Panel Luncheon 3

Practical Guidelines for Integrating Emerging Molecular Methods into Your Cytology Practice

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There are no disclosures necessary.
Practical Guidelines for Integrating Emerging Molecular Methods Into Your Cytology Practice
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Conflict of Interest

- No conflict of interest to report for Brian Collins, M.D.
- Thank Dr. Kevin Halling, Mayo Clinic, for his pioneering work in this area and his assistance in providing data and images included in this presentation.
FISH

- FISH = Fluorescent In-Situ Hybridization
- FISH is a type of cytology
- FISH is just a different way of examining cells
- Instead of using a PAP stain, one uses a FISH “stain”
- Instead of examining cells for morphologic features typical of neoplasia (by Pap stain), examine cells for chromosomal abnormalities indicative of neoplasia.
- Most tumors have chromosomal abnormalities

FISH

- General Principles
  - Probes that hybridize specific chromosomal loci
  - Use to enumerate abnormalities at the cytogenetic level
  - DAPI (counterstain) binds strongly to DNA
    - Important for the identification of abnormal nuclear morphologic features
FISH

- Preparation and Evaluation
  - Five main steps
    - Specimen collection
    - Slide preparation and pre-hybridization
    - Denaturation and hybridization to target DNA
    - Removal of non-specific bound probe
    - Assessment using fluorescence microscopy
FISH

Specimen Collection
- Wide range of specimens including cytology, peripheral blood and FFPE tissue
- For cytology, a wide range of specimen types can be used
- Urine Cytology
  - Voided urine (FDA approved for use with UroVysion™)
  - Catheterized urine, bladder wash, stoma specimens
  - Upper tract washings-includes ureters and renal pelvis
  - Fresh or fixed-ethanol, methanol, PreservCyt® or CytoLyt®

Slide Preparation and Pre-Hybridization
- Can use a variety of techniques
  - Cell suspension sediments, ThinPrep™, cytospins
- Cellularity is key
  - Highest cellularity with minimal overlap
- Monolayer preparation optimal
- Pre-hybridization
  - Important to permit FISH probe to enter cell and hybridize to the nuclear DNA target
  - Retain the cellular morphology
  - Pepsin: It is an enzyme used for DNA access
    - Problems with over and under digestion using Pepsin
**FISH**

- Denaturation and hybridization
  - Denaturation - makes the site available
    - Usually takes place at around 73°C for 3 minutes
  - Hybridization - with specific probes
    - Temperature lowered to around 37°C
    - Allows the FISH probe DNA to hybridize to its specific target
    - Usually takes 4-12 hours to complete
    - Variety of factors influence the efficiency and specificity
FISH

- Removal of non-specific bound probe
  - Slides washed to remove excess probe
    - Removes probe not specifically bound to the target
    - Inadequate wash can result in background signals
  - DAPI
    - Stains the nuclei
    - Fluoresces a blue-grey hue
    - Allows the examiner to visualize intact nuclei and recognize morphologic features
    - Think of it as a counterstain

FISH

- Probe Cocktails
  - Designed to hybridize to specific target sequences of interest
  - Chromosome enumeration probes (CEP)
    - Hybridize to repetitive DNA sequences found near the centromeres of chromosomes
    - Used to enumerate the number of copies of a given chromosome in a cell
    - Since it hybridizes to sequences with high copy numbers, provide bright signal
    - Regions tightly compacted, signals are tight rather than diffuse
FISH

- Probe Cocktails
  - Locus Specific Indicator (LSI)
    - Hybridize to unique sequences (non-repetitive DNA sequences)
    - Generally used to determine if specific genes are...
      - amplified (HER2)
      - deleted (p53 or p16)
      - translocated (BCR/ABL)
    - Hybridize to regions from 40 to 500 kb
      - Less than 40kb often produce weak signals
      - Greater than 500kb can produce diffuse signals

FISH

- Assessment using fluorescence microscopy
  - Need a fluorescence microscope
    - Goal is to cause the FISH labeled DNA probe to fluoresce and highlight the target
    - Fluorophore is a molecule that absorbs light in certain range and then re-emits as light of longer wavelength.
    - Arc lamps emit light and filters select appropriate wavelength of light
    - Typically red, green, aqua, and gold
FISH

- FISH Case Analysis
  - DAPI stain
    - Nuclear features are used to determine which cells are best examined for chromosomal abnormalities
    - DAPI features of dysplastic/neoplastic nuclei:
      - Nuclear enlargement
      - Nuclear membrane abnormalities
      - Weak or mottled staining nuclei
    - Scanning and recognition of cells are skills of cytologists
    - Screening patterns are used in FISH analysis
    - Abnormal cells are assessed for probe targets to determine if there is a specific abnormality

DAPI

- Weak
- Moderate
- Bright
Urothelial Carcinoma Diagnosis

- 2006: 50,000 new cases; 12,000 deaths; high recurrence rate
- Cystoscopy (sensitive for low grade papillary tumors; may miss CIS)
- Cytology (sensitive to high grade; may miss low grade)
- Increasingly, molecular markers, including UroVysion FISH, used to aid diagnosis and monitor recurrence
- CAP proficiency: +126 Labs

