



Molecular Diagnosis for Indeterminate Thyroid Nodules on Fine Needle Aspiration

Advances and Limitations

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Expert Rev Mol Diagn. 2013;13(6):613-623.

Abstract and Introduction

Abstract

Indeterminate thyroid lesions are diagnosed in up to 30% of fine needle aspirations. These nodules harbor malignancy in more than 25% of cases, and hemithyroidectomy or total thyroidectomy has therefore been advocated in order to achieve definitive diagnosis. Recently, many molecular markers have been investigated in an attempt to increase diagnostic accuracy of indeterminate fine needle aspiration cytology and thereby avoid unnecessary complications and costs associated with thyroid surgery. Somatic mutation testing, mRNA gene expression platforms, protein immunocytochemistry and miRNA panels have improved the diagnostic accuracy of indeterminate thyroid nodules, and although no test is perfectly accurate, in the authors' opinion, these methods will most certainly become an important part of the diagnostic tools for clinicians and cytopathologists in the future.

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence is steadily increasing.^[1] Thyroid cancer typically presents as a thyroid nodule, and up to 15% of thyroid nodules will harbor a malignant diagnosis. The prevalence of palpable thyroid nodules varies between populations and ranges from 4% to 7% within the USA, which represents approximately 10–18 million affected individuals.^[2–4] The incidence of thyroid nodules detected by ultrasound is even higher and exceeds 50% in patients over the age of 65 years.^[5] The most important established risk factor for the development of thyroid nodules is iodine deficiency, and iodine intake has been shown to be inversely correlated with the prevalence of thyroid nodules. Other epidemiologic and genetic risk factors include female sex, increasing age, smoking, previous head and neck radiation and familial predisposition.^[6,7] The gold standard for evaluating thyroid nodules remains fine needle aspiration (FNA) with subsequent cytological analysis of the harvested cells. FNA yields a final diagnosis in 70–80% of cases, and the remaining 20–30% of samples are characterized as indeterminate for malignancy.^[4,8] The National Cancer Institute sponsored a conference in 2008 to determine the use of FNA in the management of thyroid nodules and proposed a six-category scheme with the predicted probability of malignancy increasing from category II to VI: Bethesda I – nondiagnostic, Bethesda II – benign, Bethesda III – follicular lesion of indeterminate significance, Bethesda IV – follicular neoplasm or suspicious for follicular neoplasm, Bethesda V – suspicious for malignancy and Bethesda VI – malignant^[9] (Figure 1). The indeterminate categories comprise Bethesda III–V and have an approximate cancer risk of 5–10, 20–30 and 50–75%, respectively, allowing for variability between cytopathologists.^[10,11] These indeterminate nodules show a follicular growth pattern and therefore FNA is often not sufficient to distinguish between benign and malignant lesions, since diagnostic hallmarks of thyroid cancer such as vascular and capsular invasion are not detected by cytopathology or the nuclear features of papillary thyroid cancer are not adequately apparent to make a diagnosis of cancer. Therefore, thyroid nodules with indeterminate features on FNA cause a significant problem for the clinician and the patient, and currently, only surgical excision with histopathological analysis is able to provide a final diagnosis.^[12] A high volume of diagnostic surgeries is performed every year in the USA and potentially results in morbidity and higher

healthcare costs.^[3,8,13] Moreover, patients who are ultimately found to have malignant tumors but who had indeterminate FNA lesions often undergo a second surgical procedure – a completion thyroidectomy. This challenge has led many scientists to investigate the use of molecular markers in indeterminate thyroid nodules in order to improve the sensitivity and specificity of FNA cytology. Since the overlap between follicular adenomas (FA) and follicular thyroid cancer (FTC) or follicular variant of papillary thyroid cancer as well as the relatively low prevalence of known mutations in indeterminate FNA lesions pose a true challenge, only a few molecular diagnostic tests with their own limitations have entered daily clinical routine up to now. This review focuses on advances and limitations of molecular markers that seek to differentiate between benign and malignant indeterminate thyroid nodules on FNA. Only studies including indeterminate thyroid FNA lesions were included for review, since those are the only data providing information on test accuracy in a real-life clinical scenario. Four major groups of markers have been investigated: somatic mutation analysis, gene expression analysis, protein analysis and miRNA analysis.

