Chapter 15
Utilization of Molecular Markers in the Diagnosis and Management of Thyroid Nodules

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Introduction

Advances in molecular testing have made it possible to routinely incorporate molecular markers in guiding management of patients with indeterminate cytology thyroid nodules. Ultrasound and cytopathologic examination of thyroid nodules are crucially important diagnostic methodologies, and utilization of these methodologies is able to definitively classify the majority (70–80%) of thyroid nodules as benign or malignant [1, 2]. The Bethesda reporting system, first proposed in 2007 by the National Cancer Institute, provides diagnostic categories with risk stratification and recommendations for

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clinical management [3, 4]. However, 20–30% of thyroid nodules fall into one of the three indeterminate categories: atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) (Bethesda III), follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm (FN/SFN) (Bethesda IV), or suspicious for malignant cells (SMC) (Bethesda V) [4, 5].

The AUS/FLUS category carries a risk of malignancy of 5–15%; patients with AUS/FLUS nodules typically undergo a repeat fine needle aspiration (FNA) procedure. For the FN/SFN category, the risk of malignancy is 15–30%, and patients are typically recommended to undergo a diagnostic lobectomy. The SMC category carries the highest risk of malignancy at 60–70%. Patients with SMC category thyroid nodules are recommended to undergo either thyroidectomy or lobectomy [3].

On resection, the majority of indeterminate thyroid nodules are found to be benign [4–6]. Avoiding or reducing diagnostic surgeries for indeterminate nodules that turn out to be benign would be of great benefit to patients. For the 10–40% of patients with indeterminate thyroid nodules that turn out to be malignant, if the malignant nodule is greater than 1 cm in size, those patients who have undergone a diagnostic lobectomy then undergo a completion lobectomy. These patients could have benefitted from an upfront thyroidectomy rather than two separate procedures.

To increase the level of granularity in preoperative risk assessments and reduce the number of diagnostic surgeries, ancillary approaches such as molecular profiling of indeterminate nodules are increasingly being utilized. In a recent survey of practice patterns of members of the Endocrine Society, American Thyroid Association, and American Association of Clinical Endocrinologists, 38.8% of respondents obtain molecular profiles to guide management of patients with AUS/FLUS nodules, and 29.0% obtain molecular profiles for patients with FN/SFN nodules [7].

Several molecular tests are currently commercially available (Table 15.1). These tests utilize a variety of methodologies to characterize thyroid nodules by gene mutations/rearrangements, mRNA expression, or microRNA (miRNA) expression [8–12].

Table 15.1 Commercially available molecular tests for indeterminate thyroid nodules

	Mutation testing		mRNA gene expression classifier		miRNA classifier	
Test	ThyroSeq v2	ThyGenX	Thyroid Cancer Mutation Panel	Afirma	ThyraMIR (offered as reflex test if ThyGenX test is negative)	RosettaGX Reveal
Company	UPMC, via CBLPath	Interpace Diagnostics	Quest	Veracyte	Interpace Diagnostics	Rosetta Genomics
Methodology	Next-generation sequencing	Next-generation sequencing	Real-time PCR, pyrosequencing	mRNA expression array	qRT-PCR	qRT-PCR
Number of targets tested	Hotspot mutations in 14 genes and 42 types of gene fusions involving RET, PPARG, NTRK1, NTRK3, BRAF, THADA, and ALK genes	Hotspot mutations in five genes and RET/PTC1, RET/PTC3, PAX8/PPARG fusions	Hotspot mutations in four genes and RET/PTC1, RET/PTC3, PAX8/PPARG fusions	142 mRNAs	Ten miRNAs	24 miRNAs

(continued)

TABLE 15.1 (continued)

	Mutation testing		mRNA gene expression classifier	miRNA classifier
Sample requirements	1–2 drops from first FNA pass into collection tube	One dedicated FNA pass into collection tube	Needle washing in alcohol-based fixative (eg., Cytolyt), two unstained tissue slides, or four unstained FNA slides	Two dedicated FNA passes into collection tube	One dedicated FNA pass into collection tube	One stained FNA smear slide

Gene Mutation/Rearrangement Testing

Gene mutation and rearrangement testing are based upon decades of characterization of the molecular alterations responsible for driving thyroid tumorigenesis. These studies have culminated in recent large-scale sequencing projects, such as The Cancer Genome Atlas (TCGA) sequencing study, and have resulted in a comprehensive profile of the landscape of alterations in thyroid tumors.

The TCGA study of papillary thyroid cancer examined single-nucleotide variants, small indels, copy number alterations, rearrangements, mRNA expression, miRNA expression, and DNA methylation of 496 papillary thyroid carcinomas [13]. Driver alterations were identified by this analysis for 96.5% of cases [13]. Thus, driver mutations that account for nearly all papillary thyroid cancers have now been described. The majority of alterations seen in papillary thyroid carcinoma involve the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways. The knowledge gained through prior studies and large-scale sequencing projects have guided the design of gene mutation/rearrangement panels.

Seven-Gene Mutation/Rearrangement Panels

A seven-gene panel of the mutations and rearrangements most frequently seen in thyroid cancer (accounting for approximately 70% of thyroid cancers) is one approach for mutational testing. These panels typically include hotspot mutations in *BRAF*, *NRAS*, *HRAS*, and *KRAS,* and testing for the fusion genes *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARG*.

