Cytological preparations for molecular analysis.
Pre-analytical issues for EBUS TBNA specimens.

Gilda da Cunha Santos MD, PhD, FRCPC, FIAC
Associate Professor
University Health Network - Laboratory Medicine Program
Department of Laboratory Medicine and Pathobiology
University of Toronto, Ontario, Canada
EBUS – TBNA
Endobronchial Ultrasound guided TBNA

Balloon inflated with saline
Type of specimens/sampling method

EGFR mutation analysis

• Lowest insufficient rates (1)
  ✓ EBUS samples and Effusions
• Highest detection rates among cytology samples (2)
  ✓ Pleural effusions

ALK FISH

• Highest positivity for EBUS-TBNA (2011-2015: 945 cases)

Lymph node FNA algorithm – EBUS TBNA

Flow Cytometry

FTA Card

Microbiology
PCR: B and T-cell clonality

Needle Rinse

Unstained archival cytospins

ANCILLARY TECHNIQUES:
- H&E stain
- Papanicolaou stain
- Romanowsky stain
- Smears
- FISH
- MIB-1
- Ancillary techniques: EBER, IHC and Mutation analysis (carcinoma)
Cell Blocks

Advantages
• Most commonly used cytologic preparation for ancillary testing.
• Majority of ancillary studies validated on FFPE sections.
• FFPE cell blocks similar to surgical pathology FFPE blocks.
• Multiple serial sections obtained for different tests.
• Archival material.

Limitations
• Cellularity assessment difficult at the time of collection.
• Primary fixatives: Formalin, Alcohol, Residual LBC.
• Test failures with short DNA fragments (100–200 bp)(1)
• Formalin fixation sequencing artifacts.

It is recommended that 10% buffered formalin be used for tissue fixation for optimal molecular preservation, with avoidance of Bouin or any fixative containing heavy metal.”


“Properly fixed material from cytology cell block preparations is generally required for analysis, as opposed to cytology smear preparations.”


“Cytology samples are also suitable for EGFR and ALK testing, with cell blocks being preferred over smear preparations.

Type of fixative

- LBC methods: Cytolyt gave fivefold higher yield than CytoRich Red (poor results due to formaldehyde content).

- Needle rinse fixed in a formalin-free fixative (FineFix).


Cytological preparations

Cytology: multiple options for DNA extraction.
The challenges: validation of multiple protocols

Cell Block
Preparation Methods

• Simple sedimentation
• Normal saline needle rinse
  ✓ Prior to agar or plasma thrombin or Histogel
• Tissue coagulum clot
• Plasma thrombin/thrombin clot
• Agar
• Histogel
• Collodion bag
• Shandon cytoblock
• Rapid cell block
• Automated

Cell Block Methods

• Three CB techniques were compared:
  ✓ FNAB rinsed in saline and clotted with plasma and thrombin (SPT);
  ✓ FNAB rinsed in formalin and clotted with HistoGel (HG);
  ✓ FNAB rinsed in formalin, centrifuged, and the pellet captured in a collodion bag (ColB)
• ColB appears to be a superior technique for CB, yielding greater cellularity, preservation, and architecture in the majority of cases.

Cytological vs histological samples

- Cytological samples – unsatisfactory 7/147 (4.7%)
- Histological samples – unsatisfactory 44/443 (9.9%)
- No relationship – amount of DNA (DNA concentration) and outcome of genotyping (quality!)
- Unsatisfactory samples:
  - FNA smears 0/30
  - FNA cell blocks 2/43
  - Effusions (slides or smears) and Bbrushings 0/7
  - Effusions cell blocks 5/67

Cytology vs Histology
EGFR mutation analysis

Cytological samples – unsatisfactory 7/147 (4.7%)
Histological samples – unsatisfactory 44/443 (9.9%)


Canadian province-wide testing

• 1780 histology and 513 cytology specimens (2 years)
• Similar/comparable results. Test success rate similar.
• Higher detection rates in Cytology:
  ✓ Distant LN – Cytology vs Histology
    (36.0% versus 21.3%).
  ✓ Pleural fluid vs pleural core needle biopsy
    (31.1% versus 21.2%).
  ✓ Cytology/EBUS-TBNA vs histology/mediatinoscopy
    (34.4% versus 13.4%).

Cell block sections and ancillary tests

NSCLC

- H&E – 4μm
- IHC
  - Adeno vs SCC: CK7, CK5, TTF-1, p63
  - ALK IHC

Special stains:
- (sub-typing NSCLC)
  - PAS
  - Mucicarmin

FISH – 1 H&E + 3 sections (4μm)
- ALK rearrangements

EGFR Mutation Analysis
- 2 H&E pre and post “curls”
- 2 curls (20μm thick sections)

Standard of care – diagnostic work-up: total 24 sections (4μm)
IHC – Automated - Controls
Cell block sectioning

**EGFR and ALK testing at UHN**

Step 1. H&E for diagnosis 4um

Step 2. Slides for IHC
NSCLC sub-type

**EGFR mutation and ALK IHC/FISH testing**

Step 3. H&E pre-curls
4um

Step 2. Unstained slides for
ALK IHC and ALK FISH
4um

Step 4. Curls for EGFR
mutation analysis 2 (20um)

Step 5. H&E post-curls
4um

ALK IHC and ALK FISH

EGFR mutation analysis
Reporting/Cellularity

Cell block evaluation

• Overall cellularity of the sample:
  ✓ 0 – 100 cells  ✓ 750 – 1000 cells
  ✓ 100 – 250 cells  ✓ > 1000 cells
  ✓ 500 – 750 cells

• Tumour cellularity in sample:
  ✓ 0 – 50 cells  ✓ 100 – 300 cells
  ✓ 50 – 100 cells  ✓ > 300 cells

• Percentage of nucleated cells that are tumour:
• Percentage of necrosis:
Evaluation of tumor cellularity

“Evaluation of a postcurl slide is an unnecessary practice.”

