Molecular Testing for Mutations in Improving the Fine-Needle Aspiration Diagnosis of Thyroid Nodules


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Context: Thyroid nodules are common in adults, but only a small fraction of them are malignant. Fine-needle aspiration (FNA) with cytological evaluation is the most reliable tool for cancer diagnosis in thyroid nodules. However, 10–40% of nodules are diagnosed as indeterminate by cytology, making it difficult to optimally manage these patients.

Objective: The aim of this study was to establish the feasibility and role of testing for tumor-specific mutations in improving the FNA diagnosis of thyroid nodules.

Design: The prospective study included 470 FNA samples of thyroid nodules from 328 patients. At the time of aspiration, a small portion of the material was collected and tested for \( \text{BRAF} \), \( \text{RAS} \), \( \text{RET/PTC} \), and \( \text{PAX8/PPAR}\gamma \) mutations. The mutational status was correlated with cytology and either surgical pathology diagnosis or follow-up (mean, 34 months).

Results: A sufficient amount of nucleic acids were isolated in 98% of samples. Thirty-two mutations were found, including 18 \( \text{BRAF} \), eight \( \text{RAS} \), five \( \text{RET/PTC} \), and one \( \text{PAX8/PPAR}\gamma \). The presence of any mutation was a strong indicator of cancer because 31 (97%) of mutation-positive nodules had a malignant diagnosis after surgery. A combination of cytology and molecular testing showed significant improvement in the diagnostic accuracy and allowed better prediction of malignancy in the nodules with indeterminate cytology.

Conclusions: These results indicate that molecular testing of thyroid nodules for a panel of mutations can be effectively performed in a clinical setting. It enhances the accuracy of FNA cytology and is of particular value for thyroid nodules with indeterminate cytology. (J Clin Endocrinol Metab 94: 2092–2098, 2009)

Palpable thyroid nodules are common in the adult population, with an estimated prevalence in the United States of 4–7%, resulting in 10–18 million affected individuals (1–3). The incidence of thyroid nodules detectable by ultrasonography is even higher and may exceed 50% in patients over 65 yr old (2, 3). The vast majority of thyroid nodules are benign and can be managed conservatively, whereas approximately 5–15% of nodules examined by ultrasound and fine-needle aspiration (FNA) cytology are malignant (4–8). A challenge facing the physician is to distinguish between benign nodules and malignant tumors to ensure that each patient receives timely and appropriate treatment, while minimizing the risk of unnecessary intervention.

Currently, the most reliable and commonly used diagnostic test for thyroid nodules is FNA, in which cells from the nodule

Abbreviations: FLUS, Follicular lesion of undetermined significance; FNA, fine-needle aspiration; NPV, negative predictive value; PPV, positive predictive value.
are collected using a thin needle, followed by cytological examination of the harvested cells. FNA cytology accurately diagnoses a benign or malignant lesion in the majority of cases; however, 10–40% of all FNA samples are diagnosed as indeterminate for malignancy, often prompting diagnostic hemithyroidectomy (2, 5, 9–11). This is because cytological features of thyroid lesions with a follicular growth pattern are frequently not sufficiently different to distinguish between benign and malignant lesions.

Most recently, it has been proposed to subdivide the general category of indeterminate cytology into three subcategories: 1) follicular lesion of undetermined significance (FLUS); 2) follicular or oncocytic (Hürthle cell) neoplasm; and 3) suspicious for malignancy, with a predicted probability of malignancy of 5–10, 20–30, and 50–75%, respectively (12). The impact of the new reporting scheme on clinical management of patients with thyroid nodules remains to be defined. However, currently among surgically removed thyroid nodules only 8 to 56% are found to be malignant (11, 13, 14). Such a high volume of unnecessary thyroid surgeries results in additional morbidity and higher health care costs (15, 16). Moreover, patients with malignant tumors and indeterminate FNA cytology typically undergo a limited surgery, i.e., thyroid lobectomy. After the diagnosis of malignancy is established by pathological examination of the removed nodule, these patients require a second operation to complete the thyroidectomy, which is associated with additional costs and morbidity. In addition, 1–3% of nodules diagnosed as benign on FNA cytology are later found to be malignant on follow-up (false-negative FNA), and the delay in treatment places patients at risk for progression of disease during the interval before definitive diagnosis (5, 13). Therefore, additional methods to improve the sensitivity and specificity of FNA cytological diagnosis are highly desirable and could have a significant impact on clinical care.

