Disclosures

- Still don’t have any.
Learning Objectives

At the completion of this session the participants will have acquired an understanding of:

- Suitability of cytologic samples for molecular testing
- The variety of cytologic substrates for molecular testing
- How different cytologic sampling techniques differ in their potential as substrates for molecular testing
- The impact of pre-analytical processing on suitability for molecular testing
- Limitations of cell block production
Typical Statement About Cytology and Molecular

Finally, the ad hoc panel commented that there is often a limited amount of tissue for mutation testing and, therefore, endorses a relevant recommendation in ASCO’s stage IV NSCLC guideline update: “In order to obtain tissue for more accurate histologic classification or for investigational purposes, the Update Committee supports reasonable efforts to obtain more tissue than what is contained in a routine cytology specimen.” Properly fixed material from cytology cell block preparations is generally required for analysis, as opposed to cytology smear preparations. Development of more sensitive mutation analysis techniques may help in case of limited tumor material availability.

Typical Statement About Cytology and Molecular

Can Cytology Samples Be Used?

Cytology samples may be suitable for analysis but further research is needed to fully understand the clinical reliability of mutational data obtained from these samples. Until then, clinicians should be encouraged to provide tissue biopsy samples whenever possible.

Myths of Molecular Testing

- Cytologic samples are inadequate for molecular testing – why is this said?
  - Cytology results are untrustworthy
  - Cytology samples are too small
  - Cytology preparations adversely alter the sample
  - Cytology sampling not representative
    - Biased results due to lesion heterogeneity
Cytology and Quantity of Tissue
DNA Based Studies

**Conditions for detection of mutations:**

- **Quantity of DNA**
  - Minimum quantity dependent on technique used
    - 1 to 50 ng DNA (167 to 8,350 intact diploid nuclei)

- **DNA fragment length**
  - Most assays require 100 to 400 bp fragments

- **Frequency of mutant alleles**
  - Minimum frequency dependent on technique used
    - 1-5% for amplification refractory mutation system (ARMS) PCR / pyrosequencing
    - 20-30% for direct sequencing
Myth – FNA Collects Too Little Tissue

“Ideally, a sample should contain at least 200 to 400 tumour cells”

<table>
<thead>
<tr>
<th>Biopsy Techniques</th>
<th>21-g Needle Aspiration</th>
<th>19-g Needle Aspiration</th>
<th>Transbronchial Biopsy</th>
<th>CT-Guided Needle Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of cells</td>
<td>≥100</td>
<td>≥300</td>
<td>≥500</td>
<td>≥500</td>
</tr>
<tr>
<td>per biopsy/aspiration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of biopsies</td>
<td>4</td>
<td>4</td>
<td>4–5</td>
<td>2–3</td>
</tr>
</tbody>
</table>
Quantity of Cytology Samples
(Boerner – Generalizations)

- Resection specimens >>> cytology samples
  - Effusion specimens may be an exception
- Excisional biopsy >> cytology samples
  - Effusion specimens may be an exception
- Core biopsy samples – a toss-up
  - Depends on lesional characteristics
    - Some cores < cytology samples
    - Some cores > cytology samples
    - Some cores = cytology samples
Quantity of Cytology Samples
(Boerner – Generalizations)

- Depends on type of cytology sample
  - Brushings / Scrapings samples
    - Small quantity
  - Washings samples
    - Small to rarely moderate quantity
Quantity of Cytology Samples
(Boerner – Generalizations)

- **Effusion samples**
  - Exceedingly variable
    - Virtually none to massive quantity

- **FNA samples**
  - Moderate to large
    - *Exceedingly* aspirator dependent
Myth – FNA Collects Too Little Tissue

- Why the Boerner – literature contradiction?
  - Literature statements – mainly based on cell blocks
  - Cell block production is a *very ineffective process*
    - Best to assume you lose 50 to 85% of the sample
      - Cell suspension – 90+% loss
      - Tissue fragments – 50% loss, size dependent
How Many Cells Are Needed?