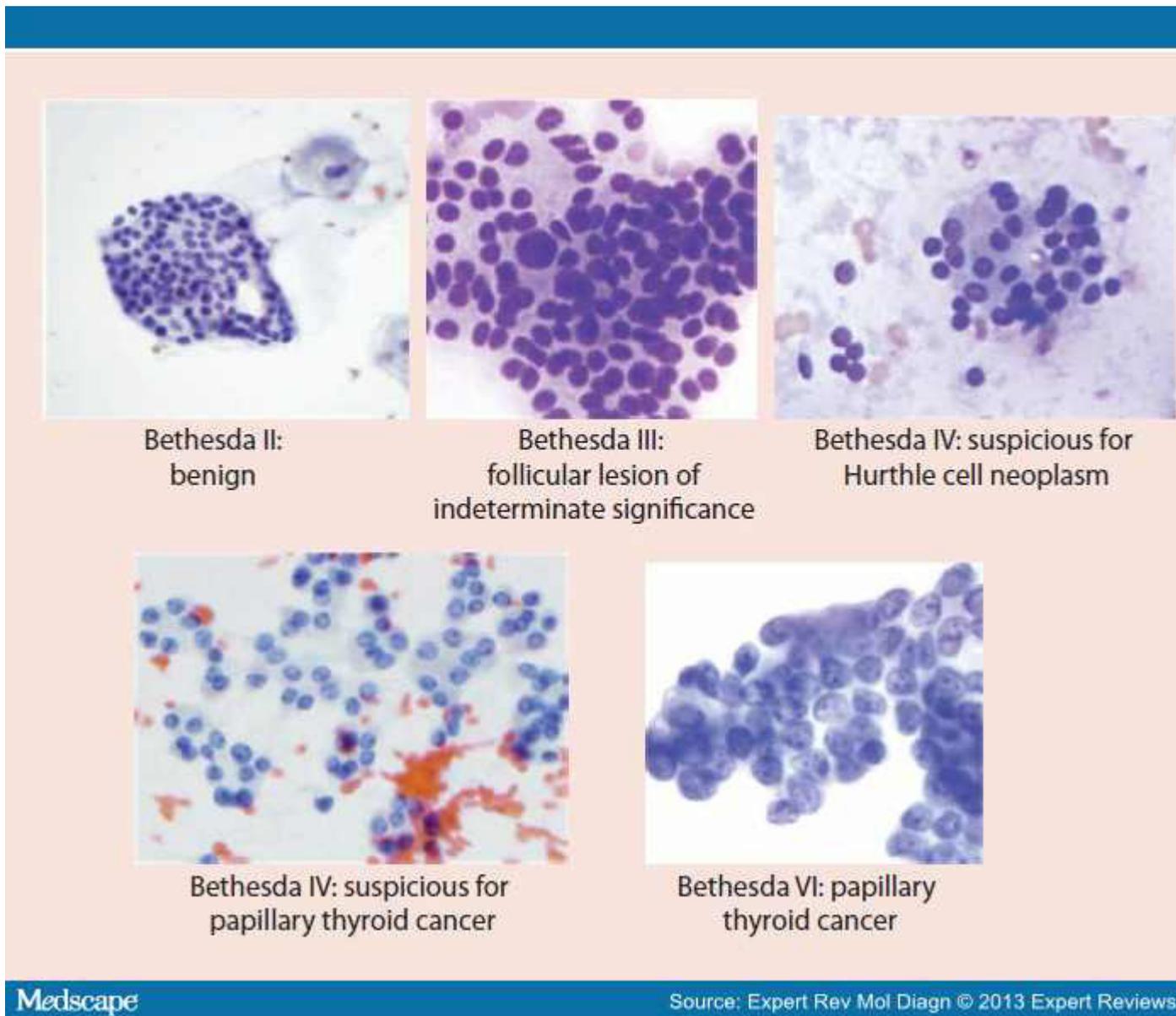


Figure 1.

Bethesda classification for thyroid nodule cytology (according to The NCI Thyroid Fine-Needle-Aspiration State of the Science Conference scheme).

Somatic Mutations

Several point mutations and gene rearrangements have been identified in thyroid cancer. The following genes carry somatic mutations in differentiated thyroid cancer and have been studied as potential tools to enhance the diagnostic accuracy of indeterminate FNA lesions – either alone or in combination as a panel: *BRAF* (the most common somatic mutation found in thyroid cancer), *RET-PTC*, *RAS* and *PAX8/PPAR-γ* ().

Table 1. Studies analyzing somatic mutations in indeterminate thyroid nodules.

Study (year)	Mutated genes	Indeterminate FNA number	Sensitivity (%)	Specificity (%)	Comments	Ref.
Nikiforova <i>et al.</i> (2011)	<i>BRAF</i> (V600E), <i>NRAS</i> , <i>HRAS</i> , <i>KRAS</i> , <i>RET/PTC1</i> , <i>RET/PTC3</i> , <i>PAX8/PPAR-γ</i>	967	57–68	96–99	Sensitivity and specificity calculated for Bethesda III, IV and V nodules	[23]
Kleiman <i>et al.</i> (2013)	<i>BRAF</i> (V600E)	310	15	100	Sensitivity and specificity calculated for <i>BRAF</i> alone	[27]
Filicori <i>et al.</i> (2011)	<i>BRAF</i> (V600E), <i>NRAS</i> , <i>HRAS</i> , <i>KRAS</i> , <i>RET/PTC1</i> , <i>RET/PTC3</i> , <i>PAX8/PPAR-γ</i>	466	35	98	Sensitivity and specificity for Bethesda III, IV and V nodules	[47]
Moses <i>et al.</i> (2010)	<i>BRAF</i> (V600E), <i>NRAS</i> , <i>KRAS</i> , <i>RET/PTC1</i> , <i>RET/PTC3</i> , <i>NTRK1</i>	137	12	98	Sensitivity and specificity calculated for Bethesda III, IV and V nodules	[22]
Ohori <i>et al.</i> (2010)	<i>BRAF</i> (V600E), <i>NRAS</i> , <i>HRAS</i> , <i>KRAS</i> , <i>RET/PTC1</i> , <i>RET/PTC3</i> , <i>PAX8/PPAR-γ</i>	117	63	100	Only Bethesda III nodules included	[48]
Cantara <i>et al.</i> (2010)	<i>BRAF</i> (V600E), <i>NRAS</i> , <i>HRAS</i> , <i>KRAS</i> , <i>RET/PTC1</i> , <i>RET/PTC3</i> , <i>PAX8/PPAR-γ</i>	95	80–87	97–100	Sensitivity and specificity calculated for Bethesda III/IV and V nodules separately	[46]

