Molecular and Other Ancillary Tests

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Background

Insights into the genomic landscape of thyroid neoplasia have improved our ability to characterize thyroid tumors on FNA cytology samples [1]. These advances have led to several clinically relevant applications over the past decade, with gradual incorporation of FNA-based molecular testing into thyroid tumor management guidelines [2–5]. Chief among these applications has been the use of molecular diagnostics for the subset of thyroid nodules with indeterminate (AUS or FN) cytology. For these nodules, molecular testing complements cytomorphologic, clinical, and sonographic

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evaluation to fine-tune their risk of malignancy and to inform clinical decisions regarding active surveillance and diagnostic or therapeutic surgery. More recently, molecular testing of thyroid FNAs has also been leveraged to identify prognostic and predictive biomarkers for patients with an established diagnosis of thyroid cancer. This chapter will briefly summarize the key molecular changes in thyroid neoplasia, highlight the established and emerging uses of ancillary molecular testing for thyroid FNA specimens, and review the various lab-developed and commercially available molecular testing platforms that are currently used in clinical practice.

Overview of Molecular Changes in Thyroid Neoplasia

To date, ancillary molecular diagnostic tests for thyroid FNAs have largely focused on nucleic acid-based testing strategies. Genomic alterations in thyroid neoplasia include variants that occur at the DNA level as well as those that result in measurable changes in mRNA or microRNA expression profiles.

DNA-Level Alterations

Large-scale tumor genotyping studies have revealed recurrent single nucleotide variants, insertions and deletions, gene fusions, and copy number alterations in thyroid neoplasms [6–11]. Many of these variants result in overactivation of the MAPK (RAS-RAF-MEK-ERK) and/or PI3K/AKT/mTOR signaling pathways. Characteristic associations between driver alterations and thyroid tumor types are illustrated in Fig. 14.1 and summarized below.

- The detection of a *BRAF* V600E mutation and other driver alterations that confer *BRAF*-like gene expression profiles (e.g., *RET*, *BRAF* fusions) are highly specific for thyroid cancer and are typically associated with classical papillary thyroid carcinoma (cPTC) and tall-cell subtype of PTC [6].
- *ALK* and *NTRK* fusions are also highly specific for PTC, usually classical type with prominent follicular pattern or infiltrative follicular variant of PTC [12]. Rare cases of primary secretory carcinoma of the thyroid harboring *ETV6::NTRK3* fusion have also been described [12, 13].
- *RAS*-like alterations (e.g., mutations in *HRAS*, *KRAS*, *NRAS*, *BRAF* K601E, *EIF1AX*, *PTEN*, *DICER1*, and gene fusions involving *PPARG* or *THADA*) can be considered molecularly indeterminate for cancer, as such alterations are found in a broad spectrum of both benign and malignant follicular-patterned neoplasms, including follicular adenoma (FA), follicular thyroid carcinoma (FTC), noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), and invasive encapsulated follicular variant of papillary carcinoma (invasive EFV-PTC) [10, 14, 15].
- Mutations in *TP53*, *TERT* promoter, *AKT1*, and *PIK3CA* are generally considered late events in thyroid tumorigenesis that are associated with clinically

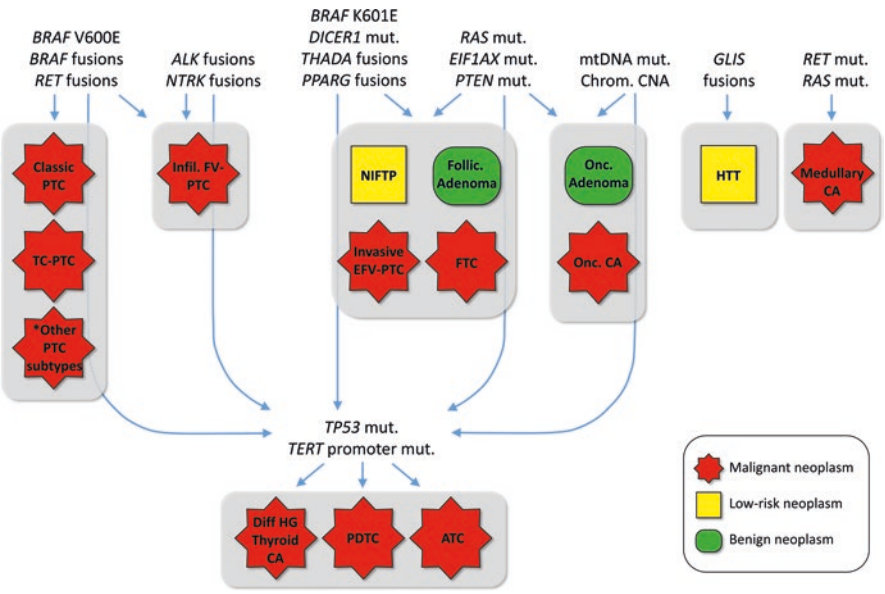

Fig. 14.1 Characteristic relationships between genomic alterations and thyroid tumor type. While *BRAF* V600E and other *BRAF*-like alterations are strongly associated with cancer, *RAS* mutations and other *RAS*-like alterations are associated with a range of benign, low-risk, and malignant neoplasms. Clinically aggressive thyroid cancers often harbor multiple mutations, including co-occurrence of driver mutations with *TP53* and/or *TERT* promoter mutations. *Other PTC subtypes with *BRAF*-like alterations include solid, diffuse sclerosing, columnar cell, hobnail, and Warthin-like subtypes. Abbreviations: *PTC* papillary thyroid carcinoma, *TC* tall-cell subtype, *Infil. FV-PTC* infiltrative follicular variant of PTC, *NIFTP* noninvasive follicular thyroid neoplasm with papillary-like nuclear features, *EFV-PTC* encapsulated follicular variant of PTC, *FTC* follicular thyroid carcinoma, *mtDNA* mitochondrial DNA, *Chrom.CNA* chromosomal copy number alterations, *Onc* oncocytic, *CA* carcinoma, *HTT* hyalinizing trabecular tumor, *Diff HG Thyroid CA* differentiated high-grade thyroid carcinoma, *PDTC* poorly differentiated thyroid carcinoma, *ATC* anaplastic thyroid carcinoma

aggressive cancers. Co-mutations of these genes with one of the driver alterations listed above are seen with increased frequency in differentiated thyroid cancers with distant metastasis as well as poorly differentiated thyroid carcinoma and undifferentiated (anaplastic) thyroid carcinoma [9, 16].

