Cytologic and Molecular Diagnosis of Thyroid Cancers

Is it Time for Routine Reflex Testing?

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The Bethesda system for standardized reporting of thyroid fine needle aspiration (FNA) cytology has positively affected the clarity of communication of results and management of patients evaluated for thyroid nodules. Problematic areas still exist in the triage of some of these samples, particularly those in the categories of "follicular lesion with atypia of uncertain significance" and "follicular lesion." The literature on molecular and genetic abnormalities in thyroid lesions is reviewed. Potentially useful markers for distinguishing currently problematic categories of FNA cytologic samples, especially nondiagnostic samples, atypia of uncertain significance, and follicular lesions, are discussed. The predictive value of the respective molecular analyses in these settings is examined. Evaluation of FNA samples with negative or suboptimal follicular cytology for Ras mutations may be useful in detecting potentially significant follicular lesions (carcinomas) but is quite low in overall yield. Cytologic samples with atypia of uncertain significance, which may include the possibility of papillary carcinomas, may be fruitfully evaluated using a panel of molecular tests for *BRAF, RET*/PTC, *PAX8/PPARG1*, and Ras. Other markers also have potential utility in the workup of thyroid lesions. An era of combined modality testing in thyroid cytology is emerging in which classical cytologic findings can be coupled with molecular data to increase the predictive power of diagnostic interpretations; however, there remains a group of atypical cytologic samples negative for known molecular markers in which the risk of malignancy is too high to simply follow expectantly. ***Cancer (Cancer Cytopathol)* 2012;120:7–17.** © *2011 American Cancer Society.*

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The incidence of thyroid cancer is increasing in some areas of the world, presumably because of increased detection of small papillary carcinomas; however, mortality rates in the United States because of thyroid cancer have not shifted significantly over time.[1] By contrast, evaluation and management of thyroid nodules have changed dramatically over the past 30 years. Two significant diagnostic advances have catalyzed this change. The first was the introduction of radionuclide scintigraphy imaging, which allowed functional classification of thyroid nodules as those considered "hot" (ie, taking up or trapping the radionuclide) and those that are "cold" (ie, failed to trap the radiotracer). This technological advance alone excluded many hot nodules from the need for further evaluation.[2] However, the remaining cold nodules, while containing an enriched proportion of neoplastic lesions, still included many benign entities. More recently, ultrasound

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examination has supplanted radionuclide scintigraphic imaging of the thyroid. The second major advance in the management of patients with thyroid nodules came with the popularization of fine needle aspiration (FNA) biopsy during the last few decades of the 20th century. This relatively simple, readily available technique could categorize some thyroid lesions into those that were clearly malignant and those that were clearly benign. However, again, there remained a large group of cases in which malignant features could not be reliably distinguished from benign proliferation.[3-5]

In October 2007, a National Cancer Institute consensus conference was held in an attempt to standardize and codify cytopathologic practices related to thyroid FNA samples and to ensure consistent communication of results to clinicians. This tiered classification system, otherwise known as the Bethesda system, provides clinicians and patients with a structure under which to interpret reported findings and make management decisions.[6] Application of the system in clinical practice has already had a salutary impact on patient management.[7,8]

Despite these advances, there are still several situations in which the best efforts at evaluation of thyroid nodules yield inconclusive results. Although it is encouraging that we can identify groups of patients with elevated risk of harboring a thyroid malignancy, for a patient in a high-risk group who undergoes "needless" surgery for a lesion ultimately found to be benign, the classification system has not provided the best outcome. Similarly, for patients in low-risk groups who ultimately are found to have a malignancy, the evaluation protocols are suboptimal. It is into this abyss that the molecular diagnostician enters with the intent of providing additional information that may reduce the requirement for needless surgery or decrease the number of low- or intermediate-risk individuals who endure uncertainty until their disease fully declares itself benign or malignant.

Molecular Alterations in Papillary Thyroid Carcinomas

Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy and, at least in the Western world, is increasing in frequency. This has been attributed to several factors, including increased detection because of the use of ultrasound evaluation, improved diagnostic recognition of follicular and other variants of PTC, increased exposure to ionizing radiation in the environment, and an increase in iodine-rich diets.[9] Evidence also suggests that benign thyroid diseases such as Hashimoto's thyroiditis, follicular adenoma, and Graves disease are present in an increased number of patients who develop PTC.

