Biomarkers & Cancer
Chemoprevention Trial

JianYu Rao, M.D.
Chief, Cytopathology
Director, Gynecological Pathology
CHEMOPREVENTION

• Administrating specific amounts of a particular natural or synthetic chemical in an attempt to identify agents that will prevent, halt or reverse the process of carcinogenesis

• The basic assumption is that treating early stages of malignant process will halt the progression of malignancy

• The key is to define early lesions, and treat the malignant field
Cancer

Precancerous Intraepithelial Lesions, (PIN, CIN, PaIN..)

Birth

Exposure to Carcinogen

Additional Molecular Event

Cancer

CHEMOPREVENTION
Multiyear progression from initiation and early precancerous lesions to invasive disease in major cancer target organs

<table>
<thead>
<tr>
<th>Normal</th>
<th>Initiated</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>CIS</th>
<th>Latent Cancer</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>20 yrs</td>
<td>PIN</td>
<td>10 yrs</td>
<td>10 yrs</td>
<td>Latent Cancer</td>
<td>3–15 yrs</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>Atypical Hyperplasia</td>
<td>14–18 yrs</td>
<td>DCIS</td>
<td>6–10 yrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>5–20 yrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung (Smokers)</td>
<td>20–40 pack-yrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>Adenoma</td>
<td>5–20 yrs</td>
<td>5–15 yrs</td>
<td>TIS</td>
<td>&lt; 5 yrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>20 yrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td>CIN I</td>
<td>9–13 yrs</td>
<td>CIN III/CIS</td>
<td>10–20 yrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
<td>Barrett’s</td>
<td>est 5–20 yrs</td>
<td>Severe Dysplasia</td>
<td>est 3–4 yrs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>HBV Infection</td>
<td>20–40 yrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Kelloff et al. 2000 (Fig. 1)
BIOMARKERS NEED TO:

- Identification of AT-RISK subjects who are also SUSCEPTIBLE to treatment:

**LEGEND:**
- White: Not at risk to develop disease
- Green: At risk of developing disease, biology A, responsive to agent X
- Red: At risk of developing disease, biology B, NOT responsive to agent X
Cancer
Precancerous Intraepithelial Lesions, (PIN, CIN, PaIN..)
Birth
Exposure to Carcinogen
Additional Molecular Event
Cancer

Surrogate End Point Markers

Genetic Suscep. Marker
Markers for Exposure
Markers of Effect
Tumor Markers

CHEMOPREVENTION
Loss of p16
Cox-2
Telomere dysfunction
Actin remodeling

Modified from Thea Tlsty, Journal of Mammary Gland Biology and Neoplasia, 2004
CRITERIA FOR SELECTING BIOMARKER

- FITS EXPECTED BIOLOGICAL MECHANISM
- BIOMARKER AND ASSAY PROVIDE ACCEPTABLE SENSITIVITY, SPECIFICITY, AND ACCURACY
- BIOMARKER IS EASILY MEASURED
- BIOMARKER MODULATION CORRELATES TO THE END POINT
  - Disease incidence (for detection marker)
  - Disease progression (for prognostic marker)
  - Response to therapy (for therapeutic marker)
ASSAY VALIDITY

- **SENSITIVITY**: % of assay-positive cases in case group
- **SPECIFICITY**: % of assay-negative cases in control group
- **PPV (Positive Predictive Value)**: % of “true” assay-positive cases out of all assay-positive cases
- **NPV (Negative Predictive Value)**: % of “true” assay-negative cases out of all assay-negative cases

These numbers can be totally meaningless – always know what kind of design used (case-control vs cohort) and what is the gold standard
OTHER ASSAY ISSUES

• BIOMARKER CAN BE OBTAINED BY NON-INVASIVE TECHNIQUES

• ASSAY IS NOT TECHNICALLY DIFFICULT

• MULTIPLE MARKERS CAN BE EVALUATED SIMULTANEOUSLY IN LIMITED SAMPLE VOLUMES

• COST
CATEGORIES OF SEM

- HISTOLOGICAL AND MORPHOMETRIC MARKERS
- PROLIFERATION, DIFFERENTIATION AND INVASION MARKERS
- SPECIFIC ONCOGENES/GROWTH REGULATORS
- MARKERS OF GENETIC AND EPIGENETIC INSTABILITY
# Potential SEMS for Breast, Colon, and Prostate

