament invasion	10% (1/10)	0% (0/10)
Loco-regional lymph node enlargement	0% (0/10)	0% (0/10)
Pulmonary metastases	100% (10/10)	40% (4/10)[b]

Li et al. J Cancer Res Clin Oncol (2004) 130: 460–468

Metastatic behavior (%)[a]	MHCC97-L (Li et al. 2001)	MHCC97-H (Li et al. 2001)	HCCLM3 (Li et al. 2003)	HCCLM6
Subcutis inoculation				
Lung	0	0	100	100
Liver, abdominal viscera[b]	0	0	0	0
Lymph nodes	0	0	0	0
Orthotopic inoculation				
Lung	40	100	100	100
Intrahepatic	0	80	100	100
Abdominal viscera	0	10	80	100
Lymph nodes	0	0	0	60
Footpad inoculation				
Lymph node	0	0	0	75
Lung	0	0	0	0
Liver, abdominal viscera	0	0	0	0

Preparation of samples for 2DE



Harvest, rinse, and pellet the cells;

or

Dissect out tissue, organ, or fluids;

- Homogenize/lyse in buffer that *unfolds the proteins w/o adding or disturbing the charges*:
  - High urea usually 5-8 M---unfolds the protein
  - Sometimes 2 M thiourea--unfolds the protein
  - 1-4% detergent--solubilizes hydrophobic components
  - Beta-mercaptoethanol or other reductant, such as TBP, DTT
  - Inhibitors: of proteases, kinases, & phosphatases
- Clarify by centrifugation to get rid of insoluble matter;
- Protein assay to know how much and how concentrated

proteomics.biosccc.uab.edu/ masspec/pdf_files/BMG744-HK-1-18-05.pdf

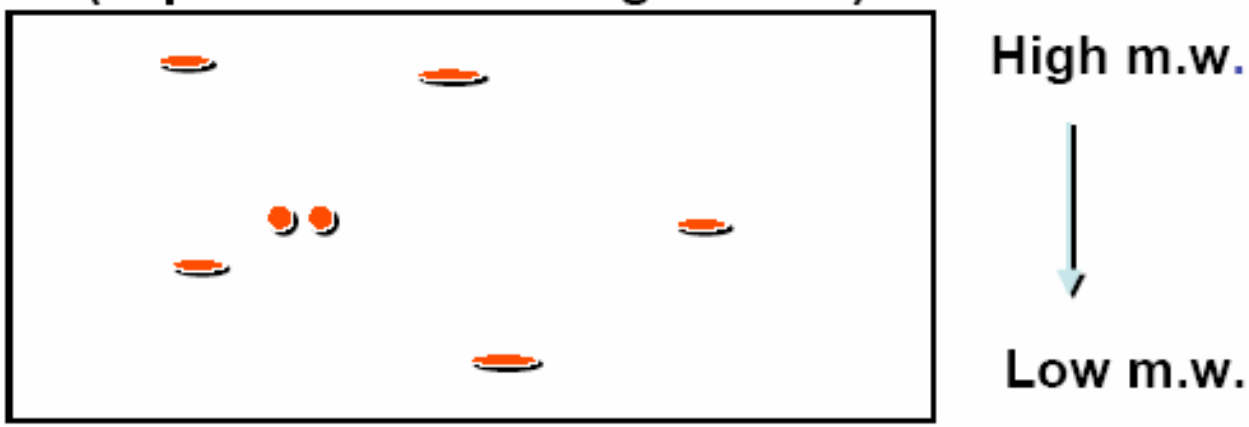
Separation of proteins by 2DE

- 1st dimension: Isoelectric focussing

(separation according to charge)

- 2nd dimension: (SDS)-PAGE

(separation according to size)