Biomarkers in bladder cancer. Histopathology 2010 Jul 57(1) 1-13
**FISH**

- Urothelial Carcinoma
- Laboratory Detection
  - Cytology
  - Antigen based methods
e.g., BTA-stat, NMP22, Immunocyt
  - Molecular genetic methods
e.g., Telomerase, microsatellite analysis
  - DNA ploidy analysis (flow cytometry/DIA)
- Cytophene genetic methods
  - Karyotype
  - Fluorescence In Situ Hybridization (FISH)

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**Table 1** Comparison between cytology and commercially available biomarker tests for use in the diagnosis and surveillance of bladder cancer. The broad range of percentages provided for sensitivity and specificity reflect differences in study sizes, assay Content, and the grade and stage of lesions studied.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Cytology</td>
<td>70-100</td>
<td>12-88</td>
<td>High specificity</td>
<td>Increased interobserver variation</td>
</tr>
<tr>
<td>BTA-Stat</td>
<td>52-93</td>
<td>24-86</td>
<td>Rapid point-of-care test</td>
<td>Low positivity (esp. for low-grade lesions)</td>
</tr>
<tr>
<td>BTA-Stat&lt;sup&gt;+&lt;/sup&gt;</td>
<td>48-85</td>
<td>61-79</td>
<td>Sensitivity comparable to in microscopy (esp. for low-grade lesions)</td>
<td>High false positive rate</td>
</tr>
<tr>
<td>NMP22</td>
<td>40-90</td>
<td>50-85</td>
<td>High specificity and sensitivity</td>
<td>High false positive rate</td>
</tr>
<tr>
<td>NMP22&lt;sup&gt;+&lt;/sup&gt;</td>
<td>46-85</td>
<td>50-81</td>
<td>Rapid point-of-care test</td>
<td>High false positive rate</td>
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<tr>
<td>ImmunoCyt</td>
<td>62-79</td>
<td>60-80</td>
<td>High sensitivity and sensitivity</td>
<td>High false positive rate</td>
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<tr>
<td>Urovysion</td>
<td>60-90</td>
<td>60-90</td>
<td>High sensitivity and specificity</td>
<td>High false positive rate</td>
</tr>
</tbody>
</table>

*Data obtained from Refs 13 and 24.*

**Biomarkers in bladder cancer.** Histopathology 2010 Jul 57(1) 1-13
Types of FISH Probes

- Chromosome enumeration probes (CEP)
- Locus specific indicator probes (LSI)

Why Might FISH be Useful for Detecting Urothelial Carcinoma?

- Urothelial carcinomas are generally aneuploid
- Urothelial carcinoma cells readily exfoliate into urine
- FISH can detect these chromosomally abnormal cells
What Do the Chromosomes of a Tumor Cell Often Look Like?

Aneuploid with extra copies of many of the chromosomes

UroVysion™

- Uses 4 FISH probes
- The use of a multi-probe cocktail increases both the sensitivity and specificity of the assay over single or dual probes
UroVysion™

Normal Cell Malignant Cell

CEP 3
SpectrumRed
Chromosome 3

CEP 7
SpectrumGreen
Chromosome 7

LSI 9p21 SpectrumGold
Chromosome 9

CEP 17
SpectrumAqua
Chromosome 17

(p16 gene)
UroVysion™

- FDA approved for:
  - Detection of recurrent tumor in patients with a history of bladder cancer (2001)
  - Evaluation of patients with hematuria for bladder cancer (2005)

Microscopic Analysis

- Normal appearing cells (do not score)
- Abnormal appearing cells - FISH normal
- Abnormal appearing cells - FISH abnormal

What Types of Cells Do You See in the Urine?

- Urothelial cells
- Squamous cells
- Inflammatory cells (mostly neutrophils)

DAPI Scanning (Cells to Ignore)

Neutrophils

Squamous Cells

Scanning for Atypical Cells

Neoplastic/Dysplastic cells generally have the following features when visualized with DAPI stain:

- Nuclear enlargement
- Nuclear irregularity
- Mottled staining (exceptions)


Scanning for Atypical Cells

Neoplastic/Dysplastic cells visualized with DAPI stain

Scoring form based on abnormal DAPI appearance

<table>
<thead>
<tr>
<th>Cell Number</th>
<th>CEP3</th>
<th>CEP7</th>
<th>CEP17</th>
<th>LSI 9p21</th>
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<tr>
<td>1</td>
<td>4</td>
<td>5</td>
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<td>10</td>
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<tr>
<td>11</td>
<td>2</td>
<td>2</td>
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<tr>
<td>etc.</td>
<td>1</td>
<td>2</td>
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<td>2</td>
</tr>
</tbody>
</table>

ROC Curve for Polysomy
FISH is More Sensitive than Conventional Urine Cytology

Use of multitarget FISH assay to diagnose bladder cancer patients with hematuria J Urol 2006 Jul 176(1) 44-7

Types of Chromosomal Abnormalities Seen with UroVysion™ in UC Patients

Polysomy

Tetrasomy
Types of Chromosomal Abnormalities Seen with UroVysion™ in UC Patients

Frequency of Different Types of Alterations

- Polysomy/tetrasomy
- Homozygous 9p21 deletion
- Trisomy

- ~95% of cases
- 1-5% of cases
- <1% of cases
### Polysomy

- **Definition:** gains of 2 or more of the 4 probes
- **Example**

<table>
<thead>
<tr>
<th>CEP1</th>
<th>CEP7</th>
<th>CEP17</th>
<th>9p21</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>5</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Polysomy

