Objectives

• Discuss the increasing demands for molecular testing on cytology specimens

• Discuss the role of Cytology in molecular testing
  – Past
  – Present
  – Future

Clinicians have many questions...

• What types of tumor do we test?
• What testing is appropriate?
• How are those tests done?
• What tissue is needed to do these tests?
• How must the tissue be collected/submitted?
• Who performs the test?
• What is the significance of these tests?
• How are results reported?
• Who reports them?
Historically, the most common application has been in thyroid cytology. The three categories of “atypia of undetermined significance (AUS),” “suspicious for a follicular neoplasm” and (to a lesser degree) “suspicious for malignancy” are often referred to by clinicians as the “indeterminate” thyroid FNA categories. They have inspired the development of molecular tests to triage patients more effectively for conservative versus surgical management and to further reduce unnecessary surgery for patients who have a benign nodule.

Some research suggests that molecular testing, particularly in AUS cases, can play a role similar to that of reflex human papillomavirus (HPV) testing for a woman with an atypical Pap test.
Molecular Testing - Thyroid

- 4 gene mutations are most common in thyroid cancer with implications for tumor diagnosis and prognosis
  - BRAF point mutation
  - RAS point mutation
  - RET/PTC rearrangement
  - PAX8/PPARγ rearrangement
- Mutually exclusive

MAPK Pathway

- These 4 common mutations are all able to activate the mitogen-activated protein kinase (MAPK) pathway
- MAPK pathway propagates signals from cell membrane receptor tyrosine kinases (RTKs) to the nucleus through a series of adapter proteins and kinases
- Regulates transcription of genes involved in cell differentiation, proliferation and survival

Available Molecular Testing

- Afirma Gene Expression Classifier (Veracyte, Inc.)
  - Microarray technology that analyses mRNA expression of 167 different genes (142 common in thyroid cancer, 25 uncommon in thyroid cancer)
  - 2 dedicated FNA passes, only for FLUS/AUS and Follicular Neoplasm (Bethesda III and IV)
  - Generates 2 possible results: benign and suspicious
  - NPV of 95%, PPV of 38% (used as a “rule out” test)
- ThyGenX est (Inerpace Diagnostics)
  - Based on previous microRNA thyroid oncogene panel by Asuragen, Inc.
  - Next Generation Sequencing (NGS) platform to identify > 100 genetic alterations across 8 genes associated with thyroid malignancy
  - 1 dedicated FNA pass, only for FLUS/AUS and Follicular Neoplasm (Bethesda III and IV)
  - ThyraMIR (analysis of 10 different microRNAs) used in conjunction with ThyGenX, when ThyGenX is negative
  - Generates a positive or negative result
  - NPV of 94%, PPV 74%
Available Molecular Testing

• ThyroSeq Test
  – ThyroSeq v1, 2012
    • Next Generation Sequencing (NGS) based gene mutation and fusion panel
defined to target 12 cancer genes with 284 mutational hot spots
    • PPV 88% (used as a “rule in” test)
  – ThyroSeq v2, 2014
    • Expanded panel of DNA alterations (14 genes, >1000 mutations) and RNAalterations (42 fusions, 16 genes for expression)
    • NPV 98%, PPV 83% (used as “rule in” and “rule out” test)
  – ThyroSeq v3, 2017
    • Further expanded panel; NGS of 112 genes providing information on >12,000mutation hotspots and >120 gene fusion products
    • NPV 97%, PPV 66% (used as a “rule in” test)

Present

Molecular Testing - Lung

• Most of our molecular efforts focus on lung cancer
• Prior to 2011 there were no therapeutic implications for classification
of lung cancers beyond small cell, and non-small cell carcinoma
• This changed dramatically with the discovery targeted therapeuticoptions, leading to a revolution in diagnosis, tissue management and treatment
• Brunt of the work shifted to cytology, because 70% of lung cancersare unresectable (advanced stage) at the time of diagnosis, makingsmall biopsies and cytology specimens the primary methods ofdiagnosis for the majority of lung cancer patients
IASLC/ATS/ERS

- Three organizations
  - International Association for the Study of Lung Cancer
  - American Thoracic Society
  - European Respiratory Society
- Held a meeting in 2011 that yielded two articles published in 2013, making major changes to lung cancer classification
  1. Need for sub-classification of lung cancers beyond SCLC/NSCLC to triage for molecular testing
  2. Reclassification of adenocarcinomas in resection specimens (e.g. adenocarcinoma with lepidic pattern vs. BAC)

