Published in final edited form as:

Head Neck. 2012 September ; 34(9): 1355–1361. doi:10.1002/hed.21818.

Predicting Malignancy in Thyroid Nodules: Molecular Advances

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Abstract

Over the last several years, a clearer understanding of the genetic alterations underlying thyroid carcinogenesis has developed. This knowledge can be utilized to tackle one of the greatest challenges facing thyroidologists: management of the indeterminate thyroid nodule. Despite the accuracy of fine needle aspiration cytology, many patients undergo invasive surgery in order to determine if a follicular or Hurthle cell neoplasm is malignant, and better diagnostic tools are required. A number of biomarkers have recently been studied and show promise in this setting. In particular, *BRAF*, *RAS*, *PAX8-PPAR γ* , microRNAs and loss of heterozygosity have each been demonstrated as useful molecular tools for predicting malignancy and can thereby guide decisions regarding surgical management of nodular thyroid disease. This review summarizes the current literature surrounding each of these markers and highlights our institution's prospective analysis of these markers and their subsequent incorporation into our management algorithms for thyroid nodules.

Keywords

thyroid nodules; molecular markers; thyroid cancer; predictors of malignancy

Introduction

Palpable thyroid nodules are a relatively common finding, detected in 4–7% of North American adults; only about 5% of these will harbor a malignancy (1). Fine needle aspiration (FNA) biopsy can be helpful in identifying malignancy (Table 1), however the biopsy can also show results that are indeterminate (10–15%) or nondiagnostic (5–15%) (2,3) and surgical excision is often necessary to obtain a definitive diagnosis. Although the risks of surgery are low in experienced hands they do occur, making any non-invasive adjunct to help diagnose malignancy an attractive option.

There are a number of patient history and physical examination characteristics that clinicians currently utilize to predict malignancy. Patients with thyroid nodules who are at extremes of age (<20 or >70 years), male, have a history of head and neck irradiation, or a positive family history are at increased cancer risk. A nodule that on physical exam is firm, fixed to surrounding structures, associated with regional lymphadenopathy or is contributing to

hoarseness or dysphagia is also concerning. Numerous ultrasonographic features including microcalcifications, intranodular vascularity, irregular borders, hypoechogenicity, a taller-than-wide shape, and a predominantly solid consistency have been identified as predictors of thyroid malignancy (4). Papini and colleagues evaluated the ultrasound characteristics of 402 nodules by multivariate logistic regression analysis and found that irregular or blurred nodular margins (RR = 16.83), an intranodular vascular pattern (RR = 14.29) and microcalcifications (RR = 4.97) were independent risk factors for malignancy (5). Another group retrospectively assessed the ultrasonographic features of over 1200 thyroid nodules in 580 patients, all imaged by a single radiologist, and found that the presence of at least one feature (microcalcifications, irregular or microlobulated margins, marked hypoechogenicity, or a taller-than-wide shape) could be used to classify a lesion as malignant with 87% sensitivity, 87% specificity and 87% accuracy (6). Thyroid nodule characterization by ultrasound is now considered routine. A patient with papillary thyroid cancer and a preoperative ultrasound demonstrating these suspicious nodule characteristics, including hypoechogenicity, microcalcifications, irregular border and a taller-than-wide shape, is shown in Figure 1.

In recent years, our institution has investigated nodule size of ≥ 4 cm as an independent criterion for diagnostic lobectomy (7), initially prompted by the high (17%) false-negative rate for benign FNA results in nodules >3 cm in size reported by Meko et al.(8). We examined the histologic findings in 223 patients who underwent thyroidectomy with a nodule ≥ 4 cm as an independent operative indication and found that 19% of those nodules were thyroid cancer on final pathology. Of the nodules diagnosed as follicular or Hurthle cell neoplasm on FNA, 33% (8/24) and 38% (3/8) were malignant on final pathology and perhaps, most provocatively, 24/71 patients (34%) who were reported to have benign cytology ultimately had a follicular neoplasm histologically (follicular adenoma, follicular carcinoma and follicular variant papillary carcinoma). Among many studies of FNA accuracy, this investigation was unusually informative in design because all patients presenting with a ≥ 4 cm thyroid mass underwent thyroidectomy allowing histologic review. Overall, a false-negative rate of 13% for cancer was observed in preoperative FNA of ≥ 4 cm nodules which is unacceptably high, and we recommend diagnostic thyroidectomy for nodules of this size.

