DIAGNOSTIC USE OF MOLECULAR MARKERS IN THE EVALUATION OF THYROID NODULES

Matthew I. Kim, MD; Erik K. Alexander, MD

ABSTRACT

Objective: To describe the molecular markers thus far evaluated for use in the care of patients with clinically relevant thyroid nodules.

Methods: We review the currently available molecular tests that have been applied to patients with thyroid nodules.

Results: In the United States, approximately 450,000 diagnostic fine-needle aspirates will be performed on patients with thyroid nodules this year in an effort to identify thyroid cancer. Unfortunately, this test is imprecise and, at times, inaccurate. Because of this, novel diagnostic testing modalities have been pursued, the most promising of which involve molecular analysis of thyroid tissue. Immunohistochemical staining, analysis for mutations and gene rearrangements, and microarray analysis have all been investigated with regard to their performance characteristics in targeted patient populations.

Conclusions: Molecular tests to evaluate thyroid nodules demonstrate variable performance characteristics. Further evaluation of available and emerging molecular tests will necessarily rely on prospective real-world test validation in the clinical setting. (Endocr Pract. 2012;18:796-802)

INTRODUCTION

The increasing prevalence of thyroid nodules detected on radiographic imaging coupled with the emergence of ultrasound-guided fine-needle aspiration (FNA) as the principal means of evaluating thyroid nodules has increased attention on the dilemma of indeterminate cytopathology (1,2). Before the advent of the Bethesda system for reporting thyroid cytopathologic findings, it was estimated that 20% to 25% of aspirates would be classified as “indeterminate” on the basis of the most widely adopted reporting schemes, with each cytologically “indeterminate” nodule estimated to carry a 20% to 30% risk of malignancy (3-5). The Bethesda classification system effectively subdivided this group into 3 distinct categories associated with differential estimates of malignant risk identified as (a) atypia of undetermined significance/follicular lesion of undetermined significance (5%-15% cancer risk), (b) follicular neoplasm/suspicious for a follicular neoplasm (15%-30% cancer risk), and (c) suspicious for malignancy (60%-75% cancer risk) (6). While this system has served to establish standards that clarify cytologic reporting, the optimal approach to and management of indeterminate nodules have yet to be defined.

The practical questions that must be answered in the course of evaluating an indeterminate nodule first center on determining whether the indeterminate nodule should be monitored expectantly or surgically removed. A separate question is whether the surgical intervention should involve removal of both lobes (near-total thyroidectomy) or be limited to removal of the involved lobe (hemithyroidectomy) with provisions for a completion thyroidectomy if the nodule proves malignant (7-9). The staged hemithyroidectomy approach offers the advantage of minimizing the risk of postsurgical hypothyroidism while also limiting...
the risk of recurrent laryngeal nerve injury. Its disadvantages are the potential need for a second operation, with the associated surgical risks and additional cost. While near-total thyroidectomy limits the operative morbidity to a single procedure, it carries an increased risk of postsurgical hypoparathyroidism and recurrent laryngeal nerve injury. Near-total thyroidectomy also invariably leads to postsurgical hypoparathyroidism requiring lifelong treatment. Consideration of either approach must be tempered by an understanding that most operations will lead to the resection of nodules ultimately proven to be histologically benign.

Although several clinical and radiographic criteria have been identified and shown to assist with prediction of benign vs malignant nodules, their utility has proven to be limited in clinical practice. Clear evidence of rapid nodule enlargement, vocal cord dysfunction, or fixation to proximate structures carries a high malignant predictive value, although they are rarely present in affected patients. Ultrasound characteristics have proven most valuable when 2 or more abnormal findings (such as microcalcifications or hypoechoic parenchyma) are present, although this also is found only in the minority. Furthermore, the modest interrater reliability of this mode of imaging is notable. Thus, ultrasonography is highly useful for stratifying cancer risk and improving the quality and accuracy of FNA, but it can preclude the need for FNA only upon identification of benign simple cysts. Or, conversely, ultrasound evaluation provides a high index of malignant suspicion when nodules demonstrating 2 or more abnormal features are identified (10, 11).

Recognition of the limitations inherent to the clinical and sonographic assessment of thyroid nodule cancer risk has prompted many to investigate the diagnostic utility of molecular markers. As price points for these diagnostic tests have fallen, cost-effectiveness analyses also suggest that the use of diagnostic molecular tests may reduce cost while improving quality-adjusted life years (12,13). Thus, the approach to cytologically indeterminate thyroid nodules is increasingly using molecular diagnostic testing. Below, we provide a description of the molecular markers thus far evaluated for use in the care of patients with clinically relevant thyroid nodules (Box 1).

