Diagnosis and Management of Thyroid Nodules and Cancer Focus on Thyroglobulin
November 16, 2012

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Professor of Endocrinology and Pathology
55 Year-Old women with Thyroid Cancer

• Diagnosed with papillary thyroid cancer 15 years before and was treated with thyroidectomy and radiation therapy.
• Treated with thyroid hormone to keep TSH about 0.05-0.1.
• Tg antibody positive and Tg recovery was 35%
• Chest film yearly negative
• Refused RAI scans
• Tg by LC/MS/MS undetectable.
Objectives

• Know how to diagnose thyroid nodules
• Know the main causes of thyroid nodules
• Know risk factors of thyroid cancer
• List your approach to distinguish benign from malignant thyroid nodules.
• What is the role of thyroglobulin measurements in follow up of thyroid cancer?
Thyroid Hormone Biosynthesis
Thyroid
Goiter Thyroiditis
Hashimoto’s Thyroiditis
Thyroid Cancer
Papillary Thyroid Cancer
Thyroid Nodules and Cancer

• Epidemiology
• Risk Factors
• Diagnostic Procedures
• Staging
• Treatment
• Follow-up
• Survival
Thyroid Cancer and Nodules

• Thyroid nodules
  – Incidence, 5-8%
  – Thyroid cancer in 5-6.5% of nodules

• Thyroid cancer
  – 0.4% of all cancer deaths
The changing incidence of thyroid cancer as related to age. [Derived from (4)].

Rivkees S A et al. Endocrine Reviews 2011;32:798-826
Incidence Rates of Thyroid Cancer in the United States, 1980–2005

- **White Women**
- **White Men**
- **Black Women**
- **Black Men**

**Overall Thyroid Cancer Incidence Rates**
- White Women: 9.59 per 100,000
- White Men: 8.00 per 100,000
- Black Women: 5.28 per 100,000
- Black Men: 1.84 per 100,000

**Papillary Thyroid Cancer**
- White Women: 3.59 per 100,000
- White Men: 2.70 per 100,000
- Black Women: 1.16 per 100,000
- Black Men: 1.10 per 100,000

**Follicular Thyroid Cancer**
- White Women: 1.10 per 100,000
- White Men: 1.10 per 100,000
- Black Women: 1.10 per 100,000
- Black Men: 0.53 per 100,000

**Medullary Thyroid Cancer**
- White Women: 0.47 per 100,000
- White Men: 0.20 per 100,000
- Black Women: 0.10 per 100,000
- Black Men: 0.15 per 100,000

**Anaplastic Thyroid Cancer**
- White Women: 0.10 per 100,000
- White Men: 0.09 per 100,000
- Black Women: 0.07 per 100,000
- Black Men: 0.05 per 100,000

* Indicates significant difference
† Indicates trend towards significance

Clinical Thyroidology Volume 21 Issue 4 2009

Enewold et al Clin Thyroidology 21:6-9, 2009
Incidence Rates According to Tumor Size since 1992-1995

- Tumor ≤1.0 cm: 50%
- 1.1-2.0 cm: 30%
- >2 cm: 20%

Increased Incidence According to Tumor Size since 1988–1991 in White Women

<table>
<thead>
<tr>
<th>Category</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤1.0 cm</td>
<td>248 *</td>
</tr>
<tr>
<td>1.1 to 2 cm</td>
<td>106 *</td>
</tr>
<tr>
<td>1.1 to 5 cm</td>
<td>113 *</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>222 *</td>
</tr>
</tbody>
</table>

Enewold et al Clin Thyroidology 21:6-9, 2009
Papillary Thyroid Cancer Incidence Rates of Tumors Larger than 1 cm and Papillary Microcarcinomas in Women

Rates per 100,000 in Women

Macrocarcinomas

<table>
<thead>
<tr>
<th>Period</th>
<th>Rate (per 100,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1978–1985</td>
<td>0.87</td>
</tr>
<tr>
<td>1986–1993</td>
<td>2.19</td>
</tr>
<tr>
<td>1994–2001</td>
<td>4.52</td>
</tr>
</tbody>
</table>

