Molecular Diagnostics in Thyroid Cytopathology

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Robert J. Monroe and Anagh Vora

Key Points

1. Molecular testing of thyroid specimens has become an important adjunct to cytopathology for the management of patients with thyroid nodules.
2. The most widely adopted clinical application of molecular testing in thyroid cytopathology is the evaluation of thyroid FNA specimens with indeterminate cytopathology, which comprise approximately 15–30 % of cases.
3. When cytologically indeterminate thyroid nodules undergo diagnostic surgery, approximately three-quarters prove to be benign, highlighting the need for molecular approaches to identify the benign nodules among this group.
4. Several molecular approaches have been proposed for the evaluation of nodules with indeterminate thyroid cytopathology, including tests that "rule in" thyroid cancer and those that "rule-out" thyroid cancer.

R.J. Monroe, M.D., Ph.D. (✉)
Veracyte, Inc., 7000 Shoreline Court, Suite 250, South San Francisco, CA 94080, USA
e-mail: rob@veracyte.com

A. Vora, M.D.
CellMax Life, Inc., 700 E. El Camino Real, #250, Mountain View, CA 94040, USA
e-mail: anagh@cellmaxlife.com

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5. An mRNA expression analysis approach (the Afirma Gene Expression Classifier) demonstrates high sensitivity (90 %) and high negative predictive (NPV) value (≥94 %) and is best utilized to rule out thyroid cancer in cytologically indeterminate nodules.
6. Mutational testing approaches demonstrate high specificity and high positive predictive value (PPV), and are best utilized to rule-in thyroid cancer and inform the choice of surgery.
7. Guidelines, including The NCCN Thyroid Carcinoma Guidelines and UpToDate, suggest that cytologically indeterminate thyroid nodules determined to have a ROM similar to cytologically benign nodules with a molecular test can be clinically observed.
8. Many other applications for molecular testing in thyroid cytopathology are available or under development utilizing a variety of technologies for identification of specific tumor subtypes, prognosis of individual tumors, and prediction of response to targeted therapies.

Introduction

Thyroid Cancer

Thyroid cancer is the most rapidly increasing cancer in the USA, with about 63,000 new cases estimated for 2014. The majority of this increase is thought to be attributed to identification of greater numbers of smaller and nonpalpable thyroid nodules through more widespread use of thyroid ultrasound as well as incidental discovery of thyroid nodules through CT, PET-CT, and MRI imaging studies of the neck for non-thyroid indications. This increased nodule detection rate has led, in turn, to increased rates of surgery and identification of thyroid cancers, including many small tumors. Another factor thought to contribute to this increase in thyroid cancer incidence is the more frequent recognition of certain sub-types of thyroid cancer by pathologists, such as the follicular variant of papillary thyroid carcinoma (FVPTC), which previously was under recognized and therefore underdiagnosed.

Environmental factors, including radiation exposure, are also thought to play a role. Overall, it is likely that a combination of these factors has contributed to the observed increase in thyroid cancer incidence. Interestingly, the increased incidence of thyroid cancer has not led to a corresponding increase in the death rate from thyroid cancer, which has been fairly stable for many years.

For the practicing cytopathologist, the increased detection of thyroid nodules through ultrasound and other imaging modalities has led to a significant increase in the number of thyroid pathology specimens submitted for evaluation. This increase has also led to greater opportunities for the cytopathologist to apply molecular testing for the analysis of these specimens. The following chapter lays out the applications for molecular testing of thyroid nodules, along with the role and appropriate use of these tests in the management of patients with thyroid nodules.

Fine Needle Aspiration (FNA)

The cytopathologic interpretation of Fine Needle Aspiration (FNA) specimens has revolutionized the management of thyroid nodules since its introduction in the USA over 40 years ago. Before thyroid FNA, nodules were typically managed surgically due to the absence of other reliable methods to distinguish benign from malignant lesions. The adoption of FNA, performed with or without ultrasound guidance, in combination with cytopathologic evaluation has enabled definitive and accurate classification of the majority of thyroid nodules (approximately 70–80 %) into benign and malignant categories. Through the use of thyroid FNA, patients with benign cytopathology can be spared unnecessary surgery and managed conservatively while those patients with malignant cytopathology can be triaged for thyroidectomy.

What about the remaining 20–30 % of thyroid nodules that are not clearly benign or malignant? An additional 5–10 % do not contain sufficient cellularity for diagnosis (6 groups of at least 10 follicular cells) and are classified as non-diagnostic/unsatisfactory. Guidelines suggest that patients with non-diagnostic results should undergo repeat FNA after an appropriate period of approximately

three months. The remaining 15–20 % of thyroid nodules fall into a group of "indeterminate" diagnoses that are not clearly benign or malignant cytologically. Historically, this indeterminate group has included nodules carrying a variety of related diagnoses such as "follicular lesion," "cellular follicular lesion," "follicular (or Hurthle cell) neoplasm," "suspicious for follicular (or Hurthle cell) neoplasm," "suspicious for malignancy," and "suspicious for papillary thyroid carcinoma" along with diagnoses mentioning "atypia" or "atypical cells" in some fashion.

In 2008, The Bethesda System for Reporting Thyroid Cytopathology was released in an attempt to standardize the terminology for reporting thyroid FNA specimens and to link the categories with estimated risk of malignancy (ROM) and suggested clinical management. In this system, diagnoses fall into one of six diagnostic categories (Table 5.1): Nondiagnostic (I), Benign (II), Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance (AUS/FLUS) (III), Follicular Neoplasm/Suspicious for Follicular Neoplasm (FN/SFN) (IV), Suspicious for Malignancy (SFM) (V), and Malignant (VI). The so-called indeterminate categories in the Bethesda System include AUS/FLUS (III), FN/SFN (IV), and SFM (V).

The Challenge of Indeterminate Cytopathology

For nodules diagnosed as nondiagnostic (I), benign (II), or malignant (VI) by cytopathology, the clinical management is straightforward. However, for those with indeterminate diagnoses (III, IV, and V), management options are less well-defined and open to interpretation. Some guidelines suggest surgical management of all indeterminate nodules because of the unacceptably high risk of malignancy in these nodules. The Bethesda system, however, suggests a set of management options specific to each indeterminate category: repeat FNA for AUS/FLUS (III), surgical lobectomy for FN/SFN (IV), and lobectomy or total thyroidectomy for SFM (V). These suggestions are driven by an estimated ROM put forth for each of these categories (Table 5.1).