**IMMUNOHISTOCHEMISTRY**

**Galectin-3**

Galectin-3 is a lectin that binds to β-galactosidase residues on cell surface glycoproteins and interacts with cytoplasmic and nuclear proteins to regulate cell growth, apoptosis, and malignant transformation (14). It has been extensively evaluated as a potential marker of thyroid malignancy in an array of studies that have measured detectable levels in aspirate samples from indeterminate nodules (15). Thus far, it has demonstrated variable performance as a marker for the detection of thyroid malignancy with sensitivity ranging from 20% to 100% and specificity ranging from 62% to 100%. One large multicenter prospective study measured galectin-3 expression in cell blocks derived from FNA samples obtained in 465 patients presenting with cytologically indeterminate nodules. All patients underwent surgical resection, and a sensitivity of 78% and specificity of 93% were reported. Among the study population, this resulted in an 82% positive predictive value and a 91% negative predictive value (16). These impressive results, however, have been difficult to reproduce and validate, especially within the United States. Because of this, widespread adoption of galectin-3 testing in thyroid nodules has been limited.

**HBME-1**

HBME-1 is a monoclonal antibody that binds an antigen present on the microvilli of mesothelioma cells. The antigen that HBME-1 recognizes has also been detected in differentiated thyroid cancer cells, leading to its development as a potential marker for well-differentiated thyroid malignancy. Studies that have evaluated the performance of HBME-1 assays on cytologically indeterminate thyroid nodules (both as an isolated marker, or as part of a panel of markers) have reported test sensitivities ranging from 79% to 87%, with specificities ranging from 83% to 96% (17,18). We and others have observed HBME-1 staining used frequently on histopathologic specimens, especially when visual microscopy raises uncertainties. HBME-1 staining of FNA cytologic specimens, however, is rarely used and is presently of limited utility.

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CK19

CK19 is a cytokeratin that is an integral component of the cytoskeleton of epithelial cells. It has been found to be up-regulated in well-differentiated thyroid cancer cells (19). However, while most aspirates from papillary thyroid carcinoma stain positive for CK19, the overlap between the levels of CK19 expression in benign follicular adenomas and malignant follicular thyroid carcinomas is substantial and limits the utility of this test as a discriminating marker (20).

Combined Immunohistochemical Panels

Efforts to evaluate combined panels of immunohistochemical markers have revealed performance that is, at best, marginally superior to any individual assays (21). Most panels have included galectin-3, HBME-1, and CK19 as principal constituents (22). The largest study of these 3 markers in combination evaluated 125 thyroid nodule FNA samples, and it demonstrated sensitivities of 92% and 80%, respectively, for galectin-3 and HBME-1, with respective specificities of 94% and 96%. Combined testing of both markers, however, increased the sensitivity for detecting malignancy to 97% while decreasing specificity to 80% (20). This suggests dual testing for both galectin-3 and HBME-1 may modify clinical care decisions, although no confirmatory, large-scale clinical trials have validated these data.

MUTATIONS AND GENE REARRANGEMENTS

BRAF

BRAF is a gene that encodes B-Raf, a serine/threonine-protein kinase that is part of the mitogen-activated protein kinase pathway regulating cell proliferation, differentiation, and apoptosis (23). Although more than 40 different BRAF mutations have been implicated in the pathogenesis of a range of malignancies, a specific activating mutation caused by substitution of glutamic acid for valine at position 600 (V600E) has been identified in up to 51% of classic papillary thyroid carcinomas and 24% of follicular variant papillary thyroid carcinomas (24,25). When detected, the positive predictive value for thyroid carcinoma is nearly 100%. However, the prevalence of the BRAF V600E mutation in follicular thyroid carcinoma is only 1% to 2%. Therefore, diagnostic testing for this mutation cannot serve as the sole, independent diagnostic marker of cancer when evaluating indeterminate nodules (26). Given its exceptionally high sensitivity for papillary carcinoma, the utility of BRAF V600E detection may prove to be its positive predictive value when included as a component in preoperative testing (27). In cases of indeterminate nodule aspirates, it has been suggested that presurgical testing for BRAF V600E may help to dictate the extent of required surgery. Tumors positive for the BRAF V600E mutation are highly likely to be malignant. Therefore, a recommendation to proceed with near-total thyroidectomy in lieu of hemithyroidectomy may be justified. Separately, BRAF V600E detection may assist prognostically, as patients with BRAF-positive papillary carcinomas have been shown to have more aggressive disease at presentation, with higher rates of extrathyroidal extension and lymph node metastasis, as well as tumor recurrence after initial treatment (24,28).