BRAF is a serine threonine kinase that plays an integral role in the MAPK pathway and is important in cell division and differentiation. Mutations in *BRAF* are seen in approximately 40–45% of papillary thyroid carcinomas [14, 15]. The most commonly seen *BRAF* mutation is the activating V600E mutation. Other mutations such as K601E or small in-frame insertions or deletions have been reported [16–19]. Additionally, activation of BRAF signaling may occur

through fusion of *BRAF* with partners such as *AKAP9*, *SND1*, or *MKRN1* [13, 20].

NRAS, *HRAS*, and *KRAS* are oncogenes frequently mutated in several tumors. Activating mutations typically occur at codon 61 (most frequently) and also at codons 12 and 13. Mutations in *NRAS*, *HRAS*, or *KRAS* have been reported in 40–50% of follicular carcinomas and 20–40% of follicular adenomas [21–24]. *NRAS*, *HRAS*, or *KRAS* mutations have also been reported in noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and invasive follicular variant of papillary thyroid carcinoma [25–27].

The fusions interrogated in seven-gene mutation/rearrangement panels are the RET/PTC1 (fusion of *RET* with *CCDC6*), RET/PTC3 (fusion of *RET* with *NCOA4*), and *PAX8/PPARG* fusions. RET/PTC1 and RET/PTC3 fusions are seen in papillary thyroid carcinomas. The incidence of which is approximately 10% of the cases (down from 20–30% incidence two decades ago [28–30]. *PAX8/PPARG* fusions are seen primarily in follicular carcinomas (30–40% of cases) [31–33]. This *PAX8/PPARG* fusion may also be seen, albeit at lower frequencies, in follicular adenomas and the follicular variant of papillary thyroid carcinomas [31–35].

All genes and rearrangements in the seven-gene mutation/rearrangement panel show high specificity and positive predictive value (PPV) for cancer (although the PPV for *NRAS*, *HRAS*, and *KRAS* is lower) [12, 36, 37]. A seven-gene mutation/rearrangement panel (or a similar eight- gene panel that also includes TRK rearrangements that occur in 5% of papillary thyroid cancers) was initially validated in three prospective studies at two institutions and was found to have a high specificity of 97–100% and high PPV of 86–100% [12, 36, 37]. In subsequent studies of similar seven-gene panels, including a single-institution retrospective study and two prospective multi-institutional studies of the Asuragen miRInform test (now currently commercially offered by Interpace Diagnostics as the ThyGenX test), similar high specificities of 86–92% and PPV of 71–80% were seen in FN/SFN thyroid nodules

[38–40]. Seven-gene mutation/rearrangement panels are commercially available from providers such as Quest Diagnostics or Interpace Diagnostics (ThyGenX). The ThyGenX test has been modified from the miRInform test to also include mutations in *PIK3CA*. *PIK3CA* alterations have been primarily described in poorly differentiated and anaplastic thyroid carcinomas [41–43].

ThyroSeq v2 Mutation/Rearrangement Panel

The ThyroSeq v2 panel is a large, next-generation sequencing (NGS)-based test. Use of NGS technology allows for the simultaneous interrogation of multiple genes in a cost-effective and high-throughput manner. This test includes the seven genes in other mutation/rearrangement panels (*BRAF*, *NRAS*, *HRAS*, *KRAS*, *RET/PTC1*, *RET/PTC3*, *PAX8/PPARG*), as well as additional genes that have been implicated in thyroid cancer: *AKT1*, *PTEN*, *TP53*, *TSHR*, *GNAS*, *CTNNB1*, *RET*, *PIK3CA*, *EIF1AX*, and *TERT* and rearrangements of *RET*, *BRAF*, *NTRK1*, *NTRK3*, *PPARG*, and *THADA* [44].

EIF1AX is a novel gene recently described to be recurrently mutated in the TCGA study of papillary thyroid carcinoma [13]. *EIF1AX* is a translational initiation factor and was seen in 2% of papillary thyroid carcinomas; however, in a subsequent study, mutations in *EIF1AX* were also identified in two follicular adenomas and one hyperplastic nodule, suggesting that the presence of *EIF1AX* mutations is not specific for carcinoma [45]. *EIF1AX* mutations have also been observed in 11% of poorly differentiated and 9% of anaplastic thyroid carcinomas [46, 47]. Interestingly, as opposed to papillary thyroid carcinoma where *EIF1AX* mutations were generally mutually exclusive with other driver mutations, in poorly differentiated and anaplastic thyroid carcinomas, a tendency toward co-occurrence of *EIF1AX* and *RAS* mutations was seen [13, 46, 47]. Further study is needed to fully elucidate the role of *EIF1AX* in thyroid cancer and to

investigate whether there may be a cooperative effect between *EIF1AX* and *RAS* in poorly differentiated and anaplastic thyroid carcinomas.

TERT promoter mutations are another important alteration present on the ThyroSeq v2 panel. *TERT* promoter mutations, located either 124 or 146 bp upstream of the start codon, have been described as recurrent alterations in follicular cell thyroid cancers; they have not been detected in medullary thyroid carcinomas or benign thyroid lesions [48–51]. These mutations, while present in well-differentiated papillary thyroid and follicular carcinomas, are present at increased frequency in aggressive tumors such as poorly differentiated carcinoma, anaplastic thyroid carcinoma, and widely invasive oncocytic carcinoma [48–51]. Furthermore, studies have found an association of *TERT* promoter mutation with increased risk for disease-specific mortality, distant metastases, and persistent disease [51].