Hereditary factors have been implicated in some cases of PTC. Patients with familial adenomatous polyposis, an autosomal dominant disorder characterized by mutation of the *APC* gene, experience a markedly increased risk of developing PTC, particularly the cribriform-morular variant.[10] PTC is also associated with Carney complex type I, an autosomal dominant disease that results from mutation of the *PRKAR1A* gene. An additional set of familial PTCs, most often displaying autosomal dominant inheritance, demonstrates linkage to various other chromosomal loci (eg, 1q21, 2q21, 8p23.1-p22, 14q31, and 19p13.2), but definitive susceptibility genes have yet to be defined.[11]

Many of the somatic mutations detected in PTC result in constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathway that is responsible for control of expression of genes involved in cell proliferation, differentiation, and survival/apoptosis. Indeed, more than 70% of PTCs harbor point mutations in *BRAF* or Ras genes or a structural chromosomal rearrangement resulting in fusion transformation of various tyrosine kinase receptors including *RET* and *NTRK1*.[12,13] *BRAF* mutations are the most commonly detected abnormality in PTC, seen in roughly 45% of sporadic adult cases but less frequently (0%-12%) in pediatric and radiation-induced tumors.. More than 90% of *BRAF* mutations in PTC are characterized by a change of valine to glutamic acid at codon 600, designated $BRAF^{V600E}$, although $BRAF^{K601E}$ has been described in adenomas and follicular-variant PTCs. A paracentric inversion on 7q resulting in fusion of the N-terminus of *AKAP9* with the C-terminus of *BRAF* also activates *BRAF* in radiation-induced PTC. *BRAF*-positive tumors usually display classical PTC features (although *BRAF* mutations are also seen in the tall cell variant, poorly differentiated thyroid cancer, and undifferentiated thyroid cancer of papillary origin) and appear to have a more aggressive course.[14]

Another frequent (20%-30%) somatic mutation found almost exclusively in PTC involves chromosomal

Table 1. Rearrangements of Ret and Ntrk Protooncogenes in Papillary Thyrroid Cancers

Rearrangement	Type of Rearrangement	Partner Gene; Name[a]
RET-PTC1	inv(10)(q11.2;q21.2)	*CCDC6*; coiled-coil domain containing 6
RET-PTC2	t(10;17)(q11.2;q23)	*PRKAR1A*; protein kinase, cAMP-dependent, regulatory type 1 alpha
RET-PTC3	inv(10)(q11.2;q11) (exon 12 of *RET*)	*NCOA4*; nuclear receptor coactivator 4
RET-PTC4	inv(10)(q11.2;q11) (exon 11 of *RET*)	*NCOA4*; nuclear receptor coactivator 4
RET-PTC5	t(10;14)(q11.2;q32)	*GOLGA5*; golgin A5
RET-PTC6	t(7;10)(q32-34;q11.2)	*TRIM24*; tripartite motif containing 24
RET-PTC7	t(1;10)(p13.1;q11.2)	*TRIM33*; tripartite motif containing 33
RET-PTC8	t(10;14)(q11.2;q22.1)	*KTN1*; kinectin
RET-PTC9	t(10;18)(q11.2;q21-q22)	*RFG9*
RET-ERC1	t(10;12)(q11.2;p13.3)	*ERC1*; ELKS/RAB6-interacting/CAST family member 1
RET-HOOK3	t(8;10)(p11.21;q11.2)	*HOOK3*; hook homolog 3
RET-TRIM27	t(6;10)(p22;q11.2)	*TRIM27*; tripartite motif containing 27
RET-PCM1	t(8;10)(p21.3-p22;q11.2)	*PCM1*; pericentriolar material 1
NTRK1-T1	inv(1)(q22;q25) (breaks in introns of both genes differ for T1, T2, and T4)	*TPR*; translocated promoter region
NTRK1-T2	inv(1)(q22;q25) (breaks in introns of both genes differ for T1, T2, and T4)	*TPR*; translocated promoter region
NTRK1-T3	t(1;3)(q21-q22;q12.2)	*TFG*; TRK-fused gene
NTRK1-T4	inv(1)(q22;q25) (breaks in introns of both genes differ for T1, T2, and T4)	*TPR*; translocated promoter region
NTRK1-TPM3	inv(1)(q22;q21.2)	*TPM3* (1q21.2); tropomyosin 3