The “ ≥ 4 cm” study observed a 38% malignancy rate among Hurthle cell lesions ≥ 4 cm (7) which is comparable to other reports that have revealed larger nodule size as a strong predictor of malignancy. Zhang and colleagues reviewed 55 consecutive patients who underwent thyroid surgery for a Hurthle cell neoplasm and while 44% of the lesions >4 cm were malignant, only 18% of those 2–4 cm in size were carcinoma (9). Similarly, a recent report from Sippel et al. evaluated 57 patients with a preoperative FNA showing Hurthle cell neoplasm and found that the risk of malignancy increased from 13% to 55% once tumors reached a size >4 cm (10). Many centers, including our own, now recommend an upfront total thyroidectomy for Hurthle cell neoplasms ≥ 4 cm or larger.

Banks and colleagues developed a predictor model to aid in the diagnosis of suspicious or indeterminate FNA samples. They first evaluated 468 patients, comparing FNA results to the final surgical histopathology and clinical characteristics, with univariate analysis, which

indicated that extremes of age (3% and 3.4% increased risk for each year < and >50 years of age, respectively), nodule size (53% and 39% increased risk for each centimeter > and <2.5 cm, respectively) and cytopathology (suspicious for papillary thyroid carcinoma had the greatest risk) were significant predictors of thyroid cancer. These 3 variables remained significant on multivariate analysis and, in combination, were predictive with 74% accuracy, and the findings were then validated when the model was prospectively applied to 135 new patients (11). This was a very nicely done study, however the predictormodel still did not pick up all of the cancers.

Unfortunately, no patient, physical exam, radiographic or FNA biopsy characteristic has provided a perfect tool for prediction of malignancy in thyroid nodules and many patients continue to require diagnostic thyroidectomy. In the last several years there has been significant interest in evaluating biomarkers as a potential diagnostic tool for thyroid nodules. This review summarizes the current body of literature regarding the use of what we consider to be the most promising molecular markers in predicting malignancy in thyroid nodules.

BRAF

BRAF is an oncogene that is implicated in a number of human cancers. It has been well-established that the development of papillary thyroid cancer (PTC) involves the activation of the MAPK signaling pathway, and *BRAF* is a strong activator of that pathway (12). A point mutation resulting in a valine to glutamate alteration at codon 600 (V600E) has been reported in up to 80% of papillary thyroid cancers (13). *BRAF* mutations are also found in thyroid lymphomas, anaplastic and poorly differentiated thyroid cancers, but not in follicular or medullary carcinomas and only rarely reported in benign hyperplastic nodules (14).

Not only is the *BRAF* V600E mutation strongly linked to PTC, but there is evidence that it is also a marker of poor prognosis. Multiple reports have demonstrated an association between this mutation and established clinicopathologic parameters considered to represent aggressive tumor behavior and poor prognosis, including local recurrence, extrathyroidal extension, multicentricity, lymph node and distant metastases, and advanced TNM stage (15,16). Elisei and colleagues retrospectively evaluated a small cohort of PTC patients with median follow-up of 15 years and demonstrated a statistically significant decreased survival in the group carrying the V600E mutation, which also served as an independent predictor of poor prognosis after multivariate analysis (17).

