Chapter 12
Molecular Diagnostics in Thyroid Cytology

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Abbreviations

ATA	American Thyroid Association
AUS/FLUS	Atypia of undetermined significance/follicular lesion of undetermined significance
BRAF	v-raf murine sarcoma viral oncogene homolog B
CALCA	Calcitonin-related polypeptide alpha
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5
cPTC	Classical papillary thyroid carcinoma
DNA	Deoxyribonucleic acid
FN/SFN	Follicular neoplasm/suspicious for follicular neoplasm
FNA	Fine-needle aspiration
FTC	Follicular thyroid carcinoma

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FV-PTC	Follicular variant of papillary thyroid carcinoma
GC	Genomic Classifier (for ThyroSeq v3)
GEC	Gene Expression Classifier (for Afirma)
GSC	Gene Sequencing Classifier (for Afirma)
HRAS	HRas proto-oncogene, GTPase
KRAS	Kirsten rat sarcoma viral oncogene homolog
KRT7	Cytokeratin 7
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MTC	Medullary thyroid carcinoma
NIFTP	Noninvasive follicular thyroid neoplasm with papillary-like nuclear features
NPV	Negative predictive value
NRAS	Neuroblastoma RAS viral oncogene
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PPV	Positive predictive value
PTC	Papillary thyroid carcinoma
PTH	Parathyroid hormone
RET-PTC1/3	Gene fusion between tyrosine kinase domain of *RET* (ret proto-oncogene) and *CCD6* gene (PTC1) or *ELE1/RFG/NCOA4* gene (PTC3)
RNA	Ribonucleic acid
ROC	Receiver operating curve
SCG3	Secretogranin III
SCN9A	Sodium voltage-gated channel alpha subunit 9
SLC5A5	Solute carrier family 5 member 5 (also known as *NIS* [sodium/iodide symporter])
SYT4	Synaptotagmin 4
TBSRTC	The Bethesda System for Reporting Thyroid Cytopathology
TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse transcriptase
TG	Thyroglobulin

TP53	Tumor protein p53
TTF-1	Thyroid transcription factor 1 (gene name: *NKX2–1*)

Key Terminology

The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC)	Standardized reporting system for thyroid fine-needle aspiration specimens, consisting of six cytomorphology-based diagnostic categories. Each category is associated with an approximate risk of cancer, which may be used to guide subsequent management decisions
Driver mutation	Refers to somatic alterations in genes (including point mutations, insertions/deletions, and gene fusions) that are responsible for the development and progression of cancer
Gene expression profiling	Analysis of the expression levels of a large panel of genes (mRNA) from cells/tissues, as a measure of the cells' biologic activity
Indeterminate cytology	Refers to the diagnostic categories within TBSRTC that are neither clearly benign nor overtly

	malignant based on cytologic features. Three categories of TBSRTC are considered indeterminate: atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular neoplasm/suspicious for follicular neoplasm (FN/SFN), and suspicious for malignancy. Most of the ancillary molecular tests described in this chapter are geared toward improving risk stratification among the lower-risk cytologically indeterminate categories (AUS/FLUS and FN/SFN)
microRNA	Short (~22 nucleotide) noncoding RNA that influences gene expression at the posttranscriptional level
microRNA expression profiling	Analysis of the expression levels of a panel of microRNAs from cells/tissues, as a measure of the cells' biologic activity
Negative predictive value (NPV)	For a medical test with a binary classification system, NPV refers to the proportion of patients with a negative test result who do not have the

	disease; i.e., percentage of "true-negative" results among all (true- and false-)negative test results. Corresponds to posttest probability of benignity if the population being tested has similar prevalence of cancer as the cohort in which a test was validated
Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)	Indolent follicular cell-derived thyroid neoplasm characterized by good demarcation, absence of invasive growth, follicular architecture, and nuclear atypia of papillary carcinoma; these tumors were formerly classified as the noninvasive subset of the encapsulated follicular variant of papillary thyroid carcinoma
Positive predictive value (PPV)	For a medical test with a binary classification system, PPV refers to the proportion of patients with a positive test result who have the disease; i.e., percentage of "true-positive" results among all (true- and false-)positive test results. Corresponds to posttest risk of disease

	if the population being tested has similar prevalence of cancer as the cohort in which a test was validated
Sensitivity	For a medical test with a binary classification system, sensitivity refers to the proportion of sick patients who are correctly identified with a positive test result. Tests with high sensitivity have low false-negative rates; consequently, a negative test result is helpful for excluding disease
Specificity	For a medical test with a binary classification system, specificity refers to the proportion of healthy patients who are correctly identified with a negative test result. Tests with high specificity have low false-positive rates; a positive test result is thus helpful for "ruling in" disease

Key Points

- Molecular diagnostics for thyroid cytology specimens is aimed at improving the risk stratification of cytologically indeterminate thyroid nodules
- Test performance can be inferred from positive and negative predictive values (PPV and NPV, respectively) reported by clinical validation studies. However, predictive values are not fixed properties of a diagnostic test. PPV and NPV vary with the prevalence of disease in the tested population
- The four commercially available molecular tests for thyroid FNAs described in this chapter all aim for a high negative predictive value to help identify cytologically indeterminate nodules that can be monitored nonsurgically
- Tests such as ThyGenX/ThyraMIR and ThyroSeq report granular estimates of cancer risk based on genotype. Therefore, the positive predictive value (where the detection of any genetic alteration in the test panel is considered a "positive" result for the purposes of statistical analysis) calculated for these tests does not necessarily reflect the full spectrum of risk stratification these tests offer in clinical practice
- Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) is an indolent tumor for which lobectomy is diagnostically necessary and therapeutically sufficient

What Is the Role of Molecular Testing in Thyroid Cytology?

FNA cytology plays an important role in the evaluation of patients with thyroid nodules. For nodules meeting clinical and ultrasonographic criteria for FNA biopsy, cytomorphologic criteria can be used to place nodules into one of the six interpretive categories outlined by the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) [1]. Each of these categories is associated with an approximate cancer risk, which in turn helps guide subsequent management decisions (Table 12.1).

Table 12.1 The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC)

Category	Risk of malignancy (%) (If NIFTP considered nonmalignant)	Risk of malignancy (%) (If NIFTP considered malignant)	Usual management
Nondiagnostic	5–10%	5–10%	Repeat FNA with ultrasound
Benign	0–3%	0–3%	Clinical and sonographic follow-up
Atypia/follicular lesion of undetermined significance (AUS/FLUS)	6–18%	10–30%	Repeat FNA, molecular testing, or lobectomy
Follicular neoplasm/suspicious for follicular neoplasm (FN/SFN)	10–40%	25–40%	Molecular testing, lobectomy
Suspicious for malignancy	45–60%	50–75%	Near-total thyroidectomy or lobectomy
Malignant	94–96%	97–99%	Near-total thyroidectomy or lobectomy

Adapted by permission from Springer Nature, Overview of Diagnostic Terminology and Reporting, Baloch et al. [1]

At the extreme ends of TBSRTC, management options are fairly straightforward (Fig. 12.1). Nodules classified as cytologically "benign" (Bethesda-II) have a low cancer risk

