New Strategies in Diagnosing Cancer in Thyroid Nodules: Impact of Molecular Markers

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Abstract
Thyroid cancer is the most common type of endocrine malignancy, with approximately 55,000 new cases diagnosed in the United States in 2012. However, thyroid nodules are much more prevalent, particularly with increased age, and only a small fraction of those are malignant. Therefore, the major clinical challenge is to reliably differentiate those nodules that are malignant and need to be treated surgically from the majority of nodules that are benign and do not require surgery. The traditional diagnostic approach to this clinical situation is ultrasound-guided fine-needle aspiration (FNA) of the thyroid nodule followed by cytologic examination, which together reliably establish the diagnosis in 70% to 80% of cases. However, in the rest of nodules the presence of cancer cannot be ruled out by FNA cytology, hampering appropriate surgical management and frequently resulting in unnecessary surgical interventions. New approaches to diagnosis of cancer in thyroid nodules are based on mutational and other molecular markers, which can be reliably detected in cells aspirated during the FNA procedure. These markers offer significant improvement in the diagnostic accuracy of FNA cytology and are poised to make a profound effect on the management of patients with thyroid nodules. In addition to the molecular markers that have recently become available for clinical use, rapid development of new sequencing techniques is expected to further improve the accuracy of cancer diagnosis in thyroid nodules and allow for a fully individualized approach to the management of patients with thyroid nodules. Clin Cancer Res; 19(9); 2283–8. ©2013 AACR.

Background
Thyroid nodules are very common, particularly in women and with increased age. The prevalence of palpable nodules in population-based studies is 3% to 4%, and the prevalence of nonpalpable nodules incidentally identified on imaging approaches 40% to 50% after the age of 60 years (1–5). Thyroid nodules are found to be even more common on high-resolution ultrasound screening using sensitive high frequency (10–13 MHz) transducers (6). Thus, as the population ages, technology improves, and the use of neck imaging becomes more routine, clinicians will be faced with a growing number of patients with thyroid nodules.

Thyroid cancer is the primary clinical concern in a patient with thyroid nodules, although only a small proportion of thyroid nodules are malignant. In euthyroid patients with an asymptomatic thyroid nodule who come for an office visit, cancer is found in approximately 5% of nodules (7). Mortality from thyroid cancer remains low, particularly in patients with stage I or II of the disease, for which the 10-year survival rate is higher than 98%. However, the survival rate decreases to 56% in those who present at a more advanced stage or with distant metastases, and therefore early diagnosis and treatment offer optimal long-term outcomes (8).

The diagnosis of thyroid cancer relies on cervical ultrasound and fine-needle aspiration (FNA) biopsy, which collects cells for cytologic examination (9, 10). Ultrasound examination detects nodules with high sensitivity, reliably determines nodule size, improves diagnostic discrimination, and guides FNA biopsy of those nodules that have suspicious sonographic features. FNA cytology is currently the most reliable diagnostic tool for evaluation of thyroid nodules. It provides a definitive diagnosis of benign or malignant thyroid disease in most cases. However, in 20% to 30% of nodules, FNA cytology cannot reliably rule out cancer, and such cases are reported as indeterminate for malignancy (11, 12). To provide uniform diagnostic criteria and better risk stratification, the National Cancer Institute Thyroid Fine-needle Aspiration State of the Science Conference held in Bethesda, Maryland, in 2007, offered a classification system for reporting thyroid cytology, which has been adopted by most large volume centers, referred to subsequently as the Bethesda system (12, 13). The Bethesda system recognizes 3 specific cytologic diagnoses of indeterminate cytology: follicular lesion of undetermined significance/atrophy of undetermined significance (FLUS/AUS); follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm (FN/SFN); and suspicious for malignant cells (SMC), with a predicted probability of cancer...
of 5% to 10%, 20% to 30%, and 50% to 75%, respectively (12). Although these cytologic diagnoses are now associated with a better-defined cancer risk, none of them reliably predict the malignant or benign outcome. Because of the lack of a definitive diagnosis for these nodules, most patients with indeterminate cytology undergo diagnostic surgery to establish histopathologic diagnosis. However, only 10% to 40% of such surgically resected thyroid nodules prove to be malignant (4, 12, 14). The diagnostic operations, with their attendant expenses and risks, could be avoided if FNA procedures could reliably establish the presurgical diagnosis of a nonneoplastic benign nodule.

In addition, an indeterminate preoperative diagnosis does not always lead to the optimal initial surgical intervention for patients who have thyroid cancer. Options for an initial surgical procedure are either thyroid lobectomy, i.e., excision of the lobe that contains the nodule while leaving the contralateral lobe intact, or total thyroidec- tomy. Thyroid lobectomy preserves thyroid function without thy- roid hormone replacement in 20% to 30% of patients, eliminates the risk of permanent hypoparathyroidism, and therefore is typically chosen as the initial surgery for nodules with indeterminate cytology if other indications for total thyroidec- tomy, such as contralateral nodules, hypothyroid- ism, or history of head and neck radiation, are absent. However, if diagnostic lobectomy confirms a cancer of more than 1 cm in size, a second surgery is typically conducted to "complete" the thyroidec- tomy.

