Nanocytology in Cancer Diagnosis

- Is this the future of cytology?

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DISCLOSURE STATEMENT

• I am a member of Scientific Advisory Board of edeixa LLC, a company interested in developing Atomic Force Microscope for cancer diagnosis. However, no data presented is supported by the company.
What is Nanocytology?

Cellular Material (Exfoliative/Aspirative)

- Molecular Expression Analysis
- Conventional Morphology Based Cytology
- Nanomechanical Analysis

Functional diagnostic information for personalized management of cancer
Outline

• General discussion of cytology in cancer diagnosis

• Review previous work on understanding the basis of cancer cell mechanics – actin remodeling

• Nanomechanical analysis of cancer cells
  – In body fluid
  – In primary tumors
  – In urine samples
  – In cancer cells treated with various drugs
Cancer Facts

• One of every two Americans will have cancer in his/her lifetime

• Most cancer deaths are the results of cancer cell invasion and metastasis

• Cancer is not one disease, but many DIFFERENT diseases
  – Heterogeneity at different levels
    • Patient
    • Tissue level (histological variation)
    • Cell level
    • Molecular level
However, there are COMMON cancer cell phenotypes

- Loss of growth control
- Invasion
- Metastasis
NORMAL  \hspace{2cm} \textbf{PREMALIGNANCY}  \hspace{2cm} \textbf{INVASION/ METASTASIS}  \\
\text{(Intraepithelial Neoplasia)}

\begin{itemize}
  \item \textbf{Initiation}
  \item \textbf{Progression}
\end{itemize}
PARADOXICAL DIFFERENTIATION
– A feature of early invasive cancer
How Do We Diagnose Cancer Currently?

Patient present with mass-related symptoms

Image studies to determine the size and nature of the mass
+ biopsy or needle aspiration

Cytopathological / Histopathological examination on cell / tissue morphology +/- biochemical/molecular analysis

Establish the Diagnosis of Cancer
The Invention of Cytology
The Most Successful Cancer Control Story To Date

- **Pap Test**
  - Detect Early Lesions

- **Colposcopy**
  - Treat Early Lesions

**Decreased incidence/mortality of cervical CA >70%**
Morphology-based cytology, including exfoliative (body fluid) / aspirative (FNA) cytology, is a simple and first line of cancer diagnostic method.

HOWEVER, cytomorphology alone has:

- Low sensitivity – false negative findings
- Qualitative and ambiguity
- Not a functional test for cancer cell behavior (metastatic/invasive potential)
- Limited value to guide personalized therapy
RESEARCH GOAL

Study biological mechanisms of cancer cell morphogenesis, focus on cytoskeletal actin remodeling

Study nanomechanical changes as a functional marker for cytology

Functional Cytology
Cytoskeletal actin remodeling in cancer cells

• Microfilament actin and its associated protein constitute over 25% of total cellular protein
• Actin remodeling is responsible for different aspects of cancer cell phenotypes, including changes of mechanical properties
• Understanding actin remodeling is important to understand cancer cell mechanics
Actin Remodeling in Cancer Cells

Morphological hallmarks of cancer cells – MANY ARE ASSOCIATED WITH ACTIN REMODELING:

- Altered N/C-ratio
- Altered membrane (cytoplasmic and nuclear)
- Loss of cell adhesion
- Increased motility/invasion/met. (paradoxical differentiation)
Model in Focal Adhesions

- VASP
- Tenuin
- F-Actin
- Zyxin
- Actinin
- p-Tyr

- Ras Sup. Family (Rac/Rho/CDC42)
- pp60^sro
- pp125FAK
- Abl

- Vinculin
- Paxillin
- Tensin
- Talin
- R/E/M

- ECM
- Integrin
- PM
- Substrate
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

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

* Relate to integrin signaling
* Implicated in apoptosis
Increased cellular F-actin is a marker of cellular differentiation