- Mitochondrial DNA mutations and recurrent chromosome-level copy number alterations are characteristic of oncocytic (formerly Hürthle cell) neoplasms [17, 18]. Additional oncogenic mutations (e.g., in *TERT* promoter, *TP53*, and *RAS* family of genes) superimposed on this background have been reported in oncocytic carcinomas [7, 8].
- Recent NGS studies of hyalinizing trabecular tumor (HTT) have identified *PAX8::GLIS3* and *PAX8::GLIS1* gene fusions, the detection of which may help distinguish HTT from papillary carcinoma on FNA cytology samples [19–21].
- Molecular alterations in medullary thyroid carcinoma include oncogenic *RET* mutations (germline or somatic) as well as somatic *RAS* mutations [22–24].

- Given these distinctive associations, genotyping-based tests for thyroid FNAs do not provide a binary "negative" or "positive" result. Instead, such tests offer a gradient of cancer probability (and information suggesting tumor type and prognosis as well) based on the type, number, and allelic frequency of the alterations that are identified.

Gene (mRNA) and microRNA Expression Alterations

A tumor's mRNA expression profile reflects which genes—and ultimately which proteins—are turned "on" or "off" to modulate cellular activity in response to various genetic, epigenetic, and environmental changes. High-throughput gene expression profiling studies have identified expression profiles that broadly distinguish benign and non-neoplastic lesions from cancer [25], as well as gene expression-based subgroups (e.g., *BRAF*-like and *RAS*-like) corresponding to particular genotypes and histologic subtypes of thyroid neoplasms [6, 10].

MicroRNAs are short (~22 nucleotide) noncoding RNAs that regulate gene expression at the post-transcriptional level. Certain microRNAs are divergently up- or down-regulated among different types of thyroid tumors [26–30]. Such differences in gene and/or microRNA expression profiles have been harnessed as a diagnostic tool for risk-stratifying cytologically indeterminate follicular cell-derived thyroid tumors on FNA samples [31]. To the extent that medullary thyroid carcinoma, parathyroid tissue, and metastases to the thyroid express genes and/or microRNAs that are distinct from follicular cell-derived tumors, expression profiling may also help identify these lesions on FNA material. The breadth of genes and microRNAs used in commercial expression profiling panels varies widely, from those that assay a small list of markers to those that interrogate thousands of genes using machine learning algorithms.

Current and Emerging Roles of Molecular Testing for Thyroid FNA Specimens

Refining Cancer Probability in Nodules Classified as AUS and FN

As discussed in Chap. 1, the usual management of thyroid nodules following FNA is informed by the implied risk of malignancy (ROM) associated with each Bethesda category. For nodules classified in the lower-risk indeterminate (AUS and FN) categories of TBSRTC, molecular testing can help refine ROM estimates and guide the decision between sonographic surveillance and diagnostic/therapeutic thyroidectomy (Fig. 14.2).

Various molecular testing formats have been applied to cytologically indeterminate thyroid FNAs, ranging from single-marker tests to extensive mutational and/or gene expression panels. While the currently available molecular tests offer different degrees of diagnostic stratification, test results can be generalized into one of three broad bins:

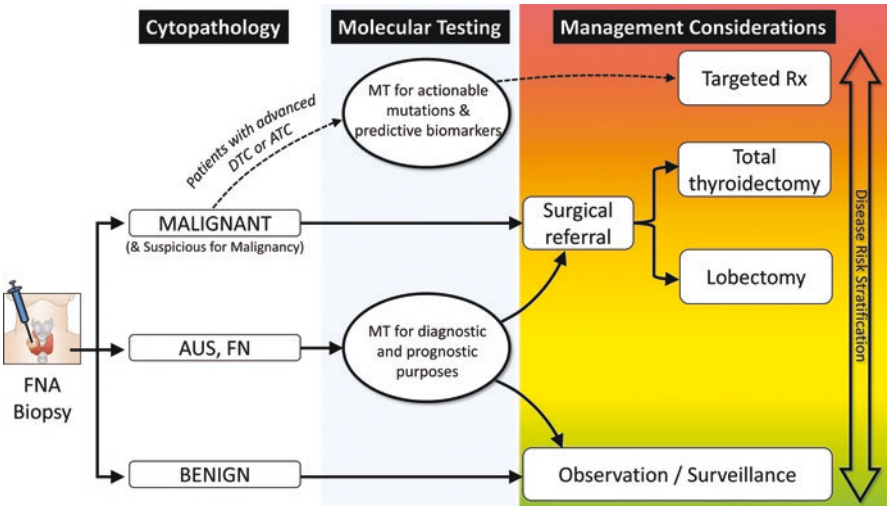

Fig. 14.2 Model summarizing the roles of FNA-based molecular testing (MT) for patients with thyroid tumors. The primary purpose of thyroid FNA-based MT over the past decade has been diagnostic/prognostic: to refine the cancer risk and guide management of lower-risk cytologically indeterminate (AUS and FN) nodules. For selected patients with advanced differentiated thyroid cancer (DTC) or anaplastic thyroid carcinoma (ATC), MT for actionable driver mutations and other predictive biomarkers can guide patients towards targeted therapies (Rx). (Model adapted from Nishino M and Krane JF. Updates in Thyroid Cytology. Surgical Pathology 2018; 11: 467–487 [32])