[a]Gene symbols as per HUGO Gene Nomenclature Committee.

rearrangements of *RET* at 10q11.2, whereby *RET* falls under the influence of the promoter of 1 of at least 15 other separate genes that are constitutively expressed in thyroid follicular cells. These rearrangements result in fusion of the tyrosine kinase domain of *RET* to the promoter of the fusion gene and aberrant ligand-independent activation of MAPK signaling. The most common rearrangements, *RET*/PTC types 1 and 3, result from paracentric inversions on 10q.[15] *RET*/PTC1-associated tumors are usually classic-type PTCs, whereas those associated with *RET*/PTC3 are more commonly solid-type PTCs.[16] Ionizing radiation is frequently correlated with *RET*/PTC rearrangements, along with younger age, although *RET*/PTC3 is also common in sporadic thyroid cancer in children. *RET*/PTC rearrangements have also been frequently described in microcarcinomas, suggesting that they are an early genetic event in tumorigenesis. These tumors have been found to be unlikely to progress to anaplastic or poorly differentiated carcinomas. Other recurrent inversions and translocations that result in *RET* gene fusion activation in PTC include *RET*/PTC types 2 and 4 through 9, *ELKS-RET*, *PCM1-RET*, *RFP-RET*, and *HOOK3-RET*. A summary of these rearrangements is presented in Table 1.

Point mutations in the *NRAS, HRAS*, and *KRAS* genes are seen in approximately 10% of PTCs, particularly follicular-variant PTCs.[17] PTCs with Ras mutations tend to be encapsulated and demonstrate less prominent nuclear features compared with PTCs lacking Ras mutations. By contrast, follicular-variant PTCs with *BRAF* mutations tend to be nonencapsulated and infiltrative.[18] Rates of lymph node metastasis from these Ras-mutated tumors have been conflictingly reported to be higher[19] and lower[20] than with PTCs lacking Ras mutations, but much of this variability may be a result of differing methods of detection and/or tumor heterogeneity.

Fusion oncoproteins resulting from paracentric inversions involving the neurotrophic tyrosine kinase I gene (*NTRK1*), at 1q22, and the *TPM3* gene, at 1q22-q23, or the *TPR* gene, at 1q25, or a t(1:3)(q21;q11) translocation involving the *NTRK1* and *TFG* genes are found exclusively in a subset of PTCs.[20] *NTRK1* is not normally expressed in thyroid tissues, whereas the 3 partner genes are ubiquitously expressed. Thus, when rearranged, the intracellular tyrosine kinase domain of *NTRK1* becomes aberrantly expressed in the fusion oncoprotein, activating the MAPK pathway and driving tumorigenesis.[21] Occurring in 5%-10% of PTCs, these rearrangements are not

associated with any particular histologic features. Prevalence of the finding does vary considerably among locations and populations.

Gene expression profiling has substantiated the histologic categorization of PTCs, demonstrating significantly different profiles among classic, follicular, and other variants.[22] In addition, several genes have been observed to be up-regulated in PTC, specifically *MET, LGALS3* (galectin-3), and *KRT19* (cytokeratin 19).[23] This information can potentially be applied in a differential diagnosis in several ways, including RNA analysis and immunohistochemistry.

