LUNG CYTOLOGY

- The importance of cytological techniques for investigation of respiratory conditions has been recognized since the earliest days of clinical cytology.
- The last few decades have shown a clear demonstration of the sensitivity and predictive value of cytodagnosis of lung tumors and an acceptance of all cytological modalities as a basis for management.
- Nowadays, in around 40% of lung cancer cases, the only material available for diagnosis and molecular testing is the cytological material.

Methods for Cytological Sampling of the Lung

- Sputum
  - Spontaneously expectorated
  - Induced
- Bronchoscopic procedures
  - Bronchial brushing
  - Bronchial washing
  - BAL
- Fine needle aspiration, including EBUS/EUS-FNA

Endobronchial Ultrasound (EBUS) guided FNA

THE PETHALS AND THORNS OF ROSE

Advantages

- Reduces the need for additional sampling (including CNB) with a lower risk of procedure complications.
- Cost-effective (fewer ancillary techniques).
- Decreases the number of passes needed for an adequate sample.
- Assists further diagnostic triage (assess whether extra-material is needed, decide how to preserve material for further ancillary studies).

<table>
<thead>
<tr>
<th>SAMPLING</th>
<th>LOCATION OF THE LESION</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPUTUM</td>
<td>70-85%</td>
</tr>
<tr>
<td>BRONCHIAL WASHING</td>
<td>70-90%</td>
</tr>
<tr>
<td>BRONCHIAL BRUSHING</td>
<td>77-90%</td>
</tr>
<tr>
<td>BAL</td>
<td>80-90%</td>
</tr>
<tr>
<td>FNA</td>
<td>80-95%</td>
</tr>
</tbody>
</table>

Gray and Kocjan, 2010; Bibbo M, 2008; Layfield et al, 1996; Rosenthal DI, 1988
**LUNG CYTOLOGY**
Morphological Aspects

- Normal Bronchial Cells
  - May be hypercellular
  - Often elongated
  - Round nuclei
  - Fine chromatin
  - Cilia

**LUNG CYTOLOGY**
Bronchial cell atypia

- Reactive
- Syncytia
- Ciliocytophthoria
- Creola bodies: hyperplastic reactive epithelium

**LUNG CYTOLOGY**
Bronchial cell atypia

- Vegetable cells
- Basal cell hyperplasia of Bronchial epithelium
- Hyperplasia of type II pneumocytes

**LUNG CYTOLOGY**
Inflammatory diseases

- Tuberculosis
- Aspergillus
LUNG CYTOLOGY
Benign tumours

Morphological Aspects

Acute irradiation effect

LUNG CYTOLOGY
Morphological Aspects

ADENOCARCINOMA

SQC

SCC

LUNG CYTOLOGY and TUMOR Typing

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>PPV, %</th>
<th>NPV, %</th>
<th>Accuracy, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC vs. non-AEC</td>
<td>100 (97.5-100)</td>
<td>100 (97.5-100)</td>
<td>100 (97.5-100)</td>
<td>100 (97.5-100)</td>
<td>100 (97.5-100)</td>
</tr>
<tr>
<td>Squamous vs. bronchiolocarcinoma</td>
<td>87 (64-97.2)</td>
<td>98 (94.6-99.6)</td>
<td>97 (64-97.2)</td>
<td>87 (64-97.2)</td>
<td>98 (94.6-99.6)</td>
</tr>
<tr>
<td>Adenocarcinoma vs. Squamous cell carcinoma</td>
<td>94 (81.5-98.6)</td>
<td>96 (86.2-96.4)</td>
<td>90 (77.5-98.3)</td>
<td>96 (86.2-96.4)</td>
<td>94 (81.5-98.6)</td>
</tr>
</tbody>
</table>

➢ Cytoplogy is a powerful tool in the diagnosis of lung cancer, in particular in the distinction of adenocarcinoma from squamous cell carcinoma.