- *Low molecular probability of cancer* similar to the ~3% ROM associated with a cytologically benign nodule, for which clinical/sonographic surveillance would be appropriate. Tests capable of "ruling out" malignancy require high sensitivity and high (typically >95%) negative predictive value (NPV) for cancer.
- *Intermediate molecular probability of cancer*. These test results are often associated with neoplasia but lack the specificity to distinguish malignant neoplasms from benign ones. If surgery is pursued, lobectomy can be diagnostically helpful and therapeutically sufficient in most cases.
- *High molecular probability of cancer* similar to the 97–99% ROM associated with a cytologically malignant diagnosis, for which thyroidectomy is generally offered for therapeutic purposes. Tests capable of "ruling in" malignancy generally include markers strongly associated with classic papillary carcinoma (e.g., *BRAF* V600E mutation and *RET* fusions).

The use and interpretation of molecular tests for cytologically indeterminate thyroid nodules raise several important caveats.

- *The **population-based** ROM estimates provided by molecular test reports do not necessarily equal an **individual patient's** risk of thyroid cancer*. With respect to molecular testing, ROM estimates are generally derived from the positive and negative predictive values (PPV and NPV) observed in the clinical validation of

the test. PPV and NPV vary with the pre-test probability of disease, as described by Bayes' theorem. The approximate prevalence of cancer among the cytologically indeterminate categories of TBSRTC (i.e., 20–32% for AUS and 25–50% for FN) is often used as a practical stand-in for pre-test probability. However, a number of patient- and nodule-specific factors (e.g., age, sex, risk factors, family history, nodule size, sonographic features, and cytologic features) also influence pre-test probability. Accordingly, clinical and radiologic context should be considered together with cytopathology when (a) selecting nodules for molecular testing and (b) interpreting the results thereof.

- *The choice of molecular test for individual practices will depend in part on regional and global differences in management paradigms, as have been historically reported between Western and Asian countries* [33, 34]. For practices with a relatively low threshold to pursue diagnostic surgery for nodules with indeterminate cytology, large multigene test panels with high NPVs would be valuable to identify those nodules that can be spared unnecessary diagnostic surgery. In contrast, for practices where clinical guidelines favor active surveillance [35], a smaller panel of markers with high PPV for cancer (e.g., *BRAF* V600E single-gene test or 7-gene panel, discussed below) may suffice for selecting nodules that warrant resection.
- *In clinical validation studies of molecular tests, tumors ranging from indolent neoplasms (NIFTP) to high-grade carcinomas have been collectively classified as "Malignant" for the purposes of calculating binary test performance metrics such as sensitivity, specificity, NPV, PPV, and ultimately, "ROM."* While such groupings are convenient for statistical analysis, they obscure the prognostic spectrum that thyroid neoplasia spans (discussed below) [2, 36].

Prognostication of Tumors Based on Molecular Profiles

The use of molecular tests on thyroid FNA samples for preoperative risk stratification can be expanded beyond primary diagnosis to include tumor prognostication as well, with respect to structural disease recurrence, distant metastasis, and cancer-related mortality. The well-characterized associations between a tumor's molecular profile and its prognosis permit stratification of tumors into low, intermediate, and high molecular risk groups (MRG) [16]. Typically, the low MRG is represented by a single *RAS* mutation or *RAS*-like variant. The intermediate MRG includes the *BRAF* V600E mutation, other *BRAF*-like variants, or copy number alterations. The high MRG profile is characterized by the co-occurrence of one of the aforementioned driver alterations together with mutations in genes such as *TERT*, *TP53*, *AKT1*, and/or *PIK3CA*; this profile helps identify a subgroup of thyroid cancers with unfavorable outcomes. While routine molecular testing is not firmly established for thyroid FNA specimens that are suspicious or positive for malignancy, knowledge of a thyroid nodule's MRG profile in such cases, together with its clinical and radiologic features, may help select surgical options (e.g., lobectomy versus upfront total thyroidectomy) that are commensurate to tumor prognosis.

Identification of Systemic Therapy and/or Clinical Trials Tailored to a Tumor's Molecular Profile

For patients with advanced stage, locally recurrent, rapidly progressive, and/or metastatic disease who are not candidates for standard surgical and/or radioactive iodine (RAI) treatment, testing for actionable driver alterations may guide the selection of systemic therapy and/or clinical trials tailored to a tumor's particular molecular profile. For anaplastic carcinoma in particular, testing for targetable alterations (e.g., *BRAF*, *NTRK*, *ALK*, *RET*, tumor mutational burden, microsatellite instability, mismatch repair deficiency) may be useful in the neoadjuvant setting to convert an unresectable or borderline-resectable tumor into one that is amenable to surgery [3, 37]. The ability to detect these alterations on cytology and small biopsy samples obviates the need for more invasive surgical procedures in this context.

Targeted therapy strategies include (a) kinase inhibitors that selectively block constitutively activated receptor or cytoplasmic kinase signaling pathways [38–40], (b) redifferentiation therapy to enhance radioactive iodine uptake in RAI-refractory tumors [41, 42], and (c) immune checkpoint inhibition [43]. Table 14.1 lists examples of drugs targeting specific molecular alterations relevant to thyroid cancer.