The ThyroSeq v2 panel also includes *TP53*, *PIK3CA*, and *AKT1* genes. These genes are associated with aggressive behavior and tumor progression, and mutations in these genes are seen more frequently in poorly differentiated and anaplastic thyroid carcinomas [41–43, 52–56]. Genes that predict benign behavior, such as activating mutations of *TSHR* and *GNAS*, are also included in the panel [57–61]. Approximately 50–80% of hyperfunctioning nodules harbor *TSHR* mutations, and approximately 3–6% of hyperfunctioning nodules harbor *GNAS* mutations [57–61]. Mutations in either *TSHR* or *GNAS* have only rarely been reported in follicular carcinomas [62].

The ThyroSeq v2 panel can additionally detect mutations in *RET* which are seen in medullary thyroid carcinoma. Both mutations associated with sporadic medullary thyroid carcinoma or MEN2B syndrome such as the activating tyrosine kinase domain mutation M918T or extracellular domain cysteine mutations associated with MEN2A syndrome or familial medullary thyroid carcinoma are detectable by the panel.

Finally, the ThyroSeq v2 panel can detect an expanded set of fusion genes. Rearrangements involving *NTRK1* and

NTRK3 are seen in 5% of papillary thyroid carcinomas [62–66]. In pediatric cases of papillary thyroid carcinoma, the incidence of *NTKR1* or *NTRK3* rearrangements may be even higher [67]. Fusions involving *THADA* have been reported in approximately 1% of papillary thyroid carcinomas [13]. Fusions involving the *ALK* gene are potentially targetable and have been seen in approximately 1–2% of papillary thyroid carcinomas, 4–9% of poorly differentiated carcinomas, 4% of anaplastic thyroid carcinomas, and 1–2% of medullary thyroid carcinomas [47, 68, 69].

ThyroSeq v2 was initially validated in a single-institution, combined retrospective and prospective study of 143 FN/SFN cytology thyroid nodules [44]. Results from this study showed overall very good performance with a specificity of 93%, sensitivity of 90%, PPV of 83%, and NPV of 96% [44]. In a follow-up, single-institution, prospective study of 465 AUS/FLUS thyroid nodules, similar good performance of the assay was seen, with sensitivity of 90.9%, specificity of 92.1%, PPV of 76.9%, and NPV of 97.2% [70].

Expression Classifier Testing

mRNA Gene Expression Classifier

The Afirma gene expression classifier (GEC), offered by Veracyte, was developed by examining the gene expression profile patterns of thyroid nodules and using this data to train a molecular classifier [71]. The Afirma classifier uses the pattern of expression of 142 genes to classify nodules into one of two categories: benign or suspicious [8, 71]. Initial validation of the Afirma test was done using 265 indeterminate nodules in a multi-institutional study [71]. Both high sensitivity (90%) and NPV (94%) were seen; lower values were seen for specificity (52%) and PPV (37%) [71]. Subsequent studies at other institutions with higher disease prevalence reported lower NPVs [72–75]. In many of these studies, the rate of true negatives or false negatives was difficult to definitively ascertain

as many patients with benign results by Afirma testing did not undergo surgical resection. A recent meta-analysis of seven studies reported a pooled sensitivity of 95.7% and pooled specificity of 30.5% [76].

In addition to the Afirma GEC, Afirma offers two malignancy classifiers, Afirma MTC and Afirma BRAF. These malignancy classifiers are recommended to be performed on thyroid nodules that have a suspicious result by the Afirma GEC or are SMC on cytology. The Afirma MTC classifier examines the expression of genes differentially expressed in medullary thyroid carcinoma. In a recent small validation study of the MTC classifier, a sensitivity of 96.3% was reported [77]. The Afirma BRAF classifier analyzes the expression of genes differentially expressed in thyroid nodules with the *BRAF* V600E mutation.

miRNA Expression Classifier

MicroRNAs (miRNAs) are small, noncoding RNAs that are important in regulation of gene expression. Studies have demonstrated the differential expression of several miRNAs in thyroid carcinoma; some have additionally shown association with prognostic features such as advanced disease or extrathyroidal extension [78, 79].

A miRNA classifier assay is offered by Rosetta Genomics (Rosetta GX Reveal). This assay examines the expression of 24 miRNAs from a single stained diagnostic smear to classify nodules as "suspicious for malignancy by miRNA profiling," "positive for medullary marker," or "benign" [80]. In a multicenter, retrospective study that utilized a total of 109 Bethesda III and IV cytology samples, this test was reported to show a sensitivity of 85%, a specificity of 72%, and an NPV of 91% [9].

The ThyraMIR test (Interpace Diagnostics) also utilizes a panel of miRNAs to classify thyroid nodules as "positive" or "negative." The ThyraMIR test analyzes the expression of ten miRNAs and is currently offered as reflex testing on nodules

that are negative by the eight-gene ThyGenX mutation/rearrangement panel. The performance of this assay, as reported in the initial validation study based on the analysis of a total of 150 Bethesda III and IV cytology samples (which did not include any cases of Hurthle cell carcinomas), is sensitivity of 57%, specificity of 92%, NPV of 82%, and PPV of 77% [40].

Comparison of Test Performance

Sensitivity measures the proportion of actual positives which are true positives, and specificity measures the proportion of negatives which are true negatives. Sensitivity and specificity reflect the performance characteristics of a test. PPV and NPV, however, will vary depending on the prevalence of disease. For example, a patient population with higher incidence of thyroid cancer than the population where the test was validated will have a lower NPV. Another factor that may affect observed NPV and PPV may be institutional differences in the malignancy rates for each indeterminate cytology category. Published test performance characteristics are summarized in Table 15.2.

