Discovery and Validation of Potential Genomic-Based Biomarkers for Asbestos Related Neoplasms

American Australian Mesothelioma Consortium and NYU Mesothelioma Biomarker Discovery Laboratory
Mesothelioma Biomarkers and Their Validation

- Introduction
- SMRP and Osteopontin
- Biomarkers in Progress
- US Validation Trial Update
- The Cappadochian Studies
American Australian Mesothelioma Consortium

• NYU School of Medicine (NYU)
  – Harvey I. Pass MD, PI

• University of Western Australia (UWA)
  – Bruce Robinson MD, PhD, Co-PI

• Peter MacCallum Cancer Institute (PMCC)
  – David Bowtell PhD
  – Andrew Holloway, PhD

• Fujirebio Diagnostics, Inc (FDI)
Asbestos-Related Thoracic Cancers

- **Pleural Mesothelioma**
  - 2500 in United States
  - 15-30 year latency period
  - Median Survival 6-13 months
  - Uniformly fatal when diagnosed after symptoms
  - $54 billion in asbestos-related claims and the estimated future liability ranges from $145 to $210 billion.
Mesothelioma Archives
NYU

- **221 MPM tumors, snap frozen**
  - 63 corresponding normal peritoneum
  - 249 sera
  - 34 plasma
  - 120 pleural effusion
  - 136 urine
  - Complete clinical demographics
- **85 Asbestos exposed**
  - All with serum, plasma, and urine
  - Complete clinical demographics
- **Over 200 lung cancers, snap frozen**
  - Corresponding normal lung
  - Corresponding serum (all); 60 with plasma
  - Complete clinical demographics
- **62 high risk for lung cancer (chemoprevention trial)**
  - All with serum and plasma
  - Complete clinical demographics
Novel Markers for Mesothelioma

• Ready for Validation
  – SMRP (MesoMark™)
    • Partnership with Fujirebio Diagnostics, Malvern Pennsylvania
  – Osteopontin

• Studies in Progress
  – MMP1 and MMP9
  – HAPLN1 (CRTL-1)
Mesothelin

- **MAb K1** demonstrated selective staining of MPM tissue and cell lines
  - Pastan et al: 1992
  - Willingham et al: 1992

- The cloned cDNA from an ovarian cDNA library encoded an antigen recognized by K1:
  - a **40-kDa glycoprotein (mesothelin)** present on the surface of mesothelial cells, MPMs, and ovarian cancers with a 69 kDa precursor
8 years later…

<table>
<thead>
<tr>
<th>TAGS ELEVATED IN MM</th>
<th>FOLD</th>
<th>MM</th>
<th>GENE</th>
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<tr>
<td>TCCTCACCAT</td>
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<td></td>
<td>vitronectin</td>
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<td>TAGGAGCAAT</td>
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<td>up-regulated by BCG-CWS</td>
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<td></td>
<td>protease, serine, 11 (IGF binding)</td>
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<td></td>
<td>plasmolin</td>
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<td>AGCTGGATGC</td>
<td>22</td>
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<tr>
<td>ATGCTCCCTG</td>
<td>21</td>
<td></td>
<td>galectin 6 binding protein</td>
</tr>
</tbody>
</table>
Serum Mesothelin Related Peptide (SMRP, Mesothelin Variant 1))

- Same N-terminal amino acid sequence as mesothelin and megakaryocyte potentiating factor.
- Most likely originates as a portion of the extracellular domain of membrane-bound mesothelin.
- Non-Quantitative “sandwich ELISA” developed with antibodies 569 and 4HR.

The antibody 569 stained 42/62 (68%) MPMs and 7/74 (10%) adenocarcinomas. All MPMs stained in a membranous pattern, and positive staining was seen in mainly epithelial components.
SMRP and Mesothelioma

- 84% sensitivity
  - 100% specificity when compared with other pleural diseases
  - 95% specificity when compared with other lung tumors
  - 83% when compared with people with asbestos exposure

Validation of SMRP in the American Cohort
Methods

• **Patient Population**
  - Serum
    • 90 MPM
    • 170 NSCLC
    • 66 Asbestos-exposed volunteers from the Center for Occupational and Environmental Medicine
    • 409 normal volunteers
  - Pleural Effusion
    • 45 MPM
    • 20 Other Cancers
    • 30 Benign

• **SMRP**
  - MesoMark™ duplicate samples

• **Statistical Analysis**
  - ROC curves
  - Kruskal-Wallis and ANOVA
# Serum Demographics