FNA: Fine needle aspiration.

***BRAF* V600E Mutations**

BRAF is the most common mutation isolated in thyroid cancer. This mutation occurs in papillary, poorly differentiated and anaplastic thyroid cancer^[14,15] and causes a V600E substitution in the *BRAF* protein, which results in neoplastic progression by aberrant activation of the MAPK pathway.^[16,17] The *BRAF* V600E mutation, along with *RET/PTC* rearrangements, are a hallmark of thyroid cancer and a vast majority of indeterminate thyroid nodules harboring either one of these two mutations are malignant on final pathology.^[18,19] Overall, the prevalence of *BRAF* mutations in indeterminate lesions varies between 15 and 40%,^[20–24] while it is estimated that at least 45% of the classic variant of papillary thyroid cancers carry this mutation.^[15,25] A report by Xing *et al.* suggest that *BRAF* may also be associated with an increased risk of extra thyroidal invasion, lymph node metastases and stage III and IV disease.^[18] Most *BRAF*-positive FNA

biopsies are found to be in the Bethesda V category, and most clinicians recommend that a Bethesda V nodule undergo a total thyroidectomy as the initial operative approach.^[26] A recent article from our group evaluating 310 indeterminate lesions for *BRAF* mutation analysis found that preoperative screening using *BRAF* mutation analysis alone did not alter initial surgical management when added to cytological analysis.^[27]

RAS Mutations

The *RAS* proto-oncogene encodes three different membrane associated GTP proteins: HRAS, KRAS and NRAS. Mutation of these domains causes increased signal transduction through both the MAPK and the PI3K/AKT pathways.^[28,29] These mutations are highly prevalent in FTC and in the follicular variant of papillary thyroid cancer (40–50%) and seldom detected in the classic variant papillary thyroid cancer (10%).^[30–32] *RAS* mutations have also been identified in benign FA; however, it is unclear whether *RAS*-positive FA have a higher chance of progression to cancer.^[33] The prevalence of this mutation in benign thyroid nodules is between 20 and 40%.^[19] The low overall prevalence of *RAS* mutations in thyroid cancers and the relatively high mutation rate in benign nodules makes *ras* mutation analysis unsuitable as a standalone test to predict malignancy in indeterminate thyroid nodules.

RET/PTC Mutations

Mutations in the *RET* oncogene can induce malignant transformation of thyroid follicular cells. This mutation is a rearrangement that causes constitutive activation and increased signaling downstream the MAPK pathway.^[34,35] These rearrangements can be found in papillary thyroid cancer as well as in benign thyroid lesions and can be associated with radiation exposure.^[36–38] There are over 12 types of *RET* rearrangements, but 80% of them are represented by *RET/PTC1* and *RET/PTC3*. The former is associated with well-differentiated histologic grade and better prognosis,^[36,39] while the latter is associated with more aggressive tumor types.^[40,41] Although initially thought to be present in over 50% of papillary thyroid cancer FNAs, overall prevalence was later discovered to be between 5 and 35%, making this mutation not suitable as a standalone screening tool for cancer.^[29,42]

PAX8–PPAR- γ Mutations

The *PAX8–PPAR- γ* fusion protein is found in 23–63% of FTCs and results in overactive cell proliferation and differentiation.^[29,33,43] The diagnostic value of this mutation as a stand-alone test to differentiate benign from malignant indeterminate FNAs is limited by its expression in 2–13% of benign thyroid lesions.^[44,45]