- Polysomic cells are rarely if ever seen in normal value studies
- The finding of polysomic cells in urine (even in small numbers) is virtually diagnostic for neoplasia
- It is the absolute number of cells (not percent) that shows the abnormality which is used to determine positivity
- 4 of 50 cells and 4 of 500 cells showing polysomy are both considered positive.
Tetrasomy

- Tetrasomic cells (signal pattern of 4,4,4,4)
- Fulfill criteria for polysomy (gains 2 or more probes)
- May occasionally be non-neoplastic cells (umbrella)
- Tumors may be tetraploid or near tetraploid
- Voided urine: 10 or more cells
- Upper tract specimens: most have some tetraploidy
  - 4 or more polysomic cells with 5 or more signals in at least one of the chromosomes (hypertetrasomic)

9p21 Deletion

- Definition: loss of both 9p21 (gold) signals
  - Homozygous loss
- 9p21(p16 gene) is commonly deleted in low grade papillary tumors
- At least 12 or more of 25 morphologically abnormal cells need to show homozygous 9p21 deletion to be called positive
- **Gold** signal is weakest of 4 probes
- Do not overinterpret 9p21 loss
Trisomy

- Definition: 3 copies of one chromosome and 2 or fewer copies of other chromosomes
- Trisomic pattern needs to be consistent

<table>
<thead>
<tr>
<th>CEP3</th>
<th>CEP7</th>
<th>9p21</th>
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<tbody>
<tr>
<td>2</td>
<td>3</td>
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<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

- Do not interpret split signals as trisomy
  - Signals should be well separated
- Cut off values for trisomy?
- Not well established
  - 10 or more cells
Trisomy
split signal problem

FISHING TO DETECT URINARY CANCERS:

DO IMAGING SYSTEMS HELP?
Why do we need image processing/analysis for UroVysion FISH?

- Time – manual interpretation up to 30 min/case.
- Shorter scan and turnaround times
- Patient care – Help reduce false negatives and false positives
- Obtain quality images for CAP archiving requirements
- Record locations of cells for re-examination
- New tool for advancement of cytology and expanded role for trained cytotechnologists

Processed Image:
- Images in different focal planes have been captured and merged.
- Signal to noise ratio optimized

BioView Duet Applications

- Automated FISH (Fluorescence)
- HC or Stain (Brightfield)
- Target FISH (IHC/stain followed by FISH)

- Cancer
  - Hematology
  - Bladder Cancer
    - Automated UroVysion FISH
    - Target FISH
  - Breast Cancer
  - Other
  - Cytogenetics
  - Sperm (IVF)
  - Rare Cells Detection
Software Analyzes and Classifies Cells Based on Nuclear Features and Signal Counts

Scans until preset # has been classified (often 250)

- Classified:
  - **Normal** (100 required to call case negative).
  - **Abnormal** (cells with 2 or more of probes in excess of 2 signals). 4 or more = positive.
  - Single Gain
  - **Zero Gold** (12 or more = positive)
  - Blood
  - Suspicious blood
  - Squamous
  - Clusters
- Unclassified

Reclassifying
Before and After Reclassification

Targeted FISH  
(aka Location Guided FISH)

Pap stain & evaluate slide by bright field using BioView to Record Cell Locations

Remove Coverslip and Destain

UroVysion FISH

Use BioView to Examine Same Cells by Fluorescence
Targeted FISH (aka Location Guided FISH)

Manual versus Image Aided

<table>
<thead>
<tr>
<th>Types of Specimens</th>
<th>Negative</th>
<th>Unsatisfactory</th>
<th>Positive</th>
<th>Equivocal</th>
<th>Total cases</th>
<th>Male</th>
<th>Female</th>
<th>Age range</th>
<th>Mean age</th>
</tr>
</thead>
<tbody>
<tr>
<td>All specimen types</td>
<td>1171</td>
<td>78</td>
<td>251</td>
<td>1</td>
<td>1909</td>
<td>1491</td>
<td>418</td>
<td>23-95</td>
<td>64</td>
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<tr>
<td>Percentages for all specimen types</td>
<td>77.50%</td>
<td>5.10%</td>
<td>11.16%</td>
<td>0.06%</td>
<td>100.00%</td>
<td>72.29%</td>
<td>27.70%</td>
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<tr>
<td>Random voided</td>
<td>1015</td>
<td>67</td>
<td>171</td>
<td>1</td>
<td>1255</td>
<td>514</td>
<td>341</td>
<td>16-60</td>
<td>63</td>
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<tr>
<td>Cathetered</td>
<td>22</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>34</td>
<td>19</td>
<td>15</td>
<td>47.85</td>
<td>77</td>
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<tr>
<td>Cystoscopyd</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>43.87</td>
<td>63</td>
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<tr>
<td>All Urine Specimens</td>
<td>1048</td>
<td>70</td>
<td>184</td>
<td>1</td>
<td>1335</td>
<td>444</td>
<td>361</td>
<td>16.15</td>
<td>71</td>
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<tr>
<td>Percentages amongst all urine specimens</td>
<td>80.30%</td>
<td>6.30%</td>
<td>14.28%</td>
<td>0.07%</td>
<td>100%</td>
<td>12.33%</td>
<td>27.69%</td>
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<tr>
<td>Bladder Washings</td>
<td>56</td>
<td>4</td>
<td>36</td>
<td>0</td>
<td>110</td>
<td>76</td>
<td>24</td>
<td>60.51</td>
<td>67</td>
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<tr>
<td>Percentages amongst bladder washings</td>
<td>60.00%</td>
<td>4.00%</td>
<td>36.00%</td>
<td>0%</td>
<td>100%</td>
<td>76.00%</td>
<td>24.00%</td>
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<td>Urine and Pelvic Washings</td>
<td>63</td>
<td>4</td>
<td>37</td>
<td>0</td>
<td>114</td>
<td>71</td>
<td>33</td>
<td>33-86</td>
<td>65</td>
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<tr>
<td>Percentages amongst urethroc and pelvic washings</td>
<td>60.57%</td>
<td>3.84%</td>
<td>36.57%</td>
<td>0%</td>
<td>100%</td>
<td>88.26%</td>
<td>31.73%</td>
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</tbody>
</table>