<table>
<thead>
<tr>
<th></th>
<th>MPM (n=90)</th>
<th>Lung Cancer (n=170)</th>
<th>Asbestos (n=66)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>71/19</td>
<td>94/76</td>
<td>61/5</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>63±1 (39-84)</td>
<td>66±1(33-87)</td>
<td>64±1(36-90)</td>
</tr>
<tr>
<td><strong>Fiber Exposure</strong></td>
<td>73/90(81%)</td>
<td>NA</td>
<td>66/66 (100%)</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>58 (64%)</td>
<td>Adenocarcinoma (64%)</td>
<td></td>
</tr>
<tr>
<td>Biphasic</td>
<td>29 (32%)</td>
<td>Squamous cell (33%)</td>
<td></td>
</tr>
<tr>
<td>Sarcomatoid</td>
<td>3 (4%)</td>
<td>Small cell (3%)</td>
<td></td>
</tr>
</tbody>
</table>

*Histology data available only on 120 of the 170 lung cancers*
### Serum SMRP

<table>
<thead>
<tr>
<th></th>
<th>MPM (n=90)</th>
<th>Lung Cancer (n=170)</th>
<th>Asbestos Exposed (n=66)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SMRP, nM</td>
<td>5.67±0.82</td>
<td>1.99±0.43</td>
<td>0.99±0.10</td>
</tr>
<tr>
<td>Range</td>
<td>(0-32 nM)</td>
<td>(0-32 nM)</td>
<td>(0-32 nM)</td>
</tr>
</tbody>
</table>

- $P<0.001$
- $P=0.173$
- $P<0.001$

### Pleural Effusion SMRP

<table>
<thead>
<tr>
<th></th>
<th>MPM (n=45)</th>
<th>Other Cancers (n=20)</th>
<th>Benign (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SMRP, nM</td>
<td>65.57±11.33</td>
<td>27.46±11.25</td>
<td>18.99±7.48</td>
</tr>
<tr>
<td>Range</td>
<td>(0-255 nM)</td>
<td>(0-140 nM)</td>
<td>(0-151 nM)</td>
</tr>
</tbody>
</table>

- $P=0.044$
- $P=0.210$
- $P<0.003$
Serum SMRP:
Age/Sex Matched Controls (n=50)

P<0.01
Serum SMRP
Mesothelioma Histology

![Graph showing Serum SMRP levels in different MPM histology types.](Image)
Serum SMRP and MPM Stage

Values Truncated at 32 nM

AsbestosExposed MPM Stage I MPM >Stage I

P=0.02 P=0.0001

P<0.0001
Serum SMRP Performance
MPM vs Normal (n=409)

AUC = 0.94
95% CI = 0.910 to 0.955

Sensitivity vs 100-Specificity

SMRP
Mesothelioma vs Normal Serum

SMRP (nM)

Normal Volunteers MPM

>1.1
Sens: 78.8
Spec: 95.8
AUC = 0.741
95% CI = 0.630 to 0.834

AUC = 0.805
95% CI = 0.734 to 0.864

AUC = 0.741
95% CI = 0.630 to 0.834

>2.0
Sens. 58.3%
Spec: 89.4%

>1.9
Sens: 60%
Spec: 89%
## Serum SMRP for MPM vs “Asbestos” Cohorts

### Summary

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Best Cut off</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robinson (2003)</td>
<td>84</td>
<td>83</td>
<td>NA</td>
</tr>
<tr>
<td>Scherpereel (2006)</td>
<td>80</td>
<td>83</td>
<td>0.93 nm</td>
</tr>
<tr>
<td>Present Study</td>
<td>60</td>
<td>89</td>
<td>1.9 nM</td>
</tr>
</tbody>
</table>
FDA and SMRP

• January 2007: limited indication reference laboratory for the “monitoring” of treatment of mesothelioma
SMRP and Treatment Monitoring

NO RECURRENCE

INTRATHORACIC RECURRENCE

INTRAABDOMINAL RECURRENCE
SMRP Conclusions

• SMRP is a reasonable single marker for mesothelioma

• The exact ranges for asbestos exposed cohorts must be studied in greater numbers of patients and in different geographies

  – *This should be done in the context of an EDRN validation trial as an initial step*
Genomic Discovery of Biomarkers

• Hypothesis
  – Affymetrix and Ingenuity Pathway Analyses can predict extracellular/secreted proteins which differ between normal mesothelium and early stage mesothelioma

• Specific Aims
  – Discover new markers in serum and plasma
  – Validate these markers using appropriate control cohorts
Methods for Discovery
Differences between Normal and Mesothelioma: All Genes