J Thorac Oncol. 2011;6: 451-458
The 2015 WHO Classification on Lung Tumors

What is new?

• A new classification for small biopsies/Cytology samples
• Wide use of IHC for classification
• Emphasis on genetics for predictive biomarkers in advanced lung cancer
• The issue of LCC, SqCC and NET
• A different approach to lung adenocarcinoma (AIS, MIA...)
• Reclassifying other tumors (NUT, hamartoma, myxoid sarcoma...)

Pathology recommendations applicable to small biopsy and cytology specimens

• For small biopsies and cytology, we recommend that NSCLC be further classified into a more specific type, such as adenocarcinoma or SqCC, whenever possible.

• We recommend that the term NSCL-NOS be used as little as possible and it be applied only when a more specific diagnosis is not possible by morphology and/or special stains.

• When a diagnosis is made in a small biopsy or cytology in conjunction with special studies, it should be clarified whether the diagnosis was established based on light microscopy alone or if special stains were required.
Metastases in lung may be single, large, multiple or lymphangitic.

In most instances a previous diagnosis of malignancy is available, and confirmation of the clinically suspected lesion is all that is required from the cytopathologist.

However, some pulmonary metastases present before the primary tumour has been identified and may require careful investigation, if inappropriate diagnosis and treatment of presumed lung primary carcinoma are to be avoided.

Adenocarcinoma, squamous cell carcinoma and small cell carcinoma are histological types that can be difficult to distinguish between primary or secondary tumours.

In recent years, cytology have been increasingly used for establishing the diagnosis of lung cancer and classifying the specific tumour type.

Accurate distinction between SCLC and NSCLC, as well as further distinction between SQCC and Adenocarcinoma has crucial therapeutic significance.

However, sometimes morphologic distinction can be difficult and ancillary techniques can help the cytopathologist in this task.
**LUNG CYTOLOGY and TUMOR TYPING**

**Expanded use of immunocytochemistry**

- In 1999 and 2004 WHO classification IHC was limited to LCNEC, sarcomatoid carcinoma and dd with mesothelioma.

- In 2015 WHO classification, IHC is recommended:
  - In biopsy/cytology samples
  - Resected specimens {solid adenocarcinoma, LCC, nonkeratinizing SqCC, sarcomatoid carcinoma, NET, NUT...}
  - More defined subtyping (for therapy)

- Minimalist antibody panel approach (the more the stains, the more difficult the diagnoses).

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**Ancillary Studies, Including Immunohistochemistry and Molecular Studies, in Lung Cytology**

**Parwise comparison of p40 and TTF1 IHC between biopsy/cellblock (BCS) samples and paired surgical specimens (SS) resulted in close correlation coefficients (by far > 0.9), revealing that BCS were as reliable as SS for rendering the definitive diagnoses.**

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**Table 5. Frequency and Accuracy of Morphologic vs. Immunohistochemistry-aided Diagnosis of Adenocarcinoma and Squamous Cell Carcinoma in Cytology**

<table>
<thead>
<tr>
<th>Method</th>
<th>Frequency</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis based on morphology</td>
<td>196 (20%)</td>
<td>95%</td>
</tr>
<tr>
<td>Diagnosis based on IHC</td>
<td>14 (9%)</td>
<td>100%</td>
</tr>
<tr>
<td>Diagnosis that needs IHC but cellularity (inadequate: NSCLC-NOS)</td>
<td>5 (7%)</td>
<td>nA</td>
</tr>
</tbody>
</table>

*J Thorac Oncol. 2011; 6: 451-458*
LUNG CYTOLOGY
Morphological Aspects

- NSCLC-NOS
  - Hypercellular
  - Large groups
  - Large cells
  - Macronucleoli, sometimes multiple
  - Cytoplasm outline frequently ill-defined

Ancillary Studies, Including Immunohistochemistry and Molecular Studies, in Lung Cytology

Role of Ancillary Studies in Fine-Needle Aspiration From Selected Tumors

Pathology Considerations for Good Practice

- Small biopsy and cytology samples should be managed not only for diagnosis but also to maximize the amount of tissue available for molecular studies.
ALGORITHM FOR ANCILLARY TESTING IN NSCLC

MUTATION STATUS TESTING WORKFLOW

EGFR Testing

P40 +

NSCLC = NOS
No Clear ADC or SQCC

TTF1 -

P40 -

TTF1 +

No ADC or SQCC

Positive

Tki inhibitors

Negative

ALK

ROS

RET

[NSP]?