Table 5.1 The Bethesda System for reporting thyroid cytopathology

Diagnostic category	Expected rate of diagnosis	Expected risk of malignancy	Evidence of risk of malignancy	Suggested management
I. Nondiagnostic or Unsatisfactory	<10 %	1–4 %	0–50 %[3,6]	Repeat FNA with ultrasound guidance
II. Benign	60–70 %	0–3 %	0.9–18 %[2,3,5,6]	Clinical follow-up
III. Atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS)	3–7 %	5–15 %	7–48 %[2,3,4,5,6]	Repeat FNA
IV. Suspicious for follicular neoplasm	8–12 %	15–30 %	14–49 %[2,3,4,6]	Surgical lobectomy
V. Suspicious for malignancy	~3 %	60–75 %	53–87 %[2,3,6]	Near-total thyroidectomy or surgical lobectomy
VI. Malignant	3–7 %	97–99 %	93–100 %[2,3,5,6]	Near-total thyroidectomy

The recommended diagnostic categories are listed with the suggested rates of diagnosis, the expected risk of malignancy, and the recommend clinical management for each category. The column listing "evidence of risk of malignancy" summarizes the ranges in ROM seen for each diagnostic category in numerous studies following the publication of TBSRTC. (Modified from Ali and Cibas with kind permission from Springer Science and Business Media)

The suggested approach of repeat FNA for AUS/FLUS (III) has been questioned in several studies reviewing the ROM in nodules with an initial diagnosis of AUS/FLUS. These studies suggest that regardless of the diagnosis of the second FNA, the ROM of the nodule remains about the same as after a single AUS/FLUS result. In other words, even if the second FNA results in a benign cytopathology diagnosis, the ROM remains at or close to the ROM of the initial AUS/FLUS diagnosis and is not reduced to the level of risk of a single benign diagnosis (3–6 %). Furthermore, these and other studies have shown that nodules with AUS/FLUS diagnoses carry a higher risk of malignancy than anticipated in the Bethesda System, very similar to the ROM for FN/SFN nodules (~20–25 %, range 7–48 %; Table 5.1 column 4). Accordingly, reconsideration of the Bethesda recommendation for repeat FNA for most patients following an initial AUS diagnosis has been suggested.

Despite the great strides that TBSRTC has made in standardizing terminology and creating a uniform set of diagnostic criteria for each category, the reproducibility of cytopathology diagnosis remains relatively poor. In a recent prospective study where locally read cytopathology cases were re-read by expert cytopathologists (inter-observer concordance), concordance of diagnostic category, particularly among the indeterminate subtypes (Bethesda III–V) was low—35 % for AUS/FLUS, 66 % for FN/SFN, and 37 % for SFM (Fig. 5.1a, b). Similarly, diagnosis of cytopathology indeterminate subtype was not highly reproducible when cytopathology cases were re-read by the same observer (intra-observer concordance) at least 30 days apart—61 % for AUS/FLUS, 78 % for FN/SFN, and 46 % for SFM (Fig. 5.1a, c). These reproducibility studies suggest that the use of Bethesda subtype diagnoses to drive clinical recommendations may not be reliable, particularly among indeterminate subtypes.

As an alternative to repeat FNA or lobectomy/thyroidectomy for indeterminate nodules, including those with AUS/FLUS (III), FN/SFN (IV), and SFM (V) diagnoses, molecular testing approaches have been developed to assist in clinical management of this challenging group of nodules. These approaches, in their application to indeterminate subtypes as a group, overcome the issue of reproducibility of cytopathology diagnosis. As a group, they have

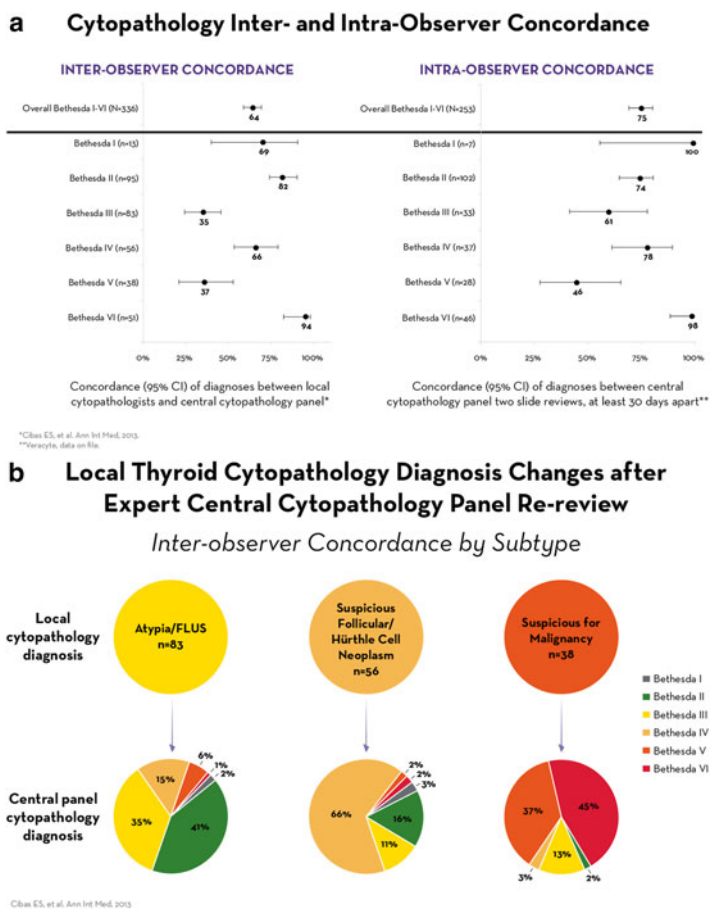

Fig. 5.1 (**a**) Overall cytopathology inter- and intra-observer concordance by Bethesda subtype. (**b**) Cytopathology inter-observer concordance by indeterminate subtype. (**c**) Cytopathology intra-observer concordance by indeterminate subtype

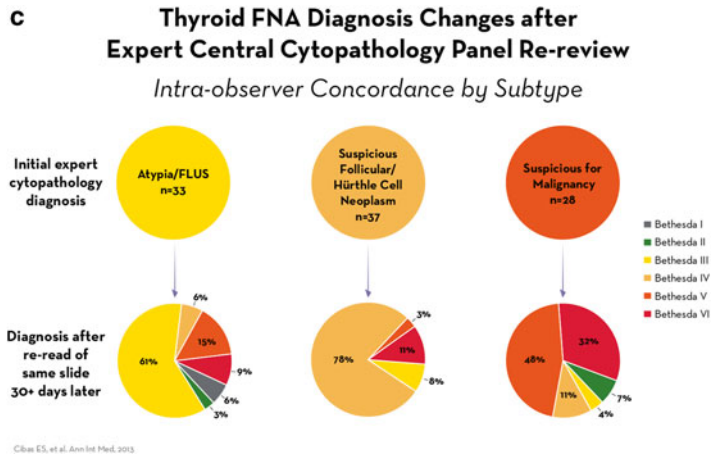

Fig. 5.1 (continued)

demonstrated the potential to identify very low risk nodules among those with indeterminate cytopathology and to thereby prevent many patients from undergoing unnecessary diagnostic thyroid surgery. In addition, some molecular tests have shown the potential to guide the choice of surgery (lobectomy vs. total thyroidectomy) in higher risk nodules, thus preventing fewer repeat/revision surgeries. The following section describes the molecular approaches being employed for the evaluation of indeterminate thyroid nodules and the optimal uses of each approach.