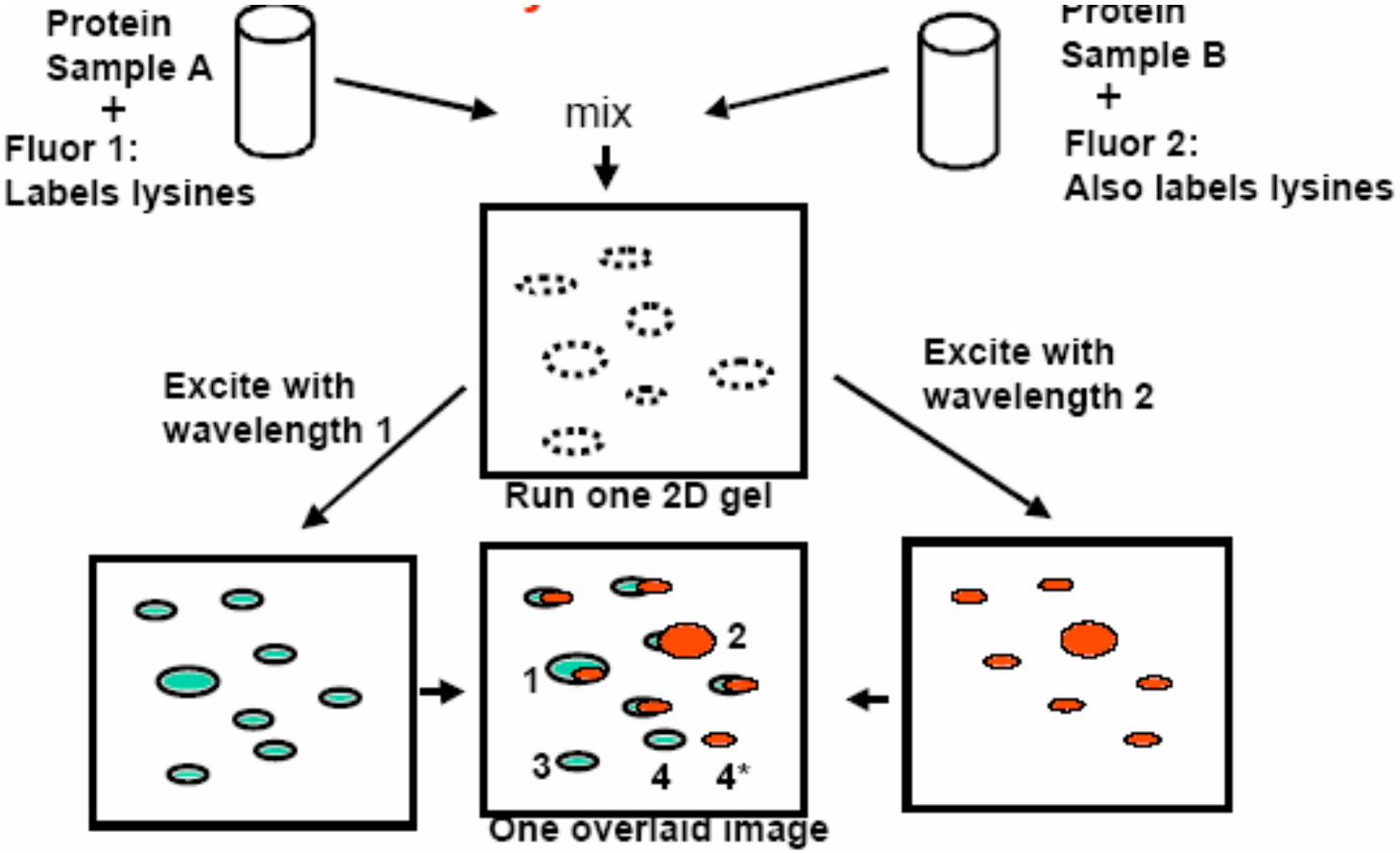


proteomics.biosccc.uab.edu/ masspec/pdf_files/BMG744-HK-1-18-05.pdf

Two Dimensional Gel Electrophoresis

- 2DE can indicate differential expression and/or posttranslational modifications
- Best at detecting proteins of high abundance
- Image analysis can be done by eye or with software
- Is susceptible to irreproducibility
- Database of 2DE images: www.expasy.org