Table 14.1 Summary of drugs targeting specific molecular alterations and possible application in clinical practice

Molecular alteration	Drugs	Clinical application
BRAF V600E mutation	Dabrafenib	Dabrafenib + trametinib (MEK inhibitor) *BRAF* V600E-mutated ATC, DTC, PDTC Redifferentiation of *BRAF* V600E-mutated PTC or PDTC
RAS mutation	Selumetinib, trametinib	Redifferentiation of RAS-mutated PTC or FTC or PDTC
RET mutation	Selpercatinib, pralsetinib	MTC
mTOR mutation	Everolimus	DTC, MTC, ATC
RET fusion	Selpercatinib, pralsetinib	RET fusion thyroid carcinoma Redifferentiation of RET-fused thyroid carcinoma
NTRK fusion	Larotrectinib	*NTRK* fusion thyroid carcinoma Redifferentiation of *NTRK*-fused thyroid carcinoma
	Repotrectinib	*NTRK* or *ALK* or *ROS* fusion thyroid carcinoma
	Entrectinib	*NTRK* or *ALK* or *ROS* fusion thyroid carcinoma
ALK fusion	Crizotinib	*ALK*-fusion thyroid carcinoma
	Repotrectinib	*NTRK* or *ALK* or *ROS* fusion thyroid carcinoma
	Entrectinib	*NTRK* or *ALK* or *ROS* fusion thyroid carcinoma
ROS1 fusion	Repotrectinib	*NTRK* or *ALK* or *ROS* fusion thyroid carcinoma
	Entrectinib	*NTRK* or *ALK* or *ROS* fusion thyroid carcinoma

Screening for Germline Alterations Associated with Hereditary Syndromes

Although thyroid FNA molecular testing is intended primarily for the detection of somatic alterations in tumor cells, these tests may identify germline mutations suggestive of hereditary cancer syndromes as well. Germline mutations associated with hereditary forms of thyroid cancer are summarized in Table 14.2 along with their

Table 14.2 Hereditary cancer syndromes associated with increased risk for thyroid cancer (*adapted from References* [44–48])

Syndrome	Germline mutation	Type of thyroid neoplasm	Incidence of thyroid lesions	Key extrathyroidal clinical features
MEN 2A and FMTC	*RET* (exons 10 and 11 most common)	MTC	90–100% (usually presents in adulthood)	MEN2A: pheochromocytoma, hyperparathyroidism, variants with cutaneous lichen amyloidosis and Hirschsprung disease FMTC: no association with pheochromocytoma or hyperparathyroidism
MEN 2B	*RET* (95% with exon 16 M918T mutation; <5% with exon 15 A883F mutation)	MTC	100% (usually presents in infancy/ childhood with early lymph node metastasis)	Pheochromocytoma, mucosal neuromas, GI ganglioneuromas, Marfanoid habitus, everted eyelids
Cowden syndrome	*PTEN*, *SDHB-D*, *KLLN* promoter methylation, *PIK3CA, AKT1, SEC23B*	PTC (classical and follicular subtypes), FTC	10%	Hamartomas and epithelial tumors of the breast, kidney, colon, endometrium and brain; mucocutaneous lesions; macrocephaly
FAP and Gardner syndrome	*APC*	Cribriform morular thyroid carcinoma	1–12% (usually women)	FAP: Multiple adenomatous polyps with malignant potential Gardner syndrome: FAP variant with extracolonic manifestations including supernumerary teeth, fibrous dysplasia of the skull, osteomas of the mandible, fibromas, desmoid tumors, epithelial cysts, hypertrophic retinal pigment epithelium, upper GI hamartomas, hepatoblastomas

Table 14.2 (continued)

Syndrome	Germline mutation	Type of thyroid neoplasm	Incidence of thyroid lesions	Key extrathyroidal clinical features
Carney complex	*PRKAR1A*	PTC, FTC, follicular adenoma	3%	Myxomas of soft tissues; skin and mucosal pigmentation (blue nevi); schwannomas, tumors of the adrenal and pituitary glands and testicle
Werner syndrome	*WRN*	FTC, PTC, ATC	18%	Premature aging; scleroderma-like skin changes; cataracts; premature graying and/or thinning of scalp hair; short stature
DICER1 syndrome	*DICER1*	Follicular nodular disease, FA, PTC, FTC, PDTC, particularly in pediatric patients	–	Pleuropulmonary blastoma; cystic nephroma; ovarian Sertoli-Leydig cell tumors

Abbreviations: *FAP* familial adenomatous polyposis, *FMTC* familial medullary thyroid carcinoma, *FTC* follicular thyroid carcinoma, *MEN* multiple endocrine neoplasia, *MTC* medullary thyroid carcinoma, *PTC* papillary thyroid carcinoma, *PDTC* poorly differentiated thyroid carcinoma, *ATC* anaplastic thyroid carcinoma

respective extrathyroidal manifestations. Awareness of the genotypes and clinical phenotypes of these syndromes can prompt genetic counseling, evaluation for germline testing, screening for associated malignancies, and consideration of screening or testing of relatives, as indicated by current guidelines.

Molecular Testing Platforms Available for Thyroid FNA Specimens

Molecular tests for thyroid FNA samples range from laboratory-developed ("in-house" or "home brew") tests to those performed in commercial reference laboratories. Laboratories performing clinical molecular tests should be certified and accredited by the appropriate national or international regulatory agencies [49]. The International Organization for Standardization (ISO) 15189 standard provides one benchmark for accreditation that is used in many countries [50]. Examples of regulatory compliance in the United States include Clinical Laboratory Improvement Amendments (CLIA) certification, College of American Pathologists (CAP) accreditation, as well as permits/licenses required by state departments of health.