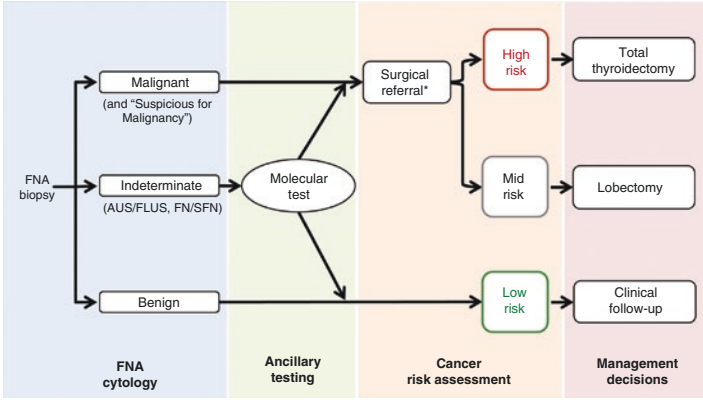


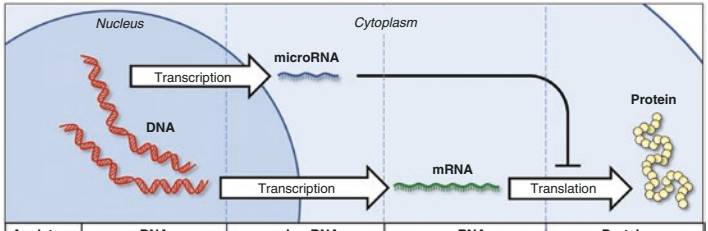
FIGURE 12.1 Simplified flowchart illustrating how cytologic and molecular testing results can guide management of thyroid nodules. The molecular tests described in this chapter are primarily indicated for aspirates classified in the "low-risk" indeterminate categories of the Bethesda System for Reporting Thyroid Cytopathology (atypia of undetermined significance/follicular lesion of undetermined significance [AUS/FLUS], follicular neoplasm/suspicious for follicular neoplasm [FN/SFN]). In this setting, ancillary molecular testing helps direct patients toward either surgical referral or clinical follow-up. Decisions regarding the extent of surgical resection (indicated by [*]) are determined by multiple factors, including (1) clinical/radiographic assessment of tumor size, extrathyroidal spread, nodal metastasis, and distant metastasis; (2) ultrasonographic and cytologic findings in the contralateral lobe; (3) patient/clinician preference; and (4) cytomorphologic or molecular features that may distinguish indolent/precancerous neoplasms from more aggressive disease. Regarding the latter, molecular tests that can detect genetic alterations characteristic of classical papillary thyroid carcinoma (e.g., *BRAF* V600E mutations, *RET-PTC1/3* fusions) could also be considered for aspirates in the higher-risk indeterminate category ("suspicious for malignancy") to help guide the extent of initial surgical resection. Abbreviations: *AUS/FLUS*, atypia of undetermined significance/follicular lesion of undetermined significance; *FN/SFN*, follicular neoplasm/suspicious for follicular neoplasm; *FNA*, fine-needle aspiration

(0–3%) and are typically followed by clinical and/or ultrasonographic observation. In contrast, nodules classified as cytologically "malignant" (Bethesda-VI, cancer risk of 94–96%) or "suspicious for malignancy" (Bethesda-V, cancer risk of 45–60%) are generally referred for surgical resection. The extent of surgery (lobectomy versus total thyroidectomy) for cytologically malignant nodules is influenced by multiple factors, including tumor size, clinical and sonographic features, and clinician/patient preference [2].

For the approximately 15–30% of thyroid aspirates that are classified in one of the indeterminate categories of TBSRTC, the decision between surgical or nonsurgical management is not as clear-cut [3]. Nodules classified as "atypia (or follicular lesion) of undetermined significance" (AUS/FLUS, Bethesda-III) or "follicular neoplasm"/"suspicious for follicular neoplasm" (FN/SFN, Bethesda-IV) have a relatively low yet non-negligible risk of malignancy, ranging from 6–18% for AUS/FLUS to 10–40% for FN/SFN [1]. Historically, surveillance by repeat FNA was an option for nodules classified as AUS/FLUS, with diagnostic lobectomy recommended for nodules that remained cytologically indeterminate on repeat FNA and/or otherwise showed worrisome clinical or sonographic features. Similarly, diagnostic lobectomy has traditionally been recommended for nodules classified as FN/SFN. However, the majority of AUS/FLUS and FN/SFN nodules that undergo surgical resection are ultimately found to be histologically benign. For these cases, surgery may be justified for diagnostic purposes but considered unnecessary from a therapeutic standpoint.

Ancillary molecular testing has emerged as a promising tool to improve risk stratification among thyroid nodules placed in these low-risk indeterminate categories of TBSRTC (Fig. 12.1). Molecular testing has dual aims in this context: (1) to identify biologically benign nodules that can be followed clinically rather than surgically and (2), for nodules that warrant resection, to help guide the extent of initial surgery (lobectomy versus total thyroidectomy). Of note, the primary indication for each of the molecular tests described herein is a cytologically indeterminate FNA. Therefore, routine microscopic evaluation of cytology slides is an essential step in determining whether ancillary molecular testing is appropriate for a thyroid nodule.

DNA, microRNA, mRNA, and proteins have all been investigated as analytes for ancillary testing on thyroid cytology specimens (Fig. 12.2). The four molecular tests that are currently offered by commercial laboratories for cytologically indeterminate thyroid FNAs are all nucleic acid-based tests and form the focus of this chapter: **Afirma Gene Expression Classifier** (Veracyte, Inc., South San Francisco, California), **RosettaGX Reveal** (Rosetta Genomics, Inc., Philadelphia, Pennsylvania), **ThyGenX/ThyraMIR** (Interpace Diagnostics, Parsippany, New Jersey), and **ThyroSeq** (University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, and CBLPath, Inc., Rye Brook, New York). These tests can be categorized by their general testing approach: (1) expression profiling for a panel of genes (mRNAs) or microRNAs, (2) genotyping for tumor-associated driver mutations and gene fusions, or (3) a combination of these methodologies (Table 12.2). Several immunohistochemical stains including HBME1, CK19, galectin-3, and *BRAF* VE1 (mutation-specific



Analyte	DNA	microRNA	mRNA	Protein
Testing approach (and examples of tests)	NGS for point mutations, small insertion/deletions (ThyGenX, ThyroSeq)	RT-PCR to examine expression pattern of panel of microRNAs (ThyraMIR, RosettaGX Reveal)	DNA microarray to examine expression pattern of panel of mRNAs (Afirma GEC) NGS for gene fusions (ThyGenX, ThyroSeq)	Immunocytochemistry to examine expression pattern of various proteins (e.g., antibodies to BRAF VE1, HBME1, CK19, Galectin-3)

FIGURE 12.2 Analytes used in ancillary testing for thyroid cytology specimens. DNA, microRNA, mRNA, and proteins have all been explored as analytes to help risk-stratify thyroid nodules with indeterminate cytology. Examples of testing approaches using each of these analytes are shown. Abbreviations: *DNA*, deoxyribonucleic acid; *RNA*, ribonucleic acid; *mRNA*, messenger RNA; *PCR*, polymerase chain reaction

Table 12.2 Comparison of testing approaches, starting materials, sample collection, and concurrent cytology review

	Afirma GEC	RosettaGX Reveal	ThyGenX/ ThyraMIR	ThyroSeq
Company (Location)	Veracyte, Inc. (South San Francisco, California)	Rosetta Genomics, Inc. (Philadelphia, Pennsylvania)	Interpace Diagnostics, Inc. (Parsippany, New Jersey)	University of Pittsburgh Medical Center (Pittsburgh, Pennsylvania) and CBLPath, Inc. (Rye Brook, New York)
Testing approach	Expression profiles of 142 mRNAs by DNA microarray	Expression profiles of 24 microRNAs by qRT-PCR	ThyGenX: Hotspot mutations in 5 genes and 3 gene fusions by targeted NGS ThyraMIR: Expression profiles of 10 microRNAs by qRT-PCR	Hotspot mutations in 14 genes and 42 gene fusions by targeted NGS
Substrate for molecular testing	Fresh cells collected into nucleic acid preservative	Fixed cells on routine cytology slides (direct smear or liquid-based cytology)	Fresh cells collected into nucleic acid preservative	Fresh cells collected into nucleic acid preservative

Minimum quantity of material required for molecular testing	2 dedicated FNA passes	1 cytology slide with sufficient cellularity for cytologic interpretation	1 dedicated FNA pass containing at least 50 ng of cellular material	1–2 drops of FNA material
Sample collection/ shipping kits provided by vendor	Yes	Yes	Yes	Yes
Cytology review	Centralized[a]	Local or centralized	Local or centralized	Local or centralized

[a]Selected centers have been authorized to submit samples for Afirma testing based on cytologic review by local cytopathologists

antibody for the *BRAF* V600E mutation) have also been explored as markers of malignancy in thyroid resection specimens. The potential utility of these antibodies in thyroid cytology specimens has been explored in a variety of studies, but they will not be discussed further in this chapter [4–11].

