Use of Molecular Markers for Cytologically Indeterminate Thyroid Nodules to Optimize Surgical Management

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Abstract The management of thyroid nodules with indeterminate cytology has traditionally been diagnostic thyroidectomy. However most nodules are benign, and for nodules that demonstrate histologic malignancy, two-stage thyroidectomy may be necessary. Molecular markers have emerged as an adjunct to preoperative FNAB cytology evaluation that can improve diagnostic accuracy. In this review, we discuss the latest methodologies and review diagnostic performance parameters of recent molecular marker techniques.

Keywords Thyroid nodule · Thyroid cancer · Thyroidectomy · Molecular markers · Mutation testing · Indeterminate biopsy · Surgical management · MicroRNA · Rearrangements

Introduction

Ultrasound (US) and fine-needle aspiration biopsy (FNAB) are the diagnostic tests currently used in thyroid nodule evaluation with the goal of accurately identifying malignancy. Thyroid cancer is rarely missed when cytology is classified as benign utilizing the six-tiered Bethesda System for Reporting Thyroid Cytopathology [1]. However, 20–25 % of FNAB cytology results are classified into one of the indeterminate Bethesda categories: atypia or

follicular lesion of undetermined significance (AUS/FLUS), follicular neoplasm (FN) or suspicious for FN, and suspicious for malignancy [2]. In a recent meta-analysis of 4,212 nodules with cytohistological correlation, the risk of malignancy in the AUS/FLUS, FN, and suspicious categories was 16, 26, and 75 %, respectively [3]. Although repeat FNAB may be helpful in nodules with lower risk AUS/FLUS FNAB results, histology is often necessary for conclusive diagnosis.

Follicular-variant papillary thyroid cancer (FV-PTC) and follicular carcinoma (FTC) are the most common malignancies associated with indeterminate FNAB results [4•, 5–7], and both are difficult to diagnose even by histology [8, 9]. FTC requires capsular and/or vascular invasion, which is not readily apparent on FNAB cytology analysis. FVPTC is the second most common variant of PTC and is associated with the multifocal and heterogeneous distribution of the characteristic nuclear features (elongation, enlargement, chromatin clearing, intranuclear grooves, and inclusions) [9]. FVPTC is most frequently the source of false-negative FNAB cytology results and intraoperative frozen section analysis [10, 11]. To further complicate interpretation of diagnostic testing, interobserver variability is up to 30 % among expert thyroid pathologists [12].

Total or near-total thyroidectomy is the recommended definitive procedure for differentiated thyroid cancer >1 cm [2], which results in permanent hypothyroidism yet is associated with a low complication rate particularly when performed by high-volume surgeons [13, 14]. The alternative surgical approach is diagnostic lobectomy, which has a lower rate of postoperative morbidity and may preserve euthyroidism; however, completion thyroidectomy (i.e., two-stage surgery) is necessary if histologic cancer is confirmed.

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To improve preoperative diagnostic discrimination within the indeterminate cytology category, a number of adjunct imaging modalities have been studied. Nodule features on US that are associated with malignancy include marked hypoechogenicity, taller-than-wide shape, microcalcifications, an irregular or spiculated margin, and mostly solid composition [15]. In a series of 180 indeterminate FNAB results with a high histologic malignancy rate of 51 %, the presence of ≥ 2 suspicious US features was associated with a 72 % risk of cancer [16]. On the other hand, 18F-FDG-PET/CT did not appear to add any diagnostic benefit in 56 nodules with indeterminate FNAB results and a 22 % malignancy rate [17]. Elastography uses US to quantify nodule compressibility by measuring shear wave propagation through the nodule [18], and it can identify malignancy with sensitivity of 80 % and specificity of 91 % [19]. Although this modality has not yet been tested in nodules with indeterminate FNAB cytology, its noninvasiveness is appealing for routine clinical use. Another noninvasive imaging technique is resonance-frequency-based electrical impedance spectroscopy (REIS), which measures electrical conductivity and capacitance differences in malignant compared to non-malignant adjacent tissue. In a recent pilot study, REIS was feasible and predicted malignant nodules with a sensitivity of 85 % and a modest specificity of 61 % [20]. However, additional study is still necessary before the technique's suitability for widespread use can be determined.