URISAT™ (degenerate sample, discarding squamous or blood, insufficient urothelial cells)
EQUIVOCAL™ = tetrasomy in voided urine or bladder wash <10 heterozygous FISH loci, tetrasomic upper tract specimen

Manual versus Image Aided

<table>
<thead>
<tr>
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<th>Mean age</th>
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<td>All specimen types</td>
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<td>Percentages</td>
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<td>37 - 82</td>
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<td>All Urine Specimens</td>
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<td>Percentages</td>
<td>71.20%</td>
<td>13.73%</td>
<td>14.60%</td>
<td>0.31%</td>
<td>100%</td>
<td>56.27%</td>
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<td>Percentages</td>
<td>64.60%</td>
<td>6.77%</td>
<td>27.96%</td>
<td>0.84%</td>
<td>100%</td>
<td>67.79%</td>
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<td>Percentages</td>
<td>63.29%</td>
<td>9.57%</td>
<td>23.40%</td>
<td>3.72%</td>
<td>100%</td>
<td>67.60%</td>
<td>32.97%</td>
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Automated Nov 2007 to April 2009

Manual versus Image Aided

<table>
<thead>
<tr>
<th>Manual vs. Image-Aided Interpretations for All Specimen Types</th>
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<td>Manual</td>
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<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
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<tr>
<td>Equivocal</td>
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<tr>
<td>Unsat</td>
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Reporting

- Positive
- Negative
- Unsatisfactory
- See comment

Positive: Numeric chromosomal aberrations associated with urothelial carcinoma identified.

Interpretative Data:
- Positive results indicate the presence of one or more numeric chromosomal abnormalities commonly associated with urothelial carcinoma, within the cells collected in this specimen. Positive results in the absence of clinical documentation of urothelial carcinoma within the bladder suggest the possibility of urothelial carcinoma or other urologic malignancy from another site (including ureter, kidney, urethra, and prostate). In this circumstance, further clinical evaluation to exclude these as a source of the abnormal cells is justified.
Reporting

- Negative: No evidence of numeric chromosomal aberrations associated with urothelial carcinoma identified.

Interprative Data:

- Negative results indicate a lack of evidence for the presence of numeric chromosomal abnormalities commonly associated with urothelial carcinoma, within the cells collected in this specimen. Negative results in the presence of other symptoms/signs of urothelial carcinoma may suggest the possibility of a false-negative test. In this circumstance, additional clinical studies to exclude urothelial carcinoma should be pursued, as clinically indicated. Although the Vysis® UroVysion™ Kit was designed to detect genetic abnormality associated with most urothelial cancers, there will be some urothelial cancers for which genetic changes cannot be detected by the UroVysion™ Test.

FISH Testing for Bladder Cancer

- UroVysion is a sensitive and specific assay for the detection of urothelial carcinoma

- FDA approved for:
  - Detection of recurrent tumor in patients with a history of bladder cancer
  - Evaluation of patients with hematuria for bladder cancer
Practical Guidelines for Integrating Emerging Molecular Methods Into Your Cytology Practice
Brian T. Collins, M.D.
Washington University in St. Louis
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FISH for the Detection of Malignancies of the Pancreatobiliary Tract
Pancreatobiliary Strictures

Distinction between malignant and benign strictures can be difficult

- By imaging, tumors often not seen due to longitudinal growth
- Inflammatory strictures look similar to malignant strictures
- Obtaining biopsies can be difficult due to tight quarters and tumor desmoplasia

Pancreatobiliary Strictures

Distinction between malignant and benign strictures can be difficult

- Large variations of sensitivity reported by cytology
  - Low sensitivity but high specificity for detecting malignancy
- Lack of malignant cells may limit sensitivity
- Morphologic overlap with carcinoma and reactive etiologies such as lithiasis, trauma, etc.
- Concern about false positive due to subsequent therapeutic surgical treatments
Cholangiocarcinoma

- Rare tumor
- Age range 50-70 years; male to female 1.5:1
- Majority of patients present with advanced, unresectable neoplasm
- 6 month survival in unresectable tumors

Pancreatic adenocarcinoma

- 5th most common cancer causing death in the US
- Surgical resection remains only hope for cure
- High percentage present with advanced stage before clinical detection
- Only 5-20% are candidates for surgical resection
- Current survival rates 5%
Collection