Rule In vs. Rule Out Tests for Evaluation of Thyroid FNAs

Overview of Rule In vs. Rule Out Tests

Historically, molecular testing in oncology has focused on the identification of DNA mutations/alterations in various cancers or cancer syndromes. For example, the presence of the *BCR-ABL* translocation in sampled leukemic cells is virtually pathognomonic for chronic myeloid leukemia, while the presence of specific

germline mutations in the *BRCA1* or *BRCA2* genes is diagnostic of Hereditary Breast and Ovarian Cancer syndrome (HBOC). These tests are examples of "rule in" tests—if positive, they are able to "rule in" a disease or syndrome. However, if negative, these tests do not necessarily "rule out" the possibility of a related disease or condition but rather simply the lack of the mutation being tested.

Rule out tests are much less common in oncology, particularly rule out molecular assays. Screening tests for serum tumor markers such as CEA, CA-125, and PSA are used by some clinicians to rule out specific types of cancer in some populations. Among molecular assays, HPV DNA testing of cervical cytology specimens can serve as a rule out test for cervical dysplasia as it has high sensitivity and high NPV—if HPV testing is negative, there is a very low likelihood of cervical dysplasia.

In thyroid cytopathology, as mentioned, the primary application for molecular testing is the evaluation of cytologically indeterminate nodules. Both rule in and rule out approaches have been developed for the evaluation of these ambiguous nodules. When applied to indeterminate thyroid nodules, the rule in tests look for presence of thyroid cancer by identifying mutations highly correlated with thyroid malignancies. In contrast, the rule out tests utilize an approach that is designed to look for the presence of benign genetic patterns in cytologically indeterminate nodules rather than the absence of specific mutations. This novel approach represents a paradigm shift in molecular oncology testing, from confirmation of malignancy through the identification of specific mutations to confirmation of benignity through the identification of specific benign genetic signatures.

Rule-In Tests

Somatic Mutations and Gene Rearrangements

A number of point mutations and gene rearrangements have been identified in thyroid cancer and can be assessed in thyroid FNA specimens. The presence of some of them conveys a near certainty of thyroid cancer, while the finding of others only raises the probability of thyroid cancer but does not exclude the possibility of a

benign tumor. Therefore, some mutations are better rule in tests than others. The most common mutations in thyroid cancer include those in the *BRAF* and *RAS* genes along with *RET/PTC* and *PAX8/PPAR-γ* rearrangements. A variety of techniques can be used for the identification of specific mutations in *BRAF* and *RAS* genes in genomic DNA purified from thyroid FNA samples, including real-time PCR (RT-PCR) assays, PCR amplification followed by DNA sequencing, and Next Generation Sequencing (NGS) approaches, among others. In the case of gene rearrangements, similar approaches can be employed following isolation of mRNA from FNA samples and conversion to cDNA.

BRAF

BRAF mutations are the most common mutations in thyroid cancer. The vast majority lead to the replacement of a valine amino acid by a glutamic acid residue at position 600 (V600E), a mutation that leads to constitutive activation of the MAPK pathway. This mutation occurs in thyroid cancers of follicular origin including papillary thyroid carcinoma (PTC), poorly differentiated thyroid carcinoma, and anaplastic thyroid carcinoma (ATC); it does not occur in follicular thyroid carcinoma (FTC) or medullary thyroid carcinoma (MTC). Among PTC and ATC, the mutation is present in about 45 % and 25 %, respectively. Among indeterminate thyroid nodules (Bethesda III and IV), the prevalence of *BRAF* mutations is low, ~2–3 %. This low incidence is not unexpected as these diagnostic categories include malignancies that less commonly carry *BRAF* mutations such as FTC and Hurthle cell carcinoma, FVPTC, and MTC. The low incidence is also explained by the finding that *BRAF*-mutated PTCs have cytologic features that cytopathologists typically recognize and classify as suspicious for malignancy or malignant (Bethesda V and VI). As a result of the low incidence of *BRAF* mutations, *BRAF* testing is not a good rule out test for thyroid cancer among Bethesda III and IV nodules. However, when the mutation is present in an indeterminate nodule, it is virtually diagnostic of malignancy as a result of the high PPV and specificity of the assay.

RAS

The *RAS* proto-oncogene encodes three different small GTPase proteins, HRAS, KRAS, and NRAS, involved in several intracellular signal transduction pathways, including the MAP kinase pathway. Mutations in the GTPase domain of the RAS proteins lead to constitutive activation of the proteins. The most common mutations involve codons 12, 13, and 61 for *KRAS* and *HRAS*, and codon 61 for *NRAS*. *RAS* mutations are highly prevalent in FTC and FVPTC (40–50 %), although very rare in PTC (10 %). *RAS* mutations are also relatively common in benign follicular adenomas (20–40 %), although it is unclear whether these *RAS*-positive adenomas are premalignant and have a higher risk of cancer progression. Overall, the prevalence of *RAS* mutations in indeterminate thyroid nodules (Bethesda III and IV) is approximately 12 %. However, as a result of the relatively high rate of *RAS* mutations in benign nodules, *RAS* mutational analysis is not an optimal test to predict malignancy in indeterminate thyroid nodules when performed as a stand-alone assay.

PAX8/PPAR-γ

The *PAX8/PPAR-γ* gene rearrangement resulting from the chromosomal translocation t(2;3)(q13;p25) is the second most common mutation in FTC (23–63 %). It is also found in approximately 5 % of Hurthle cell carcinomas and 2–10 % of follicular adenomas. Tumors with *PAX8/PPAR-γ* mutations rarely harbor concurrent *RAS* mutations, suggesting that FTCs develop through at least two distinct molecular pathways involving either *RAS* or *PAX8/PPAR-γ* mutations. *PAX8/PPAR-γ* mutated tumors tend to present at a younger age, to be smaller in size, to show a solid growth pattern, and to demonstrate vascular invasion as compared to follicular carcinomas that are negative for this mutation. Overall, the prevalence of *PAX8/PPAR-γ* mutations in indeterminate thyroid nodules (Bethesda III and IV) is very low, less than 1 %.