Several specific microRNA (miRNA) signatures have also been consistently demonstrated in PTCs, including up-regulation of miR-221, miR-222, miR-224, miR-155, miR-187, miR-181b, and miR-146b. By contrast, follicular thyroid cancers generally demonstrate up-regulation of miR-221, miR-222, miR-155, miR-187, miR-181b, and miR-224.[24] Specific miRNA profiles may play a role in the genesis of thyroid carcinomas in general and may prove of significant diagnostic value in the future.[25]

Molecular-Genetic Alterations in Follicular Thyroid Carcinomas

Follicular thyroid carcinomas (FTCs) are the second most common type of thyroid malignancy. They appear to be associated with several potential etiologic or predisposing factors, including iodine deficiency, exposure to ionizing radiation, preexisting benign thyroid disease (solitary nodule/adenoma and goiter), and some familial syndromes,[13,26] Cowden disease is a rare autosomal dominant disease caused principally by germ-line mutation of the *PTEN* gene, located at 10q23. Thyroid lesions are common in these patients, who have a 10%-20% lifetime-risk of developing thyroid cancer, usually of the follicular type.[27] Werner syndrome is a rare autosomal recessive disorder resulting from germ-line mutation of the *WRN* gene, at 8p11-p12, and is associated with FTC in about 3% of affected patients.[28] FTC is also associated with a subset of patients with Carney complex type 1, who harbor mutations in *PRKAR1A*.

Similarly to PTC, many of the somatic mutations detected in FTC affect MAPK signaling. The most common somatic mutations seen in FTC (40%-50% of cases) occur in *NRAS*, *HRAS*, and *KRAS*; *NRAS* mutations are the most frequent, followed by *HRAS* and *KRAS*. Commonly, amino acid substitutions in codons 12, 13, or 61 of Ras lead to constitutive activation of the Raf-MEK-MAPK and phosphatidylinositol-3-kinase (PI3K)/AKT cell signaling pathways, resulting in dysregulation of specific genes that promote thyroid proliferation and differentiation.

The second most common mutation found in FTC, identified in 30%-40% of cases, involves in-frame fusion of the promoter and DNA-binding domains of the thyroid transcription factor *PAX8* with the nuclear receptor domains of the *PPARG1* gene following a t(2;3)(q13;p25) rearrangement. The PAX8/PPARγ1-fusion protein results in overexpression of *PPARγ1*. However, the oncogenic mechanism of action remains under debate.[29] PAX8-PPARγ1 also likely deregulates normal PAX8 pathways in thyroid cells via novel fusion protein activities that promote thyroid carcinoma formation. Generally, *PAX8/PPARG1* rearrangement and Ras mutations are mutually exclusive, suggesting that tumors harboring these mutations follow different paths of oncogenesis. Tumors with the *PAX8/PPARG1* rearrangement have a more solid growth pattern and a higher rate of vascular invasion than tumors without the rearrangement.[30] They often demonstrate a morphologic phenotype of microfollicular, solid/trabecular growth, a thick fibrous capsule, and an immunophenotype positive for galectin-3 and/or HBME-1,[31] markers that have been reported to be occasionally useful in differentiating benign and malignant thyroid tumors.

Mutations have also been described in elements of the PI3K signaling pathway within FTCs. This pathway influences gene expression related to cell survival, proliferation, and migration and may be more important in tumor progression than tumorigenesis.[32] The *PIK3CA* gene is mutated in 6%-13% of FTCs, and gene copy numbers are increased in up to 25% of such tumors.[33,34] In addition, mutations in Ras and *PTEN* may affect this pathway. *PTEN* mutations, indentified in 6%-12% of FTCs, decrease the function of PTEN, resulting in activation of AKT and its downstream targets.[35]

Although some oncocytic follicular tumors have Ras and other mutations, a consistent pattern has not been identified. Recently, mutations in the antiapoptotic gene *GRIM19* have been identified in a number of oncocytic

follicular tumors that could play a significant role in tumorigenesis.[36]