Microcarcinomas

<table>
<thead>
<tr>
<th>Period</th>
<th>Rate (per 100,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1978–1985</td>
<td>0.14</td>
</tr>
<tr>
<td>1986–1993</td>
<td>0.81</td>
</tr>
<tr>
<td>1994–2001</td>
<td>3.94</td>
</tr>
</tbody>
</table>

Risks of Thyroid Cancer

• Age
• Children
• Adults: 20-60 years of age

Hereditary
  – Medullary
    • Ret oncogene
  – Papillary
Radiation Exposure Increases Thyroid Cancer Risk

- External irradiation of head and neck
- Radioactive fallout
- Children particularly at risk
- Screening of exposed persons is recommended vs. routine detection
- Screening includes scintiscans or ultrasound
- FNA Biopsy nodules >1cm or <1 cm if changes suggest cancer
FIG. 2. New cases of childhood (■) and adolescent (□) thyroid carcinoma in Belarus, registered yearly from 1986 to 1995.

Age Distribution Thyroid Cancer
Belarus Children

Spitzweg et al,
JCEM 86: 3327-35, 2001

Fig. 3. Age distribution of Belarus thyroid cancer patients at the time of the accident (1986). The black column (■) and the white column (□) represent patients diagnosed during childhood and during adolescence, respectively. The first column (<1 yr) includes nine patients who were in uterus at the time of the accident. The square bracket indicates the total number and percentage of thyroid cancer patients who were 5 yr old or less at the time of the accident.
# Oncogenes and TumorSuppressorGenes in Thyroid Tumors

<table>
<thead>
<tr>
<th>Neoplasm</th>
<th>Contributory genetic abnormalities</th>
<th>Genetic abnormalities of uncertain importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autonomously functioning thyroid nodule</td>
<td>TSH receptor-activating mutation; Gs-alpha mutation decreasing GTPase activity</td>
<td></td>
</tr>
<tr>
<td>Nodular goiter (colloid nodules)</td>
<td>Many nodules are monoclonal, but precise gene abnormalities are unknown</td>
<td></td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>RAS mutations</td>
<td>c-myc and c-fos overexpressed; PTEN abnormalities</td>
</tr>
<tr>
<td>Papillary thyroid carcinoma</td>
<td>RET rearrangements (RET/PTC); NTRK1 rearrangements (TRK)</td>
<td></td>
</tr>
<tr>
<td>Follicular thyroid carcinoma</td>
<td>PAX8-PPARgamma1 fusion</td>
<td></td>
</tr>
<tr>
<td>Follicular thyroid carcinoma</td>
<td>RAS mutations</td>
<td>Frequent loss of heterozygosity in genome of tumor</td>
</tr>
<tr>
<td>Anaplastic thyroid carcinoma</td>
<td>P53 mutations</td>
<td></td>
</tr>
<tr>
<td>Medullary thyroid carcinoma</td>
<td>RET activating mutations</td>
<td></td>
</tr>
</tbody>
</table>
Diagnostic Procedures

- Fine needle aspiration biopsy (FNA)
  - Improves FNA diagnostic accuracy, particularly in technically difficult lesions or cystic-solid lesions
- Ultrasound
  - Improves FNA diagnostic accuracy, particularly in technically difficult lesions or cystic-solid lesions
- Scintiscan
  - Useful for patients who are hyperthyroid
Fig. 1. Comparison between results of clinical palpation and ultrasound scanning (USS) in detection of thyroid nodules in 420 study patients. Patients were subclassified into three categories: with no thyroid nodule (group A), with a single thyroid nodule (group B), or with a multinodular goiter (group C).