When evaluating the performance of these ancillary molecular tests for cytologically indeterminate thyroid FNAs, readers should be aware of several caveats:

- Test performance is often extrapolated from its positive predictive value (PPV; corresponding to the posttest cancer risk associated with a positive test result) and negative predictive value (NPV; corresponding to the posttest probability of benignity associated with a negative test result). Importantly, PPV and NPV are not fixed properties of a test. Instead, these predictive values vary with the pretest probability of cancer in the tested population, which may differ from institution to institution [12, 13]. The prevalence of cancer among thyroid nodules classified as AUS/FLUS or FN/SFN is one estimate of the pretest cancer risk and can serve as a useful measure for determining whether the targeted test population for a particular institution is comparable to the population that was studied in the clinical validation of a molecular test.
- In clinical validation studies, the histopathologic reference diagnosis of resected thyroid nodules is typically classified in a binary manner (i.e., benign or malignant) to facilitate statistical analysis. However, this practice runs counter to evolving concepts of thyroid neoplasia as a continuum rather than a dichotomous process [2, 14].
- Similarly, clinical validation studies also confine the results of molecular tests into binary outcomes (negative or positive) to simplify statistical analysis. This approach may be apt for tests that report binary outcomes, such as the Afirma Gene Expression Classifier and Rosetta GX Reveal. However, for genotyping-based tests such as ThyroSeq or ThyGenX/ThyraMIR that offer a wide range of test results, the reduction of test results into either a "negative" or "positive" outcome for statistical purposes does not fully capture the gradation of risk estimates offered by these tests.

Expression Profiling to Risk-Stratify Indeterminate Thyroid FNAs

Histologically benign and malignant tumors show differential expression patterns of selected genes [15–18] and microRNAs [19–23]. These studies have formed the basis of ancillary tests that use proprietary algorithms to risk-stratify cytologically indeterminate thyroid FNAs based on either mRNA expression patterns (Afirma Gene Expression Classifier) or microRNA expression patterns (RosettaGX Reveal and ThyraMIR). The algorithms for these expression profiling-based tests have been optimized for high sensitivity and NPV to help "rule out" cancer among cytologically indeterminate thyroid nodules.

Afirma Gene Expression Classifier

The Afirma Gene Expression Classifier (GEC) analyzes the expression pattern of a large group of target genes using DNA microarrays (Fig. 12.3) [24]. The starting material for Afirma consists of two dedicated FNA passes collected into a vial of proprietary nucleic acid preservative solution, in addition to the FNA passes collected for microscopic cytology evaluation. If the cytology is classified as indeterminate (AUS/FLUS or FN/SFN), the concurrent sample collected for molecular testing is processed for microarray analysis. As a quality control step, the sample is first screened for the gene expression profiles of lesions that are not suited for analysis by the main GEC, including metastatic tumors (melanoma, breast carcinoma, renal cell carcinoma), parathyroid, and medullary thyroid carcinomas (MTC) (Table 12.3) [25, 26]. This screening step also includes gene expression analysis to identify samples concerning for malignant oncocytic (Hürthle-cell) thyroid tumors. Samples that trigger one of these six screening cassettes are reported as having a "suspicious" Afirma result, without subsequent analysis by the main 142-gene expression classifier. A sample that shows the expression pattern of MTC is additionally reported as "positive" for the

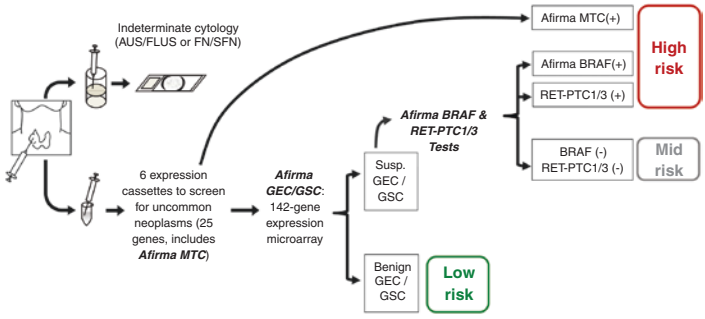


Figure 12.3 Afirma GEC/GSC and Malignancy Classifiers. See text for details. Abbreviations: *GEC*, Gene Expression Classifier; *GSC*, Gene Sequencing Classifier; *AUS/FLUS*, atypia of undetermined significance/follicular lesion of undetermined significance; *FN/SFN*, follicular neoplasm/suspicious for follicular neoplasm; *MTC*, medullary thyroid carcinoma. (Figure adapted from Nishino and Nikiforova [24] with permission from Archives of Pathology & Laboratory Medicine. Copyright 2018 College of American Pathologists)

Afirma MTC test, described further below. Specimens that pass this screening step advance to the main GEC, where the expression pattern of 142 genes is analyzed by a proprietary algorithm that classifies each FNA sample in a binary manner, as having either a "benign" or "suspicious" gene expression profile. The algorithm was trained using the gene expression profiles of histologically benign and malignant nodules.

The Afirma GEC was clinically validated in a prospective, multi-institutional study involving 129 AUS/FLUS (24% cancer prevalence), 81 FN/SFN (25% cancer prevalence), and 55 "suspicious for malignancy" (62% cancer prevalence) cases [27]. Among aspirates in the lower-risk cytologically indeterminate categories (AUS/FLUS or FN/SFN), Afirma demonstrated 90% sensitivity and ~50% specificity for cancer, corresponding to a high NPV (94–95%) for "benign" GEC results and a modest PPV (37–38%) for "suspicious" GEC results (Table 12.4) [25, 28–30]. Thus, for clinical practices where the prevalence of malignancy among AUS/FLUS and

Table 12.3 Quality controls to determine cellular composition of sample undergoing molecular analysis

	Afirma GEC	**RosettaGX Reveal**	**ThyGenX/ThyraMIR**	**ThyroSeq**
Confirmation of thyroid follicular cell sampling?	Yes: gene expression analysis for follicular cell adequacy [26]	Yes: nucleic acid extracted directly from follicular cells present on the cytology slide	Yes: gene expression analysis for thyroid follicular cells[a]	Yes: gene expression analysis for *TG*, *TTF1*, *NIS*, *KRT7* mRNAs [25]
Markers for C-cells and/or medullary carcinoma assessed?	Afirma MTC (gene expression analysis for *CALCA*, *CEACAM5*, *SCG3*, *SCN9A*, *SYT4*)	*miR-375*	*miR-375*	Gene expression analysis for *CALCA* mRNA
Markers for parathyroid assessed?	Gene expression analysis for *DMRT2*, *GCM2*, *KIDINS220*, *KL*, *PTH*, *SYCP2L*, *TMEM14B*	Unknown	Yes	Gene expression analysis for *PTH* mRNA
Others	Gene expression analysis for metastatic breast carcinoma, renal cell carcinoma, and melanoma	Unknown	Gene expression analysis for hematolymphoid markers	Gene expression analysis for *KRT20* mRNA (metastatic tumors)

[a]Personal communication from Dr. Sydney Finkelstein (Interpace Diagnostics)

Table 12.4 Performance characteristics of commercially available tests for indeterminate thyroid FNAs based on published validation studies[a]