• Specimens
  – 8 normal peritoneum
  – 7 Stage 1 mesothelioma

• Platform
  – Affymetrix U133Plus

• Analysis
  – dCHIP crossed with SAM
    • 453 genes which were significantly different
U133 Plus Unsupervised Clustering: Peritoneum vs Stage I MPM: All Genes Significantly Different
Identification of secreted proteins

- 8 NP and 7 Stage I MPM were then compared for differences in 2036 genes which code for extracellular or secreted proteins (NetAffx™)
- 669 genes were different (p<0.01)
- These 669 genes were then inputted into Ingenuity Pathway analyses which selected 330 genes for the analysis.
- 35 focus genes were chosen for the networks
16 fold elevation
Osteopontin
Actual Expression for OPN in MPM
Osteopontin Levels and Environmental Cancers: Test Populations

- 48 normal sera
- 66 asbestos-exposed
- 72 mesothelioma sera

What happens to Osteopontin in Asbestos Exposed Individuals?
Published Data: Serum OPN and MPM

- Serum OPN rises with duration of exposure and severity of radiographic asbestos changes
- Promising distinction between asbestos exposed individuals and mesotheliomas

Osteopontin New Initiatives

- Is this reproducible in plasma?

- Can you distinguish MPM from lung cancer?
Why Plasma?

- Serum worked but could be erroneous.
- Follow-up series of investigations to
  - test plasma osteopontin as a biomarker (34)
  - Measure levels in asbestos exposed (45), lung cancer (60), and smokers with dysplasia (56)
Plasma Osteopontin Levels: Thoracic Malignancies And Controls

<table>
<thead>
<tr>
<th></th>
<th>Smokers (n=56)</th>
<th>Asbestos (n=45)</th>
<th>Lung Cancer (n=60)</th>
<th>Meso (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean OPN (ng/ml)</td>
<td>40±2 (10-84)</td>
<td>42±4 (8-148)</td>
<td>271±31 (45-1575)</td>
<td>520±63 (33-1645)</td>
</tr>
</tbody>
</table>

Plasma osteopontin looks as promising as serum osteopontin for MPM
**Mesothelioma vs Asbestos Exposed**

Plasma Osteopontin

![ROC Curve](image)

Area under ROC curve = 0.980
95% CI = 0.920 to 0.997

**Lung Cancer vs High Risk Smokers**

Plasma Osteopontin

![ROC Curve](image)

Area under ROC curve = 0.988
95% CI = 0.947 to 0.998

**Lung Cancer vs Mesothelioma**

Plasma Osteopontin

![ROC Curve](image)

Area under ROC curve = 0.783
95% CI = 0.686 to 0.861

---

**Groups**

- Plasma Osteopontin (ng/ml)
  - Group 1: >109.5
    - Sens: 97.1
    - Spec: 97.8
  - Group 2: >83.9
    - Sens: 91.7
    - Spec: 98.2

**LuMesoDiag**

- <=293.0
  - Sens: 73.3
  - Spec: 73.5
Osteopontin Conclusions

• Both serum and plasma osteopontin are elevated in MPM compared to high risk asbestos controls
• Plasma Osteopontin levels are also elevated in Lung Cancer and could be confused with MPM
  – Need other markers to distinguish between the two
• The exact ranges for asbestos exposed cohorts must be studied in greater numbers of patients and in different geographies
  – This should be done in the context of an EDRN validation trial as an initial step
What about other markers? **MMP1 and MMP9**

---

**Crocidolite asbestos and SV40 are cocarcinogens in human mesothelial cells and in causing mesothelioma in hamsters**

*Barbara Kreuziger, Angela Conti*, Maurizio Marzilli, Alan H. Yang, Amina G. Emren*, Pamela Washko*, Marta Kralik-Kolarov, Brooke F. Monia*, Harvey I. Ross, and Michele Calabio*††

††Thoracic Oncology Program, Carolinas Medical Center, Charlotte, NC. 20123 Departments of *Medical Genetics and Pathology, Chicago Medical School, University of Health Sciences, Chicago, Illinois, and Departments of Thoracic Surgery, New York University, New York, NY 10016.