RAS

The RAS oncogene family is comprised of 3 genes (HRAS, KRAS, and NRAS) that encode small GTP-ase proteins involved in signal transduction. Activating mutations in these genes stimulate the mitogen-activated protein kinase and phosphatidylinositol pathways that regulate cell growth, proliferation, differentiation, mobility, and survival (29). RAS mutations may be detected in up to 40% of differentiated thyroid cancers, with a predominant distribution among follicular thyroid carcinomas and follicular variants of papillary carcinomas (30). Presently, approximately 12 uniquely different nucleotide deletions or substitutions in the HRAS, KRAS, and NRAS genes constitute most known mutations in this molecule. Similar to BRAF analysis, however, RAS mutational analysis is not sensitive or specific enough to support its singular use as an isolated molecular marker for the prediction of benign or cancerous nodules (30,31). However, RAS mutational analysis may prove to be the most powerful adjunct to visual microscopy, as many cytologically indeterminate aspirates harbor RAS mutations, suggesting cancer is likely. Interestingly, debate has emerged with regard to the meaning of RAS mutations in histologically benign follicular adenomas. While such lesions do not yet demonstrate the histopathologic hallmarks of carcinoma (such as vascular or capsular invasion), it has been suggested that RAS-positive follicular adenomas are actually carcinoma in situ, representing an intermediate stage in the malignant transformation to well-differentiated thyroid carcinoma. While plausible, this theory has yet to be definitely proven (32).

RET/PTC

The RET proto-oncogene encodes a receptor tyrosine kinase known to be involved in intrachromosomal gene rearrangements, producing fusion genes that give rise to constitutively activated forms of this signaling complex. RET/PTC1 and RET/PTC3 gene rearrangements detected in differentiated thyroid cancers produce activated forms of the RET tyrosine kinase, and they function to stimulate the mitogen-activated protein kinase and phosphatidylinositol 3-inositol pathways (33,34). These gene rearrangements have similarly been detected in follicular adenomas and other benign thyroid neoplasms at rates that compromise their utility as diagnostic markers (35). RET/PTC gene rearrangements are relatively rare in comparison to BRAF or RAS mutations. Given their low prevalence in thyroid
cancer, testing for them as a single marker is generally considered to be of only modest value.

**PAX8/PPARG**

Interchromosomal gene rearrangement that links the PAX8 gene (encoding a transcription factor) with the PPARG gene (encoding a nuclear hormone receptor involved in cell differentiation) produces a fusion gene identified as PAX8/PPARG. While the exact mechanism by which this gene rearrangement stimulates tumorigenesis is not completely understood, it is thought to be associated with inhibition of the antiproliferative activity of the PPARG receptor. This gene rearrangement has been detected in 20% to 40% of follicular thyroid carcinomas and a lower percentage of Hurthle cell carcinomas (36,37). PAX8/PPARG translocations have also been detected in follicular adenomas at a rate limiting its discriminant potential (36). Similar to above, the meaning of a PAX8/PPARG-positive follicular adenoma is uncertain. Many believe that such oncogenic activation will ultimately cause the affected nodule to behave in a malignant fashion, and therefore such lesions should be treated as though they are cancerous.

**COMBINED GENETIC ANALYSIS**

A mutation or translocation in either the BRAF, RAS, RET/PTC, or PAX8/PPARG genes is present in up to 70% of histologically proven thyroid cancers. This seminal finding has large implications for better understanding thyroid oncogenesis. Furthermore, diagnostic assessment of the 17 different mutations or translocations identified among these 4 oncogenes may prove to be of high utility. Using this premise, 2 separate studies have evaluated the performance of testing for BRAF, RAS, RET/PTC, and PAX8/PPARG in cytologically indeterminate thyroid nodules. The first study reported molecular testing in 470 consecutive FNA samples, identifying 32 mutations. These included 18 BRAF mutations, 8 RAS mutations, 5 RET/PTC gene rearrangements, and 1 PAX8/PPARG gene rearrangement (37). Surgical pathologic examination confirmed that 97% of these positive results corresponded to malignancy, revealing a positive predictive value approaching 100%. The second study limited panel testing to samples from 235 nodules referred for surgery on the basis of the identification of indeterminate or malignant cytopathologic characteristics or evidence of compressive symptoms due to the enlargement (38). Gene mutations were detected in 67 samples including 33 BRAF mutations, 23 RAS mutations, and 11 RET/PTC gene rearrangements. Surgical pathologic examination confirmed that 78% of these positive results corresponded to malignancy. Analysis revealed that when directly compared with cytopathologic findings, panel testing increased diagnostic accuracy from 83% to 93% while the combination of both cytopathologic examination and molecular analysis increased it further to 96%.