RET/PTC

RET/PTC gene rearrangements result from the fusion of the 3′ end of the *RET* gene and the 5′ end of various unrelated "*PTC*" genes. While there are over 12 types of *RET* rearrangements,

approximately 80 % are represented by *RET/PTC1* and *RET/PTC3*. As a result of *RET/PTC* rearrangements, the portion of the *RET* gene encoding the tyrosine kinase domain is fused in frame with an active promoter of the fusion partner gene. Consequently, the truncated RET receptor tyrosine kinase becomes constitutively expressed and activated, stimulating signaling of the MAP kinase pathway. *RET/PTC* rearrangements are present in approximately 20 % of PTC. They are more common in PTCs of children, young adults, and patients with a history of radiation exposure. Among indeterminate thyroid nodules (Bethesda III and IV), the prevalence of *RET/PTC* rearrangements is quite low, ~1 %. When one of the rearrangements is present, however, it is virtually diagnostic of malignancy.

Other Genes

In addition to the most common mutations in thyroid cancer described above (*BRAF*, *RAS RET/PTC* and *PAX8/PPAR-γ*), a variety of less frequent mutations involving the *TP53, PIK3CA, AKT1, CTNNB1, PTEN, GNAS, RET*, and *TSHR* genes have been identified. A role for *RET* mutations in the development of MTC is well-defined and discussed later in the chapter in the context of the MEN2 syndromes. However, further studies are needed to determine the role of mutations in many of these other genes, if any, in various thyroid cancers, even if they have well-defined roles in other malignancies. As is the case for many *RAS* mutations, other genes including *TSHR, PTEN,* and *GNAS* can be mutated in benign thyroid nodules.

Mutation Panels

Four Mutation Panel Testing

Individual DNA mutations such as those in *BRAF, RAS*, and *RET/PTC* and *PAX8/PPARG*, when performed singly, have relatively high PPVs and specificities—when detected, they accurately predict (rule in) the histological diagnosis of thyroid cancer in most cases. However, when individual mutations are absent, cancer cannot be reliably ruled out because of the low sensitivity and NPV of these markers.

What if these markers are assessed together? Several laboratories have taken this approach and offer this set of molecular markers as a combined four mutation panel for the evaluation of indeterminate thyroid FNA specimens. Furthermore, a number of studies looking at the combined performance of these markers have been conducted. A review of four studies analyzing the four mutation panel showed a mean sensitivity of 64 % for indeterminate thyroid FNAs, indicating a failure of the panel to identify 36 % of thyroid cancers in the indeterminate group. The largest study of the four mutation panel to date involved a retrospective analysis of prospectively collected thyroid FNA samples in which the mutation status was known to the clinicians, including the pathologists. The NPV of the four mutation panel for AUS/FLUS (Bethesda III) and FN/SFN (Bethesda IV) categories was 94 % and 86 %, respectively. However, the prevalence of malignancy in the Bethesda category III group (14 %) was lower than that seen in most other studies (~20–25 %). When a more typical prevalence of malignancy for AUS/FLUS of 24 % is applied, the resultant NPV declines to 89 %. A recent prospective blinded clinical validation study of the four mutation panel, the first of its kind, demonstrated a sensitivity and specificity for detection of thyroid cancer of 47 % and 87.5 % respectively in nodules with indeterminate (Bethesda III/IV) cytopathology. Given this sensitivity, the four mutation panel failed to identify 53 % of thyroid cancers. The NPV of the four mutation panel for Bethesda categories III and IV was 70 % with a prevalence of malignancy of 41 %. When a more typical prevalence of malignancy of 24 % is applied, the resultant NPV increases to 83 %.

Overall, the four mutation panel approach does not provide a sufficiently high NPV to be used as a stand-alone test to rule out cancer in indeterminate thyroid nodules and therefore to allow for conservative management and avoidance of surgery in this patient population. On the other hand, the four mutation panel serves as an excellent rule in test for thyroid cancer as a result of its high specificity and PPV. In cases where the decision has been made for surgery, but the extent of surgery may be influenced by the test result, the four mutation panel can provide valuable guidance. For example, total thyroidectomy, rather than lobectomy, might be

chosen for mutation positive nodules. By performing initial total thyroidectomies in patients with indeterminate cytology when a DNA mutation is present and thereby reducing the number of completion thyroidectomies, one group has reported the possibility of overall cost savings with the four mutation panel approach.

Next Generation Sequencing

NGS is an approach that allows for the simultaneous sequencing of thousands to millions of short nucleic acid sequences in a parallel fashion. Advances in NGS technology have led to dramatic price reductions in recent years to the point where the technology now offers a cost-effective approach relative to conventional sequencing technologies for some applications. NGS permits targeted sequencing of multiple genes or mutations, an approach that is becoming increasingly common for analysis of various tumors, including thyroid cancers. Cancer mutation panels are available on all of the major NGS platforms and include most of the common oncogenes and tumor suppressor genes implicated in the spectrum of human cancers. While the panels are being offered by individual clinical laboratories as laboratory developed tests (LDTs), only a single platform, Illumina's MiSeqDx instrument has been cleared by the FDA for clinical use.

For thyroid FNA analysis, NGS has the ability to detect both the mutations in the four mutation panel as well as other mutations from small amounts of starting material. However, simultaneous identification of both point mutations and gene rearrangements involved in thyroid cancer in a single assay has not yet been demonstrated. Point mutations are best identified through NGS analysis of genomic DNA (DNA sequencing), while translocations are best identified through NGS analysis of mRNA (RNA sequencing through targeted transcriptome analysis) or traditional RT-PCR approaches. An NGS approach called Thyroseq developed at the University of Pittsburgh Medical Center can simultaneously detect 284 mutations in 12 key cancer genes (*AKT1, BRAF, CTNNB1, GNAS, HRAS, KRAS, NRAS, PIK3CA, PTEN, RET, TP53,* and *TSHR*). In a recent study examining DNA from 228 benign and malignant samples from both surgical thyroid specimens as well as thyroid FNAs with follow-up surgical diagnosis, Thyroseq

identified at least one mutation in 68 % of all thyroid tumor types, including 19/27 PTCs (70 %), 25/30 FVPTCs (83 %), 14/18 FCs (78 %), 7/18 (39 %) Hurthle cell carcinomas, 3/10 (30 %) poorly differentiated carcinomas, 20/27 ATCs (74 %) and 11/15 (73 %) MTCs. Notably, 6 % (5/83) of the benign nodules were positive for mutations in the *RAS*, *TSHR, PTEN,* and *GNAS* genes, demonstrating that not all mutations are associated with thyroid cancer. No specific analysis of the performance of Thyroseq on indeterminate thyroid FNA samples was performed. In this regard, a second recent NGS analysis of mutations in 50 genes retrospectively assessed 34 indeterminate FNA samples with surgical follow-up. Mutations in *BRAF*, *NRAS, KRAS,* and *PTEN* were detected in 7/34 indeterminate FNA samples. The NGS test sensitivity and NPV were 71 % and 92 % respectively.