- Not well studied

- Literature

- Personal experience at UHN
  - EGFR mutations detected in cell blocks with 20-30 cells
Substrates For Molecular Testing

- Cell blocks
- Prepared slides
- Residual fixed sample
- Fresh sample
  - Really fresh
    - Hot off the press
  - Residual fresh
    - That left over stuff going moldy in your frig
- FTA Cards
DNA Recovery
(Fresh vs. CytoLyt vs. Formalin)

Sample 1

Sample 2

UHN in-house data
DNA Recovery
(Fresh vs. CytoLyt vs. Formalin vs. Time)

Sample 1

Sample 2

UHN in-house data
DNA From Slides
(Effects of Air-Drying vs Wet Fixation)

*Cancer Cytopathol.* 2013 Jul;121(7):344-53
DNA From Slides
(Effects of Fixative, Stain & Mounting Medium)

Cancer Cytopathol. 2013 Jul;121(7):344-53
DNA From Slides
(DNA Fragment Length & Cytoprep)

**TABLE 2.** Presence of Visible Bands of DNA Fragments in Extracts With Different Fixations (Median Value of 2 Batches With 3 Replicates Is Shown)\(^a\)

<table>
<thead>
<tr>
<th>Nominal Cell Count</th>
<th>Air</th>
<th></th>
<th></th>
<th></th>
<th>Spray</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>CytoRich Red</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>209 bp</td>
<td>388 bp</td>
<td>578 bp</td>
<td>760 bp</td>
<td>209 bp</td>
<td>388 bp</td>
<td>578 bp</td>
<td>760 bp</td>
<td>209 bp</td>
<td>388 bp</td>
<td>578 bp</td>
<td>760 bp</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10,000</td>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>.5</td>
<td></td>
</tr>
<tr>
<td>20,000</td>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
<td>.5</td>
<td></td>
</tr>
<tr>
<td>40,000</td>
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<td>-1</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>1.5</td>
<td>.5</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: bp, base pair.

\(^a\)Scoring: strong band = 2; distinct band = 1; indefinite = 0; no band = -1.

* Cancer Cytopathol. 2013 Jul;121(7):344-53
# Mutation Detection Equivalency

**Table 1**

EGFR and KRAS mutations in cytological samples obtained by needle washings and corresponding scraped cytological smears.

<table>
<thead>
<tr>
<th>No.</th>
<th>Site</th>
<th>Sample</th>
<th>Morphology</th>
<th>EGFR status</th>
<th>KRAS status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Needle washing</td>
<td>Cytologic smear</td>
</tr>
<tr>
<td>1</td>
<td>Lung</td>
<td>TTNA</td>
<td>SCC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>L858R</td>
<td>L858R</td>
</tr>
<tr>
<td>3</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>5</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>6</td>
<td>Lymph node</td>
<td>US-FNAB</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>7</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>8</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>L858R</td>
<td>L858R</td>
</tr>
<tr>
<td>9</td>
<td>Lymph node</td>
<td>US-FNAB</td>
<td>ADC</td>
<td>A767_V769dup</td>
<td>A767_V769dup</td>
</tr>
<tr>
<td>10</td>
<td>Lymph node</td>
<td>TBNA</td>
<td>ADC</td>
<td>E746_A750del</td>
<td>E746_A750del</td>
</tr>
<tr>
<td>11</td>
<td>Lymph node</td>
<td>TBNA</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>12</td>
<td>Lymph node</td>
<td>TBNA</td>
<td>ADC</td>
<td>E746_A750del</td>
<td>E746_A750del</td>
</tr>
<tr>
<td>13</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>L858R</td>
<td>L858R</td>
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<tr>
<td>14</td>
<td>Lymph node</td>
<td>TBNA</td>
<td>ADC</td>
<td>L858R</td>
<td>L858R</td>
</tr>
<tr>
<td>15</td>
<td>Lung</td>
<td>TTNA</td>
<td>ADC</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>