Both unnecessary and two-step surgeries can poten- tially be avoided with a more accurate preoperative diagnosis of cancer in thyroid nodules. This requires new diagnostic approaches, which can be based on molecular biomarkers of thyroid cancer. Indeed, a number of somatic mutations as well as alterations in gene expression, microRNA (miRNA) expression, and gene promoter methylation, have been described in thyroid cancer over the past few decades (15). Most common mutations that occur in papillary thyroid cancer, which is the most common type of thyroid cancer, are point mutations of the BRAF and RAS genes and RET/PTC and TRK rearrangements, all of which are able to activate the mitogen-activated protein kinase (MAPK) pathway. These mutually exclusive mutations are found in more than 70% of papillary thyroid cancers (16–19). Follicular thyroid cancer, the second most common thyroid cancer type, harbors either RAS mutations or PAX8/PPARγ rearrangements. These mutations are also mutually exclusive and identified in 70% to 75% of follicular carcinomas (20). Genetic alterations involving the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway also occur in thyroid tumors, particularly in dedifferentiated tumors (21–23). Additional mutations known to occur in poorly differe- ntiated and anaplastic carcinomas involve the TP53 and CTNNB1 genes (24). Medullary thyroid carcinomas, both familial and sporadic, frequently carry point mutations located in the RET gene or in RAS genes (25, 26). These mutational markers, as well as altered gene expression and miRNA profiles, have been explored for diagnostic use in thyroid nodules, and some are already in clinical use.

On the Horizon

Of the several types of molecular markers, gene mutation markers and, more recently, gene expression panels, are being introduced into clinical practice and are poised to make a major impact on the management of patients with thyroid nodules.

Mutational markers include BRAF and other genes fre- quently mutated in the most common types of thyroid cancer—papillary and follicular cancers. BRAF V600E is a point mutation that constitutively activates the MAPK signaling pathway and represents the most common mutation in papillary cancer. It is a highly specific diagnostic marker for thyroid cancer. In fact, a meta-analysis based of mostly prospective studies that reported more than 5,000 thyroid FNA samples tested for BRAF V600E showed that finding this mutation entailed a 99.3% risk of cancer on final histopathology (15). Importantly, 15% to 40% of nodules tested positive for BRAF had indeterminate FNA cytology, indicating that this marker can be of significant diagnostic value in these nodules (27–30). Despite high specificity for cancer, molecular testing for BRAF mutation alone has low sensitivity.

Currently, the most successful panel of mutations for thyroid FNA samples include BRAF and RAS point mutations and RET/PTC and PAX8/PPARγ rearrangements, with the possible addition of the TRK rearrangement (31–33). Such mutational panel offers a significant improvement of cancer diagnosis in thyroid nodules. The detection of any mutation is a strong predictor of malignancy in a given thyroid nodule, irrespective of the cytologic diagnosis. Specifically, BRAF, RET/PTC, or PAX8/PPARγ mutations correlate with the malignant outcome in close to 100% of cases, assuming that the analysis is conducted in a clinical laboratory and after appropriately validation of the detection techniques (31–33). On the basis of the high proba- bility of cancer in mutation-positive nodules with any type of indeterminate cytology, these patients can be treated with total thyroidec- tomy as the initial surgical approach (Fig. 1). This will allow bypassing the repeat of FNA and eliminate the need for a two-step surgery, that is, diagnostic lobectomy followed by completion thyroidec- tomy. RAS mutations have a 74% to 87% positive predictive value for cancer (31–33). The rest of the nodules that yield RAS mutations are benign follicular adenomas. However, some evidence exists that these benign nodules are prone to malignant transfor- mation (34–37), and therefore patients with RAS-mutated nodules, even when caught at a premalignant stage, are likely to benefit from surgical excision to prevent the possible progression.

The negative result of testing for the currently available mutational panels significantly decreases the risk of cancer in all categories of indeterminate cytology but does not eliminate it. Indeed, in one study that analyzed more than 1,000 nodules with indeterminate cytology, negative molecular testing decreased the risk of cancer from 27% to 14% in FN/SFN cytology and from 54% to 28% in SMC cytology (Fig. 1). Cytologic diagnosis of FLUS/AUS poses the most challenges for patient management. These patients
have a 5% to 15% probability of cancer and typically undergo repeated FNA and, if the diagnosis remains the same, are offered diagnostic lobectomy, despite the fact that the majority of them have benign hyperplastic nodules (12, 13). The most common mutation found in these nodules is RAS, followed by BRAF and PAX8/PPARγ. Detection of these mutations in nodules with FLUS/AUS cytology confers a high risk of cancer (31, 32, 38), which allows us to bypass the repeated FNA and proceed with optimal surgical management without delay. The risk of cancer in mutation-negative nodules with FLUS/AUS cytology was 6%, including a 2.3% risk of invasive cancer and a 0.5% risk of cancer spread beyond the thyroid (31). The low risk of cancer, and particularly of invasive cancer in mutation-negative nodules with FLUS/AUS cytology, expands the options for clinical management of these patients, which includes consideration for following selected patients with annual ultrasound examination in addition to diagnostic lobectomy. It is important to recognize, however, that the risk of cancer in mutation-negative nodules with FLUS/AUS or other indeterminate cytologic diagnosis may vary depending on the cytologic criteria used, which determines the overall prevalence of cancers in each category of indeterminate cytology.

