



Performance of a dual-component molecular assay in cytologically indeterminate thyroid nodules

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Abstract

Purpose Deciding whether patients with a cytologically indeterminate thyroid nodule should be referred for surgery or for active surveillance is an important challenge for clinicians. The aim of this study was to evaluate the performance of a novel dual-component molecular assay as an ancillary molecular method for resolving indeterminate thyroid nodule cytology.

Methods We selected 156 thyroid nodules from those that had undergone fine-needle aspiration processed by liquid-based cytology and surgical resection between June 2016 and December 2017. The sample set included 63 nodules cytologically classified as indeterminate, and 93 other nodules randomly selected from those with non-diagnostic, benign, suspicious, or malignant cytology. Nucleic acids from each nodule were subjected to next-generation sequencing analysis for mutation detection in 23 genes and to digital polymerase chain reaction (PCR) evaluation for miR-146b-5p expression levels.

Results Used alone, mutation analysis in the indeterminate subset (cancer prevalence: 22.5%) displayed high sensitivity (89%) and NPV (96%). In contrast, the miR-146b-5p assay offered high specificity (93%) and PPV (93%). Combined use of both analyses improved panel performance by eliminating false-negative results.

Conclusions These preliminary data suggest that a dual-component molecular test can increase the diagnostic accuracy of thyroid cytology alone by reducing the number of nodules that will be classified as indeterminate and increasing those that can be reliably classified as benign. If these findings are confirmed, this test can be considered for use in clinical practice and is expected to reduce diagnostic surgery and health care costs, and to improve patient quality of life.

Keywords Thyroid nodules · Indeterminate cytology · Mutations · NGS · miRNA · dPCR

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Introduction

Thyroid nodules are being identified with increasing frequency in clinical practice, but very few of the lesions are actually malignant (7–15% of cases) [1, 2]. Fine-needle aspiration cytology is the most reliable method for identifying these lesions preoperatively, but up to 30% of thyroid FNA samples yield indeterminate results [3]—that is, atypia or follicular lesion of undetermined significance (AUS/FLUS; Bethesda class III) or follicular neoplasm/suspicious for follicular neoplasm (FN/SFN; Bethesda class IV) [4]. A high proportion of these patients are subjected to diagnostic surgery, but only a minority of resected indeterminate thyroid nodules are histologically diagnosed as malignant, ranging from 15.9% for Bethesda class III up to 26.1% for Bethesda class IV [3, 5]. Assessment of molecular markers in FNA samples can add diagnostically valuable information to that provided by cytology, thereby improving

preoperative risk stratification for indeterminate nodules. The potential benefits would include (1) reductions in the frequency of surgical resection for benign or low-risk disease, and (2) increasing the preoperative detection of malignant thyroid nodules that should be treated by total thyroidectomy rather than diagnostic hemithyroidectomy followed by completion thyroidectomy. Over the last few years, the genomic landscape of thyroid cancer has been comprehensively investigated, thus substantially decreasing the dark matter of thyroid cancer genome [6, 7].

The knowledge thus gained and the ongoing improvements in high-throughput technologies have substantially increased the diagnostic accuracy of molecular testing for diagnosis of thyroid cancer. Two basic molecular approaches have emerged for improving the diagnostic performance of FNA cytology in the subset of indeterminate nodules: testing for somatic mutations and identification of gene and miRNA classifiers [8].

We developed a dual-component molecular assay for FNA fluid that involves next-generation sequencing (NGS)-based detection of mutations and digital polymerase chain reaction (PCR) evaluation of the expression levels of an microRNA strongly associated with thyroid cancer. Herein, we report the results of our preliminary assessment of this new tool's clinical validity as an ancillary molecular method for resolving indeterminate thyroid nodule cytology.

Materials and methods

Thyroid nodule enrollment and FNA sample collection

This retrospective study was conducted with local ethics committee approval and the written informed consent of all participants, in accordance with the principles of the Helsinki Declaration.

The 156 thyroid nodules (139 patients) subjected to molecular analysis were selected from those that had undergone FNA cytology and surgical resection at the Agostino Gemelli Hospital in Rome, Italy, between June 1, 2016 and December 31, 2017. Aspirates had been processed for cytology with the ThinPrep5000™ system (Hologic Co.) and classified according the 2014 Italian Six-tiered Reporting System for Thyroid Cytology [9]. (Because this system is similar to the Bethesda Reporting System for Thyroid Cytology [9, 10], terminology from the latter, more familiar system will be used hereafter to report cytology findings.) Any remaining cytological material was stored in Preservcyt solution (Hologic Co.). Cytological and surgical histology diagnoses were made by at least two pathologists of the staff of the hospital's Division of Anatomic Pathology and Histology.

Nodules were eligible for inclusion in the study if (1) FNA cytology and surgical histology diagnoses were available; and (2) the amount of FNA sample left after cytological assessment was sufficient for molecular analysis. The sample set selected for testing included all 63 (34.6%) of the eligible nodules (in 54 patients) that had been cytologically classified as TIR3A or TIR3B (Bethesda class III or IV, in the Bethesda Reporting System, respectively—hereafter referred to as the indeterminate subset), and 93 other eligible nodules (in 85 patients) randomly selected from those with the following cytological classifications: non-diagnostic (TIR1 or Bethesda Class I), benign (TIR2 or Bethesda Class II), suspicious (TIR4 or Bethesda Class V), or malignant (TIR5 or Bethesda Class VI).

Nucleic acid isolation

DNAs and RNAs (including microRNAs) were simultaneously isolated from the remaining cytological material of each sample using the All Prep DNA/RNA Micro kit (Qiagen) and quantified with high-sensitivity fluorescence-based assays for double-stranded DNA and RNA (Qubit®, Thermo Fisher Scientific).