All clinical laboratory tests should undergo analytical validation to establish accuracy and precision for detecting the analyte, reportable range, reference interval, analytic sensitivity, and analytic specificity. Analytical validation should be

performed for each specimen type (e.g., formalin-fixed paraffin-embedded cell-blocks, cells scraped from direct smears, fresh cells rinsed into nucleic acid preservative, etc.). In contrast, clinical validation defines the diagnostic performance characteristics of a test in a defined population (e.g., ability to distinguish benign thyroid tumors from malignant ones among nodules classified as AUS or FN). Ideally, clinical validation should be performed in prospective, blinded, multi-institutional studies to establish the diagnostic sensitivity, specificity, predictive values, and clinical utility of a test.

Tests for Oncogenic Mutations and Gene Fusions

Genotyping tests for thyroid FNA specimens have taken a variety of forms over the past decade, ranging from testing for a single variant (e.g., *BRAF* V600E mutation) to broader panels of oncogenic alterations. Traditional methods for evaluating a limited number of genomic alterations in thyroid FNA specimens include Sanger sequencing, real time PCR, allele-specific PCR, pyrosequencing, fluorescence melting curve analysis, fluorescence in situ hybridization for chromosomal rearrangements, and immunocytochemistry using mutation-specific antibodies (e.g., for the *BRAF* V600E mutation) [33, 51]. Traditional genotyping tests have been performed on various FNA sample types, including cells collected directly into nucleic acid preservative, cells lifted from direct smears, cellblocks, and residual material in liquid-based cytology samples post-slide preparation [52–56].

- *BRAF V600E mutation as single-gene test* can be incorporated into routine thyroid FNA practice [57, 58]. For patients with advanced or RAI-refractory thyroid cancer, testing for the *BRAF* V600E mutation can guide the selection of targeted therapy. The diagnostic utility of *BRAF* V600E testing alone for the risk stratification of cytologically indeterminate nodules is disputed and appears to vary geographically. In settings with an inclination towards active surveillance for cytologically indeterminate nodules and a relatively high prevalence of *BRAF* V600E among PTCs (as reported in some Asian practices), testing for this mutation alone may be cost-effective for ruling in cancer and directing patients towards thyroidectomy [33]. In Western practices, however, the relatively low sensitivity and NPV of *BRAF* V600E for thyroid cancer have limited its usefulness as a stand-alone marker for risk-stratifying nodules classified in the AUS, FN, and Suspicious for Malignancy categories [51, 59–61].
- A *7-gene test panel* comprising the most common driver mutations (involving *BRAF*, *HRAS*, *KRAS*, and *NRAS*) and gene fusions (*CCDC6::RET*, *NCOA4::RET*, and *PAX8::PPARG*) in thyroid neoplasia offers incremental improvements in refining the probability of cancer for a cytologically indeterminate thyroid nodule [53, 62]. Similar to single-gene testing for the *BRAF* V600E mutation, the use and limitations of the 7-gene panel for risk-stratifying cytologically indeterminate nodules may vary depending on the practice setting. For practices that prefer active surveillance for indeterminate nodules, detection of a *BRAF* V600E

mutation or *RET* fusion may steer management towards surgery, although these *BRAF*-like alterations are relatively infrequent compared to *RAS*-like alterations among AUS and FN aspirates [55]. In contrast, for practices that favor surgery for indeterminate nodules, the clinical impact of the 7-gene panel is less clear. Detection of one of the markers in the 7-gene panel would rule in neoplasia and only reinforce the recommendation for surgery (although the particular test result may influence extent of surgery). Moreover, a negative test result would be considered inadequate for steering nodules towards sonographic surveillance: among AUS and FN nodules in clinical validation studies, the 7-gene panel has exhibited a relatively low NPV (82–94%), corresponding to a residual cancer risk of 6–18% when the test is negative [53, 55, 63, 64].

- With the adoption of *next-generation sequencing (NGS) platforms*, massive parallel sequencing for a very large number of genomic alterations has become possible. Laboratory-developed NGS assays for cancer-related biomarkers are available for implementation in local molecular pathology laboratories, as are options to develop customized thyroid-specific NGS panels [65–67]. Different studies have demonstrated the analytical feasibility of NGS on various thyroid FNA specimen types, including the centrifuged supernatants usually discarded after the preparation of either cytospins or cell blocks [66, 68–70]. In the limited clinical validation studies reported to date for lab-developed NGS testing strategies for cytologically indeterminate thyroid FNAs, these tests showed variable NPV (81–100%) and PPV (29–81%) [67, 71, 72].

Combined Testing Platforms Offered by Reference Laboratories

In contrast to the traditional and NGS-based genotyping tests that can be performed locally in an institution's molecular pathology laboratory, several molecular diagnostic tests offered by centralized reference laboratories in the United States have emerged over the past decade: ThyroSeq® Genomic Classifier (University of Pittsburgh Medical Center and Sonic Healthcare USA, Inc.), Afirma® Genomic Sequencing Classifier & Xpression Atlas (Veracyte, Inc.), and ThyGeNEXT & ThyraMIR® (Interpace Diagnostics, Inc.). All three testing platforms combine NGS-based tumor genotyping panels with mRNA or microRNA expression profiling to varying degrees, although the core methodology and risk-stratification strategy differ among the tests (Fig. 14.3). Tables 14.3, 14.4, and 14.5 compare the methodology, pre-analytic considerations, biomarkers, and clinical validation studies for these tests.