Another test recently introduced for FNA biopsies that can help to rule out cancer in thyroid nodules is the Afirma gene expression classifier. This test is based on the expression analysis of 142 genes and uses a proprietary algorithm to assign the nodules into either benign or suspicious groups (39). The recently reported performance characteristics of this test suggest that it is helpful for ruling out the malignancy in nodules with indeterminate cytology, although the positive predictive value is lower. Indeed, in a recent study of 265 nodules with indeterminate FNA cytology, the negative predictive values of the benign test were 95%, 94%, and 85% in the nodules with FLUS/AUS, FN/SFN, and SMC cytology, whereas the positive predictive values in these groups were 38%, 37%, and 76%, respectively (40). Although the accuracy of this test in ruling out cancer was comparable with that of a mutational panel test in the FLUS/AUS cytology group, it appears to perform...
better in FNL cytology. The cancers missed by the gene expression classifier were mostly papillary thyroid cancers, although their degree of aggressiveness was not reported. This test represents an alternative molecular diagnostic tool for nodules with indeterminate cytology that may provide additional valuable diagnostic information, particularly when the goal is to rule out cancer in a given nodule and avoid surgery. The impact of this diagnostic test on clinical management of patients with thyroid nodules is expected to be further clarified in larger series of cases and in independent, not industry-sponsored studies.

In addition to the impact on cancer diagnosis in thyroid nodules, mutational markers are likely to provide significant help in preoperative assessment of cancer aggressiveness. This primarily involves BRAF V600E, which has been associated in many studies with aggressive histopathologic features of papillary carcinoma, such as extrathyroidal extension and lymph node metastases (41), as well as with tumor recurrence and tumor-related mortality (42–47). Moreover, BRAF V600E is a significant preoperative predictor of central compartment lymph node metastasis (48), and these tumors more often require reoperation for locally persistent or recurrent disease (49, 50). Therefore, patients with BRAF V600E-positive papillary cancer detected preoperatively may benefit from more extensive initial surgery. Whether prophylactic resection of the central compartment lymph nodes in PTC patients will improve outcome remains to be determined by randomized clinical trial, but preoperative BRAF status is likely to be helpful to guide the extent of both thyroidectomy and initial lymphadenectomy (51). The association between BRAF V600E and more aggressive disease characteristics has also been reported in early-stage thyroid tumors (42, 52) and thyroid microcarcinomas, which are incidentally discovered tumors of 1 cm or less in size (53–58). The use of BRAF mutational status, in combination with other features determining more adverse outcome such as patient age (59) or specific histopathologic tumor features (60), is being defined for risk stratification of both microcarcinomas and larger-size thyroid tumors. This practice will have further impact on offering more individualized surgical and postsurgical management of patients with thyroid cancer.

The use of recently developed molecular tools is expected to lead to a significant improvement in the management of patients with thyroid nodules. However, further progress in diagnostic accuracy is needed. Progress will likely be achieved in the near future due to the availability of novel technologies, such as next-generation sequencing (NGS). NGS offers simultaneous sequencing of thousands to millions of short nucleic acid sequences in a massive parallel way. It provides clear advantages over the conventional sequencing techniques, such as Sanger sequencing, by allowing sequencing of large regions of the genome with higher sensitivity of detection of various genetic variants. NGS offers sequencing at different levels of complexity, including whole-genome sequencing, whole-exome sequencing, whole-transcriptome sequencing, and targeted sequencing of multigene panels. Whereas large-scale analyses are essential for the discovery projects, it is more than likely that targeted panels will offer further advances in routine molecular diagnostics of various diseases, including thyroid cancer. In the thyroid, this approach will allow expansion of the currently existing panel through addition of multiple genetic alterations known to occur in thyroid cancer with low prevalence, such as mutations in the PIK3CA, AKT1, PTEN, and TP53 genes and chromosomal rearrangements of the BRAF and NTRK1 genes. Moreover, massive sequencing efforts involving thyroid cancer are under way by The Cancer Genome Atlas, which are likely to yield additional mutational markers for thyroid cancer. The currently available NGS platforms, such as Ion Torrent PGM (Life Technologies) and MiSeq (Illumina), should allow testing of these expanded panels using the FNA material (61). This will further improve the accuracy of cancer diagnosis in thyroid nodules with indeterminate cytology, decrease costs of testing, and offer the most individualized management of patients with thyroid nodules.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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