Deandrea et al Endocrine Pract. 8:282, 2002
Thyroid Incidentalomas

- 1-1.5 cm nodule and risk factors-biopsy
- Cancer detected in 7 of 119 >1 cm
- 5% of 450 nonpalpable nodules have cancer
- Solid, hypoechoic nodules increase likelihood of cancer
- Microcalcification increases risk of cancer to 29%

Leenhardt et al JCEM 84:24, 1999; Hagag et al Thyroid 8:989, 1998
Thyroid Ultrasound (US)

• Sensitivity, 2-3 mm
• Palpable nodule, 20-40% have additional nodules detected by US
• 20% have a nodules<1cm, not recommended for FNA unless hypoechoic or microcalcification is observed
• Nodules <1cm, 30% have non-diagnostic cytology
Thyroid Ultrasound’s Detection of Nonpalpable Thyroid Nodules

Nonpalpable recurrent papillary thyroid carcinoma Sonogram of the right thyroid lobe in the longitudinal plane from a patient who had had a left lobectomy for papillary thyroid carcinoma. Although the physical examination was normal, the sonogram shows a 8.6 mm hypoechoic nodule (arrow) that represented tumor. L = thyroid lobe. Courtesy of Manfred Blum, MD.
Benign Thyroid Epithelium
Psammoma body  Fine needle aspirate of a papillary carcinoma of the thyroid showing a psammoma body. The laminations can best be appreciated under the microscope by moving the depth of focus.
Follicular Epithelial Pattern, Adenoma
FNA Cytologic Results

- Nondiagnostic
- Benign
  - Macrofollicular
  - Colloid adenomas
  - Thyroiditis
- Suspicious or indeterminate
  - Microfollicular or follicular neoplasms
- Malignant
  - Repeat with US guidance
  - Follow
  - Surgical excision
  - Total thyroidectomy, RAI, T4
  - Follow thyroglobulin
Preoperative Diagnosis of Benign Thyroid Nodules with Indeterminate Cytology


N Engl J Med
Volume 367(8):705-715
August 23, 2012
Study Overview

• A significant fraction of fine-needle aspirates obtained from thyroid nodules are read as indeterminate.

• A new molecular test accurately predicts whether a cytologically indeterminate nodule is benign 93% of the time, permitting a conservative approach to management.
Indeterminate Cytology (Veracyte)

- 15-30% FNAs indeterminate
- Expression 167 genes
- 85/265 indeterminate malignant
- 78/85 gene-expression classifier correctly identified malignancy
- Sensitivity 92% (CL 84-97%)
- Negative predictive value, 85-95%

Conclusions

• These data suggest consideration of a more conservative approach for most patients with thyroid nodules that are cytologically indeterminate on fine-needle aspiration and benign according to gene-expression classifier results.
TABLE 1. Cytological and molecular results of ultrasonographic guided FNAB for the patients with thyroid nodules

<table>
<thead>
<tr>
<th>Cytological diagnoses</th>
<th>BRAF^{V600E} (+)</th>
<th>BRAF^{V600E} (-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>0 (0%)</td>
<td>504 (100%)</td>
<td>504 (58.5%)</td>
</tr>
<tr>
<td>ACUS</td>
<td>45 (31.9%)</td>
<td>96 (68.1%)</td>
<td>141 (16.3%)</td>
</tr>
<tr>
<td>Suspicious for FN</td>
<td>1 (10%)</td>
<td>9 (90%)</td>
<td>10 (1.2%)</td>
</tr>
<tr>
<td>Suspicious for malignancy</td>
<td>46 (85.2%)</td>
<td>8 (14.8%)</td>
<td>54 (6.2%)</td>
</tr>
<tr>
<td>Malignant</td>
<td>129 (92.1%)</td>
<td>11 (7.9%)</td>
<td>140 (16.2%)</td>
</tr>
<tr>
<td>Nondiagnostic</td>
<td>NA</td>
<td>NA</td>
<td>16 (1.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>221 (25.5%)</td>
<td>644 (74.5%)</td>
<td>865 (100%)</td>
</tr>
</tbody>
</table>

FN: Follicular neoplasm; NA: not available.