<table>
<thead>
<tr>
<th></th>
<th>Breast</th>
<th>Colon</th>
<th>Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological</strong></td>
<td>DCIS, LCIS, ADH</td>
<td>Adenomatous polyps</td>
<td>PIN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aberrant polyps</td>
<td></td>
</tr>
<tr>
<td><strong>Proliferation</strong></td>
<td>S-phase fraction</td>
<td>S-phase fraction</td>
<td>PCNA</td>
</tr>
<tr>
<td></td>
<td>Ki-67</td>
<td>Brdu Uptake, PCNA</td>
<td>Ki-67</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td>Myoepithelial (s-100 BGA, Mucin core ag)</td>
<td>BGA, Mucin core ag</td>
<td>HM Cytok</td>
</tr>
<tr>
<td></td>
<td>Vimentin), etc</td>
<td>Cytokeratins</td>
<td>BGA, actin</td>
</tr>
<tr>
<td><strong>Genetic</strong></td>
<td>Onc (erb-2, myc fos, ras)</td>
<td>Onc (ras, myc, src)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Suppressor (p53)</td>
<td>Suppressor (p53, DCC)</td>
<td></td>
</tr>
<tr>
<td><strong>Biochemical</strong></td>
<td>Estradiol</td>
<td>Ornithine Decarboxylase</td>
<td>TGF-beta, PSA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyamine</td>
<td></td>
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</table>
SEM Modulation in Chemoprevention

• Complete Phenotypic Response - idea
• Less Than Complete Phenotypic Response - Genotypic markers to distinguish chemoprevention from selecting regressing of existing disease
  – true effect is seen if post-treated lesion has less genotypic change than baseline or control
• No Response.
Genome Wide Genotypic SEM Analysis

• Monitoring pre-specified sets of genetic lesions that are strongly associated with neoplastic progression, e.g., using gene chips;
• Microarray analysis of gene expression in post-treatment and baseline lesions.
Issues in Using SEM

• The observed SEM change may not correlate with end point (cancer incidence).
• Can not measure the quality of life.
• Adverse effect may not be observed in short term SEM studies.
Lessons learned from SELDI-TOF

• Initial study on patient serum from cancer patients (ovarian, prostate, etc) versus cancer showed very promising results (nearly 100% sensitivity/specificity to separate cancer from normal)
  – Used case-control design
  – Only 2 group-comparison (cancer vs. normal)
  – No validation

• However, recent validation studies were rather disappointing
BIOMARKER STUDY DESIGN

a. Untargeted Design:

Register → Randomize

Treatment → Control

b. Untargeted Design:

Register → Test Biomarker → Biomarker + → Randomize

Treatment → Control
BIOMARKER STUDY DESIGN

Biomarker by Treatment Interaction Design:

1. Register
2. Test Biomarker
3. Stratify
   - Biomarker + Randomize
     - Treatment
     - Control
   - Biomarker - Randomize
     - Treatment
     - Control
Biomarker Based Strategy Design:

- Register
- Randomize
- Test Biomarker

Biomarker + ➔ Treatment A
Biomarker - ➔ Treatment B
No Biomarker Evaluation ➔ Treatment B
BIOMARKER STUDY DESIGN

Modified Biomarker Based Strategy Design:

- Register
- Randomize

Test Biomarker

- Biomarker + → Treatment A
- Biomarker - → Treatment B

No Biomarker Evaluation

- Randomize

Treatment A

Treatment B
Biomarker-Directed Targeted Design

• Increase the efficiency of the trial, but depends on:
  – The performance of the biomarker test (sensitivity/specificity)
  – Size of the treatment effect for target-negative patients
Actin Remodeling
As a Target for Biomarker Development
Morphological hallmarks of cancer cells:

- Altered N/C-ratio
- Altered membrane (cytoplasmic and nuclear)
- Loss of cell adhesion
- Increased motility/invasion/met.
- etc..

ALMOST ALL ARE RELATED TO ACTIN REMODELING
WHAT TO DO WITH THIS?
HYPOTHESIS/RATIONALE

• Altered cytoskeletal proteins, e.g., actin remodeling, is the foundation for malignant morphological phenotype

• Thus, signaling pathways associated actin remodeling may provide a potential target for anti-cancer drug development as well as biomarkers for a more objective assessment of malignant transformation and progression

• These targets can be identified through genomic/proteomic approach
Figure 1. Model in Focal Adhesions

- VASP
- Tenuin
- F-Actin
- Zyxin
- Actinin
- p-Tyr
- Vinculin
- Ras Sup. Family (Rac/Rho/CDC42)
- pp60
- pp125FAK
- Abl

