Tumor-microenvironment Interactions and Lung Cancer Invasiveness

Pulmonary Grand Rounds
Philippe Montgrain, M.D.
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Objectives

- Review epithelial-mesenchymal transition (EMT), and its implications for lung cancer.
- Review tumor-microenvironment interactions, the role of integrins, and their implications for lung cancer.
- Present data from our laboratory on the role of parathyroid hormone-related protein (PTHrP) in the regulation of integrins and lung cancer invasiveness.
Impact of Lung Cancer

- Kills more men and women each year than any other cancer.
- 5-year survival remains dismally low, only 15%.
Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths, by Sex, United States, 2008

**Estimated New Cases**

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>186,320</td>
<td>25%</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>114,690</td>
<td>15%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>77,250</td>
<td>10%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>51,230</td>
<td>7%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>35,450</td>
<td>5%</td>
</tr>
<tr>
<td>Melanoma of the skin</td>
<td>34,950</td>
<td>5%</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>33,130</td>
<td>4%</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>25,310</td>
<td>3%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>25,180</td>
<td>3%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>18,770</td>
<td>3%</td>
</tr>
<tr>
<td>All Sites</td>
<td>745,180</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Estimated Deaths**

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung &amp; bronchus</td>
<td>90,810</td>
<td>31%</td>
</tr>
<tr>
<td>Prostate</td>
<td>28,660</td>
<td>10%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>24,260</td>
<td>8%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>17,500</td>
<td>6%</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>12,570</td>
<td>4%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>12,460</td>
<td>4%</td>
</tr>
<tr>
<td>Esophagus</td>
<td>11,250</td>
<td>4%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>9,950</td>
<td>3%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>9,790</td>
<td>3%</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>8,100</td>
<td>3%</td>
</tr>
<tr>
<td>All Sites</td>
<td>294,120</td>
<td>100%</td>
</tr>
</tbody>
</table>

From Jemal, A. et al.  
Annual Age-adjusted Cancer Death Rates* Among Females for Selected Cancers, United States, 1930 to 2004


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Parathyroid hormone related protein (PTHrP) was discovered as the tumor-derived product associated with humoral hypercalcemia of malignancy (HHM).

It is expressed in a wide variety of malignant and normal tissues.

Named for homology between its amino terminus, PTHrP 1-34, and the comparable portion of parathyroid hormone (PTH).

PTHrP binds the same receptor as PTH, with equal affinity.
PTHrP is expressed by about 66% of non-small cell lung carcinomas (NSCLC) (Clin Cancer Res 2006; 12:499-506).

It has effects on cell growth and differentiation, apoptosis sensitivity and matrix interactions, actions that could affect cancer progression.
PTHrP Decreases Lung Cancer Cell Proliferation
Orthotopic Lung Cancer Model

- PTHrP antibody
- control antibody
- measure tumor growth

- lung cancer cell injection
Endogenous PTHrP Slows Lung Carcinoma Growth

Hastings, Cancer 92:1402-1410, 2001
Does PTHrP Impact Survival?

- Patients undergoing surgery for non-small cell lung carcinoma
- Stain lung carcinomas for PTHrP
- Analyze survival based on PTHrP status, demographic data and cancer specifics
<table>
<thead>
<tr>
<th></th>
<th>PTHrP-positive</th>
<th>PTHrP-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenocarcinoma</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>squamous carcinoma</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>large cell carcinoma</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
PTHrP Survival Benefit in Women

Tumors in Females Make More PTHrP than in Males

Montgrain et al, Cancer 2007
Established a tool to study the effects of PTHrP on lung cancer

**PTHrP positive and negative H1944 cells**

![Graph showing the expression levels of PTHrP in different cell lines.](image-url)

- **wild type**
- v1
- v2
- v15
- v80
- no PTHrP
- clones transfected with vector
- clones transfected with PTHrP
- n = 3 cell wells
- BEN cells
PTHrP-expressing clones grow more slowly