Recent advances in molecular genetics of thyroid cancer can be applied to developing new diagnostic markers for FNA samples (17, 18). Papillary carcinoma, the most common thyroid malignancy, frequently carries BRAF, RET/PTC, or RAS mutations. These mutually exclusive somatic mutations are found in more than 70% of papillary carcinomas, and some of them are associated with more aggressive tumor behavior (19–21). Follicular carcinomas, the second most common type of thyroid cancer, harbor either RAS or PAX8/PPARγ mutations, which are identified in approximately 80% of these tumors (22). Several studies have demonstrated the feasibility of detecting BRAF, RET/PTC, or RAS mutations in thyroid FNA samples and have shown that this may improve the cytological FNA diagnosis (23–28). However, these proof-of-principle studies were typically retrospective, limited to the analysis of a single mutation, and provided no information on the diagnostic accuracy of the analysis in a clinical setting.

Herein, we report the results of a large prospective two-institution study that establishes the feasibility of molecular testing of routine thyroid FNA samples and defines the diagnostic utility of a panel of mutations for clinical assessment of thyroid nodules.

**Patients and Methods**

**Study patients and FNA samples**

Altogether, 328 consecutive patients with thyroid nodules were enrolled prospectively into the study, either at the University of Cincinnati Medical Center (304 patients) or at the University of Colorado Denver School of Medicine (24 patients). All patients provided informed consent, and the study was approved by the respective Institutional Review Boards. In these patients, 470 thyroid nodules were aspirated and yielded FNA samples. The FNA procedure was performed under ultrasound guidance by an experienced thyroid surgeon or endocrinologist/radiologist using a standardized protocol for each institution. During the FNA procedure, a small portion of the aspirated material was directly collected into nucleic acids preservative solution (Roche Molecular Biochemicals, Manheim, Germany) and frozen at −80 C. At the University of Cincinnati, material from the first needle pass through the nodule was used to prepare a direct smear for cytology, and the remaining material in the needle plus the needle washing was collected into a tube containing 400 μl of the nucleic acids preservative solution. In the event that the first needle pass failed to obtain any material, the second needle pass was used for a direct smear, and the remainder was collected for molecular testing. Totally, four needle passes were taken in each case. At the University of Colorado, a dedicated pass was collected for the study (usually a fourth or fifth pass). In both centers, the harvesting of material for molecular studies was conducted in a way to ensure that it did not compromise the routine cytological evaluation. No on-site assessment of adequacy was performed at the University of Cincinnati, whereas a cytotecnologist was present to evaluate adequacy of samples at the University of Colorado.

**Nucleic acids isolation**

Total nucleic acids were isolated using magnetic glass particles (MGP) kit (Roche). The quantity of isolated DNA and RNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

**Detection of point mutations**

The DNA was tested for BRAF V600E and K601E, NRAS codon 61, HRAS codon 61, KRAS codons 12 and 13 point mutations using realtime LightCycler PCR, and fluorescence melting curve analysis. For each mutation, a pair of primers and two oligonucleotide probes were designed (Supplemental Table 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org). Amplification was performed using 5–50 ng of DNA, 40 pmol of each primer, 2 pmol of each hybridization probe, and LightCycler FastStart DNA Master HybProbe Kit (Roche). The reaction mixture was subjected to 40 cycles of PCR amplification consisting of denaturation at 95 C for 5 sec, annealing at 54 C for 20 sec, and extension at 72 C for 12 sec. Postamplification fluorescence melting curve analysis was performed by gradual heating of samples at a rate of 0.1 C/sec from 45 C to 95 C. Fluorescence melting peaks were built by plotting of the negative derivative of fluorescent signal corresponding to the temperature (−dF/dT). Melting temperatures for different mutations are listed in Supplemental Table 2. For each mutational hot spot, DNA from one or more tumors or cell lines known to carry a specific mutation was used as a positive control. The sensitivity of mutation detection by melting curve analysis was 10% of cells with a mutant allele in the background of normal cells, as established by serial dilutions of the positive controls.