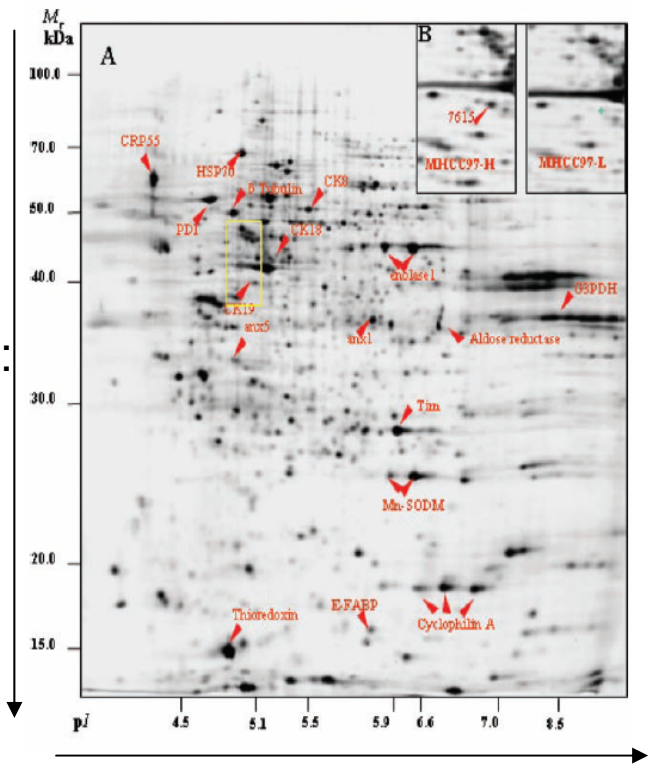
Labeling samples can improve data reliability



proteomics.biosccc.uab.edu/ masspec/pdf_files/BMG744-HK-1-18-05.pdf

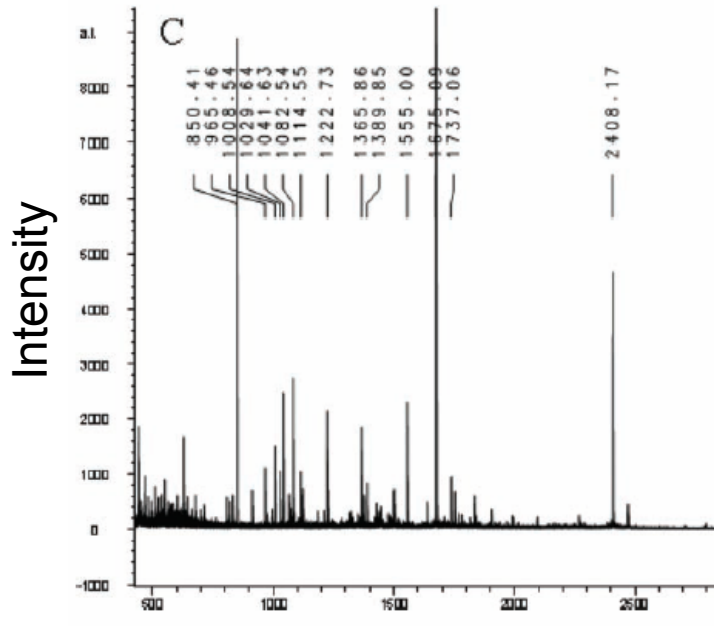
Figure 1: CK19 was identified as differentially expressed protein between MHCC97-H and MHCC97-L using 2-DE and MALDI-TOF-MS.