In summary, NGS approaches can be applied successfully to the analysis of thyroid FNA and other thyroid specimens and appear to be an effective rule-in test for thyroid cancer. When combined with assays to detect gene fusions, such as those of *RET/PTC* and *PAX8/PPAR,* through the use of RT-PCR or targeted NGS analysis of mRNA, the sensitivity for detection of thyroid cancer is expected to increase beyond the observed 68–71 % seen in initial studies, possibly to more than 80 %. However, the use of NGS as a rule out approach for indeterminate thyroid nodules requires further investigation to demonstrate clinical validity, including prospective studies of larger numbers of indeterminate FNAs with blinded and therefore unbiased surgical pathology follow-up.

MicroRNA Analyses

MicroRNAs (miRNAs) are small single stranded noncoding RNA sequences (19–25 nucleotides) that function to regulate gene expression. MiRNAs function via sequence-specific interaction with mRNA targets, binding to the 3′untranslated region and thereby suppressing translation and mRNA degradation. In addition to their function in regulation of mRNA, miRNAs have also been shown to function as tumor suppressors or oncogenes in tumor cells. MiRNAs can be detected through various molecular approaches, including microarrays and RT-PCR assays. Working with microRNAs affords several advantages over other nucleic

acids including high-stability, ability to isolate from FFPE tissues, and low input requirements for PCR assays.

In thyroid cytopathology, several studies have suggested that aberrant miRNA expression profiles may separate thyroid cancers from benign thyroid lesions. As the studies have looked at different miRNA panels, it is difficult to directly compare test performance. The most promising study assessed four miRNAs (miR-222, miR-328, miR-197, and miR-21) in 72 indeterminate thyroid FNA specimens. For the differentiation of benign from malignant thyroid nodules, sensitivity was 100 % and PPV was 90 %. These results demonstrate the promise of the miRNA approach in differentiating benign from malignant lesions in indeterminate thyroid FNA specimens. Although promising, the miRNA panels have yet to be tested on indeterminate thyroid FNAs in large, prospective, blinded, multicenter studies, and more clinical data is needed prior to the use of this approach in the clinical management of patients with indeterminate thyroid nodules. At this point, it is unclear whether miRNA panels will be most useful as a rule in or rule out approach.

Rule-Out Tests

Afirma Gene Expression Classifier (GEC)

An alternative to the rule in approach of mutation panels for the evaluation of cytologically indeterminate thyroid nodules is the rule out approach of mRNA expression analysis exemplified by the Afirma Gene Expression Classifier (GEC). Unlike the mutation panels, the GEC utilizes an approach that is designed to look for the presence of benign mRNA expression patterns in cytologically indeterminate nodules rather than the absence of specific mutations.

There are two key advantages to examining mRNA rather than DNA to distinguish benign from malignant lesions in indeterminate thyroid FNAs. First, while there are ~ 23,000 known protein-coding DNA genes, each of these may be transcribed into multiple alternatively spliced variants, with > 240,000 known mRNA isoforms. Disease-causing alterations in the DNA generally exert their effects, at least partially, on transcription, resulting in

downstream changes in the expression levels of multiple mRNA transcripts that can be measured. Second, gene expression may be impacted by lifestyle and environmental factors such that mRNA reflects additional information not discernible from DNA analysis. Thus, mRNA expression analysis has an advantage over mutation analysis in identifying gene signatures that reflect whole patterns of pathway activation resulting from both upstream mutations and environmental factors rather than alterations in a small number of genes.

The GEC was developed and clinically validated to identify benign nodules amongst those with indeterminate cytology preoperatively. Rather than relying on genes previously implicated in thyroid tumorigenesis, the design and development of the test used analysis of the whole exome to identify candidate genes most informative for the prediction of benign signatures. The resulting GEC evaluates the expression levels of 167 genes on an mRNA microarray platform that are then analyzed with a proprietary algorithm to classify indeterminate thyroid FNAs as either "Benign" or "Suspicious." Unlike some of commercially available four mutation panels for the evaluation of indeterminate thyroid nodules, extensive reagent and analytical performance studies of the GEC have been performed and published, demonstrating the reliability, robustness, and reproducibility of the assay under a variety of experimental conditions.

The GEC has been clinically validated in two independent prospective multicenter, double blind studies. The initial clinical validation publication of the GEC, performed on a set of 24 cytologically indeterminate thyroid nodule FNAs, achieved high sensitivity (100 %) and NPV (100 %). The second larger study included the largest ever prospectively collected set of thyroid FNA specimens from 3789 unique patients and 49 sites representing a mix of academic and community practices across the USA. In this study, follow-up surgical pathology was available for 265 cytologically indeterminate nodules (Bethesda III and IV). Performance of the GEC was determined by comparison of the molecular results to the surgical pathology diagnoses for each nodule based on review by a panel of thyroid experts including Dr. Juan Rosai and Dr. Virginia LiVolsi. The study demonstrated a reduction in the

ROM of cytologically indeterminate thyroid nodules (Bethesda III and IV) with "Benign" GEC (negative test) results from ~24 to ~5 %. For "Suspicious" GEC (positive test) results, the ROM of cytologically indeterminate thyroid nodules (Bethesda III and IV) was increased from ~24 to ~40 %.

Overall, the large clinical validation study of the GEC demonstrated the ability of the test to dramatically reduce the ROM for AUS/FLUS and FN/SFN subtypes to a ROM similar to that of a cytology benign diagnosis, about 5 %. In essence, the study showed the effectiveness of the GEC as a rule out test for thyroid cancer in cytologically indeterminate nodules to justify conservative management in lieu of diagnostic surgery. However, the study also concluded that the reduction in ROM for nodules classified as SFM (Bethesda V) from 62 to 15 % with Benign GEC results was insufficient to merit routine use of the test for this indeterminate subtype.

Is the test useful in the clinical setting in identifying benign nodules and preventing unnecessary surgeries? This question has been addressed by several groups who have reported their clinical experience with the GEC in routine clinical practice. In the two largest series, GEC testing of indeterminate nodules (Bethesda III and IV) led to benign results result in just over 50 % of the cases; patients with GEC Benign results were managed conservatively, with observation *in lieu* of operation, 92–94 % of the time. Most GEC benign patients in the clinical series reported to date did not undergo surgery, consistent with the purpose of the test.

An algorithm for the rule out approach of the GEC and potential clinical utility is highlighted in Fig. 5.2. Based on the 2012 estimate of 525,000 annual thyroid nodule FNAs performed in the USA and an indeterminate rate of 15–30 % (~79,000–158,000 nodules), the GEC is predicted to reclassify ~50 % of these cytologically indeterminate nodules as "Benign" (39,500–79,000 nodules). These GEC Benign nodules have a similar ROM (~5 %) as nodules with cytology benign diagnoses and are candidates for conservative management ("watchful waiting"), leading to reduction of a large number of unnecessary thyroidectomies as well as a reduction in overall health care costs. Nodules with "Suspicious" results following GEC testing carry an elevated ROM (~40 %) and are candidates for thyroid surgery, along with nodules carrying cytologic diagnoses of SFM and M (Bethesda V and VI).