The MiRInform™ Panel by Asuragen

Recently, the US FDA approved a panel comprising all the aforementioned mutations for clinical use.^[31] This diagnostic tool is marketed under the name of MiRInform. Although the commercially available panel has not been prospectively validated, several independently run prospective studies have investigated the efficacy of an identical mutation panel on indeterminate thyroid nodules.^[23,46,47] In a recent article, Nikiforova *et al.* coupled somatic mutation testing with cytological status in a cohort of 1056 indeterminate lesions. The specificity of the mutation panel was estimated to be greater than 92%, meaning that less than 8% of mutation-positive nodules turned out to be benign.^[23,47] This led to the compelling argument that patients who have a positive mutation panel should probably undergo a total thyroidectomy as the initial operation. Reported malignancy rates in this study were 5.9, 14 and 28%, respectively, in Bethesda III, IV and V biopsies with negative mutation testing. The authors also found that Bethesda III nodules with negative mutation analysis had chance of extrathyroidal extension of less than 1%. Therefore, this study suggested a more conservative approach with repeated FNAs and imaging for biopsies consistent with Bethesda III and negative mutation testing.^[23] In another study, Otori *et al.* analyzed somatic mutation analysis in 117 indeterminate thyroid lesions. All lesions were within the Bethesda III category, and the study's authors reported a malignancy rate of 7.6% with negative mutation analysis. Sensitivity and specificity of the mutation panel were 63 and 100%, respectively.^[48]

Moses *et al.* recently examined 137 Bethesda III, IV and V lesions for mutation analysis. Among the indeterminate FNAs with malignant histopathology, a total of 27 mutations were detected (13 *BRAF*, four *RET/PTC*, ten *N-RAS*), and the panel yielded a sensitivity of 12% and a specificity of 98% at differentiating benign from malignant indeterminate thyroid nodules. Interestingly, four *N-RAS* mutations were detected in benign FA in this report.^[22]

Most limitations associated with mutation analysis are ascribed to the low sensitivity of the panel. Our group recently found that when mutation analysis is negative, the overall risk of malignancy in indeterminate lesions is still 22%.^[47] This is similar to the base malignancy rate of 20–30% in indeterminate lesions regardless of mutation status,^[11] which suggests that diagnostic hemithyroidectomy may still be warranted for all mutation-negative nodules of indeterminate cytology.

Therapeutic approach based on mutation analysis has been recently examined in a cost analysis paper by Yip *et al.* evaluating mutation testing in a hypothetical cohort of patients undergoing diagnostic evaluation of thyroid nodules. The study addressed specifically follicular lesions of undetermined significance (Bethesda III) and follicular neoplasms (Bethesda IV) and hypothesized improved costs and outcomes in the diagnostic evaluation of thyroid nodules when using mutation analysis. The study's authors found that integrating mutation analysis with current American Thyroid Association algorithms is cost effective as long as the panel is offered to a cost of less than US\$870. Moreover, implementation of mutation testing led to a decrease in the number of diagnostic thyroid lobectomies (11.6 vs 9.7%), which was the factor mainly responsible for cost savings. However, the mutation panel is currently not consistently offered at that price. Costs vary from US\$600 to US\$2400, depending on whether the test is reimbursed privately, by insurance companies, or by a public entity, such as Medicare.^[49]

Gene Expression Analysis

Gene expression profiling, high throughput and computational analyzes have provided new methods to identify potential target genes or gene panels to differentiate benign from malignant indeterminate thyroid lesions. RNA-based markers can essentially be divided into single-gene analysis or multiple gene panel analysis. Although there have been reports of several RNA-based markers in the literature,^[50] only a few single markers and gene panels have been validated on indeterminate thyroid FNA samples so far.

HMGA2

HMGA2 is a nonhistone chromosomal protein that is usually highly expressed in tumor tissue only. The interaction of HMGA with DNA leads to changes in the chromatin structure involving HMGA proteins in the regulation of the expression of a high number of target genes.^[51–53] Jin *et al.* measured HMGA2 expression levels using RT-PCR in 226 FNA specimens and found that HMGA2 is 89% sensitive and 95% specific at separating benign from malignant thyroid tumors on FNA.^[54] However, the study's authors do not mention how many FNA samples were indeterminate in this study and also found that HMGA2 is not accurate at differentiating benign from malignant Hurthle cell neoplasms.

UbcH10

UBE2C (or UbcH10) is associated with destruction of cell cycle cyclins and facilitation of cell cycle progression.^[55] One study by Guerriero *et al.* examined UbcH10 expression using RT-PCR and immunohistochemistry in 84 FNA samples. All samples were indeterminate lesions with a cytopathological diagnosis of follicular neoplasm or suspicious for malignancy (Bethesda IV and V). Sensitivity and specificity for classifying indeterminate nodules remained fairly low at 72 and 67%, respectively.^[55] The study's authors suggested that UbcH10 could, however, be added as a useful adjunct to other gene panels.