MHCC97-L



Second Dimension: SDS-PAGE

First Dimension: Iso-electric focusing



Intensity

Mass/Charge

Figure 4: Western blot analysis for CK19 expression

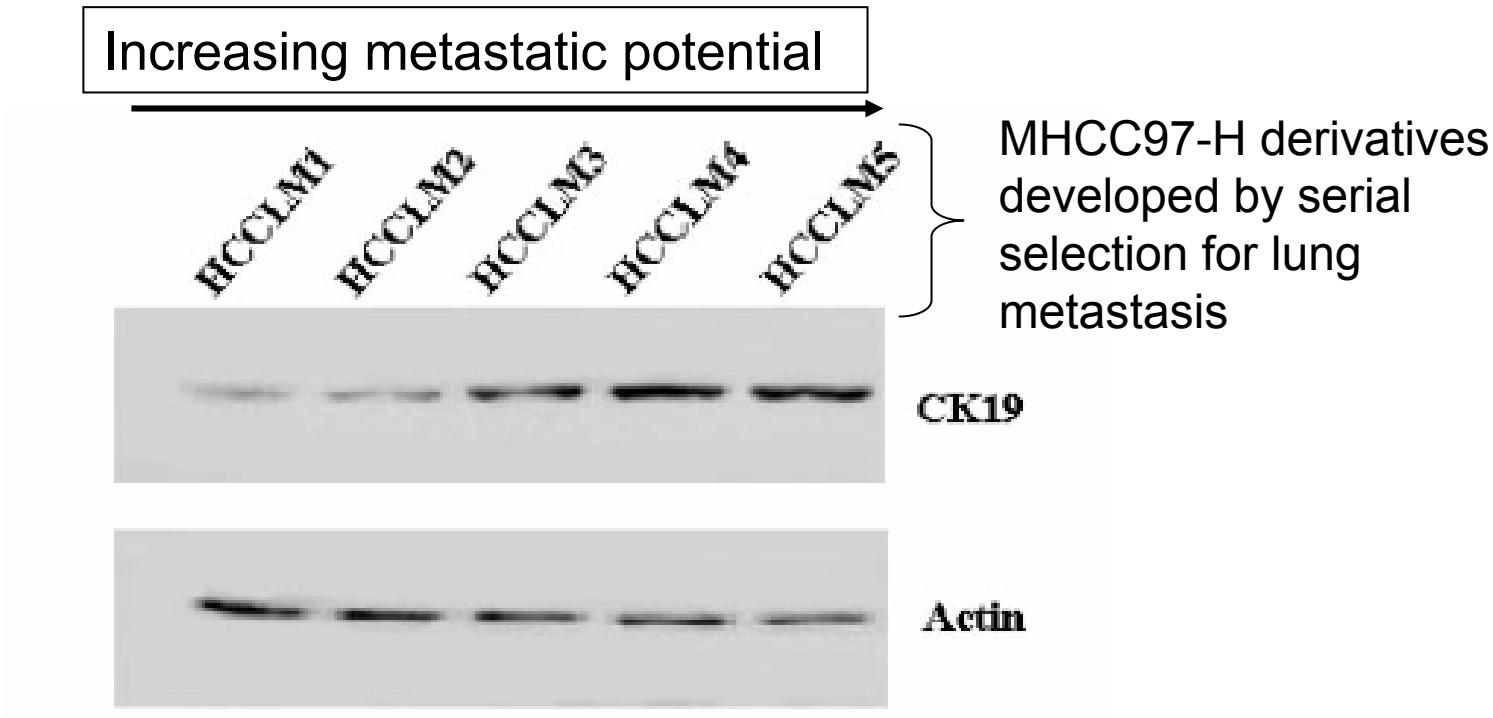


Figure 2: Two-dimensional Western blot analysis for CK19 expression

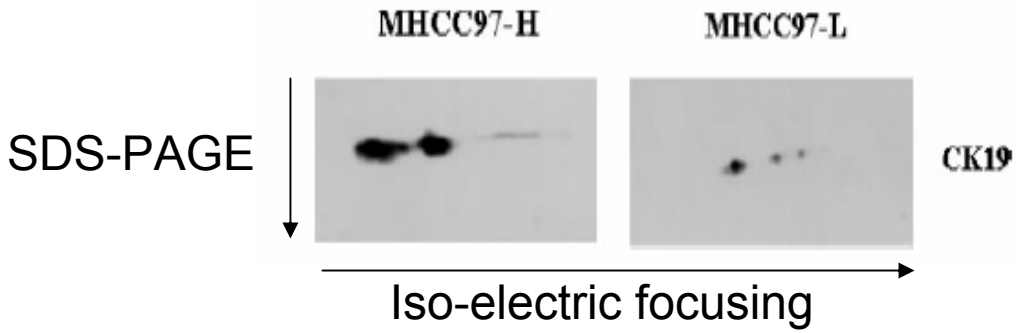
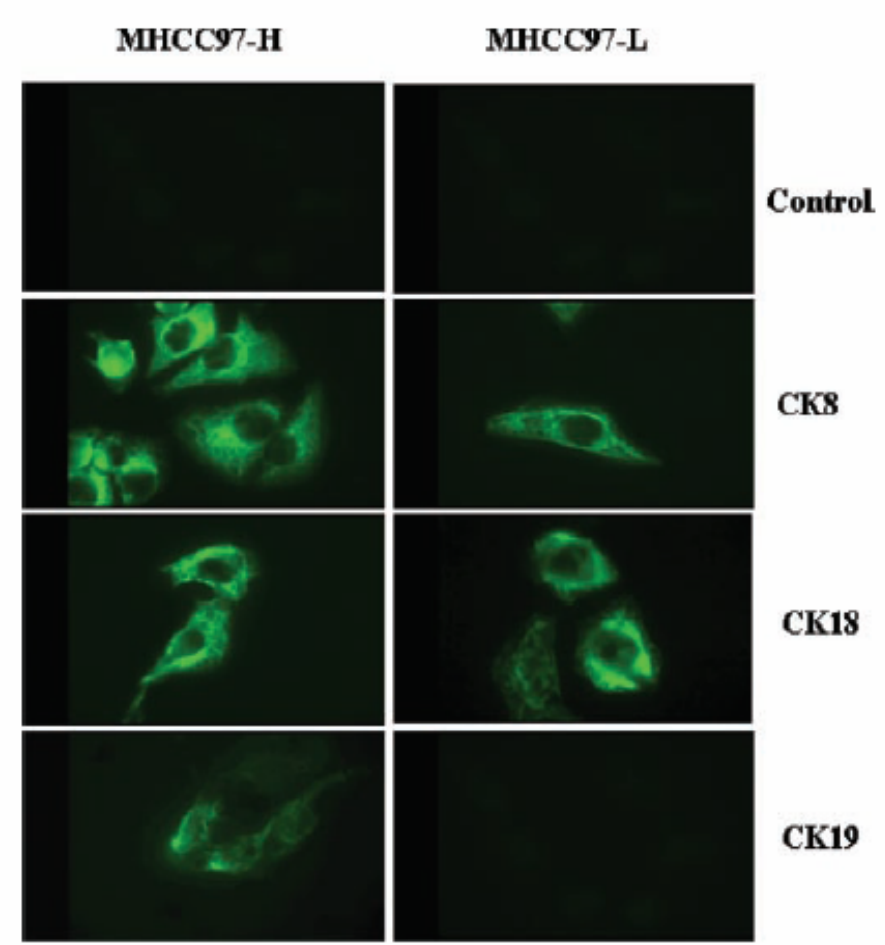


Figure 3: Immunofluorescence detection of cytokeratins



Cytokeratin

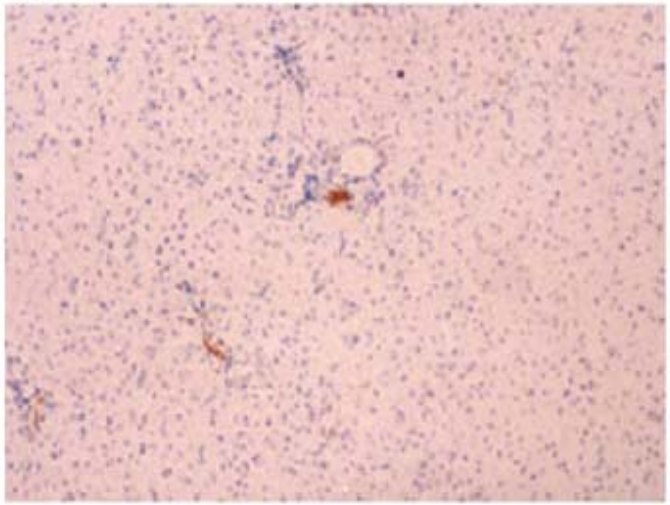
- Cytokeratins
  - Intermediate filament proteins
  - From the cytoskeleton of normal epithelial cells, and some tumor cells
  - Over 20 types of cytokeratin have been identified
  - Liver
    - Fetal liver
      - High expression of CK8, CK18, CK19
      - Upon differentiation- lose CK19
    - Mature Liver
      - CK19 is expressed in the bile ducts and is used as a biliary marker
      - Mature normal hepatocytes do not express CK19
    - Cancer cells
      - Usually retain the IF profile of their cell of origin
      - Malignant transformation may alter the cytokeratin profile of affected hepatocytes in HCC