- Gastrointestinal endoscopists most often collect cells with standard cytology brush during ERCP and then place brush in a collection container (PreservCyt™).
- Vial is sent to cytology lab for processing
  - Routine cytology
  - FISH
  - No preservative required if “in-house”

Bile Duct Brush Cytology

Cell from bile duct brush cytologic specimens can show a spectrum of changes representing (A) negative, (B) atypical, (C) suspicious, and (D) positive diagnosis.
FISH Slide Preparation

- Manual method (UroVysion package insert)
  - Using 1.0 or 1.5 cm “welled” slide
- Thin Prep Non-gyn slide
  - 2.0 cm diameter (non-gyn filters)
- Cytospin
- Hybridization process: same urine
- Not FDA approved: off label use
FISH Signal Patterns

- Disomy (Negative)
- Trisomy 7 (Equivocal)
- Polysomy (Positive)

UroVysion probe set

- CEP 3
- CEP 7
- CEP 17
- LSI 9p21

Brush Cytology
FISH Signal Pattern
FISH Signal Pattern

Pancreatobiliary FISH

- Result Reporting
- Positive
  - 5 or more cells showed gains of 2 or more chromosomes (3,7,17)
  - 10 or more cells showed trisomy for chromosomes 3 or 7
    - Usually 7, less frequently 3
  - Report: Evidence of numeric chromosomal aberrations identified. See comment.
Pancreatobiliary FISH

- **Negative**
  - No evidence of numeric chromosomal aberrations identified.

- **Unsatisfactory**
  - Specimen processed and examined. Scanning revealed that the sample prep is scanty cellular following hybridization. Suggest clinical correlation and follow-up with repeat or additional studies, as clinically indicated.

Pancreatobiliary FISH

- 436 patients; 45% with carcinoma

![Sensitivity and Specificities of RC, DIA, and FISH](chart)

Fritcher, Kipp et al. Gastroenterology 2009
Pancreatobiliary FISH

Positive Predictive Values of RC, DIA, and FISH

Cytology Atypical

Cytology Suspicious

Cytology Positive

DIA Positive

FISH Trisomy 7

FISH Polysomy

Percent

0 20 40 60 80 100

Fritcher, Kipp et al. Gastroenterology 2009

Cytology/FISH for the Detection of PB Malignancy

Figure 5. Kaplan–Meier demonstrates differences in time to carcinoma for patients by (4) fluorescence in situ hybridization (FISH) result \( P < .001 \) and (6) routine cytology result \( P < .001 \).
Cytology/FISH for the Detection of PB Malignancy

**Figure 5.** Kaplan–Meier demonstrates differences in time to carcinoma for patients by (A) fluorescence in situ hybridization (FISH) result ($P < .001$) and (B) routine cytology result ($P < .001$).

Pancreatobiliary FISH

- Conclusions
- FISH shows higher sensitivity than cytology alone for the detection of pancreatobiliary cancer
- Combined use of FISH and cytology has an improved sensitivity over either method alone
- Positive predictive value of polysomy is high
- Positive predictive value of trisomy is variable (31-100%)
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FISH in Mesothelioma

- Aggressive neoplasm of mesothelial cells lining serous surfaces:
  - Pleura: 65-70%
  - Peritoneum: 30%
  - Pericardium: 1-2%
  - Tunica vaginalis testis
- Almost universally fatal
FISH in Mesothelioma

- U.S. Incidence: ~2000-3000/year
- Age:
  - Any, highest incidence >65
- Sex:
  - Male (4-6) : Female (1)
- Risk factors:
  - Familial clustering
  - Asbestos

FISH in Mesothelioma

- Pleural Mesothelioma
- Clinical Presentation:
  - Cough, dyspnea, chest pain
  - Recurrent pleural effusion
- Gross Pathology:
  - Usually diffuse
  - Associated with pleural plaques
  - Metastases (50%): lung, LN, heart, et al.
Histologic subtype: Epithelial
Lack of extrapleural nodal involvement
Lack of positive resection margins

FISH in Mesothelioma

Early-stage treatment improves survival.
- Pleural effusions are common (54-85%).
- Evaluation is relatively non-interventional.
- Cytologic examination of effusions may facilitate early diagnosis.
FISH in Mesothelioma
Reactive or Neoplastic?

FISH in Mesothelioma
Adenocarcinoma vs Mesothelioma
FISH in Mesothelioma

Adjuvant Studies for Improving Cytology Sensitivity

- Electron Microscopy
- Immunohistochemistry
- Molecular Studies

FISH in Mesothelioma

Ultrastructual Features

© Elsevier Inc 2004 Rosai and Ackerman's Surgical Pathology 9e
FISH in Mesothelioma

Adjuvant Studies for Improving Cytology Sensitivity

- **Electron Microscopy**
  - Mesothelioma (MM) vs adenocarcinoma
- **Immunohistochemistry**
  - MM vs adenocarcinoma
  - MM vs reactive: not established
- **Molecular Studies**
  - MM vs adenocarcinoma
  - **MM vs reactive**

FISH in Mesothelioma

**Molecular Studies**

- No specific anomalies characterize MM
- A number of anomalies recur:
  - Del 1p, 3p, 6q, 9p, 22q
  - Monosomy- chromosomes 4 and 22
  - Polysomy- chromosomes 5, 7, and 20.
- Two major methods of detection:
  - Karyotype
  - FISH (fluorescence in situ hybridization)
FISH in Mesothelioma

MM: common but non-specific. Del 1p, 3p, 6q, 9p, 22q + others

Adenocarcinomas: More complex karyotypes.