Cell proliferation, % control ± SE

wild type  v1  v2  v15  v80

PTHrP-negative clones

P2  P7  P10  P25  P43

PTHrP-positive clones

n = 3-4 cell wells
n = 3-7 cell wells
n = 5 clones

P < 0.01
The Scientific Method

1. Make an Observation
2. Ask a Question
3. Form a Hypothesis
4. Conduct an Experiment
5. Accept Hypothesis
6. Reject Hypothesis
It all starts with an observation...
The Observation

PTHrP-negative H1944 cells are morphologically different than PTHrP-positive H1944 cells. They look more mesenchymal, as opposed to epithelial. Arrows show cell projections.
Expression of PTHrP is the only difference between the two cell lines.

PTHrP must be regulating something that controls cell morphology, cell-cell interaction/adhesion, and cell motility.

What???
What controls the transition from epithelial characteristics to mesenchymal characteristics? This process is known as epithelial-mesenchymal transition (EMT).

What are the molecular markers of EMT?

What is the significance of all this? Does it affect lung cancer invasiveness, metastasis, outcomes?
Epithelial-mesenchymal transition

- EMT is important in embryonic development and has been proposed to play a role in cancer. It is the conversion of cells with epithelial characteristics to the morphology, molecular signature and functional repertoire of mesenchymal cells.

- Cells lose their apical-basal polarity, assume a flattened fusiform appearance with filopodia, exchange epithelial proteins for mesenchymal markers, loosen their attachment to adjacent cells and become more motile and invasive.

- In cancer, the initial step in metastasis requires separation from neighboring cells and acquisition of an aggressive, mobile phenotype, similar to the changes that occur during EMT.

Molecular markers of EMT

- Cells typically lose cytokeratins (epithelial cytoskeletal proteins) and gain vimentin (mesenchymal cytoskeletal protein).
- Cells increase their production of fibronectin and proteases involved in matrix remodeling.
- The hallmark change is a decrease in expression of E-cadherin.

Yang and Weinberg, Developmental Cell 2008
Gravdal et al, Clin Cancer Res 2007
E-cadherin

- E-cadherin is a transmembrane glycoprotein component of the adherens junctions in epithelial cells. It participates in homophilic binding with molecules between adjacent cells, lining cells together.
- Downregulation of E-cadherin switches on EMT in many cells.

Bremnes et al, Lung Cancer 2002
Onder et al, Cancer Res 2008
Guarino et al, Pathol 2007
EMT Signaling

EMT signaling pathways frequently act on a group of transcriptional repressors (Snail, Slug, Sip1, Twist and others) that turn off expression of E-cadherin and can upregulate some mesenchymal genes.

Yang et al, Cell 2004
Extracellular matrix proteins, like collagen and fibronectin, can bind to integrins on the surface of cancer cells and trigger EMT.
Significance of EMT in cancer

- Involvement of EMT in cancer invasiveness and metastasis is postulated on the basis of morphologic changes and molecular evidence.

- In breast cancer and colorectal cancer, the tumor leading edge displays cells that have separated from their neighbors and transitioned toward a mesenchymal phenotype.

- Gene expression signatures for EMT are frequently discerned in invasive and/or metastatic cancer. Genes involved in cell-cell adhesion, such as E-cadherin and beta-catenin, are generally decreased while vimentin is upregulated.

Natalwala et al, World J Gastroenterol 2008
Guarino et al, Pathol 2007
In lung cancer, E-cadherin expression is a marker for differentiated tumors and is inversely correlated with local invasion, nodal metastasis and mortality.