	Afirma GEC [27]	RosettaGX Reveal [29]		ThyGenX/ ThyraMIR [28]	ThyroSeq (v2) [25, 30]
		Retrospective, multicenter			
Validation study design	**Prospective, multicenter**	**Entire validation set[b]**	**Agreement set[b]**	**Prospective, multicenter**	**Prospective and retrospective, single center**
Number of indeterminate FNAs	210	150	116	109	239
Prevalence of cancer (%)	24	21	12	32	26
Sensitivity (%)	90	74	100	89	90
Specificity (%)	52	74	80	85	93
NPV (%)	94	92	100	94	96
PPV (%)	37	43	41	74	81

[a]Only nodules classified as atypia/follicular lesion of undetermined significance (AUS/FLUS, Bethesda-III) or follicular neoplasm/suspicious for follicular neoplasm (FN/SFN, Bethesda-IV) are included in this table

[b]For comparison with the other tests in this table, only nodules with AUS/FLUS or FN/SFN cytology are included from RosettaGX Reveal's validation cohort. "Agreement Set" refers to subset of cases for which all three pathologists reviewing the resection specimen agreed on the reference histopathologic diagnosis

FN/SFN are similar to that of the Afirma validation cohort, the risk of cancer for a cytologically indeterminate nodule with a "benign" GEC result is ~5–6% (equivalent to 1-NPV). This low level of cancer risk is comparable to that of cytologically benign nodules, for which clinical/ultrasonographic monitoring is considered appropriate. In general, approximately 40% of patients with cytologically indeterminate thyroid nodules can avoid diagnostic surgery based on a "benign" Afirma GEC result [27, 31–36]. The remaining nodules with "suspicious" GEC results have a modest cancer risk (37–38%, corresponding to the PPV), for which a diagnostic lobectomy is generally advised. Of note, subset analyses in both the Afirma clinical validation study as well as independent post-validation studies suggest reduced specificity of the Afirma test among oncocytic (Hürthle-cell) lesions, raising concern that the test may overcall a larger proportion of histologically benign oncocytic neoplasms as having a "suspicious" GEC result relative to non-oncocytic thyroid lesions [32–34, 36].

To address the modest specificity and PPV of a "suspicious" Afirma GEC result, Veracyte offers additional tests known collectively as the Afirma Malignancy Classifiers. Afirma MTC and Afirma BRAF tests were introduced in 2014; RNA sequencing for *RET-PTC1/3* gene fusions was added to the Afirma Malignancy Classifier panel in 2017. As described above, the **Afirma MTC** is included among the screening cassettes used for quality control for the Afirma test. Afirma MTC identifies medullary thyroid carcinoma in FNA samples with high sensitivity and specificity by evaluating the expression levels of five genes: *CALCA*, *CEACAM5*, *SCG3*, *SCN9A*, and *SYT4* [37, 38]. Preoperative detection of MTC by FNA can facilitate surgical planning (total thyroidectomy with central lymph node dissection) in addition to prompting germline *RET* mutation analysis for multiple endocrine neoplasia type 2, as well as laboratory and imaging studies for metastatic disease, pheochromocytoma, and hyperparathyroidism. Patients with pheochromocytoma should undergo adrenergic blockade and adrenalectomy prior to thyroid surgery, while patients with

hyperparathyroidism can undergo parathyroid surgery at the time of thyroidectomy [39].

The other two tests that comprise the Afirma Malignancy Classifiers evaluate samples for genetic changes associated with papillary thyroid carcinoma. The **Afirma BRAF** test analyzes samples for the gene expression profile associated with the *BRAF* V600E mutation [40], while the ***RET-PTC1/3*** assay uses RNA sequencing to identify oncogenic gene fusions involving the *RET* proto-oncogene. In the context of thyroid nodules, detection of *BRAF* V600E mutation, *RET-PTC1* gene fusion, or *RET-PTC3* gene fusion has high specificity for papillary thyroid carcinoma, whereby a positive test result can help establish a malignant diagnosis preoperatively and can influence decisions regarding the extent of the initial surgical procedure.

In 2017, Veracyte released an updated version of the Afirma test known as the Gene Sequencing Classifier (GSC). In addition to the incorporation of *RET-PTC1/3* gene fusion analysis to the Malignancy Classifiers, the new Afirma GSC uses an enhanced classification algorithm with reportedly superior specificity compared to the GEC, particularly among oncocytic nodules.

Taken together, the Afirma GSC (or GEC) and Malignancy Classifiers may help stratify cytologically indeterminate aspirates into three risk levels (Fig. 12.3):

- *Low risk* for cancer based on a "benign" Afirma GSC/GEC result, for which clinical and ultrasonographic monitoring of the nodule may be sufficient
- *Intermediate risk* for cancer based on a "suspicious" Afirma GSC/GEC result (with negative Afirma Malignancy Classifier results), for which diagnostic lobectomy is generally indicated
- *High risk* for cancer based on a "suspicious" Afirma GSC/GEC result with positive Afirma Malignancy Classifier results, for which surgical resection (lobectomy versus total thyroidectomy, depending on tumor size and clinical/ultrasonographic features) is indicated

RosettaGX Reveal

MicroRNAs are small (~22 nucleotide) noncoding RNAs that regulate gene expression at the posttranscriptional level by influencing the stability and translation of mRNA. The differential expression of selected microRNAs between benign and malignant thyroid nodules [19–23, 41], together with the stability of microRNAs and their ability to be isolated from routine formalin-fixed histology or alcohol-fixed cytology samples [19, 42–44], has encouraged the development of two microRNA-based commercial assays for risk-stratifying cytologically indeterminate thyroid FNA specimens: RosettaGX Reveal and ThyraMIR. The latter is a complementary test to ThyGenX and will be discussed in more detail in the next section.

RosettaGX Reveal uses cells harvested from routinely stained direct smears or liquid-based cytology slides as the starting material for molecular testing (Table 12.2, Fig. 12.4). There are two main advantages of using routine cytology slides as the substrate for molecular testing: (1) decreased need for dedicated FNA passes to collect cells specifically for molecular testing, as the diagnostic cytology slides can be repurposed for nucleic acid extraction, and (2) decreased potential for sampling error (as can occur when separate FNA passes are performed for microscopic and molecular analysis), since nucleic acid is extracted from the same cells that are considered indeterminate by microscopic evaluation (Table 12.3). One potential drawback to this approach is the need to sacrifice a diagnostic cytology slide for molecular testing; Rosetta Genomics offers digital slide-scanning services to maintain a digital archive of the cytomorphology.

Following nucleic acid extraction, the test analyzes the expression pattern of 24 microRNAs (Table 12.5) by RT-PCR to classify each sample as “benign” or “suspicious” by microRNA profiling. The inclusion of hsa-miR-375 in the 24-microRNA panel helps identify MTC among cytologically indeterminate FNAs. In a retrospective multicenter clinical validation study involving 189 AUS/FLUS, FN/SFN, and

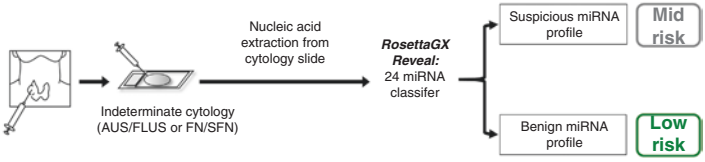


Figure 12.4 RosettaGX Reveal. See text for details. Abbreviations: *AUS/FLUS*, atypia of undetermined significance/follicular lesion of undetermined significance; *FN/SFN*, follicular neoplasm/suspicious for follicular neoplasm. (Figure adapted from Nishino and Nikiforova [24] with permission from Archives of Pathology & Laboratory Medicine. Copyright 2018 College of American Pathologists)

suspicious for malignancy aspirates (combined cancer prevalence of 32%), RosettaGX Reveal had 85% sensitivity, 72% specificity, 91% NPV, and 59% PPV for cancer [29].