Figure 5 and Table 1

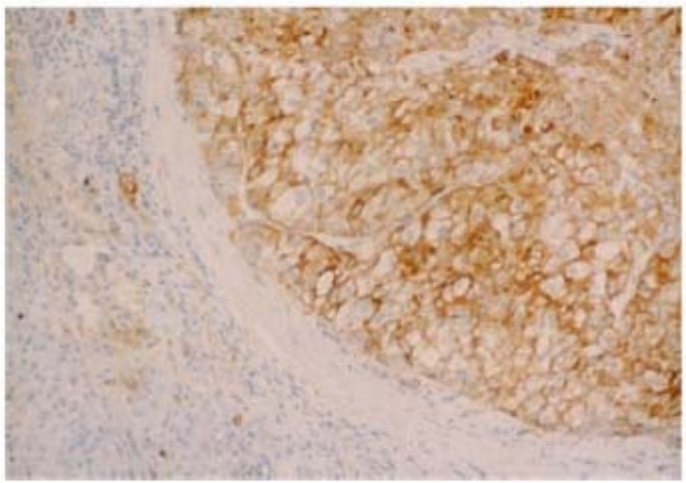
- Wanted to see if theory of CK19 expression would hold up *in vivo* (human tissues)
- Methods
  - 102 liver biopsies from HCC patients
  - Immunohistochemistry- primary AB to CK19
    - Cytokeratin 19
    - Control specimens- cavernous hemangiomas
      - Benign tumors of endothelial origin
      - Negative control
    - HCC stained with CK19
      - Considered positive for CK19 if more than 25% of the tumor cells stained for CK19
      - Supports that some cancer cells express CK19
    - HCC stained with irrelevant AB
      - Shows that positive results were not due to non-specific binding of any AB to the cancer cells
  - Subject data
    - Age
    - TNM Stage
  - Serum
    - Alpha-fetoprotein titer
    - HBAg status

Figure 5

A **Control- Hepatic hemangioma**



B **HCC with CK19 AB**



C **HCC H&E with irrevelant AB-** **serial section from B**

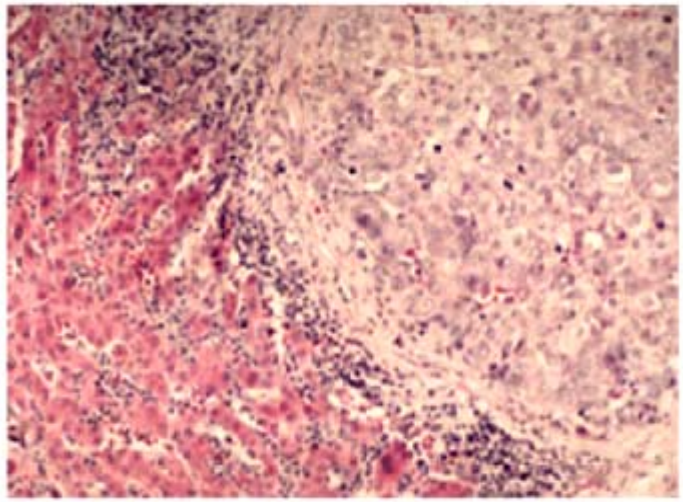


FIG. 5. Immunohistochemical study of CK19 expression in hepatocellular carcinoma specimens.

Table 1

- Evaluation of Human HCC Samples
  - Immunohistochemistry
    - Analyzed 102 HCC tumors
    - 12% (13/102) were CK19 +
  - Wanted to know what if any significant correlation there was between CK19 expression and HCC tumor characteristics

Table 1

TABLE I

Relationship between CK19 expression in tissue samples and clinicopathological characteristics of 102 HCC patients

	CK19 positive (n = 13)	CK19 negative (n = 89)	p value
Age (years, median)	54.7	49.9	>0.05[a]
HBsAg[b] positive	92.3% (12/13)	89.9% (80/89)	>0.05[c]
Tumor size			
>5 cm	69.2% (9/13)	70.8% (63/89)	
≤5 cm	30.8% (4/13)	29.2% (26/89)	>0.05[c]
AFP (>25 μg/liter)	69.2% (9/13)	76.4% (68/89)	>0.05[c]
TNM staging			
III/IV	100.0% (13/13)	69.7% (62/89)	
I/II	0.0% (0/13)	30.3% (27/89)	<0.001[c]
Capsule invasion	61.5% (8/13)	62.9% (56/89)	>0.05[c]
Satellite nodules	38.5% (5/13)	7.9% (7/89)	<0.05[c]
Vascular tumor emboli[d]	84.6% (11/13)	19.1% (17/89)	<0.001[c]

[a] Student's t test.
[b] Hepatitis B surface antigen.
[c] Fisher's exact test.
[d] Including tumor emboli in the portal vein and small blood vessels.