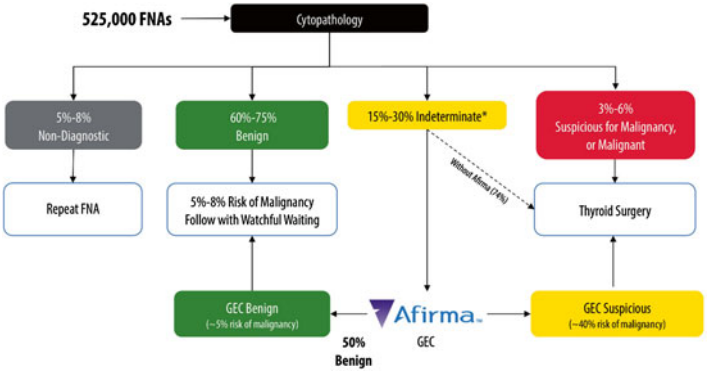

Fig. 5.2 Proposed clinical algorithm for use of the Afirma Gene Expression Classifier

Guidelines and Clinical Applications for Molecular Testing

As the use of molecular testing in thyroid cytopathology has become more widely adopted, particularly for the evaluation of cytologically indeterminate nodules, several organizations and publications including the National Comprehensive Cancer Network (NCCN) and UpToDate have included recommendations for molecular testing in their guidelines for the treatment of patients with thyroid nodules. The American Thyroid Association (ATA) and the American Association of Clinical Endocrinologists (AACE) have also recently commented on molecular testing in the context of thyroid nodule management.

NCCN

In the 2014 NCCN Guidelines (Version 2.2014) for Thyroid Carcinoma-Nodule Evaluation, the authors state: "Molecular diagnostics may be useful to allow reclassification of follicular lesions (i.e., follicular neoplasm, Hurthle cell neoplasm, atypical of undetermined significance (AUS), follicular lesions of undetermined

significance (FLUS) as they are more likely to be benign or more likely to be malignant. If molecular testing predicts a risk of malignancy comparable to the risk of malignancy seen with a benign FNA cytology (approximately 5 % or less), consider observation."

UptoDate

In the 2013 Practice Recommendation for the "Diagnostic Approach to and treatment of thyroid nodules," the authors note that for nodules with AUS/FLUS or FN/SFN cytopathology (Bethesda III and IV), "there are two approaches to the molecular characterization of FNA aspirates that are commercially available in the USA: identification of particular molecular markers of malignancy, such as BRAF and RAS mutational status, and use of high density genomic data for molecular classification (an FNA-trained mRNA classifier). The mRNA classifier measures the activity levels of 167 genes within the nodule (using the FNA aspirate). We favor using an mRNA classifier system (gene expression classifier), when available. Where available, we suggest using this classifier for evaluating patient with FNA cytology showing follicular lesion/atypia of undetermined significance or follicular neoplasm."

ATA

Although new guidelines have not been released since 2008 at the time of publication, the ATA released a draft of its proposed guidelines in June 2014 at the Endocrine Society's 96th Annual Meeting in 2014. Regarding the use of molecular markers to guide decision making in thyroid nodule management, the authors made the distinction between tests with high sensitivity and NPV and those with high specificity and PPV. They further noted that molecular markers were best used for cytological indeterminate nodules (AUS/FLUS and FN/SFN, Bethesda III/IV) in combination with clinical and sonographic features. For patients with a preference of conservative (nonoperative) management, a molecular test with

high sensitivity and NPV was recommended. For patients with a preference for surgical excision, a molecular test with high specificity and PPV was recommended, assuming it would influence the extent of surgery (hemi vs. total).

AACE

While guidelines have not been updated since the 2010 release of the AACE/AME/ETA Thyroid Nodule Guidelines, AACE recently released a "commentary" on molecular testing of thyroid nodules with indeterminate cytopathology. In this commentary, it was noted that "two principal tests are currently marketed for use to improve the malignancy risk assessment of 'indeterminate' thyroid nodules. 'Rule In' and 'Rule Out' tests attempt to confirm or exclude the presence of cancer within a thyroid nodule by means of robust positive (PPV) or negative predictive values (NPV), respectively. The Rule In tests determine the presence of single gene point mutations (*BRAF*V600E or *RAS*) or gene rearrangements (*RET*/*PTC*, *PAX8*/*PPAR*γ) that have been shown to increase the ability to predict cancer, while the Rule Out test (Afirma® gene expression classifier, GEC) utilizes a proprietary gene expression classifier (RNA expression) specifically designed to maximize the ability to define a process as benign. At present, molecular testing is meant to complement and not replace clinical judgment, sonographic assessment, and visual cytopathology interpretation."

Summary and Recommendations for Cytopathologists

These guidelines discuss the two main types of molecular testing for indeterminate thyroid nodules:

1. The "Rule In"/high specificity and PPV tests (the four mutation panel).
2. The "Rule Out"/high sensitivity and NPV tests (the Afirma GEC).

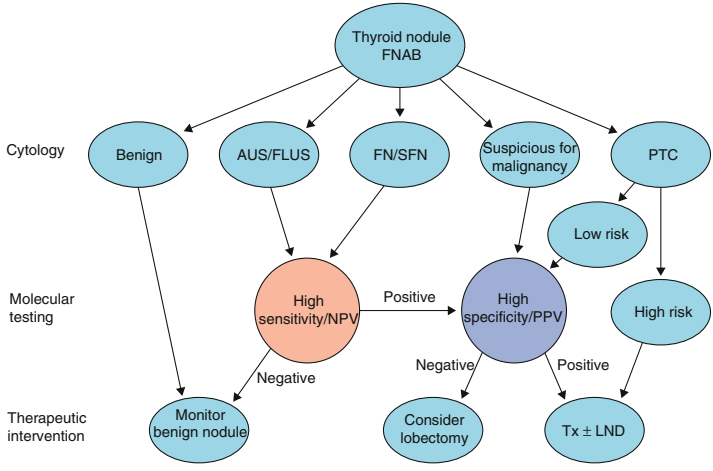

Fig. 5.3 Proposed clinical algorithm for the management of thyroid nodules on the basis of FNA cytopathology and molecular tests (kindly reproduced with permission from Lancet)

As a group, they recommend that the Rule Out approach should be used as a complement to clinical judgment, sonography, and cytopathology for evaluation of cytological indeterminate nodules (AUS/FLUS and FN/SFN, Bethesda III/IV) for patients with a preference of conservative (nonoperative) management and that such management can be considered if the result predicts a ROM comparable to the that of a benign FNA cytology. On the other hand, they suggest that the Rule In approach, as a high specificity and PPV test, is not appropriate for use as a Rule Out Test. They recommend that the Rule In approach be used in the context of patients undergoing surgery to assist in planning the extent of surgery (hemi vs total thyroidectomy).

