Molecular testing of thyroid nodules, practical considerations

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Puerto Rico Pathology
No disclosures
Thyroid Aspiration Cytology

- **Benign**: 75%
- **Atypical/follicular**: 20%
- **Malignant**: 3-7%
Malignancy risk,... and NIFTP

<table>
<thead>
<tr>
<th>Diagnostic category</th>
<th>Risk of malignancy if NIFTP ≠ CA (%)</th>
<th>Risk of malignancy if NIFTP = CA (%)</th>
<th>Usual management</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiagnostic or unsatisfactory</td>
<td>5–10</td>
<td>5–10</td>
<td>Repeat FNA with ultrasound guidance</td>
</tr>
<tr>
<td>Benign</td>
<td>0–3</td>
<td>0–3</td>
<td>Clinical and sonographic follow-up</td>
</tr>
<tr>
<td>Atypia of undetermined significance of follicular lesion of undetermined significance</td>
<td>6–18</td>
<td>10–30</td>
<td>Repeat FNA, molecular testing, or lobectomy</td>
</tr>
<tr>
<td>Follicular neoplasm or suspicious for a follicular neoplasm</td>
<td>10–40</td>
<td>25–40</td>
<td>Molecular testing, lobectomy</td>
</tr>
<tr>
<td>Suspicious for malignancy</td>
<td>45–60</td>
<td>50–75</td>
<td>Near-total thyroidectomy or lobectomybc</td>
</tr>
<tr>
<td>Malignant</td>
<td>94–96</td>
<td>97–99</td>
<td>Near-total thyroidectomy or lobectomybc</td>
</tr>
</tbody>
</table>

Adapted with permission from Ali and Cibas (7).

a Some studies have recommended molecular analysis to assess the type of surgical procedure (lobectomy vs. total thyroidectomy).

b In the case of "suspicious for metastatic tumor" or a "malignant" interpretation indicating metastatic tumor rather than a primary thyroid malignancy, surgery may not be indicated.

What should we do?

Risk of malignancy high enough to do something

Risk of malignancy low enough to be concerned about overtreatment

So far, follow up, repeat biopsy

- Up to 50% will have a negative repeat biopsy
- Increased risk even if negative in repeat biopsy? (VanderLaan, 2011; Renshaw 2010)
- Repeated indeterminate biopsy up to 27% risk of malignancy (Faquin, 2009)
Cancer is a molecular disease

Roth et al. 2018
First idea

• Let's test for mutations and see if we can identify the cancers in those indeterminate biopsies so we can identify those who need surgery (rule in approach)
• First panels based in 7 genes testing
Molecular findings in thyroid cancer

- Papillary CA: BRAF (45%), RET/PTC (20%), RAS (10%)
- Follicular CA: RAS (40%), PAX8/PPARG1 (30%)
- Medullary CA: RET (95% familial, 50% sporadic)
- But:
  - Mutations may be present in benign nodules (RAS)
  - Mutations may not be identified in malignant nodules
- Result: Mutations were not detected in most nodules, many cancers were missed by this approach and not all detected mutations led to a final cancer diagnosis

Hassell L., Gillies E., Terence S. *Cytologic and Molecular Diagnosis of Thyroid Cancers*. Cancer Cytopathology, 2011.
Second idea

- Let's test for mutations and see if we can exclude cancer in those indeterminate biopsies and identify those who do **not** need surgery (rule out approach)
And then the market race

- Tests with high specificity and PPV worked to improve sensitivity and NPV
- Tests with high sensitivity and NPV worked to improve specificity and PPV

Today's market
Molecular testing in 2021

- To stratify risk of malignancy
  - Molecular testing in category III & IV biopsies
- To tailor the surgical procedure
  - Categories V & VI
- To predict risk of progression
  - Select patients to treat vs patients to monitor in small thyroid tumors in selected patients
Stratifying risk in indeterminates

- I want to use a test to identify those who have a disease from those who don't in a given population
- Sensitivity & specificity are constant, but **predictive values depend on prevalence**

Example

- The categories III & IV in lab A have a risk of malignancy of 16%

- The categories III & IV in lab B have a risk of malignancy of 38%

Therefore, in a group of 100 cases:
- Lab A will have 16 carcinomas
- Lab B will have 38 carcinomas
Example

- If both labs use the same test, with 91% sensitivity and 68% specificity
  - Lab A will get a positive result in 15 of the 16 patients, with 1 false negative
  - Lab B will get a positive result in 35 of the 38 patients, with 3 false negatives
Example

NPV = TN / (TN + FN)

In Lab A: NPV = 44 / (44 + 1) = 98%

In Lab B: NPV = 32 / (32 + 3) = 91%

Same test will have different NPV in populations with different prevalence!
Afirma