Edited by Brian G. Druker, The Cancer Center, Philadelphia, PA, last reviewed July 21, 2006 (revised November 4, 2008)
Plasma OPN and MMP9 in mesotheliomas and asbestos exposed cohorts

Asb_OPN  Meso_OPN  Asb_MMP9  Meso_MMP9

Group

Plasma Values in ng/ml

38±4  450±51  287±38  51±10
MMP9 and OPN: MPM and Asbestos Exposed Cohorts Matched Plasma Specimens

- **MMP9**:
  - Sensitivity: 100.0%
  - Specificity: 95.5%
  - Sensitivity range: 1600 to 0
  - Specificity range: 1000 to 0

- **OPN**:
  - Sensitivity: 100.0%
  - Specificity: 95.5%
  - Sensitivity range: >86.9
  - Specificity range: <=64.5

Graphs showing ROC curves and scatter plots for MMP9 and OPN, with sensitivity and specificity values indicated.
Moreover, MMP9 is elevated in lung cancer

- MMP9 is NOT elevated in MPM
- MMP9 IS elevated in lung cancer

- Possible better discrimination between the two by combining with osteopontin?
What about other markers?
23 fold elevation
Expression of CRTL1/HAPLN1 expression data in mesothelioma patients
HAPLN1 differential expression in mesothelioma and normal pleura samples (RT-PCR)

143T 144T 219T 322T 342T 351T 367T 374T 143N 166T 172T 249T 291N 318T 336N

Loading control, PPIA
Expression of HAPLN in matched tissues (normal pleura/mesothelioma)
Mesothelioma, HAPLN1 antibodies (Genosis)
Preparations for validation of SMRP, osteopontin
Plans for EDRN Validation

• Every two week conference calls
  – Harvey Pass, BDL
  – Mark Thornquist, DMCC
  – Jackie Dahlgreen, DMCC
  – Karl Krueger, NCI

• Protocol Formulation
  – Definition of Ranges for Controls
  – ROC vs MPM
  – Retrospective/prospective studies
Validation Trial

• *Phase I*
  – Identification and assemblage of representative cohorts of individuals
    • with MPM
    • no malignancies but increased risk for MM due to asbestos exposure
    • (optionally) lung malignancies other than MM.
Mt. Sinai Selikoff Foundation

- Nationwide registry of 2900 insulators workers for which data is available up to 1994
  - Approximately 1600 are dead
  - Approximately 120 MPMs developed of which 3/5 were abdominal
Libby Montana

- Vermiculite mining in and near the city of Libby, Montana began in the 1920s and was continued by the W.R. Grace Company from 1963 until 1990. The vermiculite ore mined in Libby was contaminated with **tremolite asbestos**.
- **For the 20-year period** (1979–1998) **examined, mortality from asbestosis was approximately 40 times higher than the rest of Montana and 60 times higher than the rest of the United States.**
- Pleural abnormalities on chest radiography were seen in 17.8% of participants, 6,668 participants 18 years and older, and interstitial abnormalities were seen in less than 1% of participants undergoing chest radiography.
- The prevalence of radiographic pleural and interstitial abnormalities was highest in W.R. Grace workers: 51% (186 of 365).
- Of those participants who reported no apparent exposure, 6.7% had pleural abnormalities. Factors most strongly related to having pleural abnormalities were 1) having been a W.R. Grace/Zonolite worker, 2) having household contact with a W.R. Grace/Zonolite worker, and 3) being a male.
Libby, Montana

Vermiculite mines
PLCO (Prostate, Lung, Colon, Ovarian NCI Screening Program)

- CXR vs no CXR
  - Current, former, or never smokers
  - Minimal occupational demographics available
- 21 mesotheliomas were diagnosed
- CARET
  - multicenter randomized, doubleblinded, placebo-controlled trial examining vitamin A and β-carotene in preventing lung cancer
- Asbestos exposed cohort followed 9-17 years
- CXR, PFTs, sera at baseline

- 47 mesotheliomas developed
  - 38 asbestos arm
  - 9 smoking arm
  - 6 with serum before and after diagnosis
  - 11 with serum less than one year prior to diagnosis
Validation Trial

- **Phase 2**
  - determine what the characteristics of markers in the screening population, which will include mesothelioma cases and asbestos-exposed controls.
Validation Trial

- **Phase 2a**
  - the cut point between what the marker says is positive and negative will be established.
  - the distribution of SMRP and Osteopontin in controls will be reviewed for geographic differences and cohort differences (i.e. Libby vs Caret vs Selikoff vs New York Rom Cohort)
Validation Trial

• *Phase 2b,*
  - current cases will be examined to see what the sensitivity is to draw ROC curves
  - Important to obtain surgical cases in order to draw ROC curves for early (i.e. Stage I) cases
Validation Trial

• **Phase 2c**
  – “peri-mesothelioma” cases from the CARET and the PLCO trials will be examined for temporally related changes in the markers
### Cohort Mobilization