- ECM
- Integrin
- PM
- Substrate

- Talin
- Paxillin
- R/E/M
- Abl
ACTIN ASSOCIATED MOLECULARS IMPLICATED IN MALIGNANT TRANSFORMATION

**Oncogene signal transduction pathways**
- Ras family (GTPase):
  - Rho (stress fibers)
  - Rac (lamellipodia)
  - Cdc42 (filopodia)
- Src family (tyrosine kinase)*
  - FAK*
  - LIMK1

* Relate to integrin signaling

**Tumor Suppressor**
- Gelsolin*
- Tropomyosin/merlin
- Alpha-actinin*
- E-cadherin
- Beta-Catenin
- Vinculin
- Fodrin*
- Annexin-I

* Implicated in apoptosis
G-ACTIN  ⇄ F-ACTIN  ⇄ STRESS FIBERS
Increased cellular F-actin is a marker of cellular differentiation

Using leukemic cell lines:
- HL-60 - Transformed/Differentiable
- Daudi - Transformed/Undifferentiable
- RPMI - Nontransformed

We demonstrated that increased F-actin content is associated with cellular differentiation

(J. Rao, Cancer Res., 1990)
2. ACTIN REMODELING IN APOPTOPSIS MACHINERY
- Actin remodeling chemicals (Jas./Cytochalasin) have direct effect on apoptosis induction in HL-60 cells
3. Actin remodeling is a marker for malignant associated field changes.
Actin alteration is a field disease marker for bladder cancer

A careful mapping analysis on touch prep slides obtained from distant, adjacent and tumor tissues showed that increased G-actin is seen in over 50% of the distant field epithelial cells of cancer bearing bladder.

(J. Rao, P.N.A.S., 1993)
QFIABiomarker Profile

G-actin: Texas-Red conjugated DNase I
M344: FITC (or Rhodamin) 3-Step Immunofluorescence
DNA: Hoechst or DAPI
Abnormal G-actin in the Field Predicts Tumor Recurrence

Kaplan-Meyer Analysis

Fraction disease-free

G-actin negative

G-actin positive

$p = 0.0324$

by log-rank test

Weeks after therapy
Cellular actin levels can be used to monitor the effectiveness of chemoprevention

- Cellular F/G-actin levels in the non-tumor field epithelial cells after tumor was removed by TUR predicted the recurrence potential of the tumor.
- In addition, cellular F/G-actin levels fluctuate from abnormal to normal as results of chemopreventive effect of differentiation agent DMSO.

4. Altered actin binding protein as markers for tumor progression

TMA analysis of Gelsolin in bladder cancer

- **Ki-67 Max*Pos, P-value = 0.2**
- **P53 Max*Pos, P-value = 0.598**
- **Gelsolin Max*Pos, P-value = 0.007**
- **Ecadherin Max*Pos, P-value = 0.573**
Recurrence probability stratified by the maximum Gelsolin score in high grade tumors.
**Actin remodeling protein Gelsolin is the strongest predictor for high grade TCC**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Probability of effect*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP1MaxM</td>
<td>63.3</td>
</tr>
<tr>
<td>ATP1PosMaxM</td>
<td>70.8</td>
</tr>
<tr>
<td>ATP2MaxM</td>
<td>30.9</td>
</tr>
<tr>
<td>ATP2PosMaxM</td>
<td>9.2</td>
</tr>
<tr>
<td>KiMax</td>
<td>10.5</td>
</tr>
<tr>
<td>KiPos</td>
<td>6.5</td>
</tr>
<tr>
<td><strong>GeMax</strong></td>
<td><strong>94.4</strong></td>
</tr>
<tr>
<td>GePos</td>
<td>24.5</td>
</tr>
<tr>
<td>EcMax</td>
<td>6.3</td>
</tr>
<tr>
<td>EcPos</td>
<td>6.3</td>
</tr>
<tr>
<td>P53Max</td>
<td>38.2</td>
</tr>
<tr>
<td>P53Pos</td>
<td>22.9</td>
</tr>
</tbody>
</table>

* Results of Bayesian Model Approach on bladder TMA
SUMMARY: Actin Remodeling in Cancer

- Actin polymerization is a generalized marker for cell differentiation
- Actin dynamic regulates apoptosis morphology
- Actin depolymerization is a marker of early transformation and malignancy associate field disease
- Altered actin binding protein as markers for tumor progression
- Actin remodeling as a generalized marker for anticancer effects
GREEN TEA STORY

– Linking the studies of chemopreventive agents with biomarkers at the level of actin remodeling
• Epidemiological and animal studies showed that GTE is a potential chemopreventive agent for cancer
• Our goal is to study how GTE modulate actin remodeling in HUC model
Selective effect of GTE on transformed MC-T11 over untransformed PC