Actin remodeling in early phase of apoptotic machinery

Executioner (caspases) → Cleavage actin → G-ACTIN / DNase I → Polymerization → F-ACTIN

Other Factors → Cleavage actin related proteins

Decreased G-actin → Membrane Bleb + DNase I → DNA Fragm. → Apop. Body

Actin remodeling is a marker for malignant associated field changes

(J. Rao, et al., PNAS 1993)
Abnormal Actin Predicts Bladder Cancer Recurrence

Kaplan-Meyer Analysis

Fraction disease-free

G-actin negative

G-actin positive

p = 0.0324 by log-rank test

Weeks after therapy

Green Tea Extract modulate actin remodeling

ADHESION

MOTILITY
Annexin-1 (ANX) is the key target of GTE-induced actin remodeling
Actin remodeling in cancer cells

- Carcinogen
- Oncogene
- Growth factors
  - Apoptosis machinery
  - Increased Motility
  - EMT
  - Nanomechanics

- Rac → Rho
- G ↔ F
- ABPS

- Disrupt cell division
- Change cell shape
- Disrupt cell-cell interaction
BI-PHASIC PATTERN OF ACTIN REMODELING IN CANCER

NORMAL                  PREMALIGNANCY                  INVASIVE
                           (Intraepithelial Neoplasia)          /Met CA

Loss of ABP → Actin Depolymerization → Actin redistribution

Initiation → Progression

Differentiation (GTE, etc) → Rho/Rac/CDC42

Actin Depolymerization

Initiation

Progression
Nanomechanical Analysis Using Atomic Force Microscopy on Cytological Samples
What is Nanotechnology?

“What Nanotechnology refers broadly to a field of applied science and technology whose unifying theme is the control of matter on the atomic and molecular scale, normally 1 to 100 nanometers, and the fabrication of devices with critical dimensions that lie within that size range”

(http://en.wikipedia.org/wiki/Nanotechnology)
What is nanotechnology? (Cont.)

“The impetus for nanotechnology comes from a renewed interest in Interface and Colloid Science, coupled with a new generation of analytical tools such as the atomic force microscope (AFM), and the scanning tunneling microscope (STM)”
The promise of nanotechnology in medicine may be huge

-Nothing has been materialized yet
Atomic Force Microscopy

• Ideal for analyzing the functional role of actin remodeling in various cellular events in single living cells

• Allows functional analysis with morphology at nanometer level

• Previous studies, mostly done in in vitro cell line models showed
  – Malignant transformed cells SOFTER than non-transformed cells
Atomic Force Microscope

Diagram showing the components of an Atomic Force Microscope, including a Piezoelectric Crystal, Laser, Cantilever, Photodiode, Sample Surface, and a Computer for data acquisition and feedback generation.
Fig. 1. Schematic of an AFM tip
(a) approaching,
(b) indenting and
(c) retracting from a cell
AFM analysis of body fluid samples
Why BODY FLUID?

• Almost 20% of the body cavity effusions examined are directly or indirectly related to the presence of malignancy

• Accurate diagnosis of malignancy is important for clinical management

• The malignant cells (met.) are uniformly metastatic in nature

• Presence of morphologically similar mesothelial cells served as internal control
• **Body cavity fluid cytology is challenging:**
  – To diagnose metastatic malignancy:
    • By morphology alone, accuracy 50-70%
    • Even with a panel of markers, still <100% (70-90%)
  – For primary mesothelioma:
    • No specific diagnostic marker
    • Often surgically obtained tissue is needed to establish the diagnosis
<table>
<thead>
<tr>
<th></th>
<th>Mesothelial cells</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leu M1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mucin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Calretinin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BER-EP4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B72.3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MOC31</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
CYTOLOGY (Giemsa STAIN)

HISTOLOGY (H&E STAIN)

Normal Mesothelial cells
(Calretinin positive)

Malignant cells
(Ber-Ep4 positive)
Nanomechanics of Human Metastatic Cancer Cells in Clinical Pleural Effusions