**Detection of rearrangements**

RET/PTC1, RET/PTC3, and PAX8/PPARγ rearrangements were detected from DNA by RT-PCR with primers designed to flank the respective fusion point (Supplemental Table 1). Reverse transcription and PCR amplification were performed in one-step using QuantiTech Probe RT-PCR Kit (QIAGEN, Valencia, CA) and gene-specific primers and probes (Supplemental Table 1). Briefly, 5–50 ng of total RNA were
reverse transcribed and amplified in a 50-μl volume using Quant iTech Probe RT Mix, 40 pmol of each primer, and 2 pmol of each hybridization probe. The reverse transcription was carried out at 50 C for 30 min, followed by 40-cycle PCR amplification (denaturation at 94 C for 15 sec and annealing and extension at 72 C for 60 sec). For each rearrangement type, RNA from one or more tumors or cell lines known to carry a particular rearrangement was used as a positive control. The sensitivity of mutation detection by RT-PCR was 1% of cells carrying the rearrangement in the background of normal cells, as established by serial dilutions of the positive controls.

Direct nucleotide sequencing

All samples that tested positive for mutations were sequenced using BigDye Terminator Kit on the ABI3100 (Applied Biosystems, Foster City, CA).

Cytology review

In addition to a routine cytological evaluation of all FNA samples, cytology slides from 51 samples originally diagnosed as indeterminate were reviewed again by one cytopathologist (T.M.R.-S.) and classified into three categories as suggested by the National Cancer Institute Thyroid FNA State of the Science Conference (12).

Statistical analysis

Calculations of specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were performed using MedCalc Statistical Software version 9.6 (MedCalc Software, Mariakerke, Belgium).

Results

Evaluation of sample adequacy for molecular testing

Total nucleic acids were isolated from 470 consecutive samples collected during thyroid FNA procedure performed on 328 patients who underwent routine clinical evaluation for thyroid nodules and agreed to participate in the study (Fig. 1). The quantity of isolated nucleic acids was assessed before the molecular analysis and ranged from 250 ng to 4 μg. The quality of DNA and RNA was assessed by PCR amplification of the GAPDH gene and the GAPDH cDNA, respectively. Quality of nucleic acids was considered satisfactory when the amplification cycle threshold was less than 35 cycles, borderline at 35–38 cycles, and poor at more than 38 cycles. Using these criteria, 462 (98.3%) demonstrated satisfactory quality of DNA. Quality of RNA was satisfactory in 351 (74.7%) and borderline in 111 (23.6%) specimens. The latter samples were used for the analysis, but for each reaction the amount of template was doubled. Only eight samples (1.7%), all from the University of Cincinnati site, had insufficient and/or poor quality of nucleic acids and were excluded from the analysis.

Detection of mutations

A panel of mutations was assembled to account for most common mutations in well-differentiated follicular cell-derived thyroid cancer. It consisted of BRAF V600E/K601E, NRAS codon 61, HRAS codon 61, and KRAS codons 12/13 point mutations and RET/PTC1, RET/PTC3, and PAX8/PPARγ rearrangements. We identified 32 mutations in 462 FNA samples tested, including 18 BRAF V600E, five NRAS codon 61, three HRAS codon 61, four RET/PTC1, one RET/PTC3, and one PAX8/PPARγ. The results of molecular testing were correlated with cytology, with final surgical pathology diagnosis (when surgery was performed) or with clinical follow-up. One additional RET/PTC3 mutation was identified in a gland with several nodules and was excluded from the analysis due to difficulty in correlating with pathological findings.