1. Cell growth

2. Actin poly.

3. Cell cycle

HUC-PC

MC-T11

0 20 µg/ml 40 µg/ml 80 µg/ml
GTE induced actin polym. in MC-T11 cells resulted in an increased adhesion and decreased motility, both associated with changes of distribution of actin fibers and adhesion complex.
Effect of GTE on actin is mediated by Rho:

B. Dose effect of GTE on Rho activity

C. GTE effect on actin blocked by Rho-inhibitor C3 exoenzyme
GTE Modulates Actin remodeling by Rho activity in early transformed cells

Which ABP may be involved?
A, GTE (20μg/ml)

B, GTE (40μg/ml)

MW (kDa)

Time: 48 hr
GTE: -

Time: 24 hr
GTE: +

Time: 48 hr
GTE: +
ANX

F-act

GTE siRNA

- No + Yes (Pos) - Yes (Neg) + Yes (Neg)

Negative siRNA
Annexin-1 is regulated by epigenetic mechanism (DNA methylation)

- Annexin-1 levels are measured by RT-PCR and Annexin staining.
- GTE and 5-A treatments are shown to alter Annexin-1 expression.
- GTE alone (+) decreases Annexin-1 levels, while 5-A alone (+) increases them.
- Co-treatment of GTE and 5-A (+) shows a synergistic effect on Annexin-1 expression.
- F-actin staining is shown to remain unchanged across treatments.
Benign                  CIS
TCC-G1                TCC-G2             TCC-G3

Histology
% of cases

Neg    Weak  Strong

% of cases

Histology
Non-tumor  CIS  TCC-G1  TCC-G2  TCC-G3
In normal cells, actin depolymerization leads to differentiation, which can occur through GTE, etc. Loss of ABP can also lead to differentiation. Rho/Rac/CDC42 can cause actin redistribution, which is a key step in the progression from premalignancy to invasive cancer. The process involves transformation, actin depolymerization, and actin redistribution.

NORMAL \[\rightarrow\] PREMALIGNANCY (Intraepithelial Neoplasia) \[\rightarrow\] INVASIVE CA
Atomic Force Microscopy Study of Actin Remodeling

• A new tool for cancer research
• Ideal for analyzing the functional role of actin remodeling in various cellular events in single living cells
• Combine functional analysis with morphology at nanometer level
NEWS HEADLINES

• Nanotechnology shows cancer cells are 'softer' than normal cells
• Microscopic 'tools' can identify cancer cells by 'feel'
• Nano breakthrough in cancer detection: study
• ....
Fig. 1. Schematic of an AFM tip
(a) approaching, 
(b) indenting and
(c) retracting from a cell
Average Young’s Modulus (E) values for A549 human lung adenocarcinoma cells treated with or without (ctrl) 40 µg/mL green tea extract (GTE) for 6 and 12 hours, respectively.

Effects of GTE on the migration of A549 cells. Confluent monolayers of cells were maintained in a serum free media and a lane was scraped through the monolayers of the cells with a plastic micropipette tip. The cells were allowed to migrate across the lane at 37°C for 6 or 24 h in the presence (40 µg/ml) or absence of GTE. The distance that cells migrated into the area of the wound at different points was photographed using a computer imaging system. Top panels: GTE untreated; lower panels: GTE treated (40 µg/ml).

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Ave. Young’s Modulus (kPa) - ctrl</th>
<th>Ave. Young’s Modulus (kPa) - GTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>30.5 ± 7.2</td>
<td>38.0 ± 8.9</td>
</tr>
<tr>
<td>24</td>
<td>30.9 ± 9.0</td>
<td>94.2 ± 55.5</td>
</tr>
</tbody>
</table>
Adhesion Force Measured between Mesothelial Cells and Cancer Cells

- **Mesothelial cells**
- **Cancer cells**

**Graph:**
- **Young's Modulus (E):**
  - Counts
  - Measurement

**AFM Measurements:**
- **E**
- **AF**
Summary

• Biomarker is needed in Chemoprevention Trial to:
  – Detect early preventable lesions
  – Monitoring the efficacy

• Actin remodeling and associated cellular nanomechanical changes provide a wealth of targets for chemopreventive biomarker selection:
  – Actin change occurs in premalignant field lesion
  – Chemopreventive agents (e.g., green tea) modulates actin remodeling
  – Actin change can be detected either by traditional biochemical assays or AFM measurements of cellular nanomechanics
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