-Satellite nodules= intrahepatic metastases

Table 1

- Significant Findings
  - CK19 expression in HCC was not correlated to age, size of tumor, AFP Titer, or HBAg status
  - **100%** of CK19 + HCC were stage III/IV tumors vs. 69.7% of CK19 – tumors
    - Inference= CK19+ tumors had “poor tumor differentiation”- more aggressive/invasive stage
  - **38.5%** of the CK19+ tumors had satellite nodules (intrahepatic metastases) vs. 7.9% seen in CK19 – tumors
    - Inference= Ck19+ tumors were more invasive or of a metastatic phenotype
  - **84.6%** of the CK19 + tumors had tumor emboli in the vasculature vs. 19.1% of the CK19 – tumors
    - Inference= Ck19+ tumors were more invasive or of a metastatic phenotype

Figure 6 and Table 2

- Tumor Metastasis Animal Model
  - Nude mice (30)
    - Balb/c-nu/nu
    - Male
    - 4 wks old
    - “SPF”
    - 6 groups- 5 per group and groups only differed by the week post injection at which they were sacrificed
  - SQ injections
    - $5x10^6$ cells
    - 0.2 ml
  - HCCLM3 cells
    - Accepted model for spontaneous HCC met development
    - More efficient cell line for producing prominent lung mets
  - Blood, Tissue collection
    - CYFRA 21-1 (CK19) serum levels
      - Detected by RIA
      - Soluble fragment of CK19
      - Can be released by viable tumor cells or by apoptotic tumor cells
    - Injection site tumors were harvested and weighed
    - Pulmonary metastases were harvested, counted, weighed and evaluated histologically

Figure 6

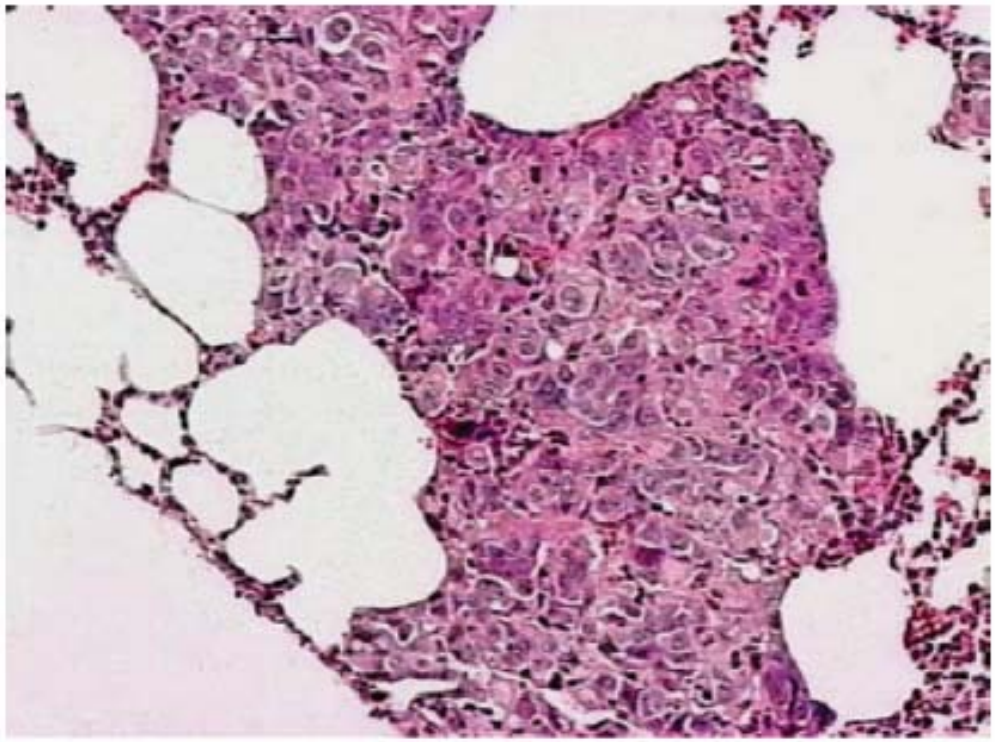


FIG. 6. **Lung metastasis in a nude mice model of human HCC.**

- All mice developed tumors and lung mets were seen after 4 weeks

Table 2

TABLE II

Relationship between serum CK19 level and lung metastasis in a nude mice model

Metastatic human HCC cell line HCCLM3 was injected into the subcutaneous region of 30 BALB/c-nu/nu nude mice, which were randomized into six groups of five mice in each group. One group of mice was sacrificed each week from the end of the second week on, and tumor weight, lung metastasis, and serum CK19 levels were determined. CK19 increased remarkably when lung metastasis occurred.