Frozen section evaluation has also been a traditional method of intraoperative pathologic evaluation in an attempt to achieve appropriate definitive initial surgery. However, with current high yield diagnostic testing including routine preoperative US-guided FNAB, the utility has diminished [21, 22], and it rarely productively alters surgical management [23]. Although practice patterns and some clinical scenarios may still direct its use, the diagnostic limitations and high costs do not support routine utilization during initial thyroid lobectomy [24, 25].

Molecular markers have emerged as a useful preoperative diagnostic adjunct, although there is currently no single test that can meet all three goals of preoperative nodule evaluation: (1) reliably identifying those nodules likely to be benign, (2) not missing significant malignancies, and (3) directing the optimal definitive initial surgical procedure.

Protein-Based Markers

Immunocytochemical (ICC) analysis of markers expressed in PTC can be helpful in risk stratifying indeterminate FNAB results, is less costly than other forms of marker testing, and uses resources that are readily available in any pathology laboratory. The three most commonly used

markers are cytokeratin-19 (CK-19), galectin-3 (Gal-3), and Hector Battifora mesothelial-1 (HBME-1), all of which have higher expression in differentiated thyroid cancers compared to benign lesions. In a recent meta-analysis, HBME-1 was the most studied ICC marker, although Gal-3 had the highest sensitivity (85 %) and specificity (90 %) for malignancy [26]. Gal-3 as an adjunct to FNAB cytology was evaluated in a multi-institutional study including 465 FNAB samples that were classified as Thy3 (FN or suspected FN) by the British Thyroid Association Guidelines [27] with a 28 % malignancy rate [28]. False-negative staining occurred in 9 % and included FVPTC, FTC, oncocytic-variant FTC, and poorly differentiated thyroid cancer. False-positive staining occurred in 25 % with an overall accuracy of 88 % [28]. Thus, using Gal-3 ICC analysis alone to exclude cancer or guide surgery is likely insufficient.

Using panels with more than one marker may increase the utility of ICC. In a small series of 115 indeterminate FNAB specimens evaluated by HBME-1 and CK-19, there were no false-negative ICC results. However, four false-positive cases occurred, and specificity was 85 %. Moreover, inconclusive ICC results were obtained in 38 % of FNAB specimens, further limiting the described technique [29]. Additional limitations of ICC are that accurate results are reliant on obtaining enough cells by FNAB, and the test is best performed on formalin-fixed, paraffin-embedded preparations, which are not the current standard method for routine cytology smears used for diagnosis.

MicroRNA Expression Analysis

MicroRNAs (miRNA) are small, non-coding, single-strand RNAs that can regulate gene expression. Dysregulation of miRNA has been associated with a number of human malignancies including leukemia, glioblastoma, breast cancer, and melanoma [30]. Differential miRNA expression can be seen in thyroid malignancies, is associated with histologic subtypes, may vary by tumor aggressiveness, and can be detected in FNAB specimens. miRNA expression patterns have been evaluated recently in FVPTC and FTC, the two histologies most often associated with indeterminate FNAB results. Dysregulation of miR-885-5p, -221, and -574-3p was identified in conventional and oncocytic FTC compared to normal thyroid tissue, and in a preliminary series of 19 FNAB specimens, analysis of these miRNAs was able to diagnose FTC with 100 % accuracy [31]. In another study of miRNA expression patterns using microarray analysis, FVPTC compared to classic PTC was characterized by dysregulation of miR-125a, -3p, -1271, and -153 [32].