Association of total number of nucleated cells and DNA yield (Kruskal-Wallis test)

<table>
<thead>
<tr>
<th>DNA yield</th>
<th>100-250</th>
<th>250-500</th>
<th>500-750</th>
<th>750-1000</th>
<th>&gt;1000</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>0.15</td>
<td>0.22</td>
<td>0.24</td>
<td>0.27</td>
<td>0.55</td>
<td>3e-05</td>
</tr>
<tr>
<td>(range)</td>
<td>(0.05-0.24)</td>
<td>(0-0.9)</td>
<td>(0.1-0.78)</td>
<td>(0.06-0.66)</td>
<td>(0.05-20.1)</td>
<td></td>
</tr>
</tbody>
</table>

Association of total number of tumor cells and DNA yield (Kruskal-Wallis test)

<table>
<thead>
<tr>
<th>DNA yield</th>
<th>1-50</th>
<th>50-100</th>
<th>100-300</th>
<th>&gt;300</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>0.24</td>
<td>0.18</td>
<td>0.24</td>
<td>0.6</td>
<td>1e-04</td>
</tr>
<tr>
<td>(range)</td>
<td>(0.05-0.78)</td>
<td>(0-5.88)</td>
<td>(0.05-0.9)</td>
<td>(0.06-20.1)</td>
<td></td>
</tr>
</tbody>
</table>

Most common ALK rearrangement: Paracentric inversion on the short arm of chromosome 2 juxtaposing the 5’ end of EML4 gene with the 3’ end of the ALK gene.
ALK Immunohistochemistry

- ALK IHC using the 5A4 antibody

  Accuracy of ALK detection on Papanicolaou-stained slides was high, with a sensitivity and specificity of almost 100% compared with ALK FISH.(a)

- Reporting results – cell blocks
  - Negative
  - Equivocal
  - Positive
  - Insufficient cells for adequate analysis.

# IASLC Atlas of ALK Testing in Lung Cancer

**Parameter** | **Recommendation**
--- | ---
Time to fixation | As short as possible, not exceeding 1 hr
Fixative | 10% neutral buffered formalin
Time of fixation | 6-48 hr
Preparation | Paraffin-embedded sections, cut at a thickness of 5 ±1 μm
Specimen storage | Tissue blocks (ideal)
Storage time for blocks | Not relevant if in proper conditions
Storage conditions for blocks | Protected from light, heat, and humidity
Storage time for cut sections | 4-6 weeks (ideal); older slides require customized protocol
Decalcification | EDTA, if necessary
• 42 y-old man. Progressive blurry vision for a year. Feeling well and run two marathons (the last 2 months before consultation). Non-smoker.
• Physical exam: Amelanotic lesion in the right choroid (?metastasis).
• CT-Thorax: Paratracheal enlarged LN. Multiple bilateral lung nodules.
• EBUS-TBNA for tissue diagnosis. Stage IV lung cancer.
- A deletion in exon 19 of the EGFR gene was detected in this sample.
- The lower limit of mutation detection using this assay is 1-5%.
- EBUS-TBNA for diagnosis.
**BRAF mutation analysis results**

- **BRAF Result:** Undetectable

- DNA was extracted from the paraffin-embedded lymph node FNA: EBUS ST 11 L, and amplified using primers specific for the normal and mutant alleles at coding nucleotides 1799T>A and 1798_1799GT>AA in exon 15 of the BRAF gene (Gene Bank NM_004333).

- The BRAF V600E/K mutation was not detected using ARMS analysis. Tumors without a BRAF V600E/K mutation will not respond to the kinase inhibitor vemurafenib.

- The lower limit of detection for this assay is 1-5%.
CB sections more successful ISH-EBER assays compared to cytospins.
Reasons for failure: loss of material on the slide and background staining.
High concordance rate with surgical specimens: usefulness of cytology for determining EBV status in patients with exhausted or no histological material available.

Patient with a previous history of tongue squamous carcinoma
New lung nodule and mediastinal LN.
CT-FNA and biopsy: SCC. Biospy: p16 faint/focal. Insufficient for HPV
Molecular analysis for HPV

• Tongue biopsy: HPV 16 positive.
• EBUS-TBNA sample: HPV 16 positive.

• HPV is not or rarely associated with NSCLC in Canadian and most likely North American patients, and P16 immunostaining is not a surrogate marker for its presence.


• hrHPV presence in a tumor with primary presentation in the lungs signifies pulmonary metastasis from a primary hrHPV-positive cancer elsewhere in the body.

Breast cancer – HER2 status

- Any specimen (cytologic specimens, needle biopsies, or resection specimens)
  - The fixation process is quick (time to fixative within 1 hour) and fixed in 10% neutral buffered formalin for 6 to 72 hours.

- ER, PR, and HER2 on FNA-CB (fixed exclusively in 10% formalin): excellent agreement for ER and HER2 and moderate agreement for PR with the corresponding tissue block.

- For both CB and TB, HER2 expression by IHC demonstrated ≥98% positive and negative concordance with FISH.

ASCO/CAP guidelines 2013 update

Opportunities and Perspectives

- Cytopathologists should provide detailed information in molecular reports about type of specimens, fixation and sample preparation (standardization still required).
- Cytopathologists are essential for alliquoting material for multiple studies for prognostic and predictive markers.
- Techniques for cell enrichment.
THANK YOU!