The Veracyte Afirma® Gene Classifier

The Veracyte Afirma gene classifier is a multigene expression classifier that uses mRNA extracted from FNAs and measures expression levels of 167 genes in order to distinguish benign from suspicious thyroid nodules. The initial trial published by Chudova *et al.* in 2010 measured mRNA expression in 137 FNA samples to determine a gene list that would accurately classify benign nodules. This gene list was then applied to 48 FNA samples, of which only 24 were indeterminate. The reported sensitivity and specificity for the validation set ranged from 85% to 100% and 40% to 73% depending on the use of a tissue-trained classifier or an FNA-trained classifier.^[56] One of the major flaws of this study was the small number of indeterminate FNAs included in the validation set. In addition, there was a complete absence of FTC and Hurthle cell adenomas or carcinomas on final histopathology of the validation set. A recently published trial by Alexander *et al.* aimed at validating the Afirma gene classifier further by applying it to 265 indeterminate thyroid nodules in a prospective multicenter trial.^[57] Of the 85 malignant nodules on final histopathology, the gene classifier predicted 78 correctly, yielding a sensitivity of 92%, a specificity of 52% and a negative predictive value (NPV) of 95% for Bethesda III, 94% for Bethesda IV and 85% for Bethesda V nodules.^[57] This study was the first report analyzing a gene expression-profiling platform in a large sample set of indeterminate FNAs. A projected 5-year cost-effectiveness analysis of the Afirma classifier from Li *et al.* used a Markov decision model based on a hypothetical cohort of adult patients and found that the treatment costs of patients undergoing molecular testing was \$10,719 compared with US\$12,171 when no molecular testing was used (at a price of US\$3200 per test). The analysis showed that cost savings were primarily related to the number of diagnostic surgeries that were avoided.^[58] However, the cost analysis based the calculations on only a single test, which of course ignores the very strong likelihood that gene expression would be retested with subsequent needle biopsies.

Protein-Based Assays

Immunocytochemistry (ICC) is widely used in most clinical laboratories and therefore theoretically represents an ideal assay for analysis of indeterminate thyroid nodules.^[59] However, interobserver interpretation and grading of ICC slides, as well as high variability in specimen processing has prevented ICC from being adopted widely in clinical practice so far. Hundreds of studies have looked at protein-based thyroid assays in the literature but only a fraction validated their utility in indeterminate FNA samples. Although the sensitivity and specificity of single protein markers are widely variable in the literature, results may be improved by combining several markers into immunohistochemistry protein panels for differentiating benign from malignant indeterminate FNA nodules.^[60]

Galectin-3

Galectin-3 is one of the most studied markers in thyroid neoplasia and well over 100 studies have been published analyzing its role in thyroid cancer.^[61] Galectin-3 is a carbohydrate-binding lectin with an affinity to galactosides. Functions of galectin-3 include cell cycle regulation, cell growth, apoptosis and tumorigenesis.^[62] A few years ago, the largest reported prospective trial analyzing galectin-3 expression by Bartolazzi *et al.* in 465 indeterminate thyroid nodules found galectin-3 ICC to have a sensitivity of 78% and specificity of 93%, with a positive predictive value (PPV) of 82% and a NPV of 91%. However, 29 out of 130 cancers were missed by the galectin-3 ICC method. The study's authors therefore concluded that galectin-3 ICC might be a complementary diagnostic tool to FNA cytology for indeterminate thyroid nodules.^[63]

HBME-1, CXCR4, CK19 & Other Markers

HBME-1 binds an antigen on the microvilli of mesothelioma cells.^[64] CXCR4 is a receptor for the chemokine C-X-C ligand 12 and is involved in chemotaxis, cell migration and cell adhesions.^[65] Both markers have been studied recently by Torregrossa *et al.* and compared with galectin-3 expression in 100 FNA samples of which 43 were indeterminate on cytology. On subgroup analysis, HBME-1 and CXCR4 taken individually or in combination, showed a diagnostic accuracy of 88 and 91%, respectively. Galectin-3, on the other hand, had a diagnostic accuracy of only 72%.^[66] One flaw of this study was the relatively small number of

indeterminate thyroid lesions included. Several other studies have looked at the accuracy of HBME-1 staining alone and found that HBME-1 ICC had a sensitivity of 79–87% and specificity of 83–96% in differentiating benign from malignant indeterminate thyroid nodules.^[66–68]

Cytokeratin 19 (CK 19) is a member of the cytokeratin family that is part of the intracytoplasmic cytoskeleton of epithelial cells.^[69] Khurana *et al.* studied CK 19 protein expression via ICC on 57 cases, of which 19 were indeterminate. Seven out of eight cases that were ultimately malignant on cytopathology had strong staining for CK 19.^[70] Saggiolato *et al.* analyzed several ICC markers individually and in combination in 125 indeterminate samples and found that CK 19 had 76% sensitivity and 90% specificity at differentiating benign from malignant indeterminate lesions. When CK 19 was combined with galectin-3 in oncocytic thyroid nodules only, sensitivity and specificity increased to 100%. However, only 24 out of 125 samples in that study had oncocytic features on subgroup analysis. The highest sensitivity and specificity in oncocytic and conventional thyroid samples was found when galectin-3 and HBME-1 ICC were combined, reaching a sensitivity of 97% and a specificity of 90%.^[68] In addition, Cochand-Priollet *et al.* recently looked at a combined HBME-1 and CK 19 ICC assay in 150 thyroid FNA samples, of which 48 were indeterminate and found a 100% sensitivity and 85% specificity at differentiating malignant from benign indeterminate thyroid nodules.^[71] Finally, Troncone *et al.* looked at cyclin D1 and D3 expression in 51 Hurthle cell FNA lesions and found 100% sensitivity and 94% specificity (PPV of 90%) at differentiating malignant from benign Hurthle cell neoplasms when both markers were combined. In that study, the ICC cut off values for predicting malignancy were determined using receiver-operating characteristic analysis.^[72] However, no study has shown the usefulness of cyclin D1 and D3 in combination with other markers at differentiating oncocytic and conventional indeterminate FNA nodules yet. Other markers such as IMP3, hTERT and retinoic acid receptor as well as retinoid X receptor subtype have been studied in thyroid neoplasms, but none of them have been validated using ICC in a cohort of indeterminate thyroid nodules at this time.^[73–75] While all these studies report quite impressive results, the variability in staining and interpretation of ICC represents the biggest impediment to the widespread adoption of these markers in clinical practice.