These data reveal the diagnostic value of multigene analysis. Such testing is available in several academic centers, while also commercially available (mirInform Thyroid, Asuragen, Inc). The test requires 1 additional needle stick, which is rinsed separately and processed in a special solution. Most clinicians view this diagnostic panel as a “rule in” test. That is, detection of a mutation supports the diagnosis of a likely malignant nodule. Surgery (and in most cases, near-total thyroidectomy) is then recommended. The fact that up to 30% of thyroid cancers do not harbor one of the above gene mutations, however, limits its negative predictive value. Regardless, in combination with FNA cytologic interpretation, analyses for BRAF and RAS mutations, as well as RET/PTC and PAX8/PPARG rearrangements, are important adjuncts to the clinical evaluation of affected patients.

**GENE EXPRESSION AND MICROARRAY ANALYSIS**

**MicroRNA Expression**

MicroRNAs are 21 to 22 nucleotide segments of non-coding RNA that have a key role in posttranscriptional gene regulation through complementary binding that mediates the translation and degradation of messenger RNA (39). Concentrations of different microRNAs present in tissue specimens have been correlated with the invasive and metastatic potential of specific malignancies (40). Numerous studies to date have sought to define differential microRNA expression signatures that could serve as a means of distinguishing benign thyroid nodules from thyroid cancer. A study that used microarray analysis evaluating tissue specimens in both follicular adenomas and follicular carcinomas demonstrated both microR-197 and microR-346 to be significantly overexpressed in thyroid cancer (41). A separate study using reverse transcription polymerase chain reaction analysis of aspirate samples confirmed that microR-221, microR-222, and microR-146b were overexpressed in papillary thyroid carcinomas compared with expression in normal thyroid tissue (42). Recent studies using microRNA expression profiles from training sets of tissue and aspiration samples have developed predictive models with diagnostic accuracies ranging from 76% to 90% when applied to validations sets (43-45). However, no large-scale, prospective, multicenter trial investigating microRNA has yet been performed. Presently, microRNA testing of thyroid aspirates is not commercially available and is offered only through research protocols.

**Microarray Analysis**

Microarray techniques seek to identify patterns of expressed RNA in the human genome that are predictive of benign or malignant thyroid disease. This technology has recently become more accessible with the advent of microarray platforms that rapidly determine the expression of all
coded human genes. Furthermore, the cost of performing this analysis has decreased. Unlike single gene mutations or rearrangements, microarray diagnostic tests have been described that involve tens to hundreds of expressed genes. Importantly, beyond simply a report of gene expression, a computational algorithm must also be used in these tests for complete analysis. This makes microarray testing less transparent when compared with other molecular markers, although it dramatically expands the diagnostic potential of such testing. As benign and malignant thyroid disease broadly includes 10 to 20 different histologic processes, microarray technology may well prove most promising for dealing with tissue heterogeneity.

The only available diagnostic microarray for use in thyroid nodule analysis is a novel gene expression classifier (Afirma) developed by Veracyte, Inc. This gene expression classifier analyzes the expression of 167 RNA transcripts from a diagnostic thyroid nodule aspirate. This test was designed with the intention of confirming a high likelihood of a benign nodule (high negative predictive value) when initial FNA cytologic findings were indeterminate. In a prospective, multicenter, blinded study enrolling 3789 patients, the performance of this gene expression classifier was validated against the criterion standard of blinded histopathology (46). Among all cytoplagnostically indeterminate thyroid nodules, the sensitivity and specificity were 92% and 52% respectively. For a study population with a cancer prevalence of 32%, the test’s negative predictive value was 93% among all cytologically indeterminate nodules. For thyroid nodules with indeterminate cytologic findings classified as atypia of undetermined significance/follicular lesion of undetermined significance, the negative predictive value was 95%; for those with cytologic findings labeled “follicular neoplasm,” the negative predictive value was 94%. Seven false-negative gene expression classifier results occurred. Independent testing of these samples revealed minimal evidence of papillary carcinoma or thyroid follicular cell content in most of these specimens. This suggests that insufficient thyroid nodule sampling (and not test performance) may be responsible for several of the false-negative results.