Sixty-nine patients underwent thyroid surgery soon after completion of the initial clinical and cytological evaluation. Treating physicians were aware of the results of molecular testing. Cytology reports as well as surgical pathology reports and glass slides were collected and reviewed. These patients (86 FNA samples) constituted a surgery correlation group (Fig. 1). The other 253 patients were initially followed conservatively. Among these, 34 patients (41 FNA) were excluded due to loss of follow-up, and the remaining 219 patients (333 FNA) constituted a follow-up correlation group. Some patients in this group underwent surgery during follow-up.

Surgery correlation group

Among 86 thyroid FNA samples collected from 69 patients who underwent surgery, diagnostic cytology was positive for malignancy in 22 samples, indeterminate (including atypical and suspicious for malignancy) in 52 samples, and negative for malignancy in 12 samples (Fig. 2). After surgery, 31 (97%) mutation-positive nodules were diagnosed as malignant on pathological examination, and one (3%) was a benign tumor.

BRAF V600E was the most frequent mutation. All 18 nodules that revealed this mutation were subsequently diagnosed as papillary carcinoma. These included 10 cases with positive cytology, seven with indeterminate cytology (Fig. 3, top panel), and one with negative cytology. RAS mutations were the second most common mutation type and involved NRAS61 and HRAS61, but not KRAS12/13 hot spots. After surgery, five of the RAS-positive nodules were found to be papillary carcinomas (all follicular variants of papillary carcinoma), two were follicular carcinomas (Fig. 3, bottom panel), and one was a follicular adenoma. The latter case, positive for HRAS codon 61 mutation, was the only one where the presence of mutation did not correlate with malignancy. All five RET/PTC rearrangements were found in nodules yielding a final diagnosis of papillary carcinoma. One nodule with PAX8/PPARγ rearrangement was a follicular carcinoma.

Overall, the detection of mutation had a strong correlation with malignant outcome independent of the cytological diagnosis. In fact, only 13 of the 32 mutation-positive FNA samples had a definitive cytological diagnosis of malignancy, whereas the rest were either indeterminate or negative for malignancy. Most of the cases in the latter group were either follicular variant of papillary carcinoma or follicular carcinoma, which are known to be difficult to diagnose with certainty by cytology. Of 52 samples with indeterminate cytology, all 15 nodules positive for mutations were malignant at surgery, whereas among 37 mutation-negative nodules, 31 were benign and six were malignant at surgery. As a result, detection of mutation in a nodule with indeterminate cytology had a 100% PPV for malignancy.

Follow-up correlation group

The follow-up for 219 patients (333 FNAs) ranged from 16 to 51 months (mean, 34 months). The patients were followed by annual ultrasound examination. During the follow-up period, 72 patients (104 FNAs) underwent thyroid surgery. Histopathological examination of the surgical material yielded diagnoses of benign hyperplastic nodule in 67 cases, incidental papillary microcarcinoma 0.1–0.3 cm in size adjacent to benign hyperplastic nodules in three cases, and papillary carcinoma in two cases. One of these two cases was a 2.2-cm follicular variant of papillary carcinoma, and another was a 2.0-cm cystic papillary carcinoma of conventional type with multiple hyperplastic nodules also present in the lobe. They were considered as false-negative by cytology and molecular analysis, although it was not entirely clear whether the initial FNA material was obtained from the malignant nodules. The three cases with papillary microcarcinoma were considered as true negatives because the largest nodules that were aspirated were benign.

The remaining 147 patients (229 FNAs) were followed by serial ultrasound observation with no change in the nodule status (124 patients) or by repeated FNA with cytology negative for malignant cells (23 patients) and no mutation found in the FNA material. These nodules were considered as negative for malignancy. The possibility of false-negative results in patients not submitted to surgery cannot be excluded, but it is expected to be low.