miRNA Analysis

miRNA are short single stranded noncoding small RNA segments that comprise from 19 to 23 nucleotides in length. These miRNAs operate via sequence-specific interaction of mRNA targets by binding the 3' untranslated region, thereby suppressing translation and mRNA decay.^[76] miRNAs have also been shown to function as tumor suppressors or oncogenes in cancerous cells and have been described as being useful for cancer prognostication and classification.^[77] miRNA molecules are relatively stable in FNA cytology and formalin-fixed paraffin-embedded tissue specimens, making these small RNA molecules ideal markers for diagnostic applications. In addition, miRNAs have been reported to be dysregulated in all human cancers, including every subtype of thyroid cancer.^[78–80] Several studies have previously documented the use of miRNA to differentiate between benign and malignant thyroid nodules.^[81–83] However, only a few studies looked at the use of miRNA in differentiating between benign and malignant thyroid nodules in indeterminate FNAs ().

Table 2. Studies analyzing miRNA expression in indeterminate thyroid nodules.

Study (year)	miRNAs	Total FNA Number	Indeterminate FNA Number	Sensitivity (%)	Specificity (%)	Comments	Ref.
Nikiforova <i>et al.</i> (2008)	187, 222, 221, 146b, 155, 224, 197	62	8	88	94	Sensitivity and specificity calculated on 62 samples	[84]
Agretti <i>et al.</i> (2012)	146b, 155, 221	141	53	88	70	Sensitivity and specificity calculated on indeterminate	[85]

						FNAs	
Shen <i>et al.</i> (2012)	146b, 221, 187, 30d	128	68	64	79	Sensitivity and specificity calculated on indeterminate FNAs	[86]
Kitano <i>et al.</i> (2012)	7, 126, 374a, let-7g	154	52	100	20	Sensitivity and specificity calculated on indeterminate FNAs	[87]
Keutgen <i>et al.</i> (2012)	328, 222, 197, 21	101	101	100	86	Specificity increased to 95% when Hurthle cell neoplasms were excluded (13 samples)	[88]
Dettmer <i>et al.</i> (2013)	885-5p, 221, 574-3p	19	19	100	100	All malignant indeterminate nodules were follicular thyroid cancers (oncocytic and conventional)	[89]

FNA: Fine needle aspiration.

One report by Nikiforova *et al.* analyzed the utility of a miRNA panel comprising seven miRNAs (mir-187, mir-222, mir-221, mir-146b, mir-155, mir-224 and mir-197) at differentiating benign from malignant thyroid pathology in 62 FNA samples. The study's authors report that if one out of the seven miRNAs was upregulated at least twofold, the sensitivity of their panel was 88%, the specificity 94% and the overall accuracy 95%. However, only eight of the 62 FNA samples that were analyzed had a diagnosis of an indeterminate lesion on cytology, and no subgroup analysis for these eight samples was available in this study.^[84]

Another report by Agretti *et al.* examined expression profiles of seven miRNAs using RT-PCR in a prospective series of 141 thyroid FNA samples. A total of 45 samples were benign, 43 samples were papillary thyroid carcinomas (PTCs) and 53 samples were from indeterminate thyroid nodules. Statistical analysis was performed to determine differences in expression of these seven miRNAs (mir-146b, mir-155, mir-187, mir-197, mir-221, mir-222 and mir-224) and to perform a prediction of malignancy based on decision-tree analysis. An increase in all miRNAs analyzed was detected in PTCs compared with benign nodules, and six out of seven miRNAs were statistically significantly increased. The decision models obtained by the authors comprised only three miRNAs (mir-146b, mir-155 and mir-221) and were highly predictive for differentiating PTCs from benign thyroid nodules (97% accuracy), with only one false-positive and one false-negative prediction. However, once applied to the set of 53 indeterminate lesions, only 59% of nodules were correctly predicted using the decision tree, with a total of 16 false-positive and six false-negative predictions. The authors hypothesized that poor RNA quality and/or low proportion of malignant cells in the FNA samples may have been the contributing factor to the poor prediction results of their decision-tree model.^[85]