Next-generation sequencing (NGS)-based mutation analysis

Genetic analysis was performed on the Ion Gene Studio S5 system (Thermo Fisher Scientific) using two custom NGS multi-gene panels (Table 1): a DNA panel that targeted single-nucleotide variants/small indels involving 19 known or putative thyroid cancer driver genes; and an RNA panel that identified 204 gene fusions involving seven known or putative thyroid cancer driver genes [6, 7, 11]. The RNA panel also included gene expression controls selected to provide quantitative and qualitative estimates of each sample's cellular composition (follicular and parafollicular thyroid cells, parathyroid cells). The mutation types, gene fusions, and gene expression controls screened in study samples are shown in Supplementary Table 1.

Two libraries were created from 15 ng of DNA and 10 ng of RNA using the Ion AmpliSeq™ Library Kit Plus and Ion Xpress™ Barcode Adapter 1–96 Kit (Thermo Fisher Scientific). Libraries were quantified by real-time PCR using the Ion Library TaqMan® Quantitation Kit on the 7900HT Fast Real Time PCR system (Thermo Fisher Scientific). Eight picomolars of pooled libraries were clonally amplified on the Ion One Touch2 System and sequenced on the Ion Gene Studio S5 (Thermo Fisher Scientific) [12].

DNA data were analyzed with software Torrent Suite v.5.10 with the plugins Coverage Analysis and Variant Caller v5.10 and annotated with Ion Reporter 5.10 and the wANNOVAR web server. Variants were prioritized on the

Table 1 Molecular assay of FNA liquid for detection of thyroid cancer

Test component	Rationale for testing	Genes tested	Test method
Mutation detection	Known TC drivers [6, 7]	<u>BRAF</u> (<i>ex15</i>), <u>CHEK2</u> , <u>EIF1AX</u> , <u>HRAS</u> , <u>KRAS</u> , <u>NRAS</u> , <u>RET</u> (<i>ex 5,8,10,11,13,14,15,16</i>), <u>TSHR</u> (<i>ex10</i>) <u>ALK</u> (<i>ex 20–29</i>), <u>ATM</u> , <u>PIK3CA</u> , <u>pTERT</u> , <u>PTEN</u> , <u>TP53</u>	NGS DNA Panel 19 genes
	Mutated in PTCs w/o known drivers [6]	<u>DICER1</u> , <u>DNMT3A</u> , <u>TG</u>	
	Mutated in TCs in our in-house database (11, data not shown)	<u>MET</u> , <u>SETD2</u>	
	Fusions involving TC driver genes [6, 7]	<u>ALK</u> , <u>BRAF</u> , <u>NTRK1</u> , <u>NTRK3</u> , <u>RET</u> , <u>PPARG</u> , <u>THADA</u>	NGS RNA Panel 7 driver genes 72 fusion partners 12 control genes
	Gene Expression Controls ^a	<u>SLC5A5</u> , <u>TPO</u> , <u>TSHR</u> , <u>TG</u> , <u>KRT7</u> , <u>KRT20</u> , <u>TTF1</u> , <u>PTH</u> , <u>CALCA</u> , <u>JUN</u> , <u>MTTP</u> , <u>TBP</u>	
miRNA expression assessment	miRNA overexpressed in TC [15–17]	<u>miR-146b-5p</u>	Digital PCR 1 microRNA

Underlined genes are tested for multiple mutations (single-nucleotide variants, small insertions/deletions AND gene fusion) *ex* exons, NGS next-generation sequencing, PTC papillary thyroid cancer, TC thyroid cancer

^aGene expression controls were selected to provide quantitative (*JUN*, *MTTP*, *TBP*) and qualitative estimates of each sample's cellular composition (follicular cells: *SLC5A5*, *TPO*, *TSHR*, *TG*, *TTF1*; epithelial cells: *KRT7*, *KRT20*; parafollicular thyroid cells: *CALCA*; parathyroid cells: *PTH*)

basis of their population frequency, quality values, and somatic status. Mutation analysis positivity was defined as the detection of at least one variant that was: (1) rare in the European-descendent population, as reflected by a minor allele frequency (AF) of <0.005; (2) a high-confidence call as reflected by a depth of coverage >500×, genotype quality >30; strand bias in variant relative to reference between 0.3 and 0.7; AF >0.05, and (3) likely to be somatic, as reflected by an AF between 0.05 and 0.40. The third criterion was waived for mutations involving *RET* since both somatic and germline alterations of this gene have been implicated in the pathogenesis of medullary thyroid cancer [13] and for known hotspots involving *BRAF* and *RAS* since clonal mutations are likely [14].

RNA data were analyzed with Ion Reporter 5.12 software (Thermo Fisher Scientific) using the workflow for gene fusion detection. Analysis was reserved for samples with a total of ≥20,000 mapped reads, at least 20% of which involved expression of thyroid follicular cell markers. The minimum read count for fusion calling was set at 20.

Digital PCR (dPCR)-based assessment of miR-146b-5p expression

Digital PCR was performed on QuantStudio 3D Digital PCR Instrument (Thermo Fisher Scientific) to measure FNA levels of miRNA-146b-5p (Table 1), which have been found to be high in thyroid cancer tissues [15–17]. Single-stranded cDNA was synthesized from 1.25 ng of total RNA using the High Capacity Reverse Transcription kit and specific stem-loop primers for miR-146b-5p (target, ID: 001097) and U6 (endogenous control, ID:001973) (Thermo Fisher Scientific). For each sample, the dPCR reaction was

prepared by mixing 1.5 μl of fivefold-diluted cDNA/miR-146b-5p, 1.5 μl of fivefold-diluted cDNA/U6