AVAILABLE TESTS

Laboratory-developed mutation analyses that test tissue and FNA samples for the **BRAF** V600E mutation are readily available through a number of academic institutions and commercial laboratories (28,47,48). The cobas 4800 **BRAF** V600 Mutation Test produced by Roche Molecular Diagnostics is currently the only US Food and Drug Administration–approved test for a specific **BRAF** mutation (49). It was developed to test melanoma tissue for the **BRAF** V600E mutation and to determine suitability for treatment with vemurafenib. It remains to be seen whether it may be feasible to test thyroid tissue with this or other similar tests. Nonetheless, laboratories certified by Clinical Laboratory Improvements Amendments can also test for this **BRAF** mutation without formal Food and Drug Administration approval. The Asurgen miRInform Thyroid Panel analyzes an FNA sample for 14 gene mutations in **BRAF**, **KRAS**, **NRAS**, and **HRAS** and for 3 gene rearrangements (**RET/PTC1**, **RET/PTC3**, and **PAX8/PPARG**) identified in thyroid malignancies (50). The report generated from testing indicates a positive or negative result for each tested mutation and gene rearrangement. The Veracyte Afirma Thyroid FNA Analysis can be performed on cytoplagnostically indeterminate nodules (51). Reports generated from testing provide results of “benign” or “suspicious” for each sample. A prospective, large-scale clinical validation of the Afirma gene expression classifier was recently published, and it provides the broadest available data among any of the thyroid nodule diagnostic tests (46).

**DISCUSSION**

With the increasing availability of accurate, reliable, and affordable tests, molecular diagnostic testing appears poised to transform the management of indeterminate thyroid nodules. As the cost continues to fall and the discriminant capability of testing continues to improve, it is anticipated that diagnostic testing using molecular marker signatures will become standard practice. Outcomes and cost-benefit analyses should favor limiting intervention to patients most likely to obtain the greatest benefit while simultaneously reducing test or treatment morbidity.

Presently, the best widely available options for the evaluation of nodules classified as indeterminate, on the basis of published data and respective positive and negative predictive values, appear to be the Veracyte Afirma Thyroid Gene Expression Classifier and combined analyses of the 17-mutation panel (including the Asurgen miRInform Thyroid test). Large-scale, prospective, and blinded investigations will be critical for full understanding of test performance. To date, only 1 such large-scale clinical trial has been reported. The transparent and multicenter nature of this trial supports its direct translation into the clinic. For other markers, publications are primarily based on small-scale, single-institution experiences and available data must be viewed with appropriate limitations.

With an identified positive predictive value of 87% to 95%, the 17-mutation assessment (provided by academic institutions or by Asuragen, Inc, miRInform™ Thyroid panel) may serve best as a diagnostic test to “rule in” suspected malignancy. When a mutation is detected, this finding will influence the decision to proceed with surgery or optimally plan the extent of surgery (52). In contrast, with an identified negative predictive value of up to 95% for nodules cytologically characterized as atypia of undetermined significance/follicular lesion of undetermined significance, the Afirma Gene Expression Classifier may better serve as...
a test to exclude possible malignancy, allowing consideration of a more conservative, nonoperative approach altogether (51,53).

CONCLUSION

Ongoing research in this field actively continues with the identification of new potential molecular markers and the publication of clinical trials demonstrating both the utility and limitations of these markers in clinical practice. Diagnostic microRNA research in particular looks to be a promising field of inquiry that may offer a complementary approach to the identification of benign or malignant molecular signatures. Beyond simply marker discovery, however, the importance of large-scale, prospective clinical validation trials should be stressed. Given the heterogeneity of thyroid histology, as well as the variation in test performance for some markers, transparent clinical trials will provide the optimal means for understanding molecular marker analysis in a real-world setting. Indeed, large-scale validation should be viewed as a critical and necessary adjunct to marker discovery. From this point into the future, our approach to any patient with a thyroid nodule should most certainly include assessment of clinical, biochemical, radiologic—and now molecular—variables.

DISCLOSURE

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