---

FISH in Mesothelioma

Improving Cytology Sensitivity

- Granados et al investigated the possibility of identifying clonal cytogenetic aberrations in effusions.
- Prospective study: 10 patients with a clinical suspicion of MM.
- Fluids submitted for cytology and cytogenetics.
- Compared to histology from biopsies.

Results

- Cytogenetics:
  - Chromosomal aberrations in 10/10 (100%)
  - Most common deletions: 1p, 3p, 6q, 9p, 22q

- Histology:
  - 9 cases positive, 1 no tissue confirmation

- Cytology:
  - 5 POS, 4 SUS, 1 NEG


Conclusions

- Cytogenetics is a useful adjunct to the diagnosis of MM in Cytology.
- Most useful in helping to confirm the suspicious* category.

* suspicious = atypia is present and raises the possibility of malignancy, but the degree of atypia or the quantity of cells is insufficient for a conclusive diagnosis.

Chiosca (2008): FISH for deletion 9p21 on paraffin-embedded tissue from 52 patients with histologically confirmed MM and 40 controls.
- Del 9p21 found in 35/52 cases (67%).
- 0/40 controls.

Few studies on MM effusions published.
Shin (2003) analyzed 17 pleural fluids from histologically confirmed MM and 17 control fluids with centromeric probes for chromosome 7 and 9.
- Anomalies found in:
  - 88% of MM cases, 0 reactive


Factor et al investigated the clinical utility of using both karyotype and FISH analysis of effusions from patients suspected of having Malignant Mesothelioma.

FISH in Mesothelioma

Effusion

Karyotype

Normal
Unsuccessful

FISH
Probes: 9p-, 22q-, 7cen

Abnormal

STOP

FISH in Mesothelioma

Karyotype

- Short-term culture of fresh cells from pleural and peritoneal fluid.

FISH

- Fixed cell suspensions from uncultured material.
- DNA probe sets to different regions on 9p, 22q and 7centromere.
- At least 50 nuclei analyzed.
- Positive values were determined by normal pleural fluids (cut off of 2/50 or 4/50 depending upon the probe).

### FISH in Mesothelioma

**Karyotype result n (%)**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unsuccessful</strong></td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Normal</strong></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Abnormal</strong></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>48</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FISH in Mesothelioma**

FISH from 33 Effusions with Unsuccessful or Normal Karyotypes

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Abnormal</th>
<th>Not done</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Karyotype Result, n(%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
<td>9</td>
<td>2</td>
<td>20 (61)</td>
</tr>
<tr>
<td>Unsuccessful</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>13 (39)</td>
</tr>
<tr>
<td>Total</td>
<td>14 (42)</td>
<td>13 (39)</td>
<td>6 (18)</td>
<td>33</td>
</tr>
</tbody>
</table>

FISH in Mesothelioma

NEG/SUS Cytology with FISH in 8 of 14 Cases (57%)

Sensitivity for detection of Mesothelioma:
- Karyotype alone: 31%
- Karyotype + FISH: 58%

Improvement in diagnostic yield: 87%

FISH in Mesothelioma

- Using FISH in conjunction with karyotype nearly doubled the sensitivity of karyotype alone in the diagnosis of Mesothelioma in effusions.
- 57% of cytologically negative or suspicious cases were positive by karyotype or FISH.
- The two tests used together are a useful adjunct to cytology alone.

FISH in Mesothelioma

- Thank you for your kind attention
Practical Guidelines for Integrating Emerging Molecular Methods Into Your Cytology Practice

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THYROID CASE PRESENTATION
65 YEAR OLD FEMALE
RIGHT THYROID NODULE
FNA SPECIMEN

Dx: Atypical (AUS)
Thyroid Ca Molecular Markers

- Cytologic examination may not provide a conclusive diagnosis (T3, T4)(TBS 5-7%?)
- Molecular markers provide more objective method for reliable diagnosis, possibly prognosis and Rx.
- To date, thyroid-cancer specific molecular marker has not been clearly identified.
- RAS/RAF, point mutations, PAX8-PPAR, RET/PTC rearrangements, TSH-receptor DNA methylation

Recent developments in the clinical application of thyroid cancer biomarkers

Daniel Shihfu, Chi-Wook Chung and Elektron Kebebew

Purpose of article

The aim of the article is to provide an update on the status of the clinical application of thyroid cancer biomarkers.

Molecular

Our understanding of the tumor biology of thyroid cancer of follicular cell origin has improved and molecular genetic technologies are providing new data that may lead to the development of novel diagnostic tests. Molecular genetic cancer biomarkers may provide more objective and reliable diagnosis, possibly prognosis and Rx.

To date, thyroid-cancer specific molecular marker has not been clearly identified.

RAS/RAF point mutations, PAX8-PPAR, RET/PTC rearrangements, TSH-receptor DNA methylation.