Driving Therapeutic Advances for NSCLC

1. Tyrosine kinase inhibitors as first-line therapy in patients with advanced lung adenocarcinoma with EGFR mutations
2. Adenocarcinomas with ALK rearrangements are responsive to Crizotinib (ALK and ROS-1 inhibitor)
3. Patients with adenocarcinoma or NSCLC-NOS are more responsive to Pemetrexed than those with squamous cell carcinoma
4. Squamous cell carcinoma is associated with life-threatening hemorrhage in patients treated with Bevacizumab (VEGF-A inhibitor)

Targeted Therapies vs. Immunomodulatory Therapies

- Targeted Therapies
  - Generally refer to tyrosine kinase inhibitors
  - Drugs that target specific subpopulation of tumor cells defined by specific mutations
    - EGFR (Erlotinib, Gefitinib, Afatinib)
    - ALK (Crizotinib, Ceritinib)
    - ROS-1 (Crizotinib)
  - New problem: Resistance
Three organizations
- College of American Pathologists
- International Association for the Study of Lung Cancer
- Association for Molecular Pathology

Recruited a working group in 2010, and published an evidence-based guideline in 2013 for standard-of-care clinical practice concerning molecular testing in lung cancer patients
- Which samples should be tested
- Which genes should be tested
- How test should be designed, validated and executed

In 2018, an updated molecular testing guideline was published, stratifying biomarkers as
- “Must-test”
- “Should test”
- Other

“Must-test” biomarkers
- Standard of care for all patients with advanced lung cancer with adenocarcinoma component who are being considered for an approved targeted therapy
- EGFR, ALK, ROS-1

“Should test” biomarkers
- Used to direct patients to clinical trials and which should be included in any large sequencing panel that is performed for lung cancer patients, but which are not required for laboratories that perform only single-gene assays
- BRAF, MET, RET, ERBB2 (Her2) and KRAS

Other
- All others are considered investigational and are not appropriate for clinical use this time

At MUSC, the Solid Tumor Cancer Panel includes the “must test” and “should test” biomarkers

Requisition explains minimum tumor burden requirement

Requisition lists tissue requirements per test
Methodology

- FFPE tissue
  - 20% tumor DNA
  - Pathologist circles tumor area on slide and estimates % of tumor DNA
  - Molecular lab takes a punch biopsy out of block in corresponding location
- Tissue processed for:
  - Polymerase Chain Reaction (PCR) or Next-Generation Sequencing (NGS) for EGFR, KRAS, BRAF, RET
  - Fluorescent In-Situ Hybridization (FISH) for ALK and ROS-1
- Direct smears (from 2018 update)
  - Diff-Quick or Pap stained slides
  - 20% tumor DNA, approx. 400 tumor nuclei
  - Cover slip is removed, whole material is scraped off the slide
  - Tissue is processed for PCR or NGS
  - FISH probes are applied to tissue

2018 Updated Molecular Guideline

- EGFR mutation
  - PCR or NGS on resection specimens or cytology (cell blocks or smears), 20% tumor DNA
  - IHC estimation of total EGFR cannot be used (tells quantity not function)
  - Resistance testing: EGFR T790M mutation, detectable in as little as 5% of viable cells
- ALK rearrangement
  - IHC, FISH, PCR or NGS (previously only FISH permitted) on resection specimens or cytology (cell blocks or smears)
- ROS-1 rearrangement
  - Rare finding in lung adenocarcinoma (2%), but results in gene fusion that can be targeted by Crizotinib
  - IHC may be used for screening only
  - FISH, PCR or NGS on cytology (cell blocks or smears)

Cell-Free DNA

- Aka: liquid biopsy
- Studies have shown that lung cancer cells shed their DNA into the circulation at levels that are detectable with modern methods (NGS, allele-specific PCR)
- Theoretical advantage
  - Derivation of circulating tumor DNA from multiple disease sites, representing an integrative measure of all sites
- Analytical methods have high specificity (3-20% false positive rate), but low sensitivity
  - Absence of mutation finding does not exclude possibility of mutation
- Currently, evidence is insufficient for the use in primary diagnosis
- Potential utility
  - Identification of EGFR T790M resistance mutation
Targeted Therapies vs. Immunomodulatory Therapies

- **Immunomodulatory Therapies**
  - Tumors exist in an immune suppressed environment
  - Testing can detect those tumors which possess targetable markers

- **Mechanism of Action**
  - Immunomodulatory therapies deactivate the “tolerance mechanisms” engaged by these markers via antigen binding
  - The “unblinded” innate immune system, can now seek and destroy the tumor cell