ACUS = atypical cells of undetermined significance

Kim et al JCEM 96: 658-64, 2011
<table>
<thead>
<tr>
<th></th>
<th>Cytology</th>
<th>BRAF&lt;sup&gt;V600E&lt;/sup&gt; mutation</th>
<th>Cytology and BRAF&lt;sup&gt;V600E&lt;/sup&gt; mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>100</td>
<td>89.6</td>
<td>89.6</td>
</tr>
<tr>
<td>Specificity</td>
<td>36.4</td>
<td>95.5</td>
<td>95.5</td>
</tr>
<tr>
<td>PPV</td>
<td>92.9</td>
<td>99.4</td>
<td>99.4</td>
</tr>
<tr>
<td>NPV</td>
<td>100</td>
<td>52.5</td>
<td>52.5</td>
</tr>
<tr>
<td>Accuracy</td>
<td>93.3</td>
<td>90.2</td>
<td>90.2</td>
</tr>
<tr>
<td>$\kappa$ value</td>
<td>0.51 ± 0.11</td>
<td>0.63 ± 0.07</td>
<td>0.63 ± 0.07</td>
</tr>
</tbody>
</table>

The permanent pathological diagnosis was used as a reference test to calculate each parameter.

Kim et al JCEM 96: 658-64, 2011
Approximately 5% of siblings of patients are also diagnosed with Thyroid Cancer.

Table 2. Stage of the Tumors Discovered by Ultrasonographic Screening of Siblings of Patients with Papillary Thyroid Cancer

<table>
<thead>
<tr>
<th></th>
<th>T1a (tumor ≤1 cm)</th>
<th>T1b (tumor &gt;1 cm and ≤2 cm)</th>
<th>T3 (tumor with extrathyroid invasion)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx</td>
<td>21</td>
<td>6</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>N1a</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>N1b</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>9</td>
<td>9</td>
<td>43</td>
</tr>
</tbody>
</table>

*a*Lymph node dissection was only performed in the case of a pre- or perioperative suspicion of metastasis, not prophylactically.

Rosario et al Thyroid 22: 805-8, 2012
# Thyroid Cancer Staging

## TNM Classification System for Differentiated Thyroid Carcinoma

### Definition

- **T1**: Tumor diameter < 1 cm
- **T2**: Primary tumor diameter 1-4 cm
- **T3**: Primary tumor diameter > 4 cm
- **T4**: Primary tumor invasion beyond the thyroid gland capsule
- **TX**: Primary tumor size unknown, but without extrathyroidal invasion

- **N0**: No metastatic nodes
- **N1a**: Ipsilateral cervical node metastases
- **N2b**: Bilateral, midline, contralateral, or mediastinal node metastases
- **NX**: Nodes not assessed at surgery

- **M0**: No distant metastases
- **M1**: Distant metastases
- **MX**: Distant metastases not assessed

### Stages

<table>
<thead>
<tr>
<th>Stage</th>
<th>Patient age &lt;45 years</th>
<th>Patient age 45 years or older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Any T, any N, M0</td>
<td>T1, NO, MO</td>
</tr>
<tr>
<td>Stage II</td>
<td>Any T, any N, M1</td>
<td>T2, NO, MO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3, NO, MO</td>
</tr>
<tr>
<td>Stage III</td>
<td></td>
<td>T4, NO, MO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Any T, N1, M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td></td>
<td>Any T, any N, M1</td>
</tr>
</tbody>
</table>
Screening compared to Routine Detection of Thyroid Cancer

• High risk populations
  – Radiation fallout
  – Head and neck irradiation
  – Family History
Prognosis: Recurrence & Cancer Death

Treatment and Management of Thyroid Cancer

- Treatment
  - Surgery
  - Radioidine
  - TSH-suppressive thyroid hormone therapy

- Monitoring
  - Serum thyroglobulin measurement
  - Whole-body $^{131}$I scanning