Table 1. Recent studies of molecular testing for common genetic changes in fine needle aspiration biopsy samples

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Study type</th>
<th>Study size</th>
<th>Genes mutated tested</th>
<th>Genomic diagnosis using IGUS</th>
<th>Diagnostic accuracy improved (percentage, number of cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaplan et al. 1994</td>
<td>36</td>
<td>Retrospective</td>
<td>36</td>
<td>BRAF, RET/PTC 1, RET/PTC 2, RET/PTC 3</td>
<td>87%, 31</td>
<td></td>
</tr>
<tr>
<td>Cibas et al. 1994</td>
<td>59</td>
<td>Retrospective</td>
<td>59</td>
<td>BRAF</td>
<td>10%, 50</td>
<td></td>
</tr>
<tr>
<td>Bove et al. 2006</td>
<td>26</td>
<td>Retrospective</td>
<td>26</td>
<td>BRAF, RET/PTC 1</td>
<td>21%, 15</td>
<td></td>
</tr>
<tr>
<td>Shi et al. 2006</td>
<td>71</td>
<td>Retrospective</td>
<td>71</td>
<td>BRAF</td>
<td>41%, 72</td>
<td></td>
</tr>
<tr>
<td>Sbab et al. 2007</td>
<td>32</td>
<td>Retrospective</td>
<td>32</td>
<td>RET/PTC 1, VEGF, BRAF, TRK</td>
<td>3%, 10</td>
<td></td>
</tr>
</tbody>
</table>

*Fine needle aspiration biopsy suggests that the needle is aspirating only. No mutations were found in the deoxyribonucleic DNA biopsy samples (n= 39, benign in 18, malignant in 20 and one did not have an operation.)
BRAF in Papillary Thyroid Carcinoma

- BRAF gene - encodes kinase that is part of the RAS/RAF/MAPK pathway
- Mutation 1799 T>A (V600E) in Exon 15 – present in 29-83% of papillary thyroid carcinoma
- Clinical Utility:
  - Which patients may have PTC, especially for indeterminate cases. Defines therapy (thyroidectomy? Radioiodine?)
  - Which patients may respond to RAF/MEK inhibitors?
  - Prognosis?

Cytospins/smears
LBC
Cell blocks (FFPE)

SSL
DNA Extraction
PCR

FFPE
Previously stained aspirate smear material

PTC FFPE Microdissection of tumor
PCR for BRAF: FRET LightCycler Assay

Sequencing confirmation of heterozygous BRAF T1799A mutation

How molecular pathology is changing and will change the therapeutic options for patients with follicular and medullary thyroid cancer

| Table 1: Summary of studies using new compounds that target tumor molecular pathways in follicular and medullary thyroid cancer

<table>
<thead>
<tr>
<th>Study</th>
<th>Compound</th>
<th>Effect</th>
<th>Target</th>
<th>Phase</th>
<th>Tumor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study A</td>
<td>PKI1105</td>
<td>Inhibition</td>
<td>BRAF</td>
<td>Phase II</td>
<td>Follicular</td>
</tr>
<tr>
<td>Study B</td>
<td>L-685,458</td>
<td>Inhibition</td>
<td>RAF</td>
<td>Phase III</td>
<td>Medullary</td>
</tr>
<tr>
<td>Study C</td>
<td>Vemurafenib</td>
<td>Inhibition</td>
<td>BRAF</td>
<td>Phase III</td>
<td>Follicular</td>
</tr>
<tr>
<td>Study D</td>
<td>Sorafenib</td>
<td>Inhibition</td>
<td>RAF</td>
<td>Phase III</td>
<td>Medullary</td>
</tr>
</tbody>
</table>

**Abstract**

The use of targeted therapies in the treatment of follicular and medullary thyroid cancer is expanding. This table summarizes studies that have used new compounds targeting molecular pathways specific to these tumors.
Information for Clinicians: Commercially Available Molecular Diagnosis Testing In The Evaluation of Thyroid Nodule FNA Specimens.

Rosenthal D, Hodak SP.

Source
Nassau University Medical Center / State University of New York - Stony Brook, Division of Endocrinology, 2201 Hempstead Tpk, East Meadow, New York, United States, 11554, (516) 296-4803, (516) 296-4804; davdeb@pol.net.

Abstract
TSHR mRNA RT-PCR, the Veracyte and Asuragen commercial methods, and the non-commercial use of BRAF, RAS, RET/PTC, and PAX8/PPARγ testing have promising roles in the diagnosis and treatment of patients with nodular thyroid disease and thyroid cancer. However, at this time experience with these methods remains limited and no test has perfect sensitivity and specificity. Peer reviewed data evaluating the diagnostic performance of these tests is increasingly available. The ATA feels that until expert consensus review of existing data, now underway by the ATA Guidelines Task Force, can be completed, no evidence based recommendation for or against the use of these methods can be made. Clinicians are therefore advised to consider the use of these genetic diagnosis methods with appropriate caution, and to remain cognizant of the limitations of the data supporting their use. Patients who are interested in the use of these tests in their own care should discuss them thoroughly with their care providers. Determining whether or not the limited data available supports the use of these methods should be considered on a case by case basis until evidence based recommendations are available.

“The ATA feels that until expert consensus review of existing data, now underway by the ATA Guidelines Task Force, can be completed, no evidence based recommendation for or against the use of these methods can be made.”