Table 1 Analysis of miRNA expression patterns in indeterminate FNAB results

	miRNA panel identified by screening?	miRNAs evaluated in FNAB specimens	No. of indeterminate FNAB specimens	Malignancy rate (%)	Spec	Sens	Acc
Keutgen et al. [35]	y	-222, -328, -197, -21	72	31	86	100	90
Shen et al. [34]	y	-146b, -221, -187, -30d	30 ^a	37	78	89	85
Mazeh et al. [36]	n	-21, -31, -146b, -187, -221, -222	12	75	100	89	90

^a Only results in the atypia of undetermined significance were included in the study

miRNA analysis of thyroid lesions was evaluated by Nikiforova et al. [33] by initial screening of 62 benign and malignant thyroid lesions with an assay of 158 human miRNAs, and differential expression patterns were observed according to histology. Furthermore, varying patterns were present among histologic subtypes and in tumors with different oncogenic mutations. When 13 FNAB specimens were evaluated using a panel of seven selected miRNAs (miR-187, -221, -222, -146b, -155, -224, and -197) and expression patterns correlated to histology, upregulation of three or more miRNAs was predictive of malignancy with 100 % specificity, 88 % sensitivity, and 98 % accuracy [33]. miRNA expression that could further augment diagnostic FNAB analysis has been further explored in three studies (Table 1). Each of the studies (including the initial study by Nikiforova et al.) used a different miRNA panel, although miR-146b, miR-221, and miR-222 were evaluated in two of the three studies [34–36]. Thus far in these small studies, microRNA analysis shows some promise in improving preoperative diagnostic discrimination, but whether these markers will readily translate into cost-effective routine use remains to be seen in larger prospective studies.

Multigene Expression Panels

Identifying gene expression patterns to differentiate benign from malignant nodules has been another studied methodology with mixed results. In a novel approach, immunohistochemical analysis was used in 173 thyroid specimens to evaluate the protein expression of ten genes, and a three-gene panel with pronounced differential expression in malignant tumors (*HMGA2*, *MRC2*, *SFN*) was identified and analyzed in 95 FNAB specimens by quantitative reverse transcriptase-polymerase chain reaction [37]. Indeterminate cytology was present in a subset of 27 FNAB specimens, and the three-gene panel predicted malignancy with 60 % sensitivity, 91 % NPV, 96 % specificity, and 75 % PPV [37].

To improve the NPV in of FNAB testing, >240,000 gene and exon transcripts were screened in 178 thyroid samples, and the expression of 167 genes was further identified as being able to reclassify nodules with

indeterminate cytology as potentially benign. The initial validation cohort included only 24 indeterminate FNAB samples, and a specificity of 73 % and sensitivity of 100 % were observed [38]. The performance of the commercially available gene-expression classifier (GEC) was recently evaluated in a study of 265 nodules with indeterminate FNAB results and compared to histology [39•]. The rate of histologic malignancy was 32 %, and the panel had a sensitivity of 92 %, NPV 93 %, specificity 52 %, and PPV 47 %. A total of 7/85 (8 %) malignancies were missed by the GEC panel; 4 were papillary (size range 0.6–1.2 cm), 2 were FVPTC (size range 1–3 cm), and 1 was a 3.5-cm oncocytic carcinoma [39•]. In the subset of nodules with AUS/FLUS and FN cytology results, the NPV was 95 and 94 %, respectively. Thus, the GEC panel reduced but did not altogether eliminate the risk of malignancy. Furthermore, the panel did not decrease the malignancy risk to being equal to a benign FNAB result, which was 3.7 % in a meta-analysis with cytohistological correlation [3]. An unreliable NPV of 85 % was reported in nodules with suspicious FNAB results, precluding GEC use for nodules with these cytology results. Moreover, the overall high false-positive rate of 53 % prevents use of GEC results to help guide appropriate surgical management [39•].