Molecular Alterations in Other Thyroid Malignancies

Poorly differentiated (insular) thyroid carcinoma is an uncommon tumor, best characterized as a tumor of follicular cells with partial loss of follicular characteristics. Heuristically, these tumors are thought to arise along 1 of 3 potential pathways: one directly from thyroid follicular epithelium, another from well-differentiated PTC, and a third from well-differentiated FTC.[37] Molecular alterations observed in these tumors include those observed in well-differentiated follicular cell–derived tumors (eg, *BRAF* and Ras) that probably represent initial inciting molecular insults and those viewed as being more specific to poorly differentiated carcinomas (eg, *TP53* and beta-catenin [*CTNNB1*]) that probably represent subsequent events associated with tumor progression. *TP53* mutations are present in about one third of poorly differentiated thyroid carcinomas and are even more frequent in anaplastic (undifferentiated) carcinomas, suggesting a role in tumor progression. Demonstration of p53 by immunohistochemistry in poorly differentiated tumors frequently correlates with the presence of the *TP53* mutation and is seen in 40%-50% of such tumors.[38]

Anaplastic carcinoma is a highly aggressive malignancy of the thyroid that has lost most evidence of follicular cell origin. It accounts for fewer than 2% of thyroid malignancies, although rates vary geographically, and characteristically it occurs in older adults. It is more frequent in individuals with a history of thyroid disease, either benign or malignant, and in those with a history of iodine deficiency or radiation exposure.[39] The molecular alterations observed in anaplastic carcinoma are similar to those in poorly differentiated carcinoma but occur at increased frequency; *TP53* mutations are seen in 50%-80% of cases[37,40] and *CTNNB1* mutations in up to 65% of cases.[41] *PIK3CA* and *PTEN* mutations are also observed in a minority of anaplastic carcinomas. In contrast with well-differentiated tumors where *PIK3CA* and *PTEN* mutations rarely coexist with others, *PIK3CA* and *PTEN* mutations are frequently present concurrently with mutations of *BRAF* and Ras, suggesting that these are also late molecular events associated with tumor progression.

Medullary carcinomas are derived from the C-cells of the thyroid and account for 3%-12% of thyroid carcinomas. The majority of tumors are sporadic, but 15%-30% are hereditary and demonstrate an autosomal dominant pattern of inheritance. Hereditary cases are classified into 3 categories: multiple endocrine neoplasia type 2A (MEN2A), associated with pheochromocytoma and parathyroid hyperplasia; MEN2B associated with pheochromocytoma, mucosal neuromas, and gastrointestinal ganglioneuromatosis, along with marfanoid habitus; and familial medullary thyroid carcinoma (FMTC), which is not associated with other tumors. All these tumors are associated with germ-line gain-of-function point mutations of the *RET* gene that lead to activation of MAPK and other signaling pathways governing cell proliferation, survival, and differentiation.[42-44] *RET* is normally expressed on thyroid C-cells, the adrenal medulla, sympathetic ganglia, and some other sites.[45] Multiple point mutations have been described that have been generally ascribed to 1 of 3 groups that correlate with age of onset and aggressiveness of the disease. The majority of MEN2A and FMTC cases are associated with mutations in the extracellular domains of *RET* and appear to facilitate constitutive dimerization and hence activation of the intracellular kinase domain. MEN2B-related *RET* mutations have only been observed in the intracellular domain, which alters the tyrosine kinase conformation in the cell.[46] Because penetrance of these mutations for thyroid cancer is nearly 100%, prophylactic thyroidectomy is the standard intervention for patients who carry a *RET* mutation; timing for this surgery often depends on individual risk assignment. Full-gene DNA sequencing or other methods targeting specific mutations of *RET* have been used to detect family members affected by or vulnerable to hereditary medullary carcinoma and has largely replaced calcitonin monitoring.

Mixed tumor types are also described in the thyroid, such as mixed medullary and PTC or mixed medullary and FTC. The numbers of such cases are limited, although sufficient to be recognized as a distinct World Health Organization category.[47] Specific molecular genetic alterations have not been widely ascribed to tumors in these categories.

See Tables 2 and 3 for a summary of molecular genetic changes associated with various thyroid carcinomas.