Strengths of gene mutation/rearrangement panels are their high specificity and PPV for malignancy. In general, these tests are being utilized to "rule in" malignancy. Strengths of gene expression classifiers are their high sensitivity and NPV. This test is being used to "rule out" malignancy. The ideal diagnostic test would have high sensitivity, specificity, NPV, and PPV and would be able to both rule in and rule out malignancy. Combination testing is one approach being pursued: for example, addition of the ThyraMIR test to the ThyGenX test gives a sensitivity of 89%, specificity of 85%, NPV of 94%, and PPV of 74%. Also, addition of the Afirma MTC or Afirma BRAF malignancy classifiers may add some specificity to the Afirma GEC, although no data regarding this has been published yet.

Of the currently commercially available tests, the ThyroSeq v2 test shows much promise as a stand-alone test that could

TABLE 15.2 Comparison of published thyroid molecular test performance characteristics

	Type of study	Cytology (Bethesda category)	Number of cases	Sensitivity	Specificity	NPV	PPV
ThyroSeq	Prospective	III	96	91%	92%	97%	77%
	Retrospective and prospective	IV	143	90%	93%	96%	83%
Afirma	Prospective	III	129	90%	53%	95%	38%
		IV	81	90%	49%	94%	37%
RosettaGX Reveal	Retrospective	III, IV	150	74%	74%	92%	43%
ThyGenX and ThyraMIR	Prospective	III	58	94%	80%	97%	68%
		IV	51	82%	91%	91%	82%

be used to both rule in and rule out malignancy, with a NPV of 96% and PPV of 83% in FN/SFN nodules and a NPV of 97.2% and PPV of 76.9% in AUS/FLUS nodules. Further understanding of thyroid pathogenesis and the potential role of cooperating genes or mutations may help in further refining the NPV and PPV of the panel. In particular, *RAS* alterations, which may be seen in carcinomas as well as benign/indolent neoplasms such as follicular adenomas or NIFTP, confer a lower risk of malignancy of 74 to 87% than other alterations such as the *BRAF* V600E mutation which has a >95% risk of malignancy [12, 36, 37]. Knowledge of the differences in risk based on the specific gene mutation present can guide patient management. Furthermore, use of large, NGS-based mutation panels would allow for the discovery of co-occurring mutations (e.g., in *TP53* or *EIF1AX*) that may prove to be associated with increased risk.

Utilizing the Results of Molecular Profiling in Clinical Management

Test performance characteristics have formed the basis of clinical algorithms to guide the use of molecular testing in guiding perioperative decision making [81]. For a positive result on seven-gene mutation/rearrangement panels, which have high specificity and high PPV, indeterminate cytology thyroid nodules may be managed with oncologic thyroidectomy. A negative result on a seven-gene mutation/rearrangement panel may be managed by observation or diagnostic thyroid lobectomy for AUS/FLUS nodules and by diagnostic thyroid lobectomy for FN/SFN or SMC nodules. Gene expression classifiers have high sensitivity and high NPV. For a benign result, observation or diagnostic thyroid lobectomy would be appropriate, and for a suspicious result on AUS/FLUS or FN/SFN nodules, at least a diagnostic lobectomy should be considered.

For the ThyroSeq v2 panel, which has good overall sensitivity, specificity, NPV, and PPV, the following clinical

algorithm can be considered (Fig. 15.1). When test is negative
for all alterations, in nodules with Bethesda III and IV cytol-
ogy (and pretest cancer probability is that expected for these
Bethesda categories), the residual probability of cancer is
expected to be 3–4%. According to the NCCN guidelines,
these patients can be followed by observation, similar to the
recommendations for benign cytology nodules. For nodules
with a positive ThyroSeq result, the type of mutation and
other test results allow one to predict the probability of can-
cer in the nodule and estimate how aggressive the cancer is,
helping to define the most appropriate surgical approach. An
isolated *RAS* or *RAS*-like mutation predicts a high probabil-
ity (~80%) of either low-risk cancer or a precancerous
tumor, NIFTP. Many of these patients can be treated with

Bethesda III-IV Cytology			
ThyroSeq v2			

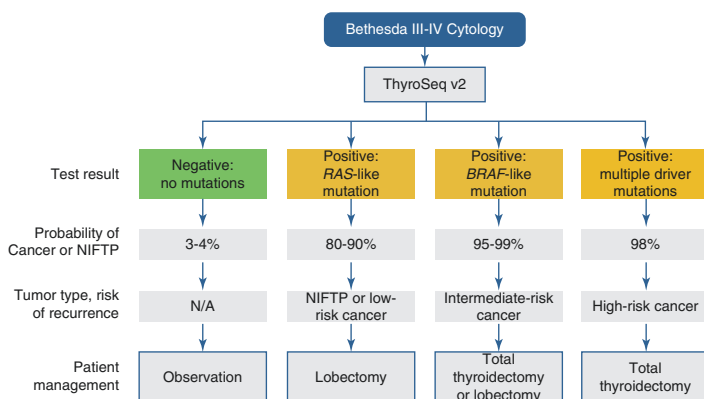|	Negative: no mutations	Positive: *RAS*-like mutation	Positive: *BRAF*-like mutation	Positive: multiple driver mutations
Test result	Negative: no mutations	Positive: *RAS*-like mutation	Positive: *BRAF*-like mutation	Positive: multiple driver mutations
Probability of Cancer or NIFTP	3-4%	80-90%	95-99%	98%
Tumor type, risk of recurrence	N/A	NIFTP or low-risk cancer	Intermediate-risk cancer	High-risk cancer
Patient management	Observation	Lobectomy	Total thyroidectomy or lobectomy	Total thyroidectomy