ALGORITHM FOR ANCILLARY TESTING IN NSCLC

Second ESMO consensus conference on lung cancer: pathology and molecular biomarkers for non-small-cell lung cancer

H. M. K. Rol, L. B. Blom, M. J. E. Eelman, A. Marchetti, T. Mani, S. Novello, K. O. Berner, R. Strobl, S. Peters, E. Klop & Pain Members,

Recommendation 1: Guidance on tissue handling

- Sample processing
- Standard fixation using formalin and formaldehyde (4% formaldehyde) is recommended [IV, A]
- Fixation time should be no less than 2 h, and no greater than 24 h [IV, A]
- Sections for biomarker testing should ideally be cut immediately before analysis [IV, A]
- Cytology samples (cell blocks, smears direct smears or liquid-based preparations) can be used reliably to detect EGFR mutations and ALK rearrangements [III, A]. At this time, a cell block is the most widely applicable cell source
- The same pathologist should, if possible, review all available tumour material from the same patient including biopsies and cytology specimens to select the most suitable for biomarker analysis [IV, A]
- A pathologist should be involved in sample preparation for DNA extraction [IV, A]
- Enrichment of samples by microarray or microdissection to maximize tumour cell content before DNA extraction is recommended [IV, A]
### Mutation frequency and morphological control

<table>
<thead>
<tr>
<th></th>
<th>Mut</th>
<th>Wild-type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without control</td>
<td>463 (38%)</td>
<td>763 (62%)</td>
<td>1226</td>
</tr>
<tr>
<td>With control</td>
<td>309 (43%)</td>
<td>408 (57%)</td>
<td>717</td>
</tr>
</tbody>
</table>

P<0.05

### Mutation frequency and tumour cell content

<table>
<thead>
<tr>
<th></th>
<th>Mut</th>
<th>Wild-type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20% tumour cells</td>
<td>7</td>
<td>20 (74%)</td>
<td>27 (4%)</td>
</tr>
<tr>
<td>&gt;20% tumour cells</td>
<td>296 (44%)</td>
<td>378 (56%)</td>
<td>674 (96%)</td>
</tr>
</tbody>
</table>

P<0.05

### % of tumour cells and mutation detection sensitivity

- Normal – G, black
- Mutation – A, green

Method sensitivity set at 20% of tumoral cells

### MUTATION STATUS TESTING WORKFLOW

1. **Slide Selection and Assessment**
   - For cases with a high tumor content (>20%) the marking of areas of tumors is unnecessary. (1 cell = 6 pg DNA)
   - Enrichment by macrodissection if necessary.

2. **Removing the Coverslip**
   - 48-72hs in xylene or substitute

3. **Collecting the Tissue**

4. **Tissue lysis and DNA Extraction**

Simple protocol for DNA extraction from archival stained FNA smears

Simple protocol for DNA extraction from archival stained FNA smears

1. Slide Selection and Assessment
   - For cases with a high tumor content (>20%) the marking of areas of tumors is unnecessary. (1 cell = 6 pg DNA)

2. Removing the Coverslip
   - 48-72hs in xylene or substitute

Enrichment by macrodissection if necessary.
Minimizing Delays in DNA Retrieval: The “Freezer Method” for Glass Coverslip Removal.

- The slide is placed flat in the freezer (at a temperature of -20ºC) for 1-3 minutes.