Table 2. Summary of Major Molecular Genetic Changes Associated With Thyroid Neoplasms

Mutation/ Rearrangement	Associated Tumors	Frequency by Tumor Type (%)	Pathway Impact or Point of Action	Benign Lesions
Ras	Follicular	45	MAP kinase	Adenoma (30%)
	Papillary	10		Nodular goiter (5%)
	Poorly differentiated	35		
	Anaplastic	50		
PAX8/PPARG1	Follicular	30-40	PPARγ1	Adenoma (7%)
	Papillary (FV)	5		
GRIM19	Hurthle cell		Apoptosis	
BRAF	Papillary (classic)	45	MAPK, ERK	Adenoma (rare)
	Poorly differentiated	20		
	Anaplastic	20		
RET/PTC	Papillary	20	RTK receptor	
PIK3CA	Follicular	<10	PI3K/AKT	
	Anaplastic	20		
PTEN	Follicular	<10	PI3K/AKT	
	Anaplastic	>10		
TRK	Papillary	< 5	NTRK receptor via MAPK	
CTNNB1	Poorly differentiated	20		
	Anaplastic	60		
TP53	Poorly differentiated	20	Cell division G1→S	
	Anaplastic	70		
APC	Papillary	< 5		

Table 3. Correlation of Differentiated Thyroid Tumor Histology and Molecular Genetic Changes

Tumor Type	Mutation/ Rearrangement (% Cases)	Morphologic Correlates
Papillary	*BRAF* (45)	Classic type; Tall cell variants
	RET/PTC (20)	PTC1-classic; PTC3-solid/micro
	APC (<2)	Cribriform-morular variant
	Ras (10)	Mostly follicular variant
	NTRK1 (<5)	
Follicular	Ras (40)	Conventional
	PAX8/PPARG1 (30)	Solid/microfollicular; Galectin-3/HBME-1 positive
	PIK3CA (<10)	
	PTEN (<10)	
	GRIM19 (<5)	Oncocytic
Medullary	*RET* (95% familial, 50% sporadic)	MEN2a/b, FMTC

Testing Platforms and Methods for Detection of Molecular Alterations of Interest in Thyroid Carcinomas

Generally, the choice of testing platforms/techniques for clinical detection of genetic alterations in thyroid cancer specimens will be determined by the sample type available for analysis and the type(s) of mutation to be analyzed. PCR-based methods are commonly employed and provide rapid, reliable, and sensitive detection of the spectrum of clinical sample types available. Qualitative assessment of single point mutations in thyroid disease, such as at codons 600 and 601 of *BRAF* and codons 12 and 13 of Ras, can be easily achieved using allele-specific PCR, PCR-RFLP, PCR-melt curve analysis, PCR-hybridization (including microarrays), Sanger sequencing and pyrosequencing, plus other methods.[48] Analytic sensitivity of these assays is important to consider because involved tumor may represent only a fraction of the available specimen. Moreover, tumors may show considerable heterogeneity in the presence of the mutation being targeted because of clonal evolution processes. Also, materials available for molecular analysis from cytological specimens and FNAs may be restricted, especially when subjecting such specimens to multiple tests. Although real-time PCR can be used for detection of such molecular lesions, such assays are generally designed to generate a qualitative (positive/negative) result rather than a quantitative result, although the high analytical sensitivity of some real-time PCR methods may be an attractive feature. Currently, quantitative molecular approaches to the detection of markers of thyroid cancer are extremely limited; however, such techniques may realize greater utility in the future for diagnosis of thyroid cancer and for

monitoring patients following treatment.[49,50] In *RET* and other genes, point mutations, deletions, and/or insertions that characterize the disease process can occur at multiple locations; these loci may be screened by using multiple PCR-conformational analyses followed by sequencing or by using a conventional multiplexed-PCR approach.

Detection of chromosomal rearrangements, such as *RET*/PTC and PAX8/*PPARG1*, relies on reverse-transcriptase PCR (RT-PCR) or fluorescence in situ hybridization (FISH). Because the break points involved in such chromosomal rearrangements often occur over considerable genetic distances, the analyte of choice for PCR-based analysis is RNA, which lacks the extensive intronic sequences present in genomic DNA. This demands careful coordination with surgical and pathology staff to assure optimal collection and processing of fresh or snap-frozen FNAs or biopsies that will be suitable for RT-PCR analysis. More often, thyroid tissues are processed by routine formalin fixation and paraffin embedding (FFPE), which may negate the possibility of RT-PCR; however, interphase FISH can be effectively used to detect chromosomal rearrangements in these FFPE thyroid tissues.