- *ThyroSeq® Genomic Classifier (GC).* ThyroSeq GC uses high-throughput targeted DNA and RNA sequencing to test for an extensive panel of mutations and gene fusions associated with thyroid neoplasia. ThyroSeq also identifies chromosomal copy number alterations associated with oncocytic neoplasms. A limited gene expression panel via RNA sequencing is also used for confirming adequate sampling of thyroid follicular cells, identifying expression profiles

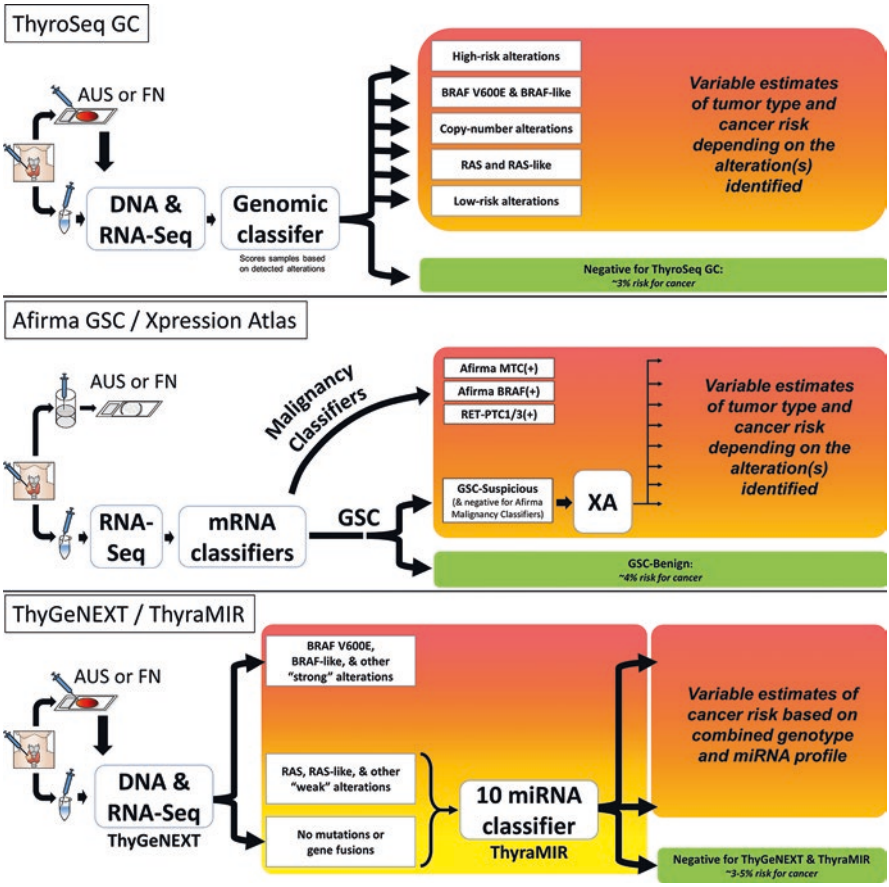

Fig. 14.3 Commercially available multigene panels and their use in risk-stratifying cytologically indeterminate (AUS or FN) thyroid nodules. Simplified schematic of ThyroSeq Genomic Classifier (GC), Afirma Gene Sequencing Classifier (GSC) and Xpression Atlas (XA), and ThyGeNEXT & ThyraMIR is shown. (Figure adapted from Nishino M and Nikiforova MN. Update on Molecular Testing for Cytologically Indeterminate Thyroid Nodules. Arch Pathol Lab Med. 2018;142(4):446–457 [73])

associated with *BRAF*-like or *RAS*-like alterations, and detecting lesions that are not derived from thyroid follicular cells, such as parathyroid, medullary thyroid carcinoma, and metastatic tumors. ThyroSeq GC is designated primarily for assigning thyroid nodules classified as AUS or FN into one of six molecular risk- and disease-stratified tiers based on the number, type, and allelic frequency of genomic and gene expression alterations that are detected. For tumors with molecular alterations, the test provides information about potential targeted therapies as well. In its clinical validation study, ThyroSeq demonstrated a NPV of 97% among AUS and FN nodules with a combined 28% prevalence of NIFTP and cancer [15]. In other words, such nodules that are negative for the ThyroSeq

Table 14.3 Comparison of methods, technology, and accepted starting materials for ThyroSeq, Afirma, and ThyGeNEXT/ThyraMIR

	ThyroSeq v3 Genomic Classifier (GC)	Afirma Genomic Sequencing Classifier (GSC) and Xpression Atlas (XA)	ThyGeNEXT and ThyraMIR
Core methodology	Tumor genotyping; detection of alterations in gene expression and chromosomal copy number	Gene expression profiling; tumor genotyping	Tumor genotyping; microRNA expression profiling
Primary technology used for the test	High-throughput DNA & RNA sequencing	High-throughput RNA sequencing	High-throughput DNA and RNA sequencing (ThyGeNEXT) RT-qPCR for microRNA profiling (ThyraMIR)
Accepted FNA sample types for nucleic acid extraction	Cellular material from FNA pass(es) collected directly into vendor's nucleic acid preservative -or- Direct smear slides (>200–300 follicular cells) -or- FFPE cellblock	Cellular material from FNA pass(es) collected directly into vendor's nucleic acid preservative	Cellular material from FNA pass(es) collected directly into vendor's nucleic acid preservative -or- Direct smear slides (>80 follicular cells) -or- FFPE cellblock

Abbreviations: *FFPE* formalin-fixed paraffin-embedded, *RT-qPCR* reverse transcription quantitative polymerase chain reaction

panel have an estimated NIFTP/cancer risk of approximately 3%, which is comparable to the NIFTP/cancer risk associated with cytologically benign nodules.