Two caveats should be considered when reviewing the validation study for RosettaGX Reveal. First, the validation study reported higher test sensitivity (98%) and NPV (99%) among an "Agreement Set" comprised of a subset of 150 cases (27% prevalence of cancer) in which all three pathologists evaluating the resection specimen (two study pathologists, in addition to the original pathologist rendering the clinical diagnosis) concurred on the reference histopathologic diagnosis. The post-unblinding exclusion of 14 encapsulated follicular variant of papillary carcinomas from the "Agreement Set" (five of which were misclassified as having a "benign" microRNA profile by RosettaGX Reveal) likely accounts for the superior test sensitivity and NPV. Secondly, the advertised performance characteristics of RosettaGX Reveal are based on a validation cohort that includes "suspicious for malignancy" FNAs. In contrast, the performance characteristics of the other three commercial molecular tests for thyroid FNAs are based on validation cases classified cytologically as AUS/FLUS or FN/SFN. For the purposes of comparison with the other tests, we provide sensitivity, specificity, NPV, and PPV calculations for RosettaGX Reveal based only on AUS/FLUS and FN/SFN cases from their validation study:

Table 12.5 List of microRNAs included in RosettaGX Reveal and ThyraMIR tests

RosettaGX Reveal	ThyraMIR
hsa-miR-31-5p	hsa-miR-31-5p
hsa-miR-222-3p	hsa-miR-222-3p
hsa-miR-146b-5p	hsa-miR-146b-5p
hsa-miR-375	hsa-miR-375
hsa-miR-551b-3p	hsa-miR-551b-3p
hsa-miR-138-5p	hsa-miR-138-1-3p
hsa-miR-486-5p	hsa-miR-139-5p
hsa-miR-23a-3p	hsa-miR-29b-1-5p
hsa-miR-574-3p	hsa-miR-155
hsa-miR-152-3p	hsa-miR-204-5p
hsa-miR-200c-3p	
hsa-miR-345-5p	
hsa-miR-5701	
hsa-miR-424-3p	
hsa-miR-3074-5p	
hsa-miR-346	
hsa-miR-342-3p	
hsa-miR-181c-5p	
hsa-miR-125b-5p	
MID-50971	
MID-20094	
MID-50976	
MID-50969	
MID-16582	

- Total AUS/FLUS and FN/SFN cases (n = 150, 21% cancer prevalence): 74% sensitivity, 74% specificity, 92% NPV, and 43% PPV
- "Agreement Set" AUS/FLUS and FN/SFN cases (n = 116, 12% cancer prevalence): 100% sensitivity, 80% specificity, 100% NPV, and 41% PPV

Thus, RosettaGX Reveal's microRNA classifier shows performance characteristics that parallel that of the Afirma GEC. Among AUS/FLUS and FN/SFN nodules, a "benign" microRNA profile is associated with a low cancer risk (0–8%, depending on which of the above subset analyses are used) and may be safe to follow by clinical observation. On the other hand, AUS/FLUS and FN/SFN nodules with "suspicious" microRNA profiles are associated with an intermediate cancer risk (41–43%), for which surgical referral should be considered (Table 12.4).

Genotyping-Based Testing Approaches

A variety of mutations and gene rearrangements in the mitogen-activated protein kinase (*MAPK*) and phosphoinositide 3-kinase (PI3K) signaling pathways have been identified in thyroid cancer [45]. Oncogenic alterations in papillary thyroid carcinomas (PTC) include mutations in *BRAF* (40–50% of PTCs) or *RAS* (10–20% of PTCs), as well as *RET-PTC1* or *RET-PTC3* gene fusions (10–20% of PTCs). Similarly, genetic alterations in follicular thyroid carcinomas (FTC) include *RAS* mutations (40–50% of FTCs) and *PAX8-PPARG* gene fusions (30% of FTCs).

Testing FNA specimens for the *BRAF* V600E mutation alone may be useful as a predictive biomarker in specific situations. In patients with advanced thyroid cancer refractory to radioactive iodine treatment, detection of the *BRAF* V600E mutation can help identify patients who may benefit from clinical trials using selective BRAF inhibitors [46–49]. Cytology specimens may be a useful substrate for *BRAF* testing in this setting, since such patients typically have recurrent/

metastatic disease or surgically unresectable thyroid cancer (e.g., undifferentiated [anaplastic] thyroid carcinoma) amenable to FNA biopsy.

From a diagnostic standpoint, a single-gene testing approach for thyroid FNAs is limited in two ways. While detection of the *BRAF* V600E mutation in a thyroid FNA can secure a diagnosis of PTC with near-100% certainty (reviewed in [50]), this mutation is infrequent (~5%) among cytologically indeterminate thyroid FNAs, for which a positive molecular testing result would have the greatest impact on management decisions [51, 52]. Secondly, testing for *BRAF* V600E alone is insufficiently sensitive for malignancy because only 40–50% of PTCs harbor this mutation; the absence of this mutation does not exclude malignancy among cytologically indeterminate nodules. Taken together, the cost-effectiveness and utility of routine *BRAF* V600E testing as a sole marker for "ruling in" or "ruling out" cancer are dubious.

Given the limitations in this single-gene testing approach, the clinical application of mutational analysis for cytologically indeterminate thyroid FNAs has largely focused on multiplexed genotyping methods. An early genotyping panel for thyroid FNAs consisted of hotspot mutations in four genes (*BRAF*, *HRAS*, *KRAS*, *NRAS*) and three gene fusions (*RET-PTC1*, *RET-PTC3*, and *PAX8-PPARG*) to help risk-stratify thyroid FNAs with indeterminate cytology. Numerous studies have evaluated the performance of this seven-marker panel for thyroid FNAs [28, 53–58]. The largest clinical validation of this panel was a single-institution prospective study involving 247 AUS/FLUS (14% prevalence of cancer) and 214 FN/SFN (27% prevalence of cancer) aspirates. In this study, the seven-marker genotyping panel was reported to have high specificity (97–99%) and PPV (87–88%) for cancer [57]. Based on these results, commercial versions of this seven-marker panel were initially marketed as tests for "ruling in" malignancy among cytologically indeterminate thyroid nodules, whereby the detection of a mutation or gene fusion could direct a patient to definitive treatment with total thyroidectomy.

However, two caveats must be considered regarding the clinical utility of this seven-marker panel. First, for genotyping-based tests such as the seven-marker panel and the others described below, the PPV reported in clinical validation studies does not capture the gradation of cancer risk estimates associated with positive test results. For instance, the *BRAF* V600E mutation and *RET-PTC1/3* gene fusions are associated with near-100% risk for papillary carcinoma in the context of thyroid FNAs. In contrast, *RAS* mutations and *PAX8-PPARG* gene fusions have been identified in a broad spectrum of benign, premalignant, and malignant follicular-patterned neoplasms (e.g., follicular adenoma, follicular carcinoma, noninvasive follicular thyroid neoplasm with papillary-like nuclear features [NIFTP], encapsulated follicular variant of papillary thyroid carcinoma) and may be best considered markers of neoplasia rather than malignancy per se [14, 45, 56, 57, 59–66]. In other words, genotyping tests offer more granular estimates of cancer risk than can be conveyed by the test's reported PPV.

Secondly, in the aforementioned validation study, the seven-marker panel demonstrated a modest sensitivity (57%–63%) for malignancy, corresponding to 86–94% NPV among AUS/FLUS and FN/SFN cases [57]. Because of the 6–14% residual cancer risk (1-NPV) associated with a negative test result, this seven-marker panel was considered clinically inadequate as a test for "ruling out" cancer for patients with cytologically indeterminate thyroid nodules. Two commercially available tests have adopted different strategies to overcome the low NPV of the seven-marker genotyping panel. **ThyGenX/ThyraMIR** combines a limited genotyping panel with a microRNA-based expression classifier to improve sensitivity and NPV for malignancy. Alternatively, **ThyroSeq** tests for a vastly expanded panel of genetic alterations to improve the sensitivity and NPV of the genotyping approach for risk-stratifying cytologically indeterminate thyroid aspirates.