Genetic Mutations and Rearrangements

The gene mutations and rearrangements frequently associated with thyroid cancer can be readily identified in FNAB specimens. The most commonly tested gene alterations are in the MAPK and PI3K-AKT pathways, which have both been implicated in thyroid carcinogenesis, and activating mutations in both of these pathways lead to downstream upregulation of tumor-promoting and cancer progression genes [40]. Mutations correlate to histology (Table 2) and can provide additional preoperative risk stratification [40–42]. Interestingly, FVPTC, which has morphologic features more consistent with PTC but biologic behavior that is often similar to FTC, shares gene alterations with both histologic types.

BRAF V600E is the most common gene alteration in thyroid carcinogenesis and can be associated with up to

Table 2 Gene alterations in thyroid cancer

Thyroid tumor	Mutations
Papillary thyroid cancer	
Conventional	<i>BRAF</i> V600E, <i>RAS</i> , <i>RET/PTC</i> , <i>PTEN</i> mutation (rare)
Follicular variant	<i>RAS</i> , <i>BRAF</i> K601E, <i>TSHR</i> , <i>PAX8-PPARG</i> , <i>RET/PTC</i> (rare),
Tall cell variant	<i>BRAF</i> V6000E
Follicular thyroid cancer	<i>RAS</i> , <i>PTEN</i> deletion or mutation, <i>PIK3-CA</i> , <i>TSHR</i> , <i>PAX8-PPARG</i>
Poorly differentiated thyroid cancer	<i>BRAF</i> V600E, <i>p53</i> , <i>RAS</i> , <i>CTNNB1</i> , <i>PIK3CA</i>
Anaplastic thyroid cancer	<i>ALK</i> , <i>p53</i> , <i>CTNNB1</i> , <i>AKT1</i> , <i>PIK3CA</i> , <i>RAS</i> , <i>BRAF</i> V600E, <i>PTEN</i> mutation
Medullary thyroid cancer, sporadic	<i>RAS</i> , <i>RET</i>

40–50 % of conventional PTC. When detected in FNAB specimens, *BRAF* V600E has >99 % PPV for PTC and may also be associated with the tall-cell variant subtype, extrathyroidal extension, lymph node metastasis, recurrence, and disease-specific mortality [42, 43•]. The additional prognostic information gained from preoperative *BRAF* V600E testing can help guide surgical management, including whether or not to perform prophylactic central compartment lymph node dissection, but this remains controversial [44]. *BRAF* K601E is the second most common *BRAF* mutation identified in thyroid cancer, but is more likely to be associated with FVPTC. In a recent study evaluating characteristics of 120 *BRAF*-positive indeterminate FNAB results, *BRAF* K601E was detected in ~50 % of the results classified as AUS/FLUS or FN, and the majority were FVPTC on histology [45].

RAS mutations are the most common gene alterations identified in indeterminate FNAB specimens and can include point mutations in *N*-, *K*-, and *H*-*RAS* hotspots in codons 12/13 and 61 [4•]. In one recent study of 63 FNAB specimens, when *RAS* was detected in preoperative FNAB cytology, histologic malignancy was present in 80–85 % of the nodules, and included either FVPTC (90 %) or FTC (10 %) [46]. Lymph node metastasis was rare, but bilateral multifocal disease was diagnosed in 50 %.

Because of the number of gene alterations involved in thyroid carcinogenesis, testing for a panel of mutations rather than a single gene provides the best sensitivity for adjunct diagnostic testing. In the initial two studies evaluating gene testing of FNAB specimens by Cantara et al. [47] and Nikiforov et al. [48], mutations were identified in 45 and 29 %, respectively, of cytology results classified as indeterminate inclusive of the suspicious category, and molecular testing added significant diagnostic sensitivity and accuracy. Nikiforov et al. then reported results from