Given the lack of FDA-approved in vitro diagnostic tests for thyroid cancer and the potential change that a positive test result triggers in the clinical management of patients, a rigorous validation process must be undertaken during all laboratory test development to assure highly specific, sensitive, and reproducible results. Although there is a desire to be able to detect small numbers of cells that carry a mutation, this must be tempered with the realization that increased false-positivity will accompany ultrasensitive detection methods. Reproducibility testing is essential for establishing clinically relevant cutoff values for all these tests. For interphase FISH, sectioning of FFPE tissues often results in nuclei with less than the full complement of probe signals. Moreover, signal patterns also may vary with section thickness. Therefore, appropriate cutoff values need to be carefully established during the validation of these tissue-based assays.

Application of Molecular Diagnostic Testing to Clinical and Cytologic Scenarios

Application of molecular testing introduces potentially costly and complex additional testing to what has been a relatively simple cascade of clinical and pathologic evaluations, albeit one with a high proportion of potentially avoidable surgical outcomes. Thus, the utility of any reflex molecular algorithm is dependent in part on both the cost of this added laboratory testing weighed against the savings of surgical and other overtreatment-related costs, discounted by any adverse outcomes due to undertreatment. Because of the need to evaluate panels of genetic markers in many cases, this cost is not trivial. Hence, several questions in management need to be addressed because this cost-benefit evaluation will differ according to the number of patients in a given cytologic category and the relative risk of malignancy in that category.

Does molecular testing of FNA samples make sense for all, some, or none of the categories of thyroid cytologic samples? And what molecular test(s) should be applied to these?

Because the risk of malignancy in cytologically negative samples is less than 3%, there is little impetus to perform molecular testing on these patients at this time. Similarly, in samples categorized as positive for malignancy, further molecular testing with diagnostic intent is superfluous, although it may be considered for other reasons mentioned below, as malignancy is confirmed in this setting in 97%-98% of cases.

Analysis for the *BRAF* mutation in atypical or indeterminate cytology samples, if positive, can be virtually diagnostic of PTC.[51,52] Likewise, clonal rearrangement of *RET*/PTC is also reasonably specific for PTC. Ras mutations are not specific for PTC or FTC, nor indeed for carcinoma, as they are also found in a number of benign conditions. But the presence of this alteration in association with other clinical or cytological features may be useful in directing further therapy. For example, Ras mutations found in a cytologically classified "follicular lesion" could represent a follicular variant of PTC or a follicular adenoma. But because this mutation may predispose a patient to the progression of adenoma to follicular carcinoma, surgical removal of such adenomas may be appropriate. In addition, the presence of the *BRAF* mutation in thyroid tumors has prognostic and therapeutic implications; the *BRAF* mutation is associated with increased aggressiveness and lack of response of recurrences to radioiodine because of impaired iodine-trapping mechanisms.[53] Moreover, novel targeted therapies to *BRAF* have been used with some success in advanced thyroid carcinomas.[54]

Table 4. Cytologic Classification and Associated Risk of Malignancy

Cytologic Category	Percent With Malignancy (Cytology Only)	Percent With Malignancy if Positive Molecular Marker	Notes
Unsatisfactory	0		
Negative	2-10	100	0.9% if negative[60]
FLUS	5-10	100	No cancers in mutation-negative group (n = 21)
Follicular lesion	20-30	100	21% cancers in mutation negative group (n = 23)
Suspicious for malignancy	50-75	100	50% (n = 7)
Positive for malignancy	98	100	

Adapted from data from Nikiforov et al.[56]

In some studies, the positive predictive value of *BRAF, RET*/PTC, and *PAX8*/*PPARG1* has been found to be 100% for PTC or FTC. Ras mutations also have a high positive predictive value at 87.5% and carry the additional value of being positive in cytologic situations that are more challenging, that is, follicular variants of PTC and FTC.[55] Thus, a panel of molecular tests that includes *BRAF, RET*/PTC, Ras, and *PAX8*/*PPARG1* has been advocated for evaluation of cytologic samples with indefinite findings for malignancy as a means of more reliably categorizing these patients. The data (see Table 4) tend to support this application, especially for those specimens categorized as "follicular lesion of uncertain significance" (FLUS).[56] Still significant numbers of malignancies remain within the "follicular lesion" and "suspicious for malignancy" groups, which means that the clinical management of molecular marker–negative patients is not significantly affected, although the type of surgery offered might differ (lobectomy vs total thyroidectomy). This could reduce the number of patients in the molecular-positive group, who would require 2 procedures for proper management of their cancer.