Take Home: THYROID

- Dx made by FNA cytology, but molecular tests may have clinical value as ancillary tests
- Indeterminate results occur – unmet need? Cost?
- BRAF point mutation - affects signal transduction
- Gene mutation detected in thyroid FNA using PCR
- Analytical sensitivity of molecular test is of paramount importance – cancer cells with mutation may be low % of sampled cells.
- Diagnostic and prognostic use: confirm malignancy, lymph node met, targeted-therapy, follow-up course

LUNG CASE
69 YEAR OLD MALE, SMOKER
3.0 CM R LUNG MASS, PET + CT-GUIDED FNA
Biomarkers for lung cancer may be used for risk stratification, early detection, treatment selection, prognostication and monitoring for recurrence.

• Benefit from sensitive and specific, noninvasive, cost-effective biomarkers

• Cytology has many advantages (and disadvantages) to obtain patient material for analysis.

• Early detection is key
Examples of Molecular Diagnostics in Cytology

FISH for early lung cancer detection in bronchoscopic specimens

EGFR amplification/mutation in NSCLC FNA specimens.

Fluorescence in situ Hybridization in Lung CA

Chromosomal alterations are hallmark of cancer cells
Sokolova, Morrison - Handbook of immunohistochemistry and in situ hybridization of human carcinomas, vol 1:2004

Multi-Target FISH Assay

- Red – LSI EGFR Probe (7p12)
- Gold - LSI C-MYC Probe (8q24)
- Green - LSI D5S23, D5S271 (5p15.2)
- Aqua - CEP 6

normal cell = two red, two gold, two green and two aqua (2R2G2G2A) signal pattern is observed.

In an abnormal cell, multiple or reduced copies of the probe signals may be observed. Copy numbers of more or less than two of any of the four probes indicate gene, region or chromosome gain or loss, respectively.
Aqua = CEP 6
Abbott/Vysis probes

Multi-Target FISH on archival bronchoscopic cytology samples

Sokolova, Morrison – Handbook of immunohistochemistry and in situ hybridization of human carcinomas, Vol 1 2004

FISH Results on Bronchoscopic cytology samples

Halling et al – Chest 2006
Examples of Molecular Diagnostics in Pulmonary Cytology

FISH for early lung cancer detection in bronchoscopic specimens

EGFR/ALK in NSCLC FNA specimens.
The EGFR Story

• Activating mutations OR amplification of the epidermal growth factor receptor (EGFR) gene (7p12.3-p12.1)

• correlate with tumor response to targeted tyrosine kinase inhibitors (TKI) gefitinib and erlotinib in a subset of patients with non-small cell lung carcinoma (NSCLC).

Identifying EGFR Mutations to Guide Therapy

• Activating mutations in Exons 18, 19, 20, 21
• Mutants bind TK inhibitors (gefitinib or erlotinib)
• About 10% of patients respond to TKI
• Identify mutations to identify patients that will respond to TKI therapy
• Evaluated assay and instruments
Identification of tumor cells. Slides were marked (A) to indicate areas rich in tumor cells (B). After marking the same areas with a diamond pen (C), the coverslips were removed, and the tumor cells were scraped into a DNA extraction buffer. Figure D shows the same area of the slide after extraction and destaining.


Amino acid sequences of Exon 19 deletion mutants in three cases compared with wild type. Activating in-frame deletion mutations were found in exon 19 in three cases. One case contained an additional mutation, A755D.

• Controversy: some studies suggest EGFR amplification better indication of response to TKI therapy than mutation status—Santos et al, Ca Cytopathol 2011;119:80-91.
• Targeted therapy (Crizotinib) with companion diagnostic (EML4-ALK) by FISH.
• KRAS gene mutation status.

Figure 2. Fluorescent in situ hybridization (FISH) in EML4-ALK-positive sample demonstrated spatial resolution of red and green probes. These probes flank the site of translocation in patients with ALK rearrangement.

Crystal – Clin Adv Heme Onc March 2011
CAP/IASLC/AMP MOLECULAR TESTING GUIDELINES FOR SELECTION OF LUNG CANCER PATIENTS FOR EGFR AND ALK TYROSINE KINASE INHIBITORS

TAKE HOME: LUNG
Whom to test: Histology matters!
• Test any tumor with lung adenocarcinoma
• May be mixed (adenosquam, adeno/small)
• No pure squamous cell, small cell ca
  – Except maybe incomplete small bx
  – Poorly diff tumors should be tested
  – Subtypes of adenoca not useful
• NSCLC not adequate
  – May need IHC

TAKE HOME: LUNG
Which sample to test?
Quality and quantity are key
A cellular FNA is better than a necrotic resection (Needle aspirates are FINE!)
Primary vs. metastasis
Quality is determinate
If mets after TKI, best to test mets
Multiple primaries
If histology differ, best both/all
TAKE HOME: LUNG

When to test?
Stage IV: at diagnosis, ASAP
Goal: 10 working days
Slow? Find a faster method
Early stage: Discuss with oncologist
If not now, save material
How much?
Sequence? EGFR first

“There appears to be sufficient tumor material in the cell block (or block) for ancillary studies. If this patient is considered a candidate for targeted chemotherapy, suggest additional molecular testing (e.g. ALK and EGFR), per current NCCN guidelines.”

Small lung bx: Place on every pathology report
You can make all the difference in the care of the patient diagnosed with lung cancer!