A study by Shen *et al.* measured expression levels of eight miRNAs (miR-146b, miR-221, miR-187, miR-197, miR-346, miR-30d, miR-138 and miR-302c) using RT-PCR in 128 FNA samples. Gene expression analyses and linear discriminant analysis (LDA) were performed in a training sample set of 60 samples to obtain a classification rule that correctly classified FNA cases as benign or malignant. A four-miRNA LDA classification rule (miR-146b, miR-221, miR-187 and miR-30d) was found to have a diagnostic accuracy of 93%, sensitivity of 93% and specificity of 94% for the training sample set. When applied to a validation set of 68 FNA samples, diagnostic accuracy was 85%, sensitivity 89% and specificity 78%. On subgroup analysis, the LDA model was applied to 30 indeterminate samples with atypical features from the validation

set and was found to have a diagnostic accuracy of only 73%, a sensitivity of 64% and a specificity of 79%. The study's authors concluded that miRNA amplification from FNA samples is feasible and that their panel of four miRNAs can accurately identify PTCs on FNA, but that it needs refinement when applied to atypical FNA lesions. One flaw on this study was that half of the FNA samples in the training set and 30 out of 68 samples in the validation set had a malignant diagnosis on cytopathology, and therefore, the number of true indeterminate FNA samples in this study amounted to only 68 samples.^[86]

In another study, Kitano *et al.* analyzed miRNA expression of miR-7, miR-126, miR-374a and let-7g using RT-PCR in a training set of 95 samples, that comprised 31 indeterminate thyroid FNA samples. A predictor model was formulated based on the most differentially expressed miRNA (miR-7) Δ Ct value which was then applied to a separate cohort of 59 FNAB samples as the validation set. The authors found that miR-7 was the best predictor for distinguishing benign from malignant FNA samples, with a sensitivity of 100%, specificity of 29%, a PPV of 36%, and a NPV of 100%, for an overall accuracy of 76%. When applied to indeterminate FNA lesions only, the predictor model had an overall accuracy of 37% with sensitivity of 100%, specificity of 20%, PPV of 25% and NPV of 100%. The study's authors concluded that the high NPV of miR-7 could potentially incite clinicians to follow patients with a benign results on the predictor model, as opposed to perform immediate diagnostic thyroidectomy. One limitation from this study is the small sample size of 21 indeterminate FNA lesions in the validation set.^[87]

Recently, our group analyzed miRNA expression levels in 101 indeterminate thyroid FNA samples. Expression of miR-222, miR-328, miR-197, miR-21, miR-181a and miR-146b was determined using RT-PCR in 29 prospectively collected indeterminate thyroid FNA lesions. A support vector machine model with four miRNAs (miR-222, miR-328, miR-197 and miR-21) was initially found to have 86% predictive accuracy in differentiating between malignant and benign indeterminate thyroid FNA nodules using cross-validation. After the support vector machine model was applied to a separate set of 72 prospectively collected FNA samples, performance increased with a reported sensitivity of 100%, a specificity of 86% and an overall accuracy of 90% in differentiating malignant from benign indeterminate FNA lesions. On subgroup analysis, the authors found that five out of the seven wrongly predicted samples were Hurthle cell nodules. When all 13 Hurthle cell lesions were excluded, overall accuracy improved to 97% with 100% sensitivity and 95% specificity.^[88] This study remains the largest miRNA analysis in indeterminate thyroid nodules to date and provides useful information on feasibility and accuracy of miRNA prediction models for indeterminate thyroid lesions.

In a recent report by Dettmer *et al.*, 38 FTCs and 10 normal thyroid tissue samples were analyzed for miRNA expression using microarray technology. On unsupervised hierarchical clustering analysis, differences in miRNA expression between normal thyroid tissue, oncocytic and conventional FTCs were demonstrated. In particular, a novel miRNA (miR-885-5p) was found to be strongly upregulated (>40-fold) in oncocytic FTCs when compared with conventional FTCs, FA and hyperplastic nodules. In this study, a classification and regression tree algorithm applied to 19 indeterminate FNA samples demonstrated that three miRNAs (miR-885-5p, -221 and -574-3p) allowed distinction of follicular thyroid carcinomas from hyperplastic nodules with 100% accuracy.^[89] Although only a small number of indeterminate FNAs were analyzed, this report provides useful information on using miRNA analysis to differentiate oncocytic and conventional FTCs from benign hyperplastic nodules.

Expert Commentary

Although FNA biopsy is an important preoperative test for thyroid nodules, it does not provide a definitive diagnosis in up to a third of patients: those with indeterminate thyroid nodules. Therefore, these patients undergo surgery with its incumbent risks and costs and only a quarter of them carry a diagnosis of cancer. Over the past decade, several molecular markers have emerged as possible diagnostic tools for solving this challenge. Somatic mutation testing, gene expression profile analysis, ICC and miRNA analysis have all been tested in indeterminate thyroid lesions with reasonable success. However, every one of these

markers has its own set of flaws, and none has yet been proven to perfectly distinguish benign from malignant indeterminate thyroid nodules on FNA. One weakness common to all these markers is the potential discordances in cytological classifications, which could be as high as 14%, even after independent expert thyroid cytopathologist review.^[57] Since this represents the reference standard against which every marker will be measured, the imperfection of interobserver agreement will naturally remain a flaw in every FNA diagnosis-based platform.