FIGURE 15.1 Algorithm for clinical management using the ThyroSeq
v2 test, based on the risk conferred by specific mutations. Negative
nodules have a risk of cancer of 3–4%, similar to that for benign
cytology nodules, and can be managed with observation. Nodules
positive for a *RAS*-like mutation have a risk of either low-risk can-
cer or NIFTP and can be managed with lobectomy. Nodules positive
for a *BRAF*-like mutation are intermediate-risk cancers and can be
managed with total thyroidectomy or lobectomy. Nodules positive
for multiple driver mutations are high-risk cancers and can be man-
aged with total thyroidectomy, with possible local lymph node dis-
section

therapeutic lobectomy, which is currently recommended for low-risk thyroid cancers. Isolated *BRAF* V600E or other *BRAF* V600E-like mutations confer a very high (>95%) probability of cancer of intermediate risk for disease recurrence. The risk may be further modified by clinical parameters; for example, small (<1 cm) tumors may still be of low risk. These patients can be treated with total thyroidectomy or lobectomy. Test positivity for multiple driver mutations is virtually diagnostic of cancer and predicts a significant risk of disease recurrence and tumor-related mortality. These patients would benefit from total thyroidectomy, with consideration for central compartment lymph node dissection.

Studies have suggested that use of molecular testing can help avoid unnecessary surgeries and can reduce the number of two-step surgeries (initial lobectomy followed by completion thyroidectomy). In a study of 471 patients with AUS/FLUS or FN/SFN cytology nodules, patients who did not have seven-gene mutation/rearrangement testing were 2.5 more times likely to require a two-step surgery [82]. Cost-effectiveness modeling studies of seven-gene mutation/rearrangement panels, gene expression classifier testing, and combined mutation and miRNA testing all show potential cost savings [83, 84].

Conclusions

Advances in technology and further elucidation of the molecular mechanisms underlying thyroid tumor pathogenesis have made possible the incorporation of molecular testing to ultrasound and cytopathologic examination in guiding the management of the patient with a thyroid nodule. Multiple tests are commercially available, and each utilizes different methodologies to profile the molecular alterations in indeterminate thyroid nodules. These tests have their own strengths and weakness; some have excellent utility in ruling in malignancy, and some have excellent utility in ruling out malignancy. Usage of these tests is increasingly being adopted in clinical practice and has potential to reduce costs by reducing

the number of unnecessary surgeries. Further advances in testing and test performance are likely to occur and may prove to have additional utility, for example, by predicting tumor aggressiveness or response to therapy.

References

1. Cooper DS, Doherty GM, Haugen BR, Kloos RT, Lee SL, Mandel SJ, et al. Revised American Thyroid Association management guidelines for patients with thyroid nodules and differentiated thyroid cancer. Thyroid. 2009;19(11):1167–214.
2. Gharib H, Papini E, Paschke R, Duick DS, Valcavi R, Hegedus L, et al. American Association of Clinical Endocrinologists, Associazione Medici Endocrinologi, and European Thyroid Association medical guidelines for clinical practice for the diagnosis and management of thyroid nodules: executive summary of recommendations. J Endocrinol Investig. 2010;33(Suppl 5):51–6.
3. Ali SZ, Cibas ES. The Bethesda system for reporting thyroid cytopathology. New York: Springer; 2010.
4. Baloch ZW, LiVolsi VA, Asa SL, Rosai J, Merino MJ, Randolph G, et al. Diagnostic terminology and morphologic criteria for cytologic diagnosis of thyroid lesions: a synopsis of the National Cancer Institute thyroid fine-needle aspiration state of the science conference. Diagn Cytopathol. 2008;36(6):425–37.
5. Baloch ZW, Fleisher S, LiVolsi VA, Gupta PK. Diagnosis of "follicular neoplasm": a gray zone in thyroid fine-needle aspiration cytology. Diagn Cytopathol. 2002;26(1):41–4.
6. Mazzaferri EL. Management of a solitary thyroid nodule. N Engl J Med. 1993;328(8):553–9.
7. Burch HB, Burman KD, Cooper DS, Hennessey JV, Vietor NO. A 2015 survey of clinical practice patterns in the management of thyroid nodules. J Clin Endocrinol Metab. 2016;101(7):2853–62.
8. Alexander EK, Kennedy GC, Baloch ZW, Cibas ES, Chudova D, Diggans J, et al. Preoperative diagnosis of benign thyroid nodules with indeterminate cytology. N Engl J Med. 2012;367(8):705–15.
9. Lithwick-Yanai G, Dromi N, Shtabsky A, Morgenstern S, Strenov Y, Feinmesser M, et al. Multicentre validation of a microRNA-based assay for diagnosing indeterminate thyroid nodules utilising fine needle aspirate smears. J Clin Pathol. 2017;70(6):500–7.

10. Bartolazzi A, Orlandi F, Saggiorato E, Volante M, Arecco F, Rossetto R, et al. Galectin-3-expression analysis in the surgical selection of follicular thyroid nodules with indeterminate fine-needle aspiration cytology: a prospective multicentre study. Lancet Oncol. 2008;9(6):543–9.

11. Keutgen XM, Filicori F, Crowley MJ, Wang Y, Scognamiglio T, Hoda R, et al. A panel of four miRNAs accurately differentiates malignant from benign indeterminate thyroid lesions on fine needle aspiration. Clin Cancer Res. 2012;18(7):2032–8.

12. Nikiforov YE, Ohori NP, Hodak SP, Carty SE, LeBeau SO, Ferris RL, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. J Clin Endocrinol Metab. 2011;96(11):3390–7.