Analysis of all FNA samples

Combining both study groups, of 419 thyroid nodules that underwent FNA biopsy and were followed until the conclusion of the study, 50 were malignant and 369 appeared benign after surgery or on mean follow-up of 34 months. The presence of any mutation was a strong indicator of malignancy, with 31 of 32 (97%) of mutation-positive nodules found to be malignant after surgery. Despite a high specificity for malignancy (99.7%), the sensitivity of the molecular testing alone was only 62%, as expected based on the fact that not all malignant tumors carry one of the mutations discovered to date.

A combination of cytology and molecular testing showed a significant improvement in the accuracy of the FNA diagnosis (Table 1). This was true for the definitive diagnosis of malignancy based on either positive cytology or detection of any mutation. Molecular testing also contributed to better predicting the probability of malignancy in nodules with indeterminate cytology and probability of a benign nodule (Table 2). Among nodules with indeterminate cytology, which had a 40% probability of malignancy based on cytology only, those positive for mutation were all malignant, whereas those negative for mutation had a 16% probability of malignant outcome. Among nodules with benign cytology, 2.1% were malignant at surgery, whereas the probability of cancer in nodules with benign cytology and no mutations decreased to 0.9%.

When 51 cytology samples from the indeterminate group were blindly reviewed again and assigned to one of the three recently proposed categories (12), 21 of them were FLUS, 23 were follicular or oncocytic (Hurthle cell) neoplasms, and seven were suspicious for malignancy. The distribution of mutations and final histopathological diagnoses in each category are summarized in Fig. 4. All mutation-positive cases of FLUS were malignant at surgery, and mutational status had a 100% accuracy in predicting the risk of a malignant or benign nodule in this category. In the follicular/oncocytic (Hurthle cell) neoplasm and suspicious for malignancy categories, the specificity of mutation detection was 100%, although the NPV and overall accuracy were lower because several mutation-negative malignant tumors were found in both categories.

Discussion

We report here the results of what is, to our knowledge, the first large prospective study aimed at determining the feasibility and diagnostic utility of molecular genetic testing in refining the FNA diagnosis of thyroid nodules. We show that testing for a panel of mutations significantly improved the accuracy of the cytological diagnosis, particularly for samples with indeterminate cytology. In this category, the detection of any mutation was highly predictive of malignancy, and should provide a strong indication for surgery.
This is of particular importance for the least worrisome category of indeterminate cytology, i.e. FLUS, where the estimated risk of malignancy is only 5–10% (12). These patients would benefit most from molecular testing, which should provide accurate prediction of malignancy, eliminate delay in surgical treatment, and avoid the costs of repeat FNA recommended to those patients (12).

The current management guidelines from the American Thyroid Association recommend radioiodine thyroid scan for patients with

TABLE 1. Performance characteristics of cytology and molecular analysis in definitive diagnosis of malignancy in FNA samples from thyroid nodules

<table>
<thead>
<tr>
<th>Diagnostic modality</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
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<tbody>
<tr>
<td>Cytology (positive for malignancy)</td>
<td>44.0</td>
<td>100</td>
<td>100</td>
<td>92.9</td>
<td>93.3</td>
</tr>
<tr>
<td>Molecular analysis (positive for mutation)</td>
<td>62.0</td>
<td>99.7</td>
<td>96.9</td>
<td>95.1</td>
<td>95.2</td>
</tr>
<tr>
<td>Cytology (positive) and molecular (positive)</td>
<td>80.0</td>
<td>99.7</td>
<td>97.6</td>
<td>97.4</td>
<td>97.4</td>
</tr>
</tbody>
</table>

Data are expressed as percentage.