This interobserver variability problem is even more pronounced when ICC is applied to FNA specimens. Cytopathologists, discordance in scoring ICC slides could be one of the major reason that none of these protein-based assays has made it to the market yet. Although the authors believe that protein markers could be a useful adjunct to molecular analysis of FNA in some limited or complicated cases, several ICC panels have been studied, and none has been found to be perfectly adequate at distinguishing

It remains unclear whether somatic mutation testing for *BRAF*, *RAS*, *RET/PTC* and *PAX8/PPAR-γ* can change the management of indeterminate thyroid lesions when the results are positive, as most of the mutations occur in Bethesda V nodules, which usually warrant a total thyroidectomy as the initial surgical approach. In addition, in the absence of evidence supporting observation as a safe clinical approach to indeterminate biopsies with low risk of malignancy (Bethesda III), diagnostic hemithyroidectomy is still warranted for indeterminate lesions with negative mutation testing. Moreover, current data do not clearly support cost-effectiveness of the routine implementation of mutation panels in the clinical management of thyroid indeterminate lesions. Lastly, impact of somatic mutation analysis on clinical care remains to be validated in prospective studies with test performance assessment in independent laboratories, FNA slide analysis by blinded pathologists and analyses of patient outcome data.

Platforms using gene expression analysis such as the Afirma test are a better approach at differentiating indeterminate nodules, since they objectively analyze gene expression levels in FNAs and therefore do not have interobserver scoring limitations, as ICC does. However, the major limitations of these gene classifiers are: the inability to pick up the great majority of malignant tumors and therefore allow adequate surgical treatment for the patient up front (e.g., total thyroidectomy); the high costs associated with the test related to the microarray chip technology and the lack of guidelines putting the gene classifier in a clinical context. This last point is crucial and several questions need to be answered. How much time needs to elapse until the clinician needs to repeat the test on a benign nodule? How can one be sure that a 'benign' nodule, such as a FA, will not turn malignant and over what time period? And finally, is it safe to observe these nodules for a long period of time based on gene expression profiling?

miRNA dysregulation has been found in virtually all subtypes of thyroid cancer and therefore represents a potential ideal marker for differentiating benign from malignant thyroid nodules. miRNA analysis conveys several advantages such as high stability of miRNA in FNAs due to small size of molecules, low miRNA concentrations from FNA extraction needed to run RT-PCR, a small number of miRNA needed to constitute a panel that accurately discriminates indeterminate nodules, easy and cheap to run miRNA RT-PCR. miRNA panel developments are currently underway and more clinical data on its accuracy for indeterminate thyroid lesions are needed and expected to emerge soon.

Five-Year View

The American Thyroid Association Guidelines Task Force is currently reviewing several commercially available gene expression markers to generate expert consensus guidelines for clinicians on when and how to use these platforms. However, as mentioned above, these tests have clear limitations and are far from perfect. More scientific progress is expected to occur in the field of differentiation of benign from malignant indeterminate nodules within the next few years. miRNA expression analysis from FNA samples, and subsequent miRNA panel developments in particular are believed to produce groundbreaking changes in improving diagnostic ability by either combining them with existing gene expression tests or functioning

on their own as a separate entity. ICC will probably play an important role in difficult-to-diagnose cases with gene expression and miRNA analysis such as oncocytic thyroid neoplasms. New technologies, such as mRNA and small RNA next-generation sequencing, are expected to further revolutionize the diagnosis and treatment of thyroid cancer by discovering additional targets and gene markers, as next-generation sequencing becomes cheaper and more readily available.

Sidebar

Key Issues

- Indeterminate thyroid nodules on fine needle aspirations represent a difficult challenge for clinicians, since up to 75% of patients undergoing surgical excision in the form of a hemithyroidectomy or total thyroidectomy will have a benign diagnosis on final histopathology.
- Several molecular marker platforms have been developed to address this problem, but no test has been shown to have perfect accuracy so far.
- Somatic mutation analysis of *BRAF*, *RET/PTC*, *RAS* and *PAX8-PPAR-γ* has high specificity but poor sensitivity at differentiating benign from malignant indeterminate thyroid lesions on fine needle aspirations. It remains unclear whether its implementation (in addition to cytological analysis) will change clinical management of indeterminate thyroid nodules.
- Immunocytochemistry of galectin-3, HBME-1, CXCR4, CK19 and other markers have been tested in indeterminate nodules with a wide range of reported accuracy.
- Gene expression platforms based on microarray analysis have been successful at detecting benign nodules, but remain suboptimal at detecting malignancy.
- miRNA expression analysis via RT-PCR is a promising method to differentiate indeterminate lesions and presents several advantages over conventional gene expression analysis.

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Papers of special note have been highlighted as:

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