• The idea: *With a very high negative predictive value, the chance of malignancy in a negative case is so low that surgery can be avoided*

• New version: Genomic Sequencing Classifier
  • Uses NGS, RNA test
  • Interrogates > 10,000 genes (nuclear and mitochondrial)
  • Special tests for Hürthle cells, medullary CA, parathyroid, and metastatic lesions
  • Analysis performed by algorithms
  • Validated with the same specimens than first version
• Valuidated in multicenter, retrospective double-blind study with 191 samples; molecular result not considered for surgery

• Alloges 96% NPV, 47% PPV, 91% Sensitivity, 68% Specificity, with 66% Benign call rate (NIFTP not included in final dx. Patel, et al. JAMA. 2018).

• Later studies reporting 76%* benign call rate, 60% PPV*, 94% specificity (Endo, et al; 2019)

• Benign call rate in 2/3 Hürthle cell lesions with 89% sensitivity

* Really? These higher numbers also reported with Thyroseq in later studies (Ohori et al; 2019)
Afirma Xpression Atlas

• Panel can be reflexed for “Suspicious” or requested in category V or VI diagnoses
• Panel of 593 genes, 905 variants, 235 fusions

From: Veracyte website.
**Sample report**

### Patient Information

<table>
<thead>
<tr>
<th>Field</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient:</td>
<td>John Doe</td>
</tr>
<tr>
<td>DOB</td>
<td>01 Jan 1960</td>
</tr>
<tr>
<td>Gender:</td>
<td>M</td>
</tr>
<tr>
<td>Lab ID:</td>
<td></td>
</tr>
<tr>
<td>MRN:</td>
<td></td>
</tr>
</tbody>
</table>

**Collection Date**: 07 Oct 2019  
**Facility Name**: University Hospital of Anytown  
**Submitting Physician**: Jane Doe  
**Treating Physician/CC**:  
**Phone**: (555) 555-5555  
**Clinical History**: No Clinical History Provided

### Results

- **Nodule**: A  
  - Thyroid, Lower Right, 5 cm

#### Afirma Genomic Sequencing Classifier

- N/A

#### Afirma Xpression Atlas

- **ETV6/NTRK3**: detected
- **BRAF p. V600E c. 1799T>A**: not detected
- **RET/PTC1, RET/PTC3**: not detected

#### Clinical Relevance

- **MTC**: negative
- **Parathyroid**: N/A

#### Risk of Malignancy

- >95%

#### Associated Neoplasms

- DTC

#### FDA Approved Therapy**

- Yes, NTRK fusion-specific therapies currently approved. See medication prescribing information for appropriate patient selection.

### Results Interpretation

The result of this 5cm Bethesda V nodule A is ETV6/NTRK3 positive. Among Bethesda II/IV nodules, an NTRK fusion suggests a risk of cancer of >95%1, and is likely higher among Bethesda V and VI nodules. This genomic alteration is associated with PTC and both BRAF V600E-like and RAS-like profiles, which include rates of lymph node metastases and extrathyroidal extension that are higher than Non-BRAF-Non-RAS-like neoplasms* 2. Clinical correlation and surgical resection should be considered.
# Our experience

<table>
<thead>
<tr>
<th></th>
<th>Puerto Rico Pathology</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant cases</td>
<td>5.5%</td>
<td>3-7%</td>
</tr>
<tr>
<td>AUS/FLUS (III)</td>
<td>8%</td>
<td>8-12%</td>
</tr>
<tr>
<td>Follicular neoplasm (IV)</td>
<td>7%</td>
<td>2-8%</td>
</tr>
<tr>
<td>All indeterminates (III &amp; IV)</td>
<td>15%</td>
<td>14-26%</td>
</tr>
<tr>
<td>Risk malignancy in cat III</td>
<td>12.5%</td>
<td>5-15%</td>
</tr>
<tr>
<td>Risk malignancy in cat IV</td>
<td>14%</td>
<td>15-30%</td>
</tr>
<tr>
<td>Risk malignancy III &amp; IV</td>
<td>13.6%</td>
<td></td>
</tr>
<tr>
<td>Afirma Benign call rate</td>
<td>64% (48/75 cases)</td>
<td>66%</td>
</tr>
<tr>
<td>Benign call rate in cat II with prior III or IV</td>
<td>74% (14/19)</td>
<td></td>
</tr>
</tbody>
</table>
ThyroSeq v3

• First with Next generation sequencing and specific mutation reporting
• Original validation studies heavily criticized (one center, biased pathologic diagnosis)
• New version tested in a double blinded multicenter trial
  • Can test samples collected in their media and FFPE tissue
  • Negative call rate 61% (Steward et al, 2018)
ThyroSeq v3