Now that *BRAF* mutation has been established as a key genetic event in the evolution of papillary thyroid cancer, it begs the question of whether it has diagnostic utility and if it can be used to optimize the surgical management of this disease. A recent study conducted at our institution compared 106 *BRAF*-positive PTC patients to 100 *BRAF*-negative controls, all of whom underwent surgical resection of their cancers with a mean follow-up of 8.8 months. A significantly higher proportion of the *BRAF*-positive patients had suspicious sonographic features on their preoperative ultrasound (50% vs. 33%, $p=0.03$), underwent total thyroidectomy as their initial surgical procedure (87% vs. 67%, $p<0.001$), had tall cell morphology (30% vs. 6%, $p<0.001$), extrathyroidal extension (57% vs. 15%, $p<0.001$),

lymphovascular invasion (40% vs. 21%, $p=0.003$), level VI lymph node metastases (36% vs. 12%, $p=0.003$), and have required reoperation for recurrent or metastatic disease (10% vs. 3%, $p=0.04$) compared to controls. (Yip et al., under review). Forty-four of the *BRAF*-positive patients had undergone preoperative *BRAF* testing on their FNA specimens, 31 of which were positive yielding a sensitivity of 70%. On final histopathology, all 31 patients had PTC (100% specificity) including 2 patients whose FNA biopsy results had inadequate yield but were *BRAF*-positive. Preoperative knowledge of *BRAF*-positive status could have altered initial surgical management in 24% of the 75 patients in this series whose preoperative *BRAF* status was either unknown or falsely negative. Our group now considers *BRAF*-positive cytology to be an independent indication for total thyroidectomy as initial surgery.

Furthermore, *BRAF* status may also guide the need for and extent of initial lymphadenectomy. At present, whether routine central compartment neck dissection (CCND) is necessary for all PTC patients remains controversial, but *BRAF* status may prove an important tool for guiding this decision. Proponents for more aggressive surgery to include CCND argue that while this approach does not improve overall survival, it does decrease the risk of locoregional recurrence which can have positive effects on quality of life, as well as allowing for more accurate staging and thus a more precise assessment of the need for postoperative adjuvant therapy (18). However, these claims have not been proven in a randomized study.

Papillary microcarcinoma, or PTC <1 cm in size, is another clinical scenario where knowledge of *BRAF* status may prove to be useful in guiding management. At present, adjuvant therapy in the form of TSH suppression and radioiodine ablation is not routinely given for microcarcinoma, yet it has been shown that some of these seemingly indolent lesions can go on to develop recurrent or metastatic disease. Reported recurrence rates have varied from 3–17%, depending on the length of follow-up in the study and whether recurrence was defined biochemically (by thyroglobulin elevation) or by local recurrence with histopathologic confirmation (19–21). One day *BRAF* testing may help identify which microcarcinomas are more aggressive and therefore direct the therapeutic approach.

Potential resistance to the conventional adjuvant therapies used to treat PTC is thought to be one of the reasons why *BRAF*-positive PTC is associated with a poorer prognosis. *In vitro* studies have documented significantly lower expression levels of the sodium iodide transporter (NIS) and TSH-receptor genes in *BRAF V600E*-positive PTC thereby limiting the effectiveness of RAI ablation and TSH suppression as treatment adjuncts (22,23). Blocking the MAPK pathway restores expression of these genes (24), and further studies to identify other targeted therapies are ongoing.

RAS and PAX8-PPAR γ

RAS is an oncogene with intrinsic GTPase activity that codes for proteins located on the inner surface of the cell membrane. When activated, *RAS* recruits other kinases ultimately leading to the transcription of growth and differentiation signals downstream. Point mutations lead to constitutive activation of these downstream signaling pathways and

missense mutations at codons 12, 13, and 61 in *K-RAS*, *N-RAS*, and *H-RAS* are reported to occur in 40–50 % of follicular carcinomas and approximately 10% of papillary thyroid carcinomas (25). *RAS* mutations are identified in 48% of benign follicular adenomas as well(26).