13. Cancer Genome Atlas Research N. Integrated genomic characterization of papillary thyroid carcinoma. Cell. 2014;159(3):676–90.

14. Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, et al. BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst. 2003;95(8):625–7.

15. Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res. 2003;63(7):1454–7.

16. Chiosea S, Nikiforova M, Zuo H, Ogilvie J, Gandhi M, Seethala RR, et al. A novel complex BRAF mutation detected in a solid variant of papillary thyroid carcinoma. Endocr Pathol. 2009;20(2):122–6.

17. Ciampi R, Nikiforov YE. Alterations of the BRAF gene in thyroid tumors. Endocr Pathol. 2005;16(3):163–72.

18. Hou P, Liu D, Xing M. Functional characterization of the T1799-1801del and A1799-1816ins BRAF mutations in papillary thyroid cancer. Cell Cycle. 2007;6(3):377–9.

19. Soares P, Trovisco V, Rocha AS, Lima J, Castro P, Preto A, et al. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene. 2003;22(29):4578–80.

20. Ciampi R, Knauf JA, Kerler R, Gandhi M, Zhu Z, Nikiforova MN, et al. Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. J Clin Invest. 2005;115(1):94–101.

21. Lemoine NR, Mayall ES, Wyllie FS, Williams ED, Goyns M, Stringer B, et al. High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. Oncogene. 1989;4(2):159–64.

22. Motoi N, Sakamoto A, Yamochi T, Horiuchi H, Motoi T, Machinami R. Role of ras mutation in the progression of thyroid carcinoma of follicular epithelial origin. Pathol Res Pract. 2000;196(1):1–7.

23. Namba H, Rubin SA, Fagin JA. Point mutations of ras oncogenes are an early event in thyroid tumorigenesis. Mol Endocrinol. 1990;4(10):1474–9.

24. Suarez HG, du Villard JA, Severino M, Caillou B, Schlumberger M, Tubiana M, et al. Presence of mutations in all three ras genes in human thyroid tumors. Oncogene. 1990;5(4):565–70.

25. Nikiforov YE, Seethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LD, et al. Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. JAMA Oncol. 2016;2(8):1098.

26. Adeniran AJ, Zhu Z, Gandhi M, Steward DL, Fidler JP, Giordano TJ, et al. Correlation between genetic alterations and microscopic features, clinical manifestations, and prognostic characteristics of thyroid papillary carcinomas. Am J Surg Pathol. 2006;30(2):216–22.

27. Zhu Z, Gandhi M, Nikiforova MN, Fischer AH, Nikiforov YE. Molecular profile and clinical-pathologic features of the follicular variant of papillary thyroid carcinoma. An unusually high prevalence of ras mutations. Am J Clin Pathol. 2003;120(1):71–7.

28. Jung CK, Little MP, Lubin JH, Brenner AV, Wells SA Jr, Sigurdson AJ, et al. The increase in thyroid cancer incidence during the last four decades is accompanied by a high frequency of BRAF mutations and a sharp increase in RAS mutations. J Clin Endocrinol Metab. 2013. https://doi.org/10.1210/jc.2013-2503.

29. Nikiforov YE. RET/PTC rearrangement—a link between Hashimoto's thyroiditis and thyroid cancer…or not. J Clin Endocrinol Metab. 2006;91(6):2040–2.

30. Zhu Z, Ciampi R, Nikiforova MN, Gandhi M, Nikiforov YE. Prevalence of RET/PTC rearrangements in thyroid papillary carcinomas: effects of the detection methods and genetic heterogeneity. J Clin Endocrinol Metab. 2006;91(9):3603–10.

31. Dwight T, Thoppe SR, Foukakis T, Lui WO, Wallin G, Hoog A, et al. Involvement of the PAX8/peroxisome proliferator-

activated receptor gamma rearrangement in follicular thyroid tumors. J Clin Endocrinol Metab. 2003;88(9):4440–5.

32. French CA, Alexander EK, Cibas ES, Nose V, Laguette J, Faquin W, et al. Genetic and biological subgroups of low-stage follicular thyroid cancer. Am J Pathol. 2003;162(4):1053–60.

33. Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn GW 2nd, Tallini G, et al. RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab. 2003;88(5):2318–26.

34. Marques AR, Espadinha C, Catarino AL, Moniz S, Pereira T, Sobrinho LG, et al. Expression of PAX8-PPAR gamma 1 rearrangements in both follicular thyroid carcinomas and adenomas. J Clin Endocrinol Metab. 2002;87(8):3947–52.

35. Nikiforova MN, Biddinger PW, Caudill CM, Kroll TG, Nikiforov YE. PAX8-PPARgamma rearrangement in thyroid tumors: RT-PCR and immunohistochemical analyses. Am J Surg Pathol. 2002;26(8):1016–23.

36. Cantara S, Capezzone M, Marchisotta S, Capuano S, Busonero G, Toti P, et al. Impact of proto-oncogene mutation detection in cytological specimens from thyroid nodules improves the diagnostic accuracy of cytology. J Clin Endocrinol Metab. 2010;95(3):1365–9.

37. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, et al. Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab. 2009;94(6):2092–8.

38. Beaudenon-Huibregtse S, Alexander EK, Guttler RB, Hershman JM, Babu V, Blevins TC, et al. Centralized molecular testing for oncogenic gene mutations complements the local cytopathologic diagnosis of thyroid nodules. Thyroid. 2014;24(10):1479–87.