	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Tumor weight (g)	0.31 ± 0.13	0.61 ± 0.12	1.43 ± 0.37	2.38 ± 0.92	3.74 ± 0.96	4.85 ± 1.02
Lung metastasis rate (%)	0	0	20	100	100	100
Median no. of lung metastases	0	0	0[a]	81	118	150
Serum CK19 (μg/liter)	4.79 ± 1.16	5.06 ± 1.21	8.55 ± 1.25	40.71 ± 9.87[b]	76.29 ± 20.00[b]	95.94 ± 19.68[b]

[a] Only one mouse had lung metastases at week 4, and the total number of lung metastases was less than five, so the median number of lung metastases was 0.

[b] $p < 0.01$, compared with serum CK19 level in week 4 (analysis of variance and q test).

Table 2

- Tumor metastases
  - Significant findings
    - After 5 weeks there was 100% rate of lung metastasis
    - Primary tumor size also continued to increase
      - Although it slowed between wks 5-7 vs. wks 2-4
    - Serum CK19 levels
      - Were significantly increased at weeks 5, 6 and 7
      - Inference= CK19 levels become elevated when metastasis is occurring
      - Caution- CK19 serum levels may be elevated if there is increase in apoptosis of tumor cells vs. increase in circulating tumor cells
        - Increase death due to outgrowing vascular supply?
        - Supported by decrease in the rate of growth of the primary tumor between wks 5-7 as compared to wks 2-4 (where the tumor size doubled each week)

Clinical Conclusions

- Proteomic techniques are useful in screening for potential biomarkers of disease
  - Comparative proteomics identified a difference in expression of CK19 in two clones from the MHCC97 cell line that differ in their ability to metastasize
- CK19 serum levels (CYFRA21-1) maybe a biomarker for tumor metastasis
  - Serum CK19 levels increase in parallel with progression of engrafted tumors
  - Previous reports have shown increased expression in other cancer states, so it may not be specific for HCC mets
- CK19 expression in tumors maybe of prognostic significance for HCC
  - Expression levels of CK19 are significantly correlated to spontaneous metastatic potentials
  - Expression was correlated to the degree of differentiation of HCC tumor
    - Expression of CK19= more poorly differentiated tumor

Commentary and Discussion

- Did this work successfully harness the strength of the “proteomic approach?”
- Why did the authors focus on CK19?
- Which proteins are most readily identified by 2DE combined with mass spec? What is the sensitivity limiting step? Are abundant proteins the most relevant?
- Is CK19 a convincing tumor marker?
- Did this work increase understanding of HCC pathogenesis or metastasis?
- What experiments might be performed for “larger impact?”
- Was the mouse metastasis model a “good” model?

Recent paper from same group

Li C, Tan YX, Zhou H, Ding SJ, Li SJ, Ma DJ, Man XB, Hong Y, Zhang L, Li L, Xia QC, Wu JR, Wang HY, Zeng R. Proteomic analysis of hepatitis B virus-associated hepatocellular carcinoma: Identification of potential tumor markers. Proteomics. 2005 Mar;5(4):1125-39.

- Compared HCC to non-HCC liver tissue
  - Didn't use LCM (???)
  - Separated proteins by 2DE
  - Identified proteins by MALDI-TOF-MS
  - Larger cohort of HBV+, HCC patients (10)
  - Identified 2 new proteins with biomarker potential

Which protein is important?

It depends on your question….

- Is your goal to diagnose the disease?
  - Biomarkers tightly correlate with disease
- Do you want to understand disease pathogenesis?
  - Proteins that are involved in mechanism
- What question did they ask?
- Lit search for “cytokeratin 19” and cancer
  - 400 Pubmed hits (primary)
  - 18 Pubmed hits (review)
    - V. Barak et al. Clinical utility of cytokeratins as tumor markers. *Clin Biochem.* 37(7):529-40. (2004)
  - 6150 Google hits