# Initial American Thyroid Association risk of recurrence classification

<table>
<thead>
<tr>
<th>Low risk</th>
<th>Intermediate risk</th>
<th>High risk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All of the following are present:</strong></td>
<td><strong>Any of the following is present:</strong></td>
<td><strong>Any of the following is present:</strong></td>
</tr>
<tr>
<td>No local or distant metastases</td>
<td>Microscopic invasion into the perithyroidal soft tissues</td>
<td>Macroscopic tumor invasion</td>
</tr>
<tr>
<td>All macroscopic tumor has been resected</td>
<td>Cervical lymph node metastases or $^{131}$I uptake outside the thyroid bed on the post-treatment scan done after thyroid remnant ablation</td>
<td>Incomplete tumor resection with gross residual disease</td>
</tr>
<tr>
<td>No invasion of locoregional tissues</td>
<td>Tumor with aggressive histology or vascular invasion (eg, tall cell, insular, columnar cell carcinoma, Hurthle cell carcinoma, follicular thyroid cancer)</td>
<td>Distant metastases</td>
</tr>
<tr>
<td>Tumor does not have aggressive histology (eg, tall cell, insular, columnar cell carcinoma, Hurthle cell carcinoma, follicular thyroid cancer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No vascular invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No $^{131}$I uptake outside the thyroid bed on the post-treatment scan, if done</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reproduced with permission from: Tuttle RM, Tala H, Shah J, et al. Estimating risk of recurrence in differentiated thyroid cancer after total thyroidectomy and radioactive iodine remnant ablation: using response to therapy variables to modify the initial risk estimates predicted by the new American Thyroid Association staging system. Thyroid 2010; 20:1341. Copyright © 2010 Mary Ann Liebert, Inc.
Logistic Analysis of Predictive Factors for Disease Recurrence

With Recurrence (N = 48)  Without Recurrence (N = 420)

<table>
<thead>
<tr>
<th>Factor</th>
<th>With Recurrence</th>
<th>Without Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age (yr)</td>
<td>32</td>
<td>40</td>
</tr>
<tr>
<td>Tg After Primary Surgery (%)</td>
<td>70.8</td>
<td>53.6</td>
</tr>
<tr>
<td>Tg Detectable after RRA (%)</td>
<td>36.4</td>
<td>9.8</td>
</tr>
<tr>
<td>Nodal Metastases (%)</td>
<td>29.2</td>
<td>11.2</td>
</tr>
<tr>
<td>Local Tumor Invasion (%)</td>
<td>20.8</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Clinical Thyroidology Volume 22 Issue 10 2010

Radioiodine ablation reduces recurrence and mortality in stage II and stage III thyroid cancer. Long-term development of recurrent disease (left panel) or death (right panel) from thyroid cancer in patients without distant metastases at presentation, who received either 131-I ablation (red dashed lines) or no ablation (blue solid lines). (Data from Mazzaferri, EL, Jhiang, SM, Am J Med 1994; 97:418.)
Clinopathologic Factors Associated with Recurrent Disease

Hazard Ratio (HR)

- Tumor size ≥ 4 cm
- Extrathyroidal Extension
- Positive TgAB2
- Shorter Disease Free Survival

Fig. 3

Clinical Thyroidology Volume 21 Issue 1 2009

The Association of Recurrent/Persistent Disease with Changing TgAb2 Values

Percent with persistent disease

Group 1
TgAb2 Decline
>50%

Group 2
TgAb2 Decline
<50%

Group 3
TgAb2 Increase

37*

Clinical Thyroidology Volume 21 Issue 1 2009

Recombinant Human TSH Use in Treatment of Thyroid Cancer

- rh-TSH can be used in patients on thyroid hormone
- Tg response can be measured
- Radioiodine therapy can be administered following its stimulation of thyroid tissue
- Are the results as good as with withdrawal of thyroid hormone and RAI therapy?
• Post thyroidectomy and radioiodine therapy serum Tg should be unmeasureable
• Thyroid hormone therapy will reduce levels in hormone responsive tissue, benign or cancerous
• Less well differentiated thyroid cancer may not produce Tg
• Antithyroglobulin antibodies interfere with Tg measurements by IA and results must be interpreted with caution and may be unreliable
Main Points of Thyroid Cancer Treatment

• Early treatment is critical to outcome
• Main location of disease: neck and lung
• Best detected by Tg under TSH stimulation
  – T4 withdrawal Tg > 10 ng/ml
  – rh-TSH Tg > 2 ng/ml
• Follow up whole body scan less useful
• Post-therapy (high dose) whole body scans are best
Thyroid Hormone’s Effect on Serum Thyroglobulin

Fig. 2. Relationship between the results of the $^{131}$I posttherapy WBS and the individual values of serum Tg both off and on L-T$_4$ before therapy. N.D., Not detectable.