<table>
<thead>
<tr>
<th>Cohort</th>
<th>MPM Serum</th>
<th>MPM Plasma</th>
<th>Lung Cancer Sera</th>
<th>Lung Cancer Plasma</th>
<th>Asbestos Controls Sera</th>
<th>Asbestos Controls Plasma</th>
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<td>Pass Archives Pre NYU</td>
<td>98</td>
<td>20</td>
<td>Published</td>
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<td>100</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Rom CVEC</td>
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<td>0</td>
<td>160</td>
<td>160</td>
<td>300</td>
<td>300</td>
<td>2003-</td>
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<tr>
<td>Sinai Selikoff</td>
<td>56*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1769</td>
<td>0</td>
<td>1981-1982</td>
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<td>Libby, Montana</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>300</td>
<td>0</td>
<td>2005-</td>
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<tr>
<td>PLCO</td>
<td>21&amp;</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Serial draws, not all with sera at the time of dx</td>
</tr>
<tr>
<td>CARET</td>
<td>47&amp;</td>
<td>47</td>
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<td>0</td>
<td>3,897</td>
<td>3,897</td>
<td>Serial draws, not all with sera at the time of dx</td>
</tr>
<tr>
<td>Wittenoon, Australia</td>
<td>50</td>
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<td>0</td>
<td>0</td>
<td>200</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*sera not drawn at time of diagnosis

&“peri-mesothelioma bloods”
Prospective Validation

- Cappadochia
- New York Asbestos Screening Protocol
  - Philanthropy
  - Combined with Low Dose Helical CT
  - Defined exposure and age for enrollment
  - Combine with action taken on marker elevation at prevalence scan or rising marker at 6 month intervals
SMRP and Osteopontin

• Cappadocia
  – Very important PROSPECTIVE opportunity

• Collaboration with Michele Carbone MD, PhD, University of Hawaii
  – Mesothelioma Pathogenesis PO1
    • No funds for biomarker development
• In the Cappadocian region of Central Anatolia, three villages, Karain, Tuzköy, and Sarihidir, with environmental exposure to erionite are known as “Erionite villages”
Up to 52% of deaths in Karain between 1970 and 1994, and 38% of deaths in Tuzköy between 1980 and 1994 were due to malignant pleural or peritoneal mesothelioma. Peritoneal mesotheliomas were more prevalent in Tuzköy (1).

Besides mesothelioma the incidence of non-mesotheliomal malignancies were found high in erionite villages.

Cancer rates in these villages is about 1000 times more than the normal rate.

MPM in Cappadocia- Genetic studies: Genetic mapping study(1)

• Analysis of a six-generation extended pedigree of 526 individuals showed that predisposition to induced MM was genetically transmitted.

• It was suggested that vertical transmission of MM occurs probably in an autosomal dominant way.

• Studies are in progress to identify the gene(s), which increase(s) the susceptibility to erionite and asbestos.

Cappadocian Studies
March and June 2006

• *Blood cannot be removed from Turkey*
• Received permission to visit the villages and draw blood
• Laboratory space used at University of Ankara for ELISA reading
• Carbone took SMRP kits from FDI and osteopontin kits from IBL to Ankara
Individuals from erionite villages from Turkey (1-58) MM (59-80) control (81-87)

Serum Mesothelin Cappadochia Group 1: March 2006

Area under the ROC curve = 0.947
Standard error = 0.034
95% Confidence interval = 0.873 to 0.985
P (Area=0.5) < 0.0001

Exposed MM

Sens: 86.4 Spec: 87.9

>2.0

Sens: 86.4
Spec: 87.9
ROC Analysis
Cappadocia June 2006

Serum Mesothelin Cappadocia Group 2: June 2006

Area under the ROC curve = 1.000
Standard error = 0.000
95% Confidence interval = 0.954 to 1.000

Cappadocia SMRP
June 2006

Exposed              MPM

>2.9
Sens: 100.0
Spec: 98.6

Area under the ROC curve = 0.817
Standard error = 0.081
95% Confidence interval = 0.715 to 0.894

June Osteopontin Turkey

Cappadocia Osteopontin
June 2006

Exposed              MPM

>45.5
Sens: 63.6
Spec: 94.2
ELISA (06/13/2006)

mesothelin (nM) vs. osteopontin (ng/ml)

Individuals from Tuzkoy (12 years ago) (1-72)
MM patients (73-80)
Control (81-88)
ROC Analysis

Cappadocia Frozen Serum

Area under the ROC curve = 0.898
Standard error = 0.075
95% Confidence interval = 0.810 to 0.955

Area under the ROC curve = 0.977
Standard error = 0.037
95% Confidence interval = 0.916 to 0.997
Partnerships for Pursuing Marker for Screening Indications

• Fujirebio Diagnostics
  – Industrial Partner in EDRN U01
  – Would pursue licensing of patent for osteopontin in asbestos related disease screening
    • Pass/Wali patent application through Wayne State University