ThyGenX/ThyraMIR

Interpace Diagnostics combines microRNA expression profiling (ThyraMIR) with a limited genotyping panel (ThyGenX) to improve the risk stratification of cytologically indeterminate thyroid aspirates (Table 12.2). This testing approach requires a dedicated FNA pass collected into a vial of proprietary nucleic acid preservative, in addition to the FNA passes required for visual cytopathology interpretation (Fig. 12.5). For nodules with indeterminate cytology, the sample collected for molecular testing is processed as follows:

- Assessment of the expression levels of genes associated with thyroid follicular cells for quality control purposes (Table 12.3).
- ThyGenX tests thyroid FNA samples for oncogenic mutations in five genes (*BRAF*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*) and three gene fusions (*RET-PTC1*, *RET-PTC3*, *PAX8-PPARG*) using a next-generation sequencing platform.
  - The detection of a *BRAF* V600E mutation or *RET-PTC1/3* gene fusion is considered virtually diagnostic of malignancy in a thyroid FNA due to the strong

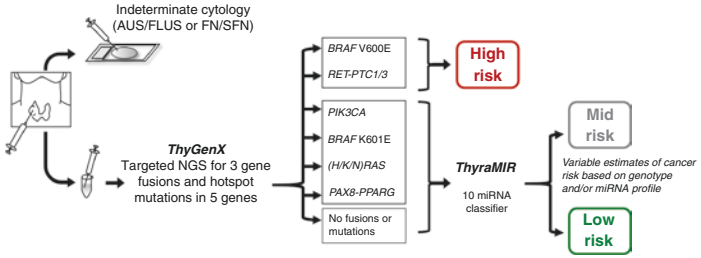


FIGURE 12.5 ThyGenX/ThyraMIR. See text for details. Abbreviations: *AUS/FLUS*, atypia of undetermined significance/follicular lesion of undetermined significance; *FN/SFN*, follicular neoplasm/suspicious for follicular neoplasm; *NGS*, next-generation sequencing; *miRNA*, microRNA. (Figure adapted from Nishino and Nikiforova [24] with permission from Archives of Pathology & Laboratory Medicine. Copyright 2018 College of American Pathologists)

association of these genetic alterations with papillary thyroid carcinoma.
  - For the remaining ThyGenX results (i.e., no mutation/fusion, *H-/K-/N-RAS* mutations, *BRAF* K601E mutation, *PIK3CA* mutations, or *PAX8-PPARG* fusion), further refinement of cancer risk is accomplished with the ThyraMIR test.

- ThyraMIR assays for the expression patterns of ten microRNAs using quantitative RT-PCR to classify samples as having either a low-risk/benign versus high-risk/positive microRNA profile. ThyraMIR's test panel includes six microRNA sequences that closely overlap with RosettaGX Reveal's panel of 24 microRNAs (Table 12.5).

In a prospective multicenter validation study of 109 AUS/FLUS and FN/SFN aspirates, the combined ThyGenX/ThyraMIR tests demonstrated 89% sensitivity and 85% specificity for malignancy [28]. The cancer prevalence in this cohort of cytologically indeterminate nodules was 32%; at this prevalence of malignancy, the NPV of the combined ThyGenX/ThyraMIR tests was 94% (Table 12.4). In other words, "double-negative" samples with negative ThyGenX results and a low-risk microRNA profile by ThyraMIR testing have an approximately 6% (1-NPV) cancer risk and may be safe to follow by clinical observation.

For statistical analysis, the validation study defined "positive" results as the detection of any mutation/fusion (by the ThyGenX test) and/or high-risk microRNA profile (by the ThyraMIR test). While this definition of test positivity yielded a PPV of 74% in the validation study, it is important to keep in mind that genotyping-based tests offer results that span a wide range of risk levels. Therefore, in clinical practice, ThyGenX/ThyraMIR may help risk-stratify cytologically indeterminate FNA samples as follows:

- *Low risk* for cancer based on the absence of a mutation or gene fusion (negative ThyGenX test) and low-risk microRNA profile (negative ThyraMIR test). Clinical and ultrasonographic monitoring of the nodule may be sufficient.

- *Intermediate risks* for cancer based on other permutations of ThyGenX results (no mutation/fusion, *H-/K-/N-RAS* mutations, *BRAF* K601E mutation, *PAX8-PPARG* fusion) and ThyraMIR results (low- versus high-risk microRNA expression patterns). For samples in this category, Interpace Diagnostics uses laboratory data to refine estimates of cancer risk, which in turn typically warrant diagnostic lobectomy.
- *High risk* for cancer based on detection of *BRAF* V600E mutation or *RET-PTC1/3* fusions by the ThyGenX test. Surgical resection (lobectomy versus total thyroidectomy, depending on tumor size and clinical/ultrasonographic features) is indicated.

ThyroSeq

In 2014, The Cancer Genome Atlas (TCGA) project published its analysis of genomic alterations of nearly 500 PTCs [67]. This comprehensive approach identified novel oncogenic alterations associated with PTC, effectively reducing the fraction of PTCs with unknown driver mutations from 25% to 3.5% [67]. Nikiforov et al. capitalized on these large-scale genomic studies to develop **ThyroSeq**, which uses targeted next-generation sequencing to assay for a broad panel of single nucleotide variants, insertions/deletions, and gene fusions associated with thyroid neoplasia (Table 12.2).

ThyroSeq requires 1–2 drops of FNA material (collected into a vial of proprietary nucleic acid preservative solution) as the substrate for molecular testing (Fig. 12.6). Gene expression analysis serves as a quality control measure to monitor the cellular makeup of the sample (Table 12.3). Expression of genes such as *TTF1*, thyroglobulin (*TG*), sodium/iodide symporter (*SLC5A5/NIS*), and cytokeratin 7 (*KRT7*) are used to confirm adequate sampling of thyroid follicular cells in the aspirate. Conversely, aspirates with expression of genes associated with parafollicular/C cells (calcitonin-related peptide alpha [*CALCA*]) or parathyroid cells (parathyroid hormone [*PTH*]) can be flagged as

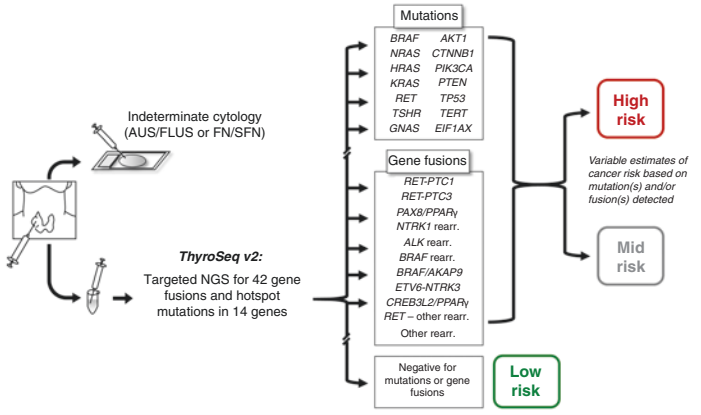


FIGURE 12.6 ThyroSeq. See text for details. Abbreviations: *AUS/FLUS*, atypia of undetermined significance/follicular lesion of undetermined significance; *FN/SFN*, follicular neoplasm/suspicious for follicular neoplasm; *NGS*, next-generation sequencing. (Figure adapted from Nishino and Nikiforova [24] with permission from Archives of Pathology & Laboratory Medicine. Copyright 2018 College of American Pathologists)

suspicious for medullary thyroid carcinoma or parathyroid sampling, respectively.