prospective mutation testing (*BRAF*, *RAS* mutations and *PAX8-PPARG*, *RET/PTC 1* and *3* rearrangements) of a separate, consecutive series of 513 indeterminate FNAB results with cytologic, molecular, and histologic correlation. The malignancy rate was 24 %, and false-positive mutation testing results were rare (11 %) [4•]. Regardless of cytology category, the risk of malignancy was 100 % when *BRAF*, *RET/PTC1*, *3*, or *PAX8-PPARG* was detected preoperatively. *RAS* positivity was the primary source of the 9/83 (11 %) false-positive testing results and was identified in 73 % of the mutation positive FNAB specimens. Overall, for indeterminate FNAB results, the MT panel had sensitivity of 61 %, NPV 89 %, specificity 98 %, and PPV 89 % [4•]. The high PPV and specificity allow accurate identification of indeterminate FNAB results that carry a high risk of cancer, and can be used to guide the appropriate extent of initial thyroidectomy and/or lymphadenectomy.

The added diagnostic utility of prospective mutation testing has been demonstrated in hypothetical decision tree modeling, which also confirmed that the added costs of preoperative mutation testing were offset by a reduction in two-stage thyroidectomy [49]. Furthermore, in analysis of patient outcomes after incorporation of prospective mutation testing, a 2.5-fold reduction in two-stage thyroidectomy for histologic clinically significant thyroid cancer ($p < 0.001$) was observed when FNAB results classified as AUS/FLUS or FN underwent preoperative mutation testing [50].

Although the risk of malignancy is lower with mutation-negative FNAB results, the risk is not yet low enough to eliminate the risk of malignancy altogether. Indeterminate FNAB results with negative mutation testing results are still associated with a 14 % risk of cancer, although this rate varies by cytology category. Next generation sequencing technology may allow for facile and cost-effective screening of more relevant mutations, and has been investigated for thyroid neoplasms. Accurate testing is possible in both paraffin-embedded and FNAB samples with concordance to conventional Sanger sequencing methods [51]. Using the Ion Torrent platform and an expanded 12-gene panel that includes 284 mutational hotspots, Nikiforova et al. [52•] were able to detect mutations in genes such as *TSHR*, *PIK3CA*, and *p53*. In addition, 2/27 conventional PTCs were noted to have coexistent *BRAF* V600E with *p53* and/or *PIK3CA*, which are mutations more likely associated with aggressive variants. The identification of such multiple mutations may provide additional preoperative prognostic information that may further help guide surgical management.

A multimodality approach to thyroid nodule evaluation will likely lead to further improvements in preoperative risk stratification. For example, *TSHR* mRNA can be detected in peripheral blood and may be a marker of

malignancy, particularly for FNAB results classified as FN. An elevated *TSHR* mRNA level >1 ng/ μ g was associated with 85 % accuracy in a study of 54 patients [53]. However, in an algorithm that also incorporated the nodule size (<3.5 cm or ≥ 3.5 cm) and number of suspicious US characteristics (hypervascularity, microcalcifications, irregular shape, and indistinct margins), the preoperative diagnostic discrimination using *TSH* mRNA increased to 91 % accuracy, 97 % sensitivity, 95 % NPV, 84 % specificity, and 88 % PPV [53]. In yet another study evaluating 230 FNAB samples classified as AUS/FLUS with negative mutation testing, there were no observed malignancies in 88 nodules <1.8 cm [54]. Whether or not combining different molecular tests selected according to high- and low-risk nodule features will also improve current algorithms remains also to be determined [40].

Conclusions

Molecular testing of indeterminate FNAB cytology augments diagnostic accuracy and improves thyroid nodule risk stratification. Current testing modalities cannot yet reliably exclude thyroid cancer and avoid missed malignancies; however, a variety of tests with high specificity are available that can help direct initial extent of surgery. Further studies are still needed with new techniques and multimodality algorithms that will allow discrimination of low- and high-risk nodules with indeterminate cytology and further optimize clinical decision-making.

Compliance with Ethics Guidelines

Conflict of Interest Linwah Yip has received grant funding from the University of Pittsburgh Medical Center.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of major importance

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