- *Afirma® Genomic Sequencing Classifier (GSC) and Xpression Atlas (XA)*. Afirma GSC uses high-throughput RNA sequencing to measure the expression levels of a broad panel of mRNA transcripts. The GSC includes biomarkers with high specificity for malignancy (e.g., gene expression profiles associated with medullary carcinoma and *BRAF* V600E-mutated papillary carcinoma, and RNA sequencing for *CCDC6::RET and NCOA4::RET* gene fusions), the detection of which is essentially diagnostic for malignancy. Expression profiles that confirm thyroid follicular cell sampling and flag sampling of non-thyroidal tissues (e.g., parathyroid tissue or metastatic tumors) are evaluated as quality control (QC) steps. RNA sequencing results that pass QC and are negative for the cancer-specific markers noted above undergo evaluation by the GSC's proprietary machine learning algorithms, which ultimately classify each sample as having either a "Benign" (low probability of malignancy) or "Suspicious" (intermediate probability of malignancy) transcriptional profile. Among AUS and FN nodules with a NIFTP/cancer prevalence of 24%, the Afirma GSC had a NPV of 96%,

Table 14.4 Comparison of biomarkers that are analyzed by ThyroSeq, Afirma, and ThyGeNEXT/ThyraMIR tests

	ThyroSeq v3 Genomic Classifier (GC)	Afirma Genomic Sequencing Classifier (GSC) and Xpression Atlas (XA)	ThyGeNEXT and ThyraMIR
Oncogenic mutations and gene fusions	112 genes (>12,000 variants and >150 gene fusions)	GSC: 1 mutation (*BRAF* V600E) and 2 fusions (RET-PTC1/3) XA: 593 genes (905 variants and 235 fusions)	13 genes (42 variants and 37 fusions)
Gene expression analysis	19 genes	10,196 genes (1115 genes for the GSC algorithm)	4 genes (housekeeping genes for QC)
microRNA expression analysis	N/A	N/A	10 microRNAs
Chromosomal copy number alterations	10 chromosomal regions	Loss-of-heterozygosity analysis	N/A
Prognostic markers	*TERT* promoter, *TP53*	*TP53*	*TERT* promoter
Markers of thyroid follicular cell sampling	mRNA of follicular cell-related genes	mRNA of follicular cell-related genes	mRNA of follicular cell-related genes
Markers of parathyroid sampling	mRNA of parathyroid-related genes	mRNA of parathyroid-related genes	N/A
Markers of medullary carcinoma	*CALCA*	*CALCA*, *CEACAM5*, *SCG3*, *SCN9A*, *SYT4*	miR-375, *RET* mutations

Table 14.5 Comparison of clinical validation studies for ThyroSeq, Afirma, and ThyGeNEXT/ThyraMIR

	ThyroSeq v3 Genomic Classifier (GC) [15]	Afirma Genomic Sequencing Classifier (GSC) [74]	ThyGeNEXT and ThyraMIR [75]
Sample source for clinical validation	Prospective, multi-institutional cohort of FNA material collected into nucleic acid preservative	Prospective, multi-institutional cohort (archival RNA samples remaining from 2012 validation study of the Afirma GEC)	Retrospective, multi-institutional cohort of archival cytology slides
# of AUS/FN cases	247	190	178
Prevalence of cancer	28%	24%	30%
Benign call rate	61%	54%	46%
Sensitivity	94%	91%	93%
Specificity	82%[a]	68%	62%[a]
NPV	97%	96%	95%
PPV	66%[a]	47%	52%[a]

[a]All test results with intermediate to high molecular probability of cancer were considered "positive" for purposes of comparing test performance

corresponding to a NIFTP/cancer probability of approximately 4% for nodules classified as "Benign" by the GSC [74].
  - While gene expression profiling remains the core methodology of the Afirma GSC, the RNA sequencing platform also permits evaluation for point mutations, insertions/deletions, and fusions involving the transcribed portion of the genome. The Afirma XA reports the detection of sequence variants with known associations with thyroid neoplasia [76–78]. Because RNA sequencing is confined the transcribed portion of the genome, *TERT* promoter mutations and other alterations in noncoding DNA are not identified by the Xpression Atlas. This test is intended for AUS and FN nodules with "Suspicious" Afirma GSC results, as well as for cytologically malignant (or Suspicious for Malignancy) aspirates for which tumor genotyping is desired for prognostic purposes and/or targeted therapy options.
- *ThyGeNEXT and ThyraMIR®*. ThyGeNEXT is a relatively focused genotyping panel that uses high-throughput DNA and RNA sequencing to identify thyroid neoplasia-related hotspot mutations in 10 genes (*ALK*, *BRAF*, *GNAS*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PTEN*, *RET*, *TERT*) and 37 types of gene fusions involving 6 genes (*ALK*, *BRAF*, *NTRK*, *PPARG*, *RET*, *THADA*). mRNA expression levels of *PAX8* and *NKX2-1* (TTF-1) genes are included among a small gene expression panel to help confirm thyroid follicular cell sampling. The detection of variants with high specificity for malignancy (e.g., *BRAF* V600E mutation, *TERT* promoter mutations, *BRAF* fusions, *RET* fusions, *ALK* mutations and fusions) is reported as positive for a "strong" driver mutation and requires no further testing. Samples that are either (1) positive for a "weak" driver alteration (typically *RAS* mutations and other *RAS*-like variants) or (2) negative for any of the alterations in the ThyGeNEXT panel are considered molecularly indeterminate for malignancy and undergo additional testing with ThyraMIR, a quantitative RT-PCR-based microRNA expression classifier. ThyraMIR determines the expression profile of 10 microRNAs that are known to be up- or down-regulated in thyroid neoplasia and classifies samples into three tiers (negative, moderate, or positive) based on their projected probability of cancer. For AUS and FN nodules with a pooled 30% prevalence of NIFTP or cancer, the combined ThyGeNEXT and ThyraMIR tests had a 95% NPV (i.e., 5% risk of NIFTP/cancer for samples that are negative for both ThyGeNEXT and ThyraMIR) [75]. For the remaining permutations of ThyGeNEXT and ThyraMIR results, the test estimates NIFTP/cancer risk based on the particular driver alteration and microRNA profile that are identified.

While each of these tests use different methods to refine the preoperative cancer risk stratification of thyroid nodules, several common themes are emerging as these commercially available multigene tests have evolved over the past decade:

- *Combined testing approaches* that use aspects of multiplexed genotyping panels and gene or microRNA expression profiling.