A recent review elegantly summarizes these approaches in a proposed clinical algorithm for the management of thyroid nodules (Fig. 5.3). In this algorithm, patients with cytologically indeterminate nodules falling in the AUS/FLUS and FN/SFN categories undergo testing with a "high sensitivity/NPV" test (ie, the GEC). Those with negative/benign results proceed to conservative management/monitoring, while those with suspicious/positive results

are then candidates for further testing with a "high specificity/PPV" test (ie, BRAF, four mutation panel) along with those with "Malignant (PTC)" or "Suspicious for Malignancy" cytological diagnoses. If the high specificity/PPV test is negative, lobectomy is recommended; if positive, total thyroidectomy is recommended (with or without lymph node dissection).

Given that the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) was released before these molecular approaches became widely available, the management "suggested" for AUS/FLUS and FN/SFN (Bethesda III/IV categories) should be revised to include molecular testing, particularly rule-out tests such as the GEC. Cytopathologists, in light of their greater understanding of diagnostic testing and molecular diagnostics in general, can assist greatly in educating endocrinologists, radiologists, surgeons, and other physicians managing patients with thyroid nodules in the selection of the appropriate tests for the evaluation of indeterminate thyroid nodules.

Other Applications of Molecular Testing in Thyroid Cancer and Future Directions

Introduction

In addition to the ability to influence treatment decisions in patients with indeterminate nodules, there are several other compelling current and future applications for molecular testing in thyroid cytopathology These include tests to predict tumor response to specific therapies ("companion diagnostics") as well as tests to provide information on prognosis, tumor subtype, and recurrence. Some of the questions molecular testing can address include:

What is the risk of recurrence?
What is the tumor related mortality?
Should radioactive iodine be used? If so, at what dose?
What therapeutic drug targets are present and mutated?
What therapeutic or combination of therapeutics should be given?
What is the type and subtype of thyroid cancer?
What surgery is most appropriate?
Has the tumor recurred? If so, has the tumor changed?

Prognostic Markers

Given that most thyroid cancers are curable, even in the context of metastatic or recurrent disease, a major challenge in thyroid cancer is determining the extent and aggressiveness of therapy. There is a clear need for prognostic markers to guide treating physicians in the type and extent of treatment, including type and extent of surgery and RAI (including dose). The choice of initial surgery can impact whether a patient requires additional surgery or thyroid hormone replacement while the choice to use RAI has consequences relating to side effects and can determine how much can be given in the future, in the case of recurrent disease.

BRAF

In addition to their role as "rule-in" tests in the diagnosis of thyroid cancer, mutational markers have been linked to tumor behavior and prognosis. The *BRAF* V600E mutation in particular has been the subject of numerous studies. Many have shown an association of the V600E mutation with aggressive histopathologic features in papillary carcinomas, such as extrathyroidal extension, lymph node metastasis, tumor size, multifocal disease, and increased tumor stage along with an increased incidence of tumor recurrence of tumor-related mortality. As a result of these findings, many clinicians choose to treat *BRAF* V600E-positive tumors more aggressively. For example, if patients with *BRAF* V600E-positive tumors are detected preoperatively, they may benefit from more extensive initial surgery. Other studies have not shown a definite association of the *BRAF* V600E mutation with a negative prognosis. The reason for the variability in findings relating to prognosis is not known. However, it is possible that rather than the presence of *BRAF* V600E alone it if the coexistence of *BRAF* V600E with other mutations that more accurately determines prognosis.

Other Mutational Markers

The prognostic role of other mutational markers is less clear than for *BRAF*. Controversial data have been reported for the prognostic role of *RET/PTC* rearrangements in PTC. *RET/PTC3* has

been correlated with more aggressive histopathologic features, specifically a larger tumor size, solid variant, and a more advanced stage at diagnosis. In contrast, *RET/PTC1* rearrangement does not appear to correlate with any clinicopathologic characteristics of PTC. Overall, there is no consensus regarding the clinical prognostic value of the presence of *RET/PTC* rearrangements at this time. Similarly, *PAX8/PPAR-γ* mutated tumors have been correlated in some studies with a younger age, a smaller size, a solid growth pattern, and an increased incidence of multifocal capsular invasion or vascular invasion as compared to follicular carcinomas that are negative for this mutation. However, there is no evidence that *PAX8/PPAR-γ* status predicts outcome in follicular thyroid cancer. Larger and more comprehensive outcome analysis will be necessary to better define the prognostic value of both *RET/PTC* and *PAX8/PPAR-γ* rearrangements in thyroid cancer.

Summary

In summary, the only well-established prognostic marker in thyroid cancer is the *BRAF* V600E mutation, which appears to predict more aggressive disease and is being used to inform decisions on the extent of surgery and treatment. In guiding clinical colleagues, the cytopathologist should advise treating physicians that preoperative *BRAF* testing may be indicated if the result would impact the choice of surgery.

Predictive Markers

Response to RAI

First line therapy for differentiated thyroid carcinoma following thyroidectomy is RAI. At this time, there are no molecular markers that predict response to RAI. Rather, response to RAI is predicted by avidity of thyroid tumors to iodine determined through RAI scans. If available, such markers would be useful in guiding decisions on treatment and, for those predicted not to be responsive, sparing unnecessary radiation exposure and its side effects as well as associated costs.

Companion Diagnostics and Therapeutic Targets

Most patients (~85 %) with differentiated thyroid carcinomas are cured with surgery, RAI, and TSH suppression. A small percentage of patients develop or present with metastases and are more difficult to treat. When metastases have RAI avidity, prognosis is better, and further RAI may be used. However, when multiple doses of RAI have been tried or the patient has non-RAI avid disease, other options such as systemic therapy with targeted agents or cytotoxic chemotherapy are needed.

In such situations, drugs targeting tyrosine kinases (tyrosine kinase inhibitors) such as sorafenib, sunitinib, pazopanib, lenvatinib, and vandetanib have shown promise. The targets of these drugs include VEGFR1, VEGFR2, VEGFR3, RET, FGFR1, PDGFR-Beta, c-kit, and BRAF. To this point, only sorafenib has been approved by the FDA for the treatment of patients with locally recurrent or metastatic, progressive differentiated thyroid cancer that no longer responds to RAI treatment. Furthermore, companion diagnostic molecular tests assessing the mutational status of these tyrosine kinases have not been developed for selection of patients likely to respond to sorafenib, other TKIs, or other therapeutic targets in thyroid cancer such as EGFR, histone deactylases, PPARγ, and cyclooxygenase 2. The identification of specific mutations in the genes encoding these proteins that confer either responsiveness or resistance to specific targeted agents promises to advance the effectiveness of these treatments in the future.