TABLE 2. Probability of cancer in thyroid nodules depending on the results of cytological and molecular analysis

<table>
<thead>
<tr>
<th>Results of cytology and molecular analysis</th>
<th>Cancer probability (%)</th>
</tr>
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<tbody>
<tr>
<td>Positive cytology and positive for mutation</td>
<td>100</td>
</tr>
<tr>
<td>Indeterminate cytology alone</td>
<td>40.4</td>
</tr>
<tr>
<td>Indeterminate cytology and positive for mutation</td>
<td>100</td>
</tr>
<tr>
<td>Indeterminate cytology and negative for mutation</td>
<td>16.2</td>
</tr>
<tr>
<td>Negative cytology alone</td>
<td>2.1</td>
</tr>
<tr>
<td>Negative cytology and negative for mutation</td>
<td>0.9</td>
</tr>
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indeterminate cytology and, if the nodule in question is not hyperfunctioning, thyroid lobectomy or total thyroidectomy should be considered (5). In light of the finding in this study, patients with nodules that yield indeterminate cytology and are positive for mutation would be strong candidates for total thyroidectomy, particularly when BRAF or RET/PTC mutation is detected. If surgical treatment is considered for nodules with indeterminate cytology that are negative for mutations, a reduced risk of malignancy would seem to suggest lobectomy as an appropriate initial surgical approach. If one would follow this algorithm in managing patients enrolled in this study, the application of the molecular analysis would have decreased the need for a second surgery in 30% of patients with an indeterminate cytology. Furthermore, no inappropriate total thyroidectomies would have been performed.

Importantly, in the current study, cells were harvested for molecular testing only after ensuring that adequate material had been collected for routine cytology. This approach may have diminished the chances for mutation detection in some cases, but it ensured that cytological examination was not compromised. Even with these limitations, the molecular testing refined or established the diagnosis of malignancy in 19 of 328 patients enrolled in the study.

As expected, BRAF mutation was the most common, and its detection had a 100% PPV for papillary carcinoma. RAS mutations were the second most common finding and also appeared to be of high diagnostic value. These mutations are found in thyroid carcinomas and also in benign follicular adenomas and some hyperplastic nodules (29–35). The current study, which tested samples from a series of consecutive thyroid nodules followed prospectively, indicates that finding a RAS mutation in an FNA sample confers a 87.5% probability of malignancy, including a 62.5% probability of a papillary carcinoma and a 25% probability of a follicular carcinoma. The high risk of malignancy provides justification for recommending surgery to all patients with RAS-positive nodules. One of eight (12.5%) RAS-positive nodules was a benign follicular adenoma, and therefore in this case the molecular test was false-positive. However, RAS-positive “benign” follicular adenomas may be precursor lesions for RAS-positive follicular carcinomas (22). Moreover, if the transition to carcinoma occurs, the presence of RAS mutations is associated with worse prognosis and propensity for further conversion to undifferentiated carcinoma (36, 37). Therefore, surgical removal of RAS-positive follicular adenomas may be justifiable to prevent this putative progression. The particular value of testing for RAS is that it identifies tumors that are most difficult to diagnose by FNA cytology alone, i.e. follicular variant of papillary carcinoma and follicular carcinoma.

This study also provides evidence that molecular testing of FNA samples with negative cytology may be diagnostically meaningful and decreases the false-negative rate. However, routine testing of all samples with negative cytology is unlikely to be cost effective. Therefore, further studies are needed to identify a set of clinical and possibly imaging criteria that would determine which nodules with negative cytology should be rescreened using molecular testing.

In addition to the diagnostic information, the preoperative detection of mutations may provide helpful prognostic information and refine patient management. This may be particularly true for the BRAF mutation because those patients should be considered as candidates for total thyroidectomy even if cytology is not definitively malignant. This would eliminate the need for intraoperative pathology consultation and subsequent second surgery to complete thyroidectomy, reducing costs and additional morbidity. In many studies, BRAF mutation appears to serve as an independent marker of aggressiveness of papillary thyroid carcinomas and correlate with the frequency of lymph node metastases (21, 38). Further studies are needed to determine whether patients with BRAF mutation diagnosed in the FNA material would benefit from more extensive surgery, such as a prophylactic central compartment lymph node dissection. Finally, because targeted therapy for thyroid cancers with multikinase inhibitors is under active development (39, 40), the detection of mutations in the FNA material may be helpful in the future to guide mutation-specific targeted therapies that can be initiated preoperatively or in those patients who are not surgical candidates.

Acknowledgments

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