Bremnes et al, Lung Cancer 2002
Sibanuma et al, Lung Cancer 1998
Bremnes et al, J Clin Oncol 2002
E-cadherin in lung cancer

A) All 193 patients
B) 95 Squamous cell
C) 83 adenocarcinoma

Solid line = high E-cadherin expression
Dashed line = intermediate E-cadherin expression
Dotted line = no to low E-cadherin expression

Table 5. Membranous E-Cadherin and Catenin Expression in Univariate Analysis (log-rank test)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Marker Expression</th>
<th>Patients (%)</th>
<th>Mean Survival* (months)</th>
<th>5-Year Survival (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Cadherin</td>
<td>Negative-low</td>
<td>11</td>
<td>27</td>
<td>35</td>
<td>.0002</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>35</td>
<td>58</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>54</td>
<td>75</td>
<td>70</td>
<td></td>
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<tr>
<td>β-Catenin</td>
<td>Negative-low</td>
<td>8</td>
<td>32</td>
<td>44</td>
<td>.009</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>33</td>
<td>68</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>59</td>
<td>67</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>γ-Catenin</td>
<td>Negative-low</td>
<td>31</td>
<td>56</td>
<td>54</td>
<td>.049</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>38</td>
<td>64</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>31</td>
<td>74</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>α-Catenin</td>
<td>Negative-low</td>
<td>11</td>
<td>48</td>
<td>48</td>
<td>.043</td>
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<tr>
<td></td>
<td>Intermediate-high</td>
<td>83</td>
<td>68</td>
<td>62</td>
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<tr>
<td>p120</td>
<td>Negative-low</td>
<td>61</td>
<td>63</td>
<td>61</td>
<td>.62</td>
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<tr>
<td></td>
<td>Intermediate</td>
<td>33</td>
<td>68</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>6</td>
<td>61</td>
<td>67</td>
<td></td>
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</tbody>
</table>

NOTE: Prognostic immunohistochemical parameters were used as predictors for disease-specific survival in 193 NSCLC patients.

*Mean survival is provided because median survival was not reached for all groups.
Integrins

In addition to their involvement in EMT, integrins mediate interactions with the extracellular matrix (ECM).

How a carcinoma grows, and its invasiveness, depend in part on these tumor-ECM interactions.

Integrins are transmembrane proteins composed of heterodimeric complexes of $\alpha$ and $\beta$ subunits.

At least 18 different $\alpha$ and 8 $\beta$ subunits have been described. These units can combine into over 20 $\alpha\beta$ dimers, each differing in affinity for the various ECM proteins.
Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ predominantly recognize collagen, $\alpha 3\beta 1$ is a laminin receptor and $\alpha 5\beta 1$ and $\alpha v\beta 3$ have greatest specificity for fibronectin.

Ivaska and Heino, Cell Mol Life Sciences 2000
Integrins and invasiveness

In the lung, collagens and laminins are localized to the alveolar and bronchial epithelial basement membrane, while fibronectin is situated in the interstitial stroma and is normally not exposed to the epithelium.

The profile of integrins expressed by a tumor cell may determine its growth pattern. A good example is the difference between invasive adenocarcinoma of the lung and bronchioloalveolar carcinoma (BAC).

BAC is a subtype of lung adenocarcinoma defined by “lepidic” growth. The tumor expands within the airway boundaries along an intact interstitial framework with no evidence of stromal, vascular or pleural invasion. The prognosis for pure BAC is better than for invasive adenocarcinomas.

Gil and Martinez-Hernandez, J Histochem Cytochem 1984
Integrins and invasiveness

The profile of integrins expressed by BAC differs from the profile for other adenocarcinomas and could contribute to the differences in growth pattern.

The predominant integrins made by BAC recognize collagen and laminin, such as $\alpha 1\beta 1$ and $\alpha 3\beta 1$.

As a result, BAC tumors possess integrins that would mediate adherence to the airway basement membrane and facilitate “lepidic” growth.

Koukoulis et al, Hum Pathol 1997
In contrast, invasive adenocarcinomas produce integrins that recognize fibronectin, such as α5β1 and αvβ1.

Their integrin profile would favor growth through the stroma, where fibronectin is found.

Furthermore, α5β1 and αvβ1 stimulate signaling pathways that favor aggressive growth.

For example, ligation of α5β1 by growth on fibronectin activates Akt and mTOR signaling in human lung adenocarcinoma cells, leading to more rapid proliferation, diminished apoptosis and increased migration.

Han et al, Int J Cancer 2004
Han et al, Cancer Res 2006
Significance of integrins in lung cancer

Integrins may have a role in regulating lung carcinoma invasiveness.