Physicians have been especially interested in finding drugs to treat MTC, as thyroid hormone-based treatments (including RAI) are not effective against these cancers. Both vandetanib and cabozantinib are targeted TKIs approved by the FDA for the treatment of patients with advanced MTC. To this point, companion diagnostics have not been developed for stratification of MTC patients into groups of responders vs. non-responders for these therapeutics. However, the existence of these agents for MTC highlights the need for accurate diagnosis.

Tumor Subtyping

Identification of Medullary Thyroid Carcinoma and Other Thyroid Cancer Subtypes

The cytological diagnosis of MTC is challenging as it is uncommon and its cytological features overlap with those of other thyroid neoplasms, including follicular neoplasms and Hurthle cell neoplasms. In approximately 50 % of cases, cytopathology may not make the specific diagnosis of MTC, instead labeling FNAs as indeterminate (AUS/FLUS or FN/SFN) or malignant/suspicious for malignancy without raising the possibility of MTC. A preoperative diagnosis of MTC impacts the patient preoperative evaluation, including evaluation for multiple endocrine neoplasia type 2 (MEN2) and associated *RET* mutation status, concomitant pheochromocytoma and hyperparathyroidism. Additionally, surgical management is altered to include a minimum of total thyroidectomy and central neck dissection. When MTC is not identified preoperatively, inappropriate surgery is often selected with less than half (46 %) of MTC patients receiving the optimal initial surgery. As a consequence, patients with MTC often face potential second surgeries for removal of the remaining thyroid and performance of a central neck dissection, with associated cost, risks, diagnostic delays, and patient anxiety.

As the preoperative identification of MTC is crucial for clinical management, preoperative MTC testing is appropriate in some circumstances where there is a possibility of MTC. Serum calcitonin can be useful but has low specificity for MTC below 500 ng/L. Immunohistochemistry can also be an effective way to rule in or rule out MTC if material is available for a cell block and a small panel of stains including calcitonin and thyroglobulin, at a minimum. However, if serum calcitonin is not sufficiently elevated or if immunohistochemistry testing is not possible or equivocal, an alternative approach is the Afirma MTC Classifier, an mRNA gene expression analysis approach that analyzes expression levels of five genes in parallel with the GEC. Originally, the MTC classifier was one of a series of small gene sets termed “cassettes” designed to assist the GEC in the identification of less commonly encountered lesions that can present clinically and sonographically as

thyroid nodules. In addition to MTC, these cassettes recognize parathyroid tissue and metastatic tumors including renal cell carcinoma, breast carcinoma, and melanoma.

Recently, the MTC classifier became available either in parallel with the GEC for cytologically indeterminate thyroid FNAs or as a stand-alone test for SFM/M thyroid FNAs (Bethesda V/VI). In the context of the original validation study of the GEC, there were 2 MTCs and 263 non-MTCs among histologically confirmed specimens. 0/263 non-MTC specimens and 2/2 MTC specimens were positive for the MTC classifier, suggesting high specificity/PPV and high sensitivity/NPV. A follow-up abstract reporting on 43 patients that were positive for the MTC classifier with clinical follow-up found 42 cases confirmed as MTC (39 with surgical pathology and 3 with elevated serum calcitonin), for an overall PPV of 98 %. The single false positive MTC classifier result was found in a case of an intrathyroidal paraganglioma, a distinct but related neuroendocrine neoplasm with overlapping gene expression. Based on the available data, the MTC classifier therefore appears to be a test with both high PPV and high NPV that can accurately predict the presence of MTC in the context of FNAs that are cytologically indeterminate (Bethesda categories III and IV) as well as those that are suspicious for malignancy or malignant (Bethesda V and VI).

Patients either diagnosed with MTC or determined to have a high suspicion of MTC preoperatively through cytopathology, serum calcitonin, or the MTC classifier should be evaluated for the presence of MEN2 through RET mutation analysis. MEN2 is an inherited, autosomal dominant disorder consisting of three syndromes, MEN2A, MEN2B, and Familial Medullary Thyroid Carcinoma (FMTC), all of which result in a high lifetime risk of developing medullary thyroid carcinoma, due to mutations within the *RET* gene. The identification of patients with one of the MEN2 syndromes preoperatively is important, as previously mentioned, for proper surgical management, including evaluation of associated pheochromocytoma and hyperparathyroidism and handling of unintentionally devascularized parathyroid glands during surgery. *RET* mutation analysis is typically performed by targeted PCR and sequencing approaches and offered on whole blood specimens by the major national reference laboratories.

The identification and subtyping of other non-follicular lesions in thyroid nodule FNA specimens is most commonly approached through immunohistochemical analysis of cell block preparations. IHC allows for the diagnosis of parathyroid as well as metastatic lesions. In the evaluation of some FNAs, as discussed for MTC, in which cytologic material for IHC is not available and additional diagnostic information is needed, the GEC can be helpful in raising suspicion for parathyroid, renal cell carcinoma, breast carcinoma, or melanoma. However, further investigation of the clinical validity of these cassettes is needed to justify their use outside of the context of indeterminate thyroid FNAs.

Future Directions

The future of molecular testing in thyroid cytology holds great promise for continued improvements in the care of patients with thyroid nodules and thyroid cancer. Advances in molecular testing of thyroid nodules with indeterminate cytopathology is expected to improve upon current rule out (high sensitivity and NPV) approaches exemplified by the GEC to provide for concurrent rule in (high specificity and PPV) capabilities.

In addition, molecular testing approaches providing more specific information on tumor behavior and prognosis are likely to be developed, analogous to commercially available molecular tests that predict risk of recurrence and/or aggressive behavior in breast, colon, and prostate cancers, among others. Such tests could be used to predict which thyroid cancers would be the "bad players" meriting aggressive treatment and which would be the "good players" with low probabilities of aggressive behavior or metastasis, possibly candidates for conservative management, similar to the watchful waiting approach employed in prostate cancer.

Finally, future advances in molecular testing in thyroid cytopathology are expected to lead to the development of companion diagnostics that allow for stratification of patients into likely responders and non-responders for various targeted therapies. Such tests could be performed on cytologic specimens obtained from recurrent or metastatic lesions, such as lymph nodes, to assess changes in the mutation status that would impact

therapeutic choices and management. For diagnosis and management of thyroid cancer, as well as other malignancies, the ability to derive information from small samples, such as FNAs or other cytologic specimens, holds great potential for both improving patient care and lowering costs to the health care system.

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