These data illustrate (again) the importance of sensitivity along with specificity in any testing algorithm. Cytology alone is quite sensitive in detecting follicular-cell-derived neoplasms. It is quite specific in classifying those cases not needing further evaluation, that is, a high negative predictive value. A molecular panel as noted above has a very high specificity for malignant lesions but does yet not have a particularly good negative predictive value when applied to the group of cytologically indeterminate cases.

Consideration is also being given to adding miRNA evaluation to the panel of mutation markers described above in the hopes of further improving the negative predictive value to a clinically usable level. An alternative approach, also directed at indeterminate cytologic samples, based on evaluation of miRNA analysis, has been developed and marketed by a for-profit venture and validated by a number of participating academic and private institutions in the United States.[57,58] Also, serum biomarker approaches to the evaluation of thyroid carcinoma (including those based on the molecular markers discussed above) show promise.[59]

The "positive for malignancy" cytologic category will include both differentiated thyroid carcinomas of the papillary type and the medullary type, together with poorly differentiated and anaplastic tumor types. Generally, the latter 2 categories will not benefit significantly from further molecular testing in terms of diagnosis or prognosis. If a medullary carcinoma is recognized cytologically as an index case, further evaluation of the patient, or the tumor, for detection of *RET* mutation can be useful if other family members are to be screened for potential germ-line abnormalities. Prospective studies to evaluate the further utility of *PTEN* or *NTRK1* mutations have not been applied to cytologic samples. However, given the relatively low prevalence of these mutations, the expected added value of these assays would appear to be low.

Application of molecular testing to surgical pathology specimens has some added value in the setting of an atypical thyroid follicular lesion with patchy or focal features suggesting PTC, where other methods (IHC and routine H&E) are inconclusive. Demonstration of a mutation such as Ras, *RET*/PTC, or *BRAF* might support

making the diagnosis of carcinoma and giving further appropriate treatment. Encapsulated lesions like these have consistently been shown to contain the mutation, even though the cytologic alterations are present only focally. Another use of molecular evaluation in surgical pathology might be in directing therapy more individually. Being able to use *BRAF*-mutation status to predict responsiveness to conventional radioiodine treatment or to a specific targeted therapeutic agent could be a valuable adjunct to traditional diagnostic reporting.

Summary and Conclusions

The distinct, and largely mutually exclusive, mutation pathways observed in PTC (mostly *RET*/Ras/*BRAF*/MAPK) versus FTC (mostly due to mutations of Ras or *PAX8*/*PPARG1* translocation) provide an opportunity to use various molecular markers in improving diagnostic accuracy. However, the clinical sensitivity and specificity of individual markers are often too low to provide clinical utility, as the combined negative predictive values leave significant clinical gaps in management. The evaluation of indeterminate thyroid FNA cytologic samples with a panel of molecular tests shows significant promise in allowing further risk stratification for the presence of malignancy. The probability of malignancy for patients with an indeterminate FNA cytology is about 40%.[6] A molecular panel of tests including *BRAF*, *PAX8*/*PPARG1*, Ras, and *RET* is able to identify a group of patients with virtually 100% probability of malignancy, thus helping to expedite them toward surgical or other therapies. However, in using such a molecular panel, up to 30% of thyroid cancers will have no mutation detected, a proportion too high to ignore clinically.[54] Hence, the problem remains about what to do with indeterminate cytologic samples that are negative for the panel of molecular tests we have identified. A testing algorithm with a very high negative predictive value is still needed in order to inspire confidence in the path of watchful waiting or inaction.

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