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 U.S. 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 detect 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 cytological examination, which reliably establish the diagnosis in 70-80% 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 novel 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.
Background

Thyroid nodules are very common, particularly in women and with increased age. The prevalence of palpable nodules in population-based studies is 3-4% and the prevalence of non-palpable nodules incidentally identified on imaging approaches 40-50% after the age of 60 (1-5). Thyroid nodules are found to be even more ubiquitous 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 and more routine, clinicians will be faced with a growing number of patients with thyroid nodules.

Thyroid cancer is the primary clinical concern in patients with thyroid nodules, although only a small proportion of thyroid nodules is malignant. In an euthyroid patient with an asymptomatic thyroid nodule who comes for an office visit, cancer is found in ~5% of nodules (7). Mortality from thyroid cancer remains low, particularly in patients with stage I-II of the disease where 10-year survival is >98%. However, it falls to 56% in those who present at 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 20-30% of nodules, FNA cytology cannot reliably rule out cancer and such cases are reported as indeterminate for malignancy (11, 12). In order to provide uniform diagnostic
criteria and better risk stratification, the Bethesda NCI Thyroid Fine-needle Aspiration State of the Science Conference in 2007 offered a classification system for reporting thyroid cytology, which has been adopted by most large volume centers (12, 13). The Bethesda system recognizes three specific cytological diagnosis of indeterminate cytology, i.e. follicular lesion of undetermined significance/atypia 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 5-10%, 20-30%, and 50-75%, 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 10-40% of such surgically resected thyroid nodules will prove to be malignant (4, 12, 14). The diagnostic operations, with their attendant expenses and risks, could be avoided if FNA procedure could reliably establish the pre-surgical diagnosis of a non-neoplastic benign nodule.

Additionally, an indeterminate preoperative diagnosis does not always lead to the optimal initial surgical intervention for patients who have thyroid cancer. Options for initial surgical procedure include either thyroid lobectomy, i.e. excision of the lobe that contains the nodule while leaving the contralateral lobe intact, or total thyroidectomy. Thyroid lobectomy preserves thyroid function without thyroid hormone replacement in 20-30% 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 thyroidectomy, such as contralateral nodules, hypothyroidism, or history of head and neck radiation, are absent.
However, if diagnostic lobectomy confirms a cancer >1 cm in size, a second surgery is typically performed to “complete” the thyroidectomy.

Both unnecessary and two-step surgeries can potentially 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, miRNA expression, and gene promoter methylation have been described in thyroid cancer over the last 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 \textit{BRAF} and \textit{RAS} genes and \textit{RET/PTC} and \textit{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 cancer type, harbor either \textit{RAS} mutations or \textit{PAX8/PPARγ} rearrangement. These mutations are also mutually exclusive and identified in 70-75\% of follicular carcinomas (20). Genetic alterations involving the PI3K/AKT signaling pathway also occur in thyroid tumors, particularly in de-differentiated tumors (21-23). Additional mutations known to occur in poorly differentiated and anaplastic carcinomas involve the \textit{TP53} and \textit{CTNNB1} genes (24). Medullary thyroid carcinomas, both familial and sporadic, frequently carry point mutations located in the \textit{RET} gene or in \textit{RAS} genes (25, 26). The 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.

\textbf{On the Horizon}
Out of 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 frequently mutated in the most common types of thyroid cancer, i.e. 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, the meta-analysis based of mostly prospective studies that reported >5,000 of thyroid FNA samples tested for $BRAF$ V600E showed that finding this mutation entailed a 99.3% risk of cancer on final histopathology (15). Importantly, 15-40% 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 $PAX8/PPAR\gamma$ 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 cytological diagnosis. Specifically, $BRAF$, $RET/PTC$ or $PAX8/PPAR\gamma$ mutations correlate with the malignant outcome in close to 100% of cases, assuming that the analysis is performed in a clinical laboratory and after appropriately validation of the detection techniques (31-33). Based on the high probability of cancer in mutation-positive nodules with any type of indeterminate cytology, these patients can be treated with total thyroidectomy as the initial surgical approach (Fig. 1). This will allow
bypassing the repeat of FNA and eliminate the need for a two-step surgery, i.e. diagnostic
lobectomy followed by completion thyroidectomy. \textit{RAS} mutations have a 74-87\% positive
predictive value for cancer (31-33). The rest of the nodules yielded \textit{RAS} mutations are benign
neoplasms, i.e. follicular adenomas, although some evidence exists that they are prone to
malignant transformation (34-37), and therefore patients with \textit{RAS}-mutated nodules, even when
captured at a pre-malignant 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 >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). Cytological diagnosis of FLUS/AUS poses the most
challenges for patient management. These patients have a 5-15\% 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/PPARY. Detection of these mutations in nodules with FLUS/AUS cytology confers a high
risk of cancer (31, 32, 38), which allow to bypass the repeat of 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 thyroid (31). The low risk of cancer, and particularly of invasive cancer in
mutation-negative nodules with FLUS/AUS cytology expands the options for the 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 determine 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. It is based on the expression analysis of 142 genes and uses the 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 indeterminate cytology nodules, although the positive predictive value is lower. Indeed, in a recent study of 265 nodules with indeterminate FNA cytology, the negative predictive value of the benign test was 95%, 94%, and 85% in the nodules with FLUS/AUS, FN/SFN, and SMC cytology, while the positive predictive value in these groups was 38%, 37%, and 76%, respectively (40). Although the accuracy of this test in ruling out cancer was comparable with that of mutational panel in the FLUS/AUS cytology group, it appears to be significantly 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, which 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 a 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 a significant preoperative predictor of central compartment lymph node metastasis(48) and these tumors more often require re-operation for the 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 been also 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 will have further impact on offering more individualized surgical and post-surgical management of patients with thyroid cancer.

The use of recent molecular tools is expected to lead to a significant improvement in the management of patients with thyroid nodules. However, further progress in the diagnostic accuracy is needed. It will likely to 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 technique, such as Sanger sequencing, by allowing to sequence 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 multi-gene panels. Whereas large-scale analyses are essential for the discovery projects, it is more than likely that targeted panels will offer further advance in routine molecular diagnostics of various diseases, including thyroid cancer. In the thyroid, such approach will allow to expand the currently existing panel by adding multiple genetic alterations known to occur in thyroid cancer with low prevalence, such as mutations in the \( \text{PIK3CA}, \text{AKT1}, \text{PTEN}, \) and \( \text{TP53} \) genes and chromosomal rearrangements of the \( \text{BRAF} \) and \( \text{NTRK1} \) genes. Moreover, massive sequencing efforts involving thyroid cancer is under the way by TCGA, 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 to test 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.


Figure legends

**Figure 1.** Cancer risks and algorithm for clinical management of patients with cytologically indeterminate thyroid nodules based on the results of mutational analysis (Based on the results reported in ref. (31)). Bethesda categories of indeterminate cytology: FLUS/AUS - follicular lesion of undetermined significance/atypia of undetermined significance; FN/SFN - follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm; SMC - suspicious for malignant cells.
Thyroid nodule
Fine needle aspiration

Cytologic diagnosis

FLUS/AUS 14%
FN/SFN 27%
SMC 54%

Testing for panel of mutations (BRAF, RAS, RET/PTC, PAX8/PPARγ)

Mutational status
Negative Positive
Negative Positive
Negative Positive

Cancer risk after mutational testing
6% 88%
14% 87%
28% 95%

Clinical management
Observation vs. Repeat FNA vs. Lobectomy
Diagnostic lobectomy
Diagnostic lobectomy

Total thyroidectomy

Figure 1:
Clinical Cancer Research

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