FTA Cards

Cancer Cytopathol. 2012 Jun 25;120(3):206-14
FTA Cards for EGFR

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cell Block</th>
<th>FTA Cards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGFR</td>
<td>KRAS, Exon 2</td>
</tr>
<tr>
<td>FNA-SS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>3</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>4</td>
<td>WT</td>
<td>PCR failed</td>
</tr>
<tr>
<td>5</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>6</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>7</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>8</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>9</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>10</td>
<td>DEL</td>
<td>PCR failed</td>
</tr>
<tr>
<td>11</td>
<td>WT</td>
<td>L858R</td>
</tr>
<tr>
<td>12</td>
<td>DEL</td>
<td>WT</td>
</tr>
<tr>
<td>13</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>14</td>
<td>WT</td>
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<td>15</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>16</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>17</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>18</td>
<td>DEL (2+)</td>
<td>WT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>EGFR</th>
<th>KRAS, Exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>WT</td>
<td>K12 (AGT)</td>
</tr>
<tr>
<td>H3255</td>
<td>WT</td>
<td>L858R</td>
</tr>
<tr>
<td>H358</td>
<td>WT</td>
<td>K12 (TGT)</td>
</tr>
<tr>
<td>HCC-827</td>
<td>DEL</td>
<td>WT</td>
</tr>
</tbody>
</table>

FNA indicates fine-needle aspiration; FNA-SS, FNA surgical specimens; WT, wild-type; NA, not available (not collected); PCR, polymerase chain reaction; DEL, deletion mutations in exon 19; L858R, a point mutation at codon 858 in exon 21.

* Cancer Cytopathol. 2010 Dec 25;118(6):450-6 *
Tumour Purity
Purity of Tumour

- Tumour:Normal (T:N) ratio
  - Depends on type of cytology sample
    - Brushings / Scrapings – low T:N ratio
    - Washings – low T:N ratio
    - Effusion – exceedingly variable T:N ratio
      - 0 to 1.0 (virtually all tumour)
    - FNA – high T:N ratio
      - FNA enriches for tumour and is depleted of stroma
        - Natural “micro-dissection”
Purity of Tumour

- How to improve tumour purity
  - Get an FNA
  - “Micro-dissection”
    - Prepared slides
    - Cell blocks
      - At UHN we do not routinely micro-dissect cell blocks
My Preferences for Molecular Testing

- Each case requires its own judgement
- General guidelines
  - Fresh sample first
    - Hot off the press
    - The old stuff in the frig
  - Residual alcohol fixed sample
  - Cell block material
  - Scrape a slide
    - The destruction of slide is the hold back
Fixative for Cell Blocks for DNA Studies

- 10% neutral buffered formalin
- Pre-alcohol fixed + 10% NBF
- Avoid exposure to fixatives containing
  - Picric acid (Bouin’s)
  - Mercury (B5, Zenker’s, Helly’s, Ridley’s)
  - Low pH fixatives
  - Phenols
  - Heavy metals (zinc)
UHN Cell Block Protocol
Does Molecular Testing Work on Cytology

The World is Changing
(But still have a way to go)

4.2: Expert Consensus Opinion.—Cytologic samples are also suitable for EGFR and ALK testing, with cell blocks being preferred over smear preparations.

Thank you

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University Health Network
Associate Professor, University of Toronto
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Which of the following statements regarding cytology samples and molecular testing is true?

1. FNA samples are of low cellularity and diluted by abundant normal elements.
2. Cells scraped from previously prepared cytology slides are suitable for DNA testing.
3. DNA recovery from fresh (unfixed) effusion samples is poor.
4. FFPE cell block is a better choice for DNA testing than residual sample in alcohol.
Which of the following chemicals has the least impact on DNA recovery for mutation testing?

1. Formalin
2. Ethanol
3. Picric acid
4. Xylene based mounting media
5. Fixatives containing heavy metals