PAX8-PPAR γ refers to a fusion protein resulting from a balanced translocation of the peroxisome proliferator-activated receptor γ gene (*PPAR γ*) and the paired box gene 8 (*PAX8*). The fusion protein is oncogenic, in that its formation suppresses *PPAR γ* 's usual function as a regulator of transcription, but its exact role in thyroid cancer oncogenesis remains unclear. Rearrangements of *PAX8-PPAR γ* have been demonstrated in up to 63% of follicular carcinomas, but not in PTCs or follicular adenomas (27).

Nikiforova and colleagues were the first to evaluate the correlation between *RAS* and *PAX8-PPAR γ* point mutations within follicular thyroid tumors. Of the 33 follicular thyroid carcinomas (FTC) evaluated, 49% were *RAS*-positive and 36% were *PAX8-PPAR γ* -positive. Only 3% had both mutations suggesting that *RAS* and *PAX8-PPAR γ* are two separate oncogenic pathways (26). When the clinicopathologic features of the *RAS*-positive FTC were compared with those of the *PAX8-PPAR γ* -positive FTC, they found that the *RAS*-positive tumors presented later in life, were larger and displayed a wide range of invasiveness while the *PAX8-PPAR γ* -positive tumors presented at a younger age, were smaller, and the majority had overtly invasive pathology at presentation. Interestingly, Hürthle cell lesions in this study (adenomas and carcinomas) rarely exhibited either a *RAS* mutation or a *PAX8-PPAR γ* rearrangement lending support to the growing body of evidence that Hürthle cell neoplasms are unique entities rather than simply a subtype of follicular lesions. The authors concluded that since 85% of conventional FTC exhibit one of these two mutations, testing FNA specimens for their presence can enhance diagnostic accuracy.

Nikiforov et al, recently reported results for a prospective study of 470 FNA samples from 328 consecutive patients who were tested for *BRAF*, *RAS*, *PAX8-PPAR γ* and *RET/PTC*, and correlated the results of mutation status with cytology, surgical pathology, or clinical follow-up results. The patients were followed for a mean of 34 months. The *BRAF* and *RAS* point mutations were detected utilizing real time PCR and fluorescence melting curve analysis, while detection of *RET/PTC* and *PAX8-PPAR γ* rearrangements was done using reverse transcription-PCR technology. A total of 32 mutations were identified and 97% of mutation-positive nodules were papillary or follicular carcinoma on final surgical pathology. The sensitivity of molecular testing alone was 62%, but rose to 80% when combined with cytology(28).

Based on this experience, we now routinely test all FNA specimens with results in the categories of indeterminate (follicular or oncocytic neoplasm), inadequate yield, and lesion of undetermined significance for a panel of biomarkers that includes *NRAS61*, *HRAS61*, *KRAS12/13*, *BRAF*, *RET/PTC1* and *3* and *PAX8-PPAR γ* . We have found that detection of the latter three in a FNA specimen correlates to a near 100% probability of cancer whereas detection of *RAS* correlates with malignancy in 83–87% of cases. Molecular testing is also proving to be particularly helpful among thyroid lesions classified as “follicular lesion of undetermined significance” (FLUS). At the University of Pittsburgh Medical Center, 121

FLUS cases with molecular analysis (*BRAF*, *RAS*, *RET/PTC1* and *PAX8-PPAR γ*) and available histologic correlation were reviewed. Twelve cases had positive molecular results and each of them was a papillary thyroid carcinoma on final pathology (PPV=100%)(Ohori et al., under review). While the current recommendation for FLUS is to repeat the FNA, molecular testing may ultimately direct clinicians when to move directly to diagnostic surgery.

MicroRNAs

Recently, a class of RNAs that can function as gene expression regulators have been identified. These microRNAs (miRNAs) are small, single-stranded, noncoding RNA molecules and are important in embryonic development. There has been increasing evidence documenting their involvement in tumorigenesis as well. Decreased expression of some miRNAs may lead to oncogene upregulation and increased expression may lead to tumor suppressor gene downregulation. Since microRNAs have been implicated in cellular growth, differentiation, and apoptosis (29), it is not surprising that aberrations in miRNA expression have been implicated in the development and progression of a number of human cancers including lung cancer (30), breast cancer (31), sarcoma (32), gastric cancer (33) and lymphoma (34). MiRNAs are expressed differentially depending not only on their tissue of origin but also the growth characteristics of that tissue (neoplastic versus benign) and it has been suggested that miRNA profiles may be utilized to determine a tumor's cellular origin.