Pacini et al JCEM 86:4092-97, 2001
**Figure 2**

Kloos et al JCEM 90:5047-57, 2005
Figure 3

Kloos et al JCEM 90:5047-57, 2005
<table>
<thead>
<tr>
<th>Tumor Positive</th>
<th>Tumor Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg&gt;2 N=20</td>
<td></td>
</tr>
<tr>
<td>16 TP</td>
<td>4 FP</td>
</tr>
<tr>
<td>PPV 80%</td>
<td></td>
</tr>
<tr>
<td>Tg≤2 N=87</td>
<td></td>
</tr>
<tr>
<td>2† FN</td>
<td>85 TN</td>
</tr>
<tr>
<td>NPV 98%</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity: 89%
Specificity: 96%

<table>
<thead>
<tr>
<th>Tumor Positive</th>
<th>Tumor Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg&gt;0.5 N=5</td>
<td></td>
</tr>
<tr>
<td>4 TP</td>
<td>1 FP</td>
</tr>
<tr>
<td>PPV 80%</td>
<td></td>
</tr>
<tr>
<td>Tg≤0.5 N=102</td>
<td></td>
</tr>
<tr>
<td>14† FN</td>
<td>88 TN</td>
</tr>
<tr>
<td>NPV 86%</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity: 22%
Specificity: 99%

---

Figure 4
Kloos et al JCEM 90:5047-57, 2005
Summary

- Thyroid nodules are common
  - About 10% > 1 cm are malignant
- Thyroid cancer is more common in women
- Thyroid FNA and Ultrasound are most useful in diagnosis
- Staging correlates with prognosis
- Early treatment reduces risk of recurrence and death
Summary (continued)

• Tg measurement is highly useful in follow up for recurrence, particularly after rh-TSH or withdrawal from thyroid hormone

• Triple therapy
  – Includes near total thyroidectomy, RAI and TSH suppression with T4
  – greatly reduces recurrence rates and death.
Triple Antibody Tg Assay

• Antisera to three epitopes was produced
• Rationale binding to epitope site not used by AntiTgAb
• Assay compared well with other Tg assays
• Did not overcome the AntiTgAb interference
Quantitative Real Time RT-PCR Assay for the Detection of Thyroid Specific mRNAs

Cindy Meadows
Supervisor Molecular Hematopathology & Genetics
ARUP Laboratories
Thyroglobulin and thyroid cancer: part 2 of presentation: analytical method and performance

Alan L. Rockwood, Mark M. Kushnir, A. Wayne Meikle
Pathology Grand Rounds
University of Utah
16 November 2012
Learning objectives for part 2

• Relevant concepts of tandem mass spectrometry and liquid chromatography-tandem mass spectrometry (LC-MS/MS)
• Use of peptide generated by tryptic digestion of thyroglobulin as surrogate for Thyroglobulin analysis (SISCAPA technique)
• Application of these principles to analysis of thyroglobulin
Thyroglobulin

- Thyroglobulin is dimer-protein
- Molecular weight: 660,000
- Thyroglobulin synthesized only in thyroid
- 19 epitopes for antibody binding
Thyroglobulin Autoantibodies (Tg-AAb) Interfere with Measurement of Tg

- Currently immunoassays (IAs) are the only methodology for Tg testing
- Immune based diagnostic tests for Tg use capture and detection antibodies
- IAs work for samples from patients who don’t have Tg-AAb
- Presence of Tg-AAb causes false-negative results in Tg immunoassays

http://www.medscape.com
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http://www.medscape.com
Mass Spec Concepts
What a Mass Spectrometer Measures

• Mass to charge ratio \((m/z)\), or roughly speaking the molecular weight
  – What is the compound?