In support of this, $\alpha_5$ expression is associated with an increased risk of metastasis to lymph nodes in NSCLC (Han, Lung Cancer 2003).

The factors that regulate subtype-dependent integrin expression in lung cancer are not well characterized.
PTHrP and Integrins

- PTHrP exerts a number of actions that affect carcinoma growth, including effects on integrin expression and matrix metalloproteinase expression.

- In prostate carcinoma, breast carcinoma and colon carcinoma cells, PTHrP upregulates collagen- and laminin- specific integrin subunits, including $\alpha_1$, $\alpha_3$, $\alpha_6$, $\beta_1$ and $\beta_4$.

References:
- Hastings, Respir Physiol Neurobiol 2004
- Shen and Falzon, Regul Pept 2003
- Shen and Falzon, Mol Cell Endocrinol 2003
- Shen and Falzon, Regul Pept 2005
<table>
<thead>
<tr>
<th>PTHrP-negative</th>
<th>PTHrP-positive</th>
</tr>
</thead>
</table>

PTHrP-negative H1944 cells are morphologically different than PTHrP-positive H1944 cells. They look more mesenchymal, as opposed to epithelial. Arrows show cell projections.
The Hypothesis

- PTHrP inhibits EMT. This will be evidenced by higher expression of E-cadherin in the PTHrP-producing cells.

- PTHrP results in an integrin profile that favors less aggressive growth.
Initial experiments

- Analyze human metastasis proteins by qPCR array (RT2Profiler PCR Array System, SABiosytems).
- Confirm findings with Western blotting.
- Determine changes in integrin levels with PTHrP.
- Adhesion assays (to fibronectin, collagen, laminin).
- Motility assays.
qPCR array

PTHrP resulted in changes in gene expression that could reduce lung cancer invasiveness and/or inhibit EMT.
PTHrP upregulates E-cadherin

E-cadherin was increased and vimentin decreased in 3 PTHrP-positive H1944 clones compared to 4 PTHrP-negative clones.
PTHRP decreases vimentin

Vimentin immunofluorescence was markedly decreased in PTHrP-positive compared to negative H1944 cells.
PTHrP affects integrin profile

PTHrP-positive H1944 cells expressed lower levels of $\alpha_5$ and $\alpha_v$ integrin subunits compared to the control cells. In contrast, $\beta_4$ was unaffected.
PTHRP affects integrin profile

Since integrins $\alpha_5$ and $\alpha_v$ are decreased by PTHrP, one would expect PTHrP-positive cells to adhere less tightly to fibronectin.

Adhesion assays: 96-well plates are precoated with ECM proteins and blocked. Cells at constant density are plated in each well and allowed to adhere in the incubator for a constant period. Unattached cells are gently washed away and attached cells are stained with Hoechst 33342 (H33342), a membrane permeant dye that binds DNA. H33342 is then measured with a fluorescent plate reader and the relationship between cell number and fluorescence intensity is determined.
PTHrP decreases adhesion to ECM

PTHrP-positive H1944 clones (open circles) adhered to a lesser extent to fibronectin and Matrigel than did PTHrP-negative clones (closed circles).
Similar results in another cell line

MV522 lung adenocarcinoma cells
Summary

- PTHrP increases E-cadherin and decreases vimentin. This supports our hypothesis that PTHrP inhibits EMT and reduces lung cancer invasiveness.

- PTHrP decreases expression of integrins α5 and αv, which bind fibronectin in the lung stroma and can signal through Akt and mTOR.

- PTHrP reduces adhesion of lung cancer cells to ECM proteins.
H1944 lung cancer cells demonstrate morphology and molecular signatures consistent with EMT. Ectopic expression of PTHrP inhibits EMT. PTHrP also downregulates integrin expression, particularly the αvβ3 fibronectin receptor, and reduces adhesion to fibronectin and Matrigel. Thus, PTHrP is associated with changes toward a less invasive, less motile profile, effects that could lead to a reduction in metastasis and improved survival.
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