Several recent studies have evaluated the miRNA expression profiles within different histological types of thyroid cancer. Visone and colleagues used microarray analysis to compare the miRNA expression profile of 10 human anaplastic thyroid cancers (ATC) to 10 normal thyroid tissue samples. They discovered a significant decrease in miR-30d, miR-125b, miR-26a and miR-30a-5p in ATC compared to the normal thyroid tissue, suggesting a "miRNA signature" associated with ATC. Interestingly, a downregulation of miRNAs, rather than an upregulation, has generally been found in the setting of ATC, suggesting that they function as tumor suppressor genes in undifferentiated thyroid cancer (35,36). Findings like these naturally prompt investigation of miRNAs as potential therapeutic targets. One study has already documented that alteration in miRNA activity can change the way tumor cells respond to chemotherapeutic agents (37). Similar work directed at thyroid cancer holds much promise, particularly for the universally lethal anaplastic subtype for which current therapeutic options are limited.

Conversely, for papillary thyroid cancer (PTC), studies have more commonly reported an upregulation of miRNAs. For example, Chen et al. reported significant overexpression of miR-221, -222 and -146b in PTCs (38). Israel et al. designed a novel method of quantifying the overall effect of miRNAs on their target genes, and found a global downregulation of their target genes in PTC (39). Regarding follicular thyroid cancers (FTCs), Weber et al. concluded that both miR-197 and 346 play a role in FTC carcinogenesis after demonstrating significant overexpression of these miRNAs in FTCs when compared to follicular adenomas (40).

At our institution there has been a great deal of focus in evaluating not only the microRNA expression profiles of various thyroid neoplasms, but also on the utilization of miRNAs as potential diagnostic biomarkers. Nikiforova and colleagues utilized RT-PCR to quantify expression levels of 148 miRNAs in 60 thyroid neoplasms including PTC, MTC, FTC, ATC, poorly differentiated cancers (PDC) and follicular adenomas (FA). FROM WHERE DID SHE GET THE CELLS? – these were patient samples so less concern about integrity of thyroid cancer cell lines. Thirty-two percent (47/148) of the miRNAs analyzed were consistently upregulated in the neoplasms when compared to normal thyroid tissue, and 38% (57/148) were downregulated. They further identified seven miRNAs that were consistently overexpressed in all tumors of follicular cell origin, whereas MTC had a distinct profile of highly expressed miRNAs, which is not surprising given the unique C-cell origin of these tumors. Using the subset of 7 miRNAs overexpressed in differentiated thyroid cancers, 62 FNA specimens were analysed to determine if the panel could be a useful diagnostic adjunct to cytology and 100% of patients with cancer on final pathology showed a 2-fold overexpression of at least one of the miRNAs, whereas only 25% of those with a hyperplastic nodule had any significant miRNA overexpression. When >3 miRNAs in the panel were overexpressed at least 2-fold, malignancy could be predicted with 98% accuracy (41).

In summary, the discovery of miRNAs just over a decade ago is one of the most exciting developments in the field of biomarkers and holds significant promise in its application as a diagnostic tool in thyroid cancer and may eventually lead to significant therapeutic advances, especially for ATC.

Loss of Heterozygosity

Loss of heterozygosity (LOH) can be a key step in oncogenesis leading to a loss of tumor suppressor gene function, thereby permitting uncontrolled cell growth. Since LOH is a relatively late event in tumorigenesis, it is an attractive molecular marker for distinguishing follicular carcinoma from adenoma. LOH has been examined by several groups in follicular thyroid lesions (REFS) with what results? WE WERE NOT THE FIRST.