### PD-1/PD-L1 Pathway

- Helps tumor cells hide from the immune system
- Programmed (cell) death protein 1 (PD-1)
  - Type I transmembrane cell receptor expressed primarily on activated mature T cells, B cells, monocytes and other antigen presenting cells (APCs)
- Programmed death ligand 1 (PD-L1)
  - Membrane antigen present on tumor cells
- Binding of PD-1 to PD-L1 results in the inhibition of T-cell receptor-mediated lymphocyte proliferation, acting as a limiting factor in inflammatory responses and a mediator of peripheral tolerance
- Plays a role in many types of tumors
  - NSCLC *Classical Hodgkin Lymphoma
  - Melanoma # Urothelial cancer
  - HNSCC # Gastric cancer
**PD-1 and PD-L1**

- Various degrees of expression
  - Some malignancies have more PD-1, some have more PD-L1
  - Prognostic role of PD-1/PD-L1 expression in malignancy remains unclear
- There is no direct, predictable, universally proportionate relationship that applies to every tumor type
  - Individual tumors dictate potential for response
- In NSCLC, this pathway has become crucial
  - PD-L1 expression dictates response to anti-PD-1 therapy
  - Immunomodulatory therapy targeting this pathway is now a first-line therapy for advanced NSCLC

**Immunomodulatory Therapies**

- **PD-1 inhibitors**
  - Pembrolizumab (Keytruda)
  - Nivolumab (Opdivo)

- **PD-L1 inhibitors**
  - Atezolizumab (Tecentriq)

**New York Times - April 16, 2018**

- [Image of news article]
Methodology

- PD-L1 expression by immunohistochemical stain
  - Clone specific
  - 22C3 (Keytruda), 28-8 (Opdivo), SP142 (Tecentriq)
- FFPE tissue
  - Not validated on decalcified tissue
  - Minimum 100 tumor nuclei
  - <3 year old tissue; PD-L1 expression drops after that
- Interpretation
  - Scored by a pathologist at NeoGenomics using a manual microscope
  - Expressed as a tumor proportion score (TPS)
    - High expression: ≥ 50% TPS
    - Expressed: 1-49% TPS
    - No Expression: <1% TPS

Future

Molecular Testing – GI Cancer

- Immunomodulatory therapy is bridging a transition from lung cancer to other malignancies
  - PD-1/PD-L1 inhibition therapy already has a wide application
- Future questions regard increased demand for test previously done on surgical specimens
- Recent experience with pancreatic adenocarcinoma
- Example:
  - Keytruda website states that there may be a benefit for patients with malignancies shown to have microsatellite instability-high (MSI-H) or have mismatch repair deficiencies (dMMR)
**MSI and MMR**

- Errors of DNA replication are inevitable
- Specialized systems that identify and correct mismatched pairs of DNA and act as “proofreaders” are called mismatch repair (MMR) systems
- Microsatellite instability is the molecular fingerprint of a deficient MMR system
  - Key proteins form heterodimers that recognize and remove DNA errors
  - The loss of MMR proteins leads to an accumulation of DNA replication errors, a phenomenon known as microsatellite instability, and eventually to somatic mutations

**The Connection**

**Methodology**

- Microsatellite Instability (MSI)
  - Current gold standard is molecular testing
  - DNA extraction from FFPE tissue
  - PCR amplification of selective microsatellites
  - Analysis of fragment size by gel electrophoresis or automated sequencing
  - Analysis of 5 mononucleotide repeat markers
    - 2 mononucleotide repeats (BAT26, BAT25), 3 dinucleotide repeats (D5S346, D2S123D17S250)
  - MSI-H (high frequency): 2 or more markers show instability in >30% loci tested
  - MSI-L (low frequency): 1 marker shows instability in 10-30% of loci tested
  - Compared to DNA extracted from healthy tissue that is microsatellite stable (MSS)
  - Sensitive but not specific, ex: will miss 5% of Lynch syndrome mutations
Methodology

• Mismatch Repair (MMR)
  – Done by immunohistochemistry (IHC)
  – Less labor intensive, more cost effective than PCR
  – Excellent concordance with PCR
  – >90% sensitive, 100% specific, 100% PPV for MSI-H
  – IHC on FFPE tissue (surgical or cell block)
  – Requires 30% tumor DNA
    • 4 commercially available antibodies
      • MLH1, MSH2, MSH6 and PMS2
    – Microscopically examined by a pathologist
    • Lymphocytes and any normal epithelium serve as positive internal controls

MSI/MMR currently

• MSI is preferentially done on surgical resection specimens
  – Easy to sample benign and malignant tissue at the same time
• MMR can be done on cytology specimens, as only tumor tissue is required
  – Possible future ideas for MSI, sampling buccal mucosa at time of EBUS

Take Home Points

• Molecular testing is no longer optional for a majority of malignancies
• Testing demands on cytology specimens will only increase
• Most likely, all tests will be available by NGS
References


"Thank you!
Questions!"