- After removing the slide from the freezer and wearing eye protection, immediately place the tip of a scalpel blade under one corner edge of the coverslip, lift up the coverslip and remove it.

- After removing the coverslip allow the now uncoverslipped slide to return to room temperature before soaking in xylene for 1 to 2 minutes until the remaining mounting media has been removed.

- The slide can be then sent for microdissection for collecting cells for DNA extraction.

Methods for mutation screening

- Allele-specific PCR
- HRM
- ARMS PCR
- Others...

- Fragment analysis
- SSCP
- Sanger Sequencing
- Others...

More demanding in DNA quantity and quality
More sensitive

Less demanding in DNA quantity and quality
Less sensitive
**ALK (Anaplastic Lymphoma Kinase)**

- ALK translocations → up-regulation of ALK protein
  - Anaplastic large cell lymphoma
  - Inflammatory myofibroblastic tumor
  - Non-small cell lung carcinoma (NSCLC)
    - genetic translocation of ALK to echinoderm microtubule-associated protein-like 4 (EML4)
    - chimeric protein (EML4-ALK):
      - constitutive tyrosine kinase activity

**ALK gene translocation in NSCLC**

- 1-7% of NSCLC patients:
  - younger age and never or light smoking
  - high-stage disease
- Adenocarcinomas (ADCs)
- ALK+: no EGFR mutations
- Crizotinib: inhibitor of tyrosine kinase activity
  - potential treatment efficacy

**Detection of ALK rearrangements**

- Fluorescence in situ hybridization (FISH)
  - ALK dual colour break-apart probe (Abbott Molecular)
  - detect rearrangements in chromosome 2p23
- Immunohistochemistry (IHC)
  - detection of the chimeric ALK protein
  - clones: D5F3 (Cell Signaling); 5A4 (Novocastra)
  - rapid and relatively inexpensive
  - bright field microscopy: tumor morphology
Practical aspects of FISH testing for EML4-ALK

- At least 50 tumour cells should be counted for accurate results.
- FISH is considered positive when at least one set of orange and green signals is > 2 signal diameters apart or there is a single orange signal without a corresponding green signal in addition to fused (normal) signals.
- A sample is considered negative for ALK rearrangement if there are <5 positive cells (<10%) and positive if there are >25 positive cells (>50%).
- A sample is considered equivocal if 10-50% of cells are positive. In this case a 2nd reader should evaluate the slide and if the average of 2 readings contains at least 15% of positive cells the case is considered positive.

Savic S and Bubendorf L, Acta Cytol 2012; 56: 611-621

Cases (n = 523)
- FISH positive (n = 20)
- FISH negative (n = 503)

IHC positive (n = 18)
- 18
- 0

IHC negative (n = 505)
- 2
- 503

Arch Pathol Lab Med. 2014; 138:1449-1458

Detection of ALK-positive NSCLC using ICC

Detection of ALK-Positive Non–Small-Cell Lung Cancers on Cytological Specimens

High Accuracy of Immunocytochemistry with the 5A4 Clone

Sparenzi S, MD,* Bronte Rude, MD,* Joachim Dachold, MD,‡ Iva Trivoni, MD,‡ Audrey Bensaccol,* Betty Bussière,* Bruno Grisli,* Michelle Herzog,* Ellen Oehmann, MD,* and Lukas Bubendorf, MD*

Comparative ALK IHC and FISH testing

Diagnostic algorithm for ALK testing
What should be tested?

A major clinical challenge is prospectively determining the status of multiple clinically relevant genes in tumor DNA before starting therapy.

Multi-test single assay!
Conclusions

- Small samples obtained by minimally invasive methods exploited morphological and molecular information are the model biopsies of the future.

- Various techniques using different types of cytological samples and preparations have shown promising results, with similar or higher accuracy and sensitivity when compared with surgical specimens.

- Presently, with the rapid development of personalized treatment every lung carcinoma should be tested for available biomarkers and every effort should be made to spare the tissue for molecular testing.