The list of genetic alterations included in the ThyroSeq test panel has evolved with updated versions of the test. The most comprehensive clinical validation of ThyroSeq to date has involved single-center studies using ThyroSeq v2, which includes 42 types of gene fusions and mutational hotspots in 14 different genes in its test panel [25, 30]. These validation studies have included a combination of prospectively and retrospectively analyzed thyroid FNA samples. Among 239 nodules with indeterminate cytology (96 AUS/FLUS and 143 FN/SFN, with a combined cancer prevalence of 26%), ThyroSeq v2 had high sensitivity (~90%) and specificity (~93%) for malignancy, corresponding to a NPV of 96% and PPV of 81% (Table 12.4). Independent reports of ThyroSeq v2 performance in actual clinical practice support the high NPV of the test [68–70]. At the same time, these post-validation

studies indicate that the test's PPV for cancer may be lower (22–63%) than the 81% PPV that was initially reported in the clinical validation study. The lower PPV of a "mutation-positive" ThyroSeq v2 result in these studies may be explained in part by the prevalence of histologically benign or premalignant neoplasms that harbor *RAS*, *RAS*-like, and *EIF1AX* mutations [68–71].

As discussed above, interpretation of PPV is challenging for genotyping-based tests because the type of mutation factors heavily into posttest cancer risk. Mutations in *RAS* and related ("*RAS*-like") pathways may be considered a marker of neoplasia but appear to be less specific for malignancy, given the detection of these genetic changes in a range of benign, premalignant, and malignant follicular-patterned neoplasms. In contrast, *BRAF* V600E mutations and related ("*BRAF*-like") genetic alterations help rule in malignancy with near-100% specificity among indeterminate thyroid FNAs due to their strong association with papillary thyroid carcinoma. Additionally, *TERT* promoter mutations and *TP53* mutations – particularly when they co-occur with *BRAF*-like or *RAS*-like driver alterations – have been associated with clinically aggressive thyroid cancers, including undifferentiated (anaplastic) thyroid carcinoma and poorly differentiated thyroid carcinoma [72–79]. Finally, the allelic frequency with which a mutation/fusion is detected in a FNA sample may also inform posttest cancer risk, to the extent that a genetic alteration present at a low level implies an early step in the clonal evolution of a neoplasm.

Taken together, broad targeted genotyping panels like ThyroSeq v2 can help triage thyroid nodules by risk level, as follows:

- *Low risk*: Nodules that are negative for all mutations/fusions in the test panel or positive for a marker associated with benignity may be safe to monitor by clinical observation due to a very low (3–4%) risk of cancer.
- *Intermediate risks*: For nodules with isolated *RAS*, *RAS*-like, or *EIF1AX* mutations, diagnostic lobectomy may be suitable as the initial surgical approach, given the moderate risk of cancer in this setting.

- *High risk*: For nodules with *BRAF* V600E mutation or *RET-PTC1/3* gene fusion, surgical resection (lobectomy versus total thyroidectomy, depending on tumor size and clinical/sonographic features) is indicated due to the virtually 100% risk of papillary thyroid cancer associated with these alterations. The detection of *TP53* or *TERT* promoter mutations, particularly in concert with other alterations in the panel, may indicate a biologically aggressive cancer.

ThyroSeq v3, offered commercially since in 2017, makes two major updates to the test: (a) expansion of the number of genes in the test panel to 112 (compared to 56 genes in ThyroSeq v2) and (b) analysis of several genomic regions for copy-number alterations that are associated with thyroid cancer [67].The thyroidectomy specimens used for the training set for ThyroSeq v3 were also enriched for oncocytic (Hürthle-cell) nodules, with the goal of improving the preoperative distinction between nonneoplastic, benign neoplastic, and malignant Hürthle-cell tumors.

Each type of genetic alteration in the test panel is assigned a point value commensurate to its association with malignancy, as determined from review of the published literature as well as from analysis of internal and publically searchable databases. This weighted point-based system allows for the integration of all genetic alterations in a sample (or lack thereof) into a single "Genomic Classifier" (GC) score [80]. In the analytic validation study for ThyroSeq v3, authors used receiver operating curve (ROC) analysis to establish a GC cutoff for optimal sensitivity and specificity for malignancy. GC scores below this threshold are reported as "negative" (favoring benignity), while samples at or beyond the cutoff are reported as "positive." Using this GC cutoff, ThyroSeq v3 demonstrated 98.0% specificity and 90.9% sensitivity for malignancy among an analytic validation cohort of 175 thyroid FNA samples that was enriched for cancer (52.6% prevalence of cancer). As with previous versions of ThyroSeq, the genotype and allelic frequency of genetic alterations should provide additional risk

stratification among GC "positive" cases. Clinical validation of ThyroSeq v3 in a prospective, blinded, multicenter study is in progress at this time.

Is One Ancillary Molecular Test Superior to the Others?

There is no evidence to date that one of the commercially available tests described in this chapter is superior to any of the others. On the one hand, direct head-to-head comparisons between these tests using a common validation cohort are currently lacking, due in part to the prohibitive costs associated with such a study. While the NPV and PPV reported by the clinical validation studies for each test reflect test performance to a degree, the differences in test design as well as differences in the composition of their respective validation cohorts limit meaningful comparison across studies. For these reasons, the latest management guidelines from the American Thyroid Association (ATA) do not endorse a specific molecular test for thyroid FNAs with indeterminate cytology [2].

With these caveats in mind, one emerging viewpoint is that the various approaches for molecular testing of thyroid FNA samples may be fundamentally similar from the standpoint of patient care. A high NPV for ruling out cancer remains a shared and vital goal for all four molecular tests reviewed in this chapter: the ability to identify biologically benign nodules preoperatively can triage appropriate patients toward clinical observation, thereby avoiding thyroid lobectomy for purely diagnostic purposes.

Genotyping tests offer a high degree of granularity in their results compared to the binary outcomes of gene expression-based tests; yet, for clinical decision-making, the granular genotyping results are typically binned into broader risk categories to help patients and clinicians choose between clinical observation and surgical management (and for the latter, to guide the extent of initial thyroid surgery). As a case

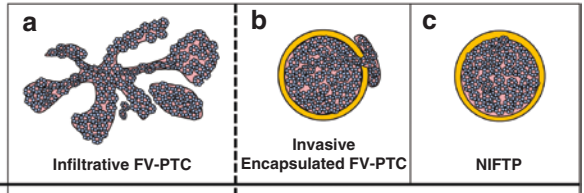
in point, the detection of *RAS* and *RAS*-like mutations in FNA samples by genotyping tests such as ThyroSeq – while providing insight into the phenotype and molecular biology of a patient's thyroid nodule – generally leads to similar risk-based management recommendations (diagnostic lobectomy) as a "suspicious" Afirma GEC result.

The addition of markers that help "rule in" malignancy such as the *BRAF* V600E mutation (in the form of the Afirma BRAF test) and *RET-PTC1/3* gene fusions to Afirma's test panel further supports the notion that the different molecular tests for thyroid FNAs appear to converge with respect to their ability to stratify cytologically indeterminate aspirates as being either high, intermediate, or low risk for cancer (Fig. 12.1).

Ancillary Molecular Testing for Thyroid FNAs in the NIFTP Era

In recent years, there has been a trend toward more conservative treatment options for carefully selected low-risk thyroid neoplasms [2]. The recent nomenclature revision regarding noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) underscores ongoing efforts to classify and manage thyroid neoplasms commensurate to their risk of recurrence and/or metastasis [14].

Historically, thyroid tumors demonstrating a follicular architecture and the nuclear atypia of papillary carcinoma were classified as the "follicular variant" of papillary thyroid carcinoma (FV-PTC). However, the term "FV-PTC" itself encompasses tumors with diverse biologic and clinical characteristics; subclassification of these tumors relies mainly on histopathologic evaluation of tumor circumscription and invasion (Fig. 12.7). FV-PTCs with diffuse, infiltrative growth into the adjacent thyroid parenchyma (**Infiltrative FV-PTC**, Fig. 12.7a) are similar to classical papillary thyroid carcinoma (cPTC), with a tendency to be driven by *BRAF*-like alterations and a predilection for local recurrence and cervical lymph node metastasis [81–84].