- FNA Sample QA
- Acellular
- STOP

1. FNA Cellular Composition
   - Thyroid Follicular Cells
     - Parathyroid cells
     - C-cells
     - Non-Thyroidal
   - Parathyroid Lesion
   - Medullary Carcinoma
   - Inadequate/Non-thyroid

2. NGS Analysis, DNA and RNA, 112 genes
3. Genomic Classifier
   - SNV
   - Indels
   - Gene Fusion
   - Gene Expression
   - CNV

- ThyroSeq
  - Negative
  - Positive
  - Detailed Genomic Findings
# ThyroSeq v3

<table>
<thead>
<tr>
<th>Bethesda category of cytology</th>
<th>Bethesda III</th>
<th>Bethesda IV</th>
<th>Bethesda III + IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cytology (95% CI)</td>
<td>cytology (95% CI)</td>
<td>cytology (95% CI)</td>
</tr>
<tr>
<td>No of cases</td>
<td>154</td>
<td>93</td>
<td>247</td>
</tr>
<tr>
<td>Disease Prevalence</td>
<td>23%</td>
<td>35%</td>
<td>28%</td>
</tr>
</tbody>
</table>

**ThyroSeq v3 performance:**

<table>
<thead>
<tr>
<th></th>
<th>Bethesda III</th>
<th>Bethesda IV</th>
<th>Bethesda III + IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>91% (77-97%)</td>
<td>97% (85-100%)</td>
<td>94% (86-98%)</td>
</tr>
<tr>
<td>Specificity</td>
<td>85% (77-90%)</td>
<td>75% (63-84%)</td>
<td>82% (75-87%)</td>
</tr>
<tr>
<td>PPV</td>
<td>64% (50-77%)</td>
<td>68% (54-80%)</td>
<td>66% (56-75%)</td>
</tr>
<tr>
<td>NPV</td>
<td>97% (92-99%)</td>
<td>98% (89-100%)</td>
<td>97% (93-99%)</td>
</tr>
</tbody>
</table>

ThyroSeq v3

Diagram:
- Indeterminate FNA Cytology
  - ThyroSeq
  - MTC, parathyroid, mets
  - Test Result:
    - Negative: No alterations
    - Currently Negative: LR alterations
    - Positive: RAS-like mutation CNA, GEA
    - Positive: BRAF-like alterations
    - Positive: HR alterations
  - Probability of Cancer or NIFTP:
    - 3-4%
    - <10%
    - 40-80%
    - 95-100%
    - 98-100%
  - Tumor type, risk of recurrence:
    - N/A
    - NIFTP or low-risk cancer
    - NIFTP or low-risk cancer
    - Intermediate-risk cancer
    - High-risk cancer
  - Individualized patient management:
    - Observation
    - Active surveillance
    - Lobectomy
    - Total thyroidectomy or lobectomy
    - Total thyroidectomy +/- LND

MTC - medullary thyroid carcinoma, LR - low risk, HR - high risk, CNA - copy number alterations, GEA - gene expression alterations, LND - lymph node dissection
CLINICAL HISTORY
FNA cytology: FN/SFN (Bethesda IV)

THYROSEQ® GC RESULTS SUMMARY
RIGHT UPPER THYROID FNA

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Probability of Cancer or NIFTP</th>
<th>Potential Management</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSITIVE</td>
<td>High (~99%)</td>
<td>Surgical excision*</td>
</tr>
</tbody>
</table>

*See interpretation below for details

INTERPRETATION
- BRAF V600E mutation was identified in this sample without other high-risk mutations.
- BRAF V600E is associated with a very high (~99%) probability of papillary thyroid carcinoma or related cancers.
- Risk of cancer recurrence associated with an isolated BRAF V600E mutation is intermediate for tumors >1cm and may be low for tumors <1cm.
- Surgical management may include total thyroidectomy or lobectomy, depending on tumor size and other clinical factors.
- Patient management decisions must be based on the independent medical judgment of the treating physician. Molecular test results should be taken into consideration in conjunction with all relevant imaging and clinical findings, patient and family history, as well as patient preference.