Our institution examined 17 benign and malignant follicular neoplasms for loss of heterozygosity (LOH) at the von Hippel Lindau (VHL) gene locus; VHL is a tumor suppressor gene that plays an important role in the evolution of both sporadic and familial endocrine tumors. DNA was extracted from each lesion and the PCR products were analysed in a semiquantitative fashion to detect LOH at the VHL locus, which was present in 13% of follicular thyroid cancers and none of the benign follicular adenomas, and which also was strongly correlated with tumor recurrence and death. Four of the 7 cancers that recurred had LOH while 3 FTC that did not recur did not have LOH at the VHL locus(42).

A subsequent study looked at a more extensive panel of tumor suppressor genes, including VHL, p53, PTEN, p16, APC, 1p and NF2 and once again, a correlation between LOH and poor disease outcome was illustrated (43). Fractional allelic loss (FAL) was significantly higher in the malignant compared to the benign follicular tumors (56% vs. 14%, $p<0.001$). Widely invasive follicular carcinomas had a significantly higher mean FAL compared to

minimally and angioinvasive carcinomas (86% vs. 44%, $p=0.023$). These studies suggest that LOH analysis of follicular neoplasm can both predict the presence of malignancy and also identify the carcinomas that may necessitate a more aggressive therapeutic strategy.

Conclusion

Management of thyroid nodular disease requires the integration and interpretation of clinical, radiographic and cytologic variables to achieve optimal patient outcomes. A number of clinicopathologic predictors and diagnostic models have been identified, but none are able to predict malignancy in a thyroid nodule with 100% accuracy and at present, a significant proportion of patients still require diagnostic surgery. To improve diagnostic accuracy, a number of biomarkers have recently been evaluated and hold much promise. This review has outlined the current understanding of what we believe to be the most promising markers (Table 2). We present in Figure 2 a management algorithm for use of molecular markers in thyroid nodule diagnosis. These molecular markers also provide exciting possibilities for targeted therapeutics in the management of thyroid cancer.

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Figure 1.

Ultrasound of a $1.2 \times 1.1 \times 1.1$ cm nodule in the left mid to upper pole of the thyroid gland, depicting an irregular border, microcalcifications, hypoechogenicity and a taller-than-wide shape. On final pathology, this was a 1.2 cm papillary thyroid cancer with extrathyroidal extension. (IJV = internal jugular vein)

Figure 2.

University of Pittsburgh Medical Center's current algorithm for the evaluation of thyroid nodules.

FNA=fine needle aspiration

FLUS=follicular lesion of undetermined significance

CCND=Central Compartment Node Dissection

*Indications for Total Thyroidectomy: family history of thyroid cancer, personal history of head and neck irradiation, hypothyroidism, symptomatic goiter, contralateral dominant nodule

Table 1

National Cancer Institute Thyroid Fine-Needle Aspiration State of the Science Conference (3)

Cytology Classification	Risk Category	Risk of Malignancy
Benign	Low	<1%
Follicular lesion of undetermined significance	Low	5–10%
Follicular or Hürthle cell neoplasm	Low - Intermediate	20–30%
Suspicious for malignancy	Intermediate - High	50–75%
Malignant	High	100%
Nondiagnostic	N/A	N/A

Table 2

Summary of molecular diagnostic adjuncts for follicular cell-derived thyroid cancer subtypes.

Thyroid Cancer Subtype	Frequency (45)	Associated Molecular Changes	Associated microRNAs
Papillary	80%	BRAF mutation	221
		RAS mutation	222
		RET-PTC rearrangement	146b
Follicular	11%	RAS mutation	197
		PAX8-PPAR γ rearrangement	346
		Loss of Heterozygosity	
Hürthle	3%	PAX8-PPAR γ rearrangement	N/A
Anaplastic	2%		30d
		BRAF mutation	125b
		RAS mutation	26a
		P53	30a-5p