Intranuclear pseudoinclusions	Relatively frequent	None to rare	
Molecular alterations	"BRAF-like"	"RAS-like"	
Low-magnification appearance	Infiltrative; no encapsulation	Well-circumscribed and/or encapsulated	
Invasion	Parenchymal infiltration ± vascular invasion	Capsular and/or vascular invasion	No
Clinical behavior	Similar to classical-type PTC	Similar to FTC	Indolent

FIGURE 12.7 Follicular-patterned thyroid neoplasms with the nuclear atypia of papillary carcinoma: a comparison of pathologic, molecular, and clinical features. In the past, the term "follicular variant of papillary thyroid carcinoma" (FV-PTC) has been applied to each of these three tumors. Studies over the past decade have identified pathologically, molecularly, and clinically distinctive subcategories among these tumors: (**a**) infiltrative FV-PTC, (**b**) invasive encapsulated FV-PTC, and (**c**) NIFTP. Abbreviations: *NIFTP*, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; *PTC*, papillary thyroid carcinoma; *FTC*, follicular thyroid carcinoma

In contrast, FV-PTCs that are encapsulated or otherwise well-demarcated from the surrounding thyroid parenchyma bear more molecular and clinical resemblance to follicular adenoma/carcinoma rather than cPTC. Encapsulated/well-demarcated FV-PTCs with capsular or vascular invasion (**Invasive Encapsulated FV-PTC**, Fig. 12.7b) have a predilection for distant metastasis via hematogenous spread, similar to follicular carcinomas [85]. On the other hand, encapsulated/well-demarcated FV-PTCs with no evidence of capsular or vascular invasion have an exceptionally indolent clinical course, akin to follicular adenomas [14, 83, 85–91]. Given the very low malignant potential of these tumors, the noninvasive subset of encapsulated/well-demarcated FV-PTC was recently reclassified as "noninvasive follicular thyroid neoplasm with papillary-like nuclear features" (Fig. 12.7c). NIFTP may be

considered a precursor to its invasive counterpart. Such tumors are adequately treated by thyroid lobectomy and generally do not require completion thyroidectomy or radioactive iodine treatment [14, 85]. Careful adherence to the histopathologic criteria for NIFTP (Table 12.6) is essential to maintain the reproducibility and very low malignant potential of the NIFTP diagnosis [14, 92].

Genotyping studies of NIFTP have identified mutations in *RAS*, *BRAF* (K601E), and *EIF1AX*, as well as chromosomal rearrangements involving *THADA* or *PAX8-PPARG* [14, 93]. These alterations are similar to those of other follicular-patterned thyroid tumors such as follicular adenoma, follicular carcinoma, and invasive encapsulated FV-PTC and distinct from the "*BRAF*-like" genetic alterations characteristic of

Table 12.6 Histopathologic inclusion and exclusion criteria for the diagnosis of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP)

Inclusion criteria for NIFTP	**Exclusion criteria for NIFTP**
Encapsulation or clear demarcation	Capsular or vascular invasion
Predominantly follicular growth pattern	True papillary architecture
Nuclear score of 2 or 3[a]	>30% solid, insular, or trabecular architecture
	Psammoma bodies
	Features of tall cell or columnar cell variant of PTC
	Tumor necrosis
	>3 mitoses per 10 high-power (400x) fields

[a]Nuclear score refers to a three-point scoring system for assessing the nuclear atypia. One point is assigned for each of the following: (a) nuclear size and shape [enlargement, elongation, overlapping], (b) nuclear membrane irregularities [irregular contours, grooves, pseudoinclusions], and (c) chromatin changes [pallor/clearing, margination of chromatin to membrane]

cPTC and infiltrative FV-PTC [50, 67, 81, 84, 88, 94, 95]. Importantly, NIFTP and invasive encapsulated FV-PTC have overlapping molecular features, and the only distinguishing feature between NIFTP and invasive encapsulated FV-PTC to date is the histologic detection of capsular or vascular invasion in the latter (similar to the distinction between follicular adenoma and follicular carcinoma). Consequently, the diagnosis of NIFTP can only be made on resection specimens following histologic examination of the entire tumor periphery to exclude invasive growth [14, 92]. For these reasons, lobectomy is considered diagnostically necessary but therapeutically sufficient for NIFTP.

What Are the Cytologic Features of NIFTP?

As its name suggests, NIFTP is characterized by a follicular growth pattern and the presence of "papillary-like nuclear features," both of which can be seen to varying degrees in FNA cytology specimens. Retrospective studies have shown that nuclear atypia (nuclear enlargement and crowding, nuclear contour irregularity, nuclear molding, and chromatin pallor) can help distinguish aspirates of NIFTP from those of benign follicular nodules (i.e., follicular adenomas or adenomatous/hyperplastic nodules) [96–98]. Cytoarchitectural and/or nuclear features may also help distinguish aspirates of NIFTP from cPTC and infiltrative FV-PTC. Architecturally, aspirates of NIFTP yield a predominantly microfollicular cellular arrangement, in contrast to the papillary architecture or sheetlike groups characteristic of cPTC [99,100]. Furthermore, nuclear contour irregularity is generally limited in NIFTP compared to cPTC or infiltrative FV-PTC, with most cases of NIFTP showing rare or no intranuclear cytoplasmic pseudoinclusions [84, 100–103]. These observations are in keeping with retrospective analyses showing that aspirates of NIFTPs (or equivalent tumors with their former name, noninvasive encapsulated FV-PTC) are usually classified in one of the indeterminate categories of TBSRTC (AUS/FLUS, FN/SFN,

or suspicious for malignancy) rather than as "malignant" [97, 101, 104–111]. Thus, for aspirates with microfollicular architecture and modest nuclear atypia, recognition of the possibility of NIFTP and judicious use of these indeterminate categories for such cases may help encourage lobectomy rather than total thyroidectomy as the initial surgical approach.

Of note, reliable cytologic distinction between NIFTP and invasive encapsulated FV-PTC is not possible due to overlapping architectural and nuclear features [82, 84, 97, 99, 100]. As described above, the only distinguishing feature between these two tumors to date remains the histologic detection of capsular and/or vascular invasion.

What Are the Implications of the NIFTP Nomenclature Change on Thyroid FNA Molecular Testing?

The four commercially available molecular tests for thyroid FNAs discussed in this chapter were developed and clinically validated prior to the NIFTP nomenclature revision, at a time when noninvasive encapsulated FV-PTCs were by and large considered malignant tumors. Not surprisingly, these ancillary molecular tests often classify aspirates of NIFTPs as abnormal. Retrospective studies have reported NIFTPs among tumors identified as having "suspicious" Afirma GEC results [111–115] or *RAS*/"*RAS*-like" genetic alterations by genotyping tests such as ThyroSeq [60, 70, 111, 113].

Some authors have suggested that these molecular testing results should be considered false-positive outcomes when detected in NIFTPs and have recommended revalidation of these tests in view of the NIFTP reclassification [116]. However, there are counterarguments to conflating NIFTP with nodules demonstrating overtly benign histology. In contrast to most benign follicular nodules, NIFTPs currently require surgical management (i.e., lobectomy) for diagnostic

and therapeutic purposes [14, 117]. In this light, the detection of NIFTPs as abnormal by molecular testing seems to be well-suited with current recommendations for diagnostic lobectomy for nodules with "suspicious" Afirma GEC results or *RAS*/"*RAS-like*" genotyping results.

Conclusions

Molecular and clinicopathologic studies have contributed to an increasingly nuanced model of thyroid neoplasia in recent years. The emergence of molecular diagnostics for thyroid FNAs reflects a larger trend toward a more risk-stratified approach to the diagnosis and management of thyroid neoplasms.

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