DETAILED RESULTS
Specimen cellularity/adequacy for interpretation: ADEQUATE

<table>
<thead>
<tr>
<th>Marker Type</th>
<th>Marker Result</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene mutations</td>
<td>BRAF</td>
<td>c.1799T&gt;A 23%</td>
</tr>
<tr>
<td>Gene fusions</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Gene expression</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Parathyroid</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Medullary/C-cells</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

AF=Variant Allele Frequency
ThygeNEXT/ThyraMIR

- First tests for DNA and RNA markers with high specificity using NGS
  - Also targets mutations with prognostic/therapeutic implications
- If negative, tests for miRNA
  - Non coding RNA implicated in gene expression regulation
  - Their expression profiles have been implicated in pathophysicsiology of cancer
<table>
<thead>
<tr>
<th>DNA mutation panel</th>
<th>RNA panel (# fusions)</th>
<th>ThyraMIR® miRNA classifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK</td>
<td>ALK (2)</td>
<td>miR-29b-1-5p</td>
</tr>
<tr>
<td>BRAF</td>
<td>BRAF (3)</td>
<td>miR-31-5p</td>
</tr>
<tr>
<td>GNAS</td>
<td>NTRK (8)</td>
<td>miR-138-1-3p</td>
</tr>
<tr>
<td>HRAS</td>
<td>PPARg (5)</td>
<td>miR-139-5p</td>
</tr>
<tr>
<td>KRAS</td>
<td>RET (14)</td>
<td>miR-146b-5p</td>
</tr>
<tr>
<td>NRAS</td>
<td>THADA (5)</td>
<td>miR-155</td>
</tr>
<tr>
<td>PIK3CA</td>
<td></td>
<td>miR-204-5p</td>
</tr>
<tr>
<td>PTEN</td>
<td></td>
<td>miR-222-3p</td>
</tr>
<tr>
<td>RET</td>
<td></td>
<td>miR-375</td>
</tr>
<tr>
<td>TERT</td>
<td></td>
<td>miR-551b-3p</td>
</tr>
<tr>
<td>mRNA controls:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKX2-1, PAX8, TBP, USP33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ThygeNext/ThyraMIR

- Can test from specimen in their transport media or from slides
- Claims 95% sensitivity and 90% specificity, with NPV of 97% and PPV of 75% with the combination testing (adjusted prevalence of disease, Lupo et al, 2020)
- Negative call rate 46% and moderate call rate 28% in recent clinical validation (Lupo et al, 2020)
- Test with less supporting literature
In practice, not all AUS are equal

- These two nodules have different pre-test probabilities of malignancy, both for the FNA and for molecular testing.
- If both have indeterminate cytology, a negative molecular test may NOT have the same NPV for each nodule.

In practice, not all AUS are equal

- AUS/FLUS cases with nuclear atypia - higher risk of PTC
- AUS/FLUS cases with architectural atypia only - lower risk of PTC
In practice - for the clinician

• What do I want?
  • Reassurance that the nodule is benign to avoid surgery?
    • Need a test with high sensitivity and high NPV
    • All commercially available claim to do this
  • Confirmation that it is malignant for definitive surgery?
    • Need a test that identifies high risk mutations

• What do I need?
  • Know the risk of malignancy in the indeterminate results I get
  • Other factors affecting the pre-test probability of malignancy
In practice - for the pathologist

- What is my proportion of indeterminate cases?
  - Am I dumping suspicious or positive cases in the indeterminate category?
    - Will decrease my NPV for molecular testing
  - Am I dumping negative cases in the indeterminates?
    - Some of those will get positive molecular test and then unnecessary surgery, will also increase costs.

- Do I have an idea of the risk of malignancy of my indeterminates?
Other uses for molecular

- **Prognosis:**
  - Coexistence of BRAF with PIK3CA, AKT1, TERT, or TP53 marker for increased aggressiveness
  - May use to select patients with microcarcinomas for surgery vs monitoring? (ATA 2015)

- **Diagnosis!**:
  - BRAF V600E mutation excludes NIFTP; maybe ETV6-NTRK3?
  - Mutations for which specific therapies are available (currently three FDA approved)
Considerations / take home notes

• Growing literature that molecular testing can help in triaging indeterminate thyroid nodules

• Specific higher risk mutations are now reported by most commercially available tests, but some require it to be requested

• Molecular tests are NOT perfect, false positives and false negatives do occur, correlate with other data, F/U patients according to guidelines

• As a rule, molecular tests should not be repeated in the same nodule (cytology in a previously tested nodule may, in certain circumstances)
Considerations / take home notes

• Patients requesting molecular in benign nodules
  • NPV of category II is 97%
  • Benign molecular will only add 2.5% certainty
  • But will have 32% false positives (at 68% specificity)

• Availability of molecular testing may increase the indeterminate dx by the pathologists, which will increase the false positive molecular results and number of surgeries

• Xpression Atlas of Afirma in category V or VI: a negative results does not mean benign pathology or reduced risk!

• Possibility of a NIFTP diagnosis, effect in different validation studies, patient education