39. Eszlinger M, Piana S, Moll A, Bosenberg E, Bisagni A, Ciarrocchi A, et al. Molecular testing of thyroid fine-needle aspirations improves presurgical diagnosis and supports the histologic identification of minimally invasive follicular thyroid carcinomas. Thyroid. 2015;25(4):401–9.

40. Labourier E, Shifrin A, Busseniers AE, Lupo MA, Manganelli ML, Andruss B, et al. Molecular testing for miRNA, mRNA, and DNA on fine-needle aspiration improves the preoperative diagnosis of thyroid nodules with indeterminate cytology. J Clin Endocrinol Metab. 2015;100(7):2743–50.

41. Garcia-Rostan G, Costa AM, Pereira-Castro I, Salvatore G, Hernandez R, Hermsem MJ, et al. Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer Res. 2005;65(22):10199–207.

42. Hou P, Liu D, Shan Y, Hu S, Studeman K, Condouris S, et al. Genetic alterations and their relationship in the phosphatidylinositol 3-kinase/Akt pathway in thyroid cancer. Clin Cancer Res. 2007;13(4):1161–70.

43. Ricarte-Filho JC, Ryder M, Chitale DA, Rivera M, Heguy A, Ladanyi M, et al. Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. Cancer Res. 2009;69(11):4885–93.

44. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, et al. Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. Cancer. 2014;120(23):3627–34.

45. Karunamurthy A, Panebianco F, Hsiao S, Vorhauer J, Nikiforova M, Chiosea SI, et al. Prevalence and phenotypic characteristics of EIF1AX mutations in thyroid nodules. Endocr Relat Cancer. 2016;23(4):295–301.

46. Kunstman JW, Juhlin CC, Goh G, Brown TC, Stenman A, Healy JM, et al. Characterization of the mutational landscape of anaplastic thyroid cancer via whole-exome sequencing. Hum Mol Genet. 2015;24(8):2318–29.

47. Landa I, Ibrahimpasic T, Boucai L, Sinha R, Knauf JA, Shah RH, et al. Genomic and transcriptomic hallmarks of poorly differentiated and anaplastic thyroid cancers. J Clin Invest. 2016;126(3):1052–66.

48. Landa I, Ganly I, Chan TA, Mitsutake N, Matsuse M, Ibrahimpasic T, et al. Frequent somatic TERT promoter mutations in thyroid cancer: higher prevalence in advanced forms of the disease. J Clin Endocrinol Metab. 2013;98(9):E1562–6.

49. Liu T, Wang N, Cao J, Sofiadis A, Dinets A, Zedenius J, et al. The age- and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas. Oncogene. 2013;33(42):4978–84.

50. Liu X, Bishop J, Shan Y, Pai S, Liu D, Murugan AK, et al. Highly prevalent TERT promoter mutations in aggressive thyroid cancers. Endocr Relat Cancer. 2013;20(4):603–10.

51. Melo M, Rocha AG, Vinagre J, Batista R, Peixoto J, Tavares C, et al. TERT promoter mutations are a major indicator of poor

outcome in differentiated thyroid carcinomas. J Clin Endocrinol Metab. 2014. https://doi.org/10.1210/jc.2013-3734.

52. Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP. High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. J Clin Invest. 1993;91(1):179–84.

53. Donghi R, Longoni A, Pilotti S, Michieli P, Della Porta G, Pierotti MA. Gene p53 mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland. J Clin Invest. 1993;91(4):1753–60.

54. Dobashi Y, Sugimura H, Sakamoto A, Mernyei M, Mori M, Oyama T, et al. Stepwise participation of p53 gene mutation during dedifferentiation of human thyroid carcinomas. Diagn Mol Pathol. 1994;3(1):9–14.

55. Ho YS, Tseng SC, Chin TY, Hsieh LL, Lin JD. p53 gene mutation in thyroid carcinoma. Cancer Lett. 1996;103(1):57–63.

56. Takeuchi Y, Daa T, Kashima K, Yokoyama S, Nakayama I, Noguchi S. Mutations of p53 in thyroid carcinoma with an insular component. Thyroid. 1999;9(4):377–81.

57. Fuhrer D, Holzapfel HP, Wonerow P, Scherbaum WA, Paschke R. Somatic mutations in the thyrotropin receptor gene and not in the Gs alpha protein gene in 31 toxic thyroid nodules. J Clin Endocrinol Metab. 1997;82(11):3885–91.

58. Trulzsch B, Krohn K, Wonerow P, Chey S, Holzapfel HP, Ackermann F, et al. Detection of thyroid-stimulating hormone receptor and Gsalpha mutations: in 75 toxic thyroid nodules by denaturing gradient gel electrophoresis. J Mol Med. 2001;78(12):684–91.

59. Parma J, Duprez L, Van Sande J, Hermans J, Rocmans P, Van Vliet G, et al. Diversity and prevalence of somatic mutations in the thyrotropin receptor and Gs alpha genes as a cause of toxic thyroid adenomas. J Clin Endocrinol Metab. 1997;82(8):2695–701.

60. Garcia-Jimenez C, Santisteban P. TSH signalling and cancer. Arq Bras Endocrinol Metabol. 2007;51(5):654–71.

61. Nishihara E, Amino N, Maekawa K, Yoshida H, Ito M, Kubota S, et al. Prevalence of TSH receptor and Gsalpha mutations in 45 autonomously functioning thyroid nodules in Japan. Endocr J. 2009;56(6):791–8.

62. Leeman-Neill RJ, Kelly LM, Liu P, Brenner AV, Little MP, Bogdanova TI, et al. ETV6-NTRK3 is a common chromosomal rearrangement in radiation-associated thyroid cancer. Cancer. 2013;120(6):799–807.