- *High negative predictive value* for identifying nodules with molecular profiles associated with a very low probability of cancer, for which clinical/sonographic surveillance would be appropriate.
- Positive test results that cover *a range of cancer probabilities and tumor phenotypes*, including identification of biomarkers associated with increased risk of aggressive clinical behavior (e.g., metastasis and extrathyroidal extension).
- Inclusion of *actionable oncogenic driver alterations* in the genotyping panel.

A single-institution randomized clinical trial showed no significant differences in the diagnostic performance of ThyroSeq GC and Afirma GSC among nodules classified as AUS or FN [79]. On balance, each of these commercially available tests appears to provide similar information to the patient and treating physician for guiding clinical management decisions.

Notably, these commercially available tests are currently centralized in the United States and generally have high prices that may limit their accessibility to patients in countries with national health systems that do not cover the cost of the test [80]. Furthermore, most of the literature that has been published to date on these three commercially available tests have come from North American adult patient populations. Given the relatively high ROM for AUS reported in Asian compared to Western series [34], the NPV and PPV for these molecular tests may need to be adjusted accordingly when used in populations that differ from those represented in clinical validation studies.

Conclusions and Future Directions

Molecular testing offers an opportunity to refine the probability of malignancy for cytologically indeterminate nodules and may offer additional insights into tumor type, prognosis, and expression of predictive biomarkers on FNA cytology samples. Implementation of molecular testing in routine thyroid FNA practice and the selection of a particular testing platform will vary across practice settings. Test cost and accessibility are key considerations, as are regional and global differences in clinical practice, tolerance of risk and uncertainty, and thresholds for shifting from active surveillance to surgery [34, 35, 81]. If thyroid FNA molecular testing is to be used for clinical purposes, results must be integrated with each nodule's sonographic characteristics, cytologic features, patient characteristics, and patient's treatment preferences.

Looking ahead, the growing international adoption of TBSRTC will provide further opportunities to compare the safety profiles and the cost-effectiveness of different approaches to using molecular testing in the preoperative evaluation of thyroid nodules. Additional future directions include the inclusion of molecular data for estimating ROM for the indeterminate TBSRTC categories [82, 83], as well as the integration of molecular testing results as quality assurance metrics in cytopathology laboratory management [84, 85].

Sample Reports

The integration of molecular testing results into cytopathology reports is not standardized, given the differences in testing practices and assay platforms between cytopathology laboratories [86]. In general, cytopathologic diagnosis using TBSRTC categories should be made independently of molecular results. Molecular test results—whether issued as a part of the original cytopathology report, reported as an addendum (as shown in the examples below), or provided in a separate molecular pathology report—should be accompanied by an explanation of their clinical significance vis-à-vis probability of malignancy, tumor phenotype, prognosis, and/or therapeutic implications, as applicable.

Example 1 Positive for Low-Risk Mutation
FOLLICULAR NEOPLASM.
Cellular aspirate with follicular cells in microfollicular groups. Colloid is scant.
ADDENDUM: Molecular Test Result: *NRAS p.Q61R.*
Note: This mutation is associated with a 70–80% probability of cancer with low recurrence risk (usually follicular carcinoma or encapsulated follicular variant of papillary carcinoma) or pre-malignant neoplasm (NIFTP). Follicular adenomas typically comprise the remainder of tumors with this molecular profile. Surgical referral should be considered.

Example 2 Positive for Intermediate-Risk Mutation
ATYPIA OF UNDETERMINED SIGNIFICANCE.
AUS-Nuclear.
Scattered histiocytoid cells with nuclear atypia, present in a background of proteinaceous material and macrophages.
ADDENDUM: Molecular Test Result: *BRAF p.V600E.*
Note: *BRAF* p.V600E mutation is associated with a > 95% probability of papillary carcinoma. This mutation is associated with an intermediate risk of cancer recurrence. Surgical referral is advised, with consideration of oncologic thyroidectomy in the appropriate clinical and radiologic context.

Example 3 Positive for High-Risk Mutations
MALIGNANT.
Papillary thyroid carcinoma.
ADDENDUM: Molecular Test Result: *BRAF p.V600E* and *TERT C228T.*
Note: The presence of both *BRAF* and *TERT* mutations is associated with a > 95% probability of malignancy. This molecular profile is seen in more aggressive tumors with a high risk for disease recurrence. Surgical referral is advised, with consideration of oncologic thyroidectomy in the appropriate clinical and radiologic context.

Example 4 Negative for Oncogenic Alterations
ATYPIA OF UNDETERMINED SIGNIFICANCE.
AUS-other.
Hypocellular aspirate with follicular cells in microfollicular groups.
ADDENDUM: Molecular Test Result: *Negative for oncogenic alterations*.
Note: Based on clinical validation studies, the risk of malignancy is associated with an approximately [*]% risk of cancer. Nodules with a <5% risk of cancer are generally suitable for observation or surveillance in the appropriate clinical and radiologic context.
**Risk of cancer can be estimated by calculating 1 minus the NPV, as determined by the clinical validation of the test. Laboratories should confirm whether the prevalence of cancer among AUS nodules in a particular practice is within range of those analyzed in the clinical validation study.*

Example 5 Advanced Thyroid Cancer with Targetable Alteration
MALIGNANT.
Undifferentiated (anaplastic) thyroid carcinoma.
Note: By immunocytochemistry, tumor cells are positive for PAX8 and negative for thyroglobulin and TTF-1.
ADDENDUM: Molecular Result: *BRAF p.V600E.*
Patients with *BRAF p.V600E* mutated anaplastic carcinoma are eligible for the combination therapy with *BRAF* and MEK inhibitors.

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