• Relative abundance
  – How much is there?
Tandem Mass Spectrometer

MS 1

MS 2

Precursor ion
Fragmentation (CAD)
Product ion
Tandem Mass Spectrometer

MS 1

Fragmentation (CAD)

MS 2

Product ion

Precursor ion
Tandem Mass Spectrometer

MS 1

Precursor ion

Fragmentation (CAD)

Product ion

MS 2
Tandem Mass Spectrometer

MS 1

Precursor ion

Fragmentation (CAD)

Product ion

MS 2
Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion

m/z, Da

Intensity, cps

100 200 300 400 500 600 700 800 900 1000 1100 1200

Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion: 636.3 Da


Intensity, cps

m/z, Da
Product Ion Mass Spectrum of Tg-specific Peptide
Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion

m/z, Da

Intensity, cps


Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion

Intensity, cps

m/z, Da


Y5

185.3
213.0
280.5
339.4
355.3
541.3
530.2
612.3
726.1
1059.3
142.1
155.0
155.2
169.9
186.0
232.8
306.0
341.4
354.1
797.4
311.3
592.2
574.6
518.1
502.1
599.7
714.5
225.3
245.3
296.0
280.5
541.3
530.2
612.3
726.1
1059.3
120.1
158.0
225.3
311.3
120.1
186.0
232.8
306.0
341.4
354.1
797.4
311.3
592.2
574.6
518.1
502.1
599.7
714.5
225.3
245.3
296.0
280.5
541.3
530.2
612.3
726.1
1059.3
120.1
158.0
225.3
311.3
Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion

m/z, Da

Intensity, cps


Y₄

m/z, Da

100  200  300  400  500  600  700  800  900  1000  1100  1200

636.3

185.3  213.0  280.5  339.4

355.3  541.3

530.2

1059.3

912.5

797.4
Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion

636.3

m/z, Da

Intensity, cps


y3

1059.3

Product Ion Mass Spectrum of Tg-specific Peptide

Precursor ion

m/z, Da
Intensity, cps


100  200  300  400  500  600  700  800  900  1000  1100  1200
Adding a liquid chromatograph to a tandem mass spectrometer

- Molecules characterized by three physical properties
  - Chromatographic retention time
  - Parent ion mass
  - Daughter ion mass
Adding a liquid chromatograph to a tandem mass spectrometer

- Molecules characterized by three physical properties
  - Chromatographic retention time
  - Parent ion mass
  - Daughter ion mass

- Even more selective for quantitative analysis than tandem mass spectrometry alone
Digestion by trypsin
Trypsin cuts proteins at Arg and/or Lys residues
Trypsin cuts proteins at Arg and/or Lys residues

Products are peptides of specific compositions
Tryptic Peptides from Proteins

Protein $\xrightarrow{\text{trypsin}}$ Peptides

A selected tryptic peptide can act as a surrogate marker for the protein
• Tryptic digest of Thyroglobulin produces more than 400 peptides
Tryptic Digest of Thyroglobulin

- Tryptic digest of Tg produces more than 400 peptides
- One or more peptides selected and measured as surrogate for Tg
Tryptic Digest of Thyroglobulin

- Tryptic digest of Tg produces more than 400 peptides
- One or more peptides selected and measured as surrogate for Tg

Sequence: VIFDANAPAVR
Tryptic Digest of Thyroglobulin

• Tryptic digest of human serum contains over 14,000,000 different peptides

• Concentration of Tg peptides in tryptic digest of human serum billion fold lower than most abundant peptides

• Affinity purification using anti-peptide antibody (SISCAPA)

← Needle in a haystack
SISCAPA
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

Destroys antibodies
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

1. Sample Creation
   - Protein Sample
     - Digest

2. Standardization
   - Peptides
     - Load
       - Antibody Support
         - Elute
           - LC-MS/MS

3. Enrichment
   - Wash
     - Unbound Peptides

4. Detection

   Produces Tg-specific peptides
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

Peptides serve as surrogates for thyroglobulin
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

Internal standard added to sample
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

Internal standard compensates for variability in processing and detection
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

We swap the this sequence by adding internal standard before digesting sample
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

We swap the this sequence by adding internal standard before digesting sample
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

Antibody specifically targeted for one peptide extracts and purifies the targeted peptide
SISCAPA: using tryptic digests and LC-MS/MS to measure protein concentration

Purified peptide undergoes quantitative analysis by liquid chromatography-tandem mass spectrometry.
Earlier, Hoofnagle developed MS-based method at U of Washington