63. Greco A, Pierotti MA, Bongarzone I, Pagliardini S, Lanzi C, Della PG. TRK-T1 is a novel oncogene formed by the fusion of TPR and TRK genes in human papillary thyroid carcinomas. Oncogene. 1992;7(2):237–42.

64. Greco A, Mariani C, Miranda C, Lupas A, Pagliardini S, Pomati M, et al. The DNA rearrangement that generates the TRK-T3 oncogene involves a novel gene on chromosome 3 whose product has a potential coiled-coil domain. Mol Cell Biol. 1995;15(11):6118–27.

65. Martin-Zanca D, Hughes SH, Barbacid M. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature. 1986;319(6056):743–8.

66. Radice P, Sozzi G, Miozzo M, De Benedetti V, Cariani T, Bongarzone I, et al. The human tropomyosin gene involved in the generation of the TRK oncogene maps to chromosome 1q31. Oncogene. 1991;6(11):2145–8.

67. Prasad ML, Vyas M, Horne MJ, Virk RK, Morotti R, Liu Z, et al. NTRK fusion oncogenes in pediatric papillary thyroid carcinoma in northeast United States. Cancer. 2016;122(7):1097–107.

68. Kelly LM, Barila G, Liu P, Evdokimova VN, Trivedi S, Panebianco F, et al. Identification of the transforming STRN-ALK fusion as a potential therapeutic target in the aggressive forms of thyroid cancer. Proc Natl Acad Sci U S A. 2014;111(11):4233–8.

69. Ji JH, Oh YL, Hong M, Yun JW, Lee HW, Kim D, et al. Identification of driving ALK fusion genes and genomic landscape of medullary thyroid cancer. PLoS Genet. 2015;11(8):e1005467.

70. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, et al. Impact of the multi-gene thyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance cytology. Thyroid. 2015;25(11):1217–23.

71. Chudova D, Wilde JI, Wang ET, Wang H, Rabbee N, Egidio CM, et al. Molecular classification of thyroid nodules using high-dimensionality genomic data. J Clin Endocrinol Metab. 2010;95(12):5296–304.

72. Alexander EK, Schorr M, Klopper J, Kim C, Sipos J, Nabhan F, et al. Multicenter clinical experience with the Afirma gene expression classifier. J Clin Endocrinol Metab. 2014;99(1):119–25.

73. Harrell RM, Bimston DN. Surgical utility of Afirma: effects of high cancer prevalence and oncocytic cell types in patients with indeterminate thyroid cytology. Endocr Pract. 2014;20(4):364–9.

74. Marti JL, Avadhani V, Donatelli LA, Niyogi S, Wang B, Wong RJ, et al. Wide inter-institutional variation in performance of a molecular classifier for indeterminate thyroid nodules. Ann Surg Oncol. 2015;22(12):3996–4001.

75. McIver B, Castro MR, Morris JC, Bernet V, Smallridge R, Henry M, et al. An independent study of a gene expression classifier (Afirma) in the evaluation of cytologically indeterminate thyroid nodules. J Clin Endocrinol Metab. 2014;99(11):4069–77.

76. Santhanam P, Khthir R, Gress T, Elkadry A, Olajide O, Yaqub A, et al. Gene expression classifier for the diagnosis of indeterminate thyroid nodules: a meta-analysis. Med Oncol. 2016;33(2):14.

77. Pankratz DG, Hu Z, Kim SY, Monroe RJ, Wong MG, Traweek ST, et al. Analytical performance of a gene expression classifier for medullary thyroid carcinoma. Thyroid. 2016;26(11):1573–80.

78. Chou CK, Chen RF, Chou FF, Chang HW, Chen YJ, Lee YF, et al. miR-146b is highly expressed in adult papillary thyroid carcinomas with high risk features including extrathyroidal invasion and the BRAF(V600E) mutation. Thyroid. 2010;20(5):489–94.

79. Chou CK, Yang KD, Chou FF, Huang CC, Lan YW, Lee YF, et al. Prognostic implications of miR-146b expression and its functional role in papillary thyroid carcinoma. J Clin Endocrinol Metab. 2013;98(2):E196–205.

80. Benjamin H, Schnitzer-Perlman T, Shtabsky A, VandenBussche CJ, Ali SZ, Kolar Z, et al. Analytical validity of a microRNA-based assay for diagnosing indeterminate thyroid FNA smears from routinely prepared cytology slides. Cancer Cytopathol. 2016;124(10):711–21.

81. Ferris RL, Baloch Z, Bernet V, Chen A, Fahey TJ 3rd, Ganly I, et al. American Thyroid Association statement on surgical application of molecular profiling for thyroid nodules: current impact on perioperative decision making. Thyroid. 2015;25(7):760–8.

82. Yip L, Wharry LI, Armstrong MJ, Silbermann A, McCoy KL, Stang MT, et al. A clinical algorithm for fine-needle aspiration molecular testing effectively guides the appropriate extent of initial thyroidectomy. Ann Surg. 2014;260(1):163–8.

83. Labourier E. Utility and cost-effectiveness of molecular testing in thyroid nodules with indeterminate cytology. Clin Endocrinol. 2016;85(4):624–31.

84. Yip L, Farris C, Kabaker AS, Hodak SP, Nikiforova MN, McCoy KL, et al. Cost impact of molecular testing for indeterminate thyroid nodule fine-needle aspiration biopsies. J Clin Endocrinol Metab. 2012;97(6):1905–12.