- Studied 7 pleural effusion samples
  - 4 with metastatic cancer (2 non-small cell ca of lung, 1 breast, and 1 pancreas)
  - 3 benign

- Cells collected underwent short-term ex-vivo culture (24 hr)

- Each sample selected 8 “probable” cancer vs 8 “probably” benign cells for AFM measurement

- Three force-displacement curves recorded for each cell (Young’s modulus, E), yielding 24 values of E for each cell type per clinical specimen

## Patient Characteristics and Cytological Diagnosis versus Mechanical Measurements (E).

<table>
<thead>
<tr>
<th>Case #</th>
<th>Age/Sex</th>
<th>Clinical History</th>
<th>Cytological Diagnosis of Pleural Fluid*</th>
<th>Stiffness (kPa): “Tumor”</th>
<th>Stiffness (kPa): “Normal”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52/Fem  ale</td>
<td>Non-small cell carcinoma of the lung</td>
<td>Positive for metastatic malignant cells</td>
<td>0.57 ± 0.15</td>
<td>2.0 ± 0.76</td>
</tr>
<tr>
<td>2</td>
<td>60/Fem  ale</td>
<td>Non-small cell carcinoma of the lung</td>
<td>Positive for metastatic malignant cells</td>
<td>0.50 ± 0.15</td>
<td>2.07 ± 0.81</td>
</tr>
<tr>
<td>3</td>
<td>49/Fem  ale</td>
<td>Breast ductal adenocarcinoma</td>
<td>Positive for metastatic malignant cells</td>
<td>0.50 ± 0.15</td>
<td>1.94 ± 0.84</td>
</tr>
<tr>
<td>4</td>
<td>85/Male</td>
<td>Pancreatic adenocarcinoma</td>
<td>Positive for metastatic malignant cells</td>
<td>0.55 ± 0.11</td>
<td>0.55 ± 0.14</td>
</tr>
<tr>
<td>5</td>
<td>40/Male</td>
<td>Liver cirrhosis</td>
<td>Negative for malignant cells</td>
<td>—</td>
<td>1.78 ± 0.70</td>
</tr>
<tr>
<td>6</td>
<td>47/Male</td>
<td>Fever and hepatic failure</td>
<td>Negative for malignant cells</td>
<td>—</td>
<td>1.81 ± 0.83</td>
</tr>
<tr>
<td>7</td>
<td>92/Fem  ale</td>
<td>Anasarca peripheral edema</td>
<td>Negative for malignant cells</td>
<td>—</td>
<td>2.07 ± 0.98</td>
</tr>
</tbody>
</table>
Young's Modulus E and Adhesion Force Measured between Mesothelial Cells and Cancer Cells

Metastatic tumor cells ("tumor"):  
\[ \langle E_{\text{tumor cells}} \rangle \approx 0.51 \pm 0.13 \text{kPa} \]

Benign mesothelial cells ("normal"):  
\[ \langle E_{\text{normal cells}} \rangle \approx 0.52 \pm 0.11 \text{kPa} \]

*Cell stiffness appears to be the same for both the "tumor" and "normal" cells?
Develop a simple cytospin method for AFM study and comparing that to the ex vivo culturing method.
Study the effect of Green Tea Extract (GTE) on Nanomechanics in Metastatic Tumor Cells from Body Fluid
Table 2. 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.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Ave. Yong's Modulus (kPa) - ctrl</th>
<th>Ave. Yong's Modulus (kPa) - GTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.31±0.07</td>
<td>0.38±0.09</td>
</tr>
<tr>
<td>24</td>
<td>0.31±0.09</td>
<td>0.94±0.56</td>
</tr>
</tbody>
</table>

Figure 7. 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.
Histograms of A549 Cell Mechanics Data for ctrl and GTE negative (-) and positive (+) transfection with siRNA Annexin-I:

(a) 

\[ \langle E \rangle = 0.74 \pm 0.36 \text{ kPa} \]

\[ \langle E \rangle = 2.67 \pm 0.94 \text{ kPa} \]

\[ \langle E \rangle = 0.60 \pm 0.31 \text{ kPa} \]

\[ \langle E \rangle = 0.67 \pm 0.44 \text{ kPa} \]
The Effect of Therapeutic Agents on Cell Stiffness Measurements

- GTE and chemotherapeutic agents on cancer cells from pleural effusion samples (n=10)
  - 4 ovarian ca
  - 3 lung ca
  - 2 breast ca
  - 1 benign
Clinical Fluid of Positive Samples for GTE 60ug/ml Treated at 48hr
Nanomechanical Analysis of Urine Samples
Urine Cytology

• Important complementary test with cystoscopy for bladder cancer detection

• Difficult with overall accuracy < 50%

• Numerous biomarkers developed in the past, some FDA approved, all suffer either low sensitivity or specificity
Determination of optimal cyto-centrifugation time on Young’s modulu’s E measurement in human urine samples
Effect of sample storage time on Young's modular E in different types of epithelial cells in normal urine samples

**Graph**

- **Time**
  - 0 hour
  - 4 hours
  - 24-28 hours
  - 56 hours

- **Young modural E (Pa)**
  - Squamous cells
  - Urothelial cells

**Data**

- Squamous cells:
  - 0 hour: Approximately 200 Pa
  - 4 hours: Approximately 400 Pa
  - 24-28 hours: Approximately 600 Pa
  - 56 hours: Approximately 1000 Pa

- Urothelial cells:
  - 0 hour: Approximately 100 Pa
  - 4 hours: Approximately 200 Pa
  - 24-28 hours: Approximately 300 Pa
  - 56 hours: Approximately 400 Pa
Young’s Modulus (kPa)

<table>
<thead>
<tr>
<th>Cytology</th>
<th>History</th>
<th>Sample #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>Pos</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>Pos</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>Neg</td>
<td>Yes</td>
<td>4</td>
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<td>16</td>
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<tr>
<td>Neg</td>
<td>No</td>
<td>17</td>
</tr>
<tr>
<td>Neg</td>
<td>No</td>
<td>18</td>
</tr>
</tbody>
</table>
Cyto: Positive  
History: Yes

Clinical urine cells
W390-77-19

Counts
Young's mouduls, E(kPa)

Cyto: Negative  
History: Yes

Clinical urine cells
C08-32348

Counts
Young's mouduls, E(kPa)

Cyto: Negative  
History: No

Clinical urine cells
C08-31964

Counts
Young's mouduls, E(kPa)

Asymptomatic control
Nanomechanical analysis of primary tumor cells obtained from FNA
### Young’s Modulus: Primary Tumor Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Young’s Modulus (kPa)</th>
<th>Number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a _Tumor</td>
<td>0.70 ± 0.19</td>
<td>8</td>
</tr>
<tr>
<td>3a - Tumor</td>
<td>0.68 ± 0.28</td>
<td>22</td>
</tr>
<tr>
<td>3b - Benign</td>
<td>1.84 ± 0.54*</td>
<td>8</td>
</tr>
</tbody>
</table>

*Note the significant increase in Young’s modulus for this sample*
Young's Modulus – Primary Breast Tumor Samples from 3 Different Patients

Counts

Young's Modulus, E (kPa)

Sample 1a
Sample 3a
Sample 3b
NORMAL    PREMALIGNANCY (Intraepithelial Neoplasia)    INVASION    METASTASIS
Summary

• Nanomechanical analysis can be performed in variety of clinical cytological samples

• It may provide a functional quantitative test to supplement the existing morphology/molecular based analysis

• More studies need to be done....
Challenges of AFM-based nanomechanical measurements

• Requires fresh sample/cells
• Cell selection is a issue
• AFM is a expensive machine
• Mechanical measurement is still a tedious process
• Lack of standard data handling/analysis scheme
Advancing Cytology from Morphology to Function Level
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