– Insufficiently sensitive for quantitation of Tg at concentrations representative of recurrence of TC

– Low throughput

– Uses nano-HPLC separation

Until recently, MS based methods for Tg were not generally available in clinical laboratories
LC-MS/MS, new method overview

- Tg enrichment/depletion of unrelated proteins
- Every step of sample preparation optimized for enhanced sensitivity/specificity

![Diagram showing the steps of the method:]

1. 500 uL of serum
2. Tg enrichment/depletion
3. Denature/reduce proteins
4. Digest proteins
5. Affinity purification of peptide SISCAPA
6. 2D LC-MS/MS
LC-MS/MS, new method – novel aspect

- Tg enrichment/depletion of unrelated proteins
  - Rabbit anti-thyroglobulin antibody added (if you can’t lick ‘em, join ‘em)
  - Precipitation of IgG and IgG-bound thyroglobulin using ammonium sulfate
  - Rinse precipitate and re-dissolve

- Tg enrichment/depletion

- 500 uL of serum
- Denature/reduce proteins
- Digest proteins
- Affinity purification of peptide SISCAPA
- 2D LC-MS/MS
LC-MS/MS, new method – novel aspect

- Isotopically labeled internal standard added prior to digestion
  - “Winged” peptide
    - xxxx-VIFDANAPVAVR-xxxx
  - Winged peptide helps control for recovery during digestion

500 uL of serum
Tg enrichment/depletion
Denature/reduce proteins
Digest proteins
Affinity purification of peptide SISCAPA
2D LC-MS/MS
Method performance
Chromatogram of Tg-specific Peptide in Patient Sample Containing 5 ng/mL of Tg

Tg peptide

Internal standard

Clean chromatograms
No surprises here.
We like boring calibration curves.
**Imprecision**

<table>
<thead>
<tr>
<th>Tg concentration, ng/mL</th>
<th>Within-run CV, %</th>
<th>Between-run/day CV, %</th>
<th>Total CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>6.75</td>
<td>3.67</td>
<td>7.69</td>
</tr>
<tr>
<td>5.7</td>
<td>6.87</td>
<td>5.96</td>
<td>9.10</td>
</tr>
<tr>
<td>14.8</td>
<td>6.56</td>
<td>5.40</td>
<td>8.50</td>
</tr>
<tr>
<td>399</td>
<td>3.56</td>
<td>1.71</td>
<td>3.95</td>
</tr>
</tbody>
</table>

CVs are quite good, especially considering complexity of method.
LC-MS/MS: Our Method vs. University of Washington Method for Tg-AAb Positive and Tg-AAb Negative Samples

Thyroglobulin method comparison (AAb negative samples)

\[ y = 1.17x - 1.81 \]

\[ S_{y/x} = 8.14 \]

\[ r = 0.951 \]

Thyroglobulin method comparison (AAb positive samples)

\[ y = 1.23x + 0.15 \]

\[ S_{y/x} = 0.475 \]

\[ r = 0.916 \]
Our LC-MS/MS Method vs. Access® Beckman Coulter Method for Tg-AAb Negative Samples

\[ y = 1.01x + 1.67 \]
\[ r = 0.974 \]

\( N = 73 \)
Our LC-MS/MS Method vs. Access® Beckman Coulter Method for Tg-AAb Positive Samples

\[ N = 105 \]
Our LC-MS/MS Method vs. Access® Beckman Coulter Method for Tg-AAb Positive Samples
Conclusions

• Serum Tg is the best marker for follow-up of patients with Differentiated Thyroid Carcinoma
• No commercial immunoassays for measuring Tg in samples of patients positive for Tg-AAb
• This LC-MS/MS method allows accurate measurement of Tg in presence of Tg-AAb
• Sensitivity of this method is likely adequate for detection of the recurrence of thyroid cancer
• Method is in routine use for testing autoantibody positive samples (>3000 samples so far)
Acknowledgements

• Andy Hoofnagle: External collaborator on this project and developer of an earlier related method for thyroglobulin

• William Roberts: to whom this presentation is dedicated, for many encouraging and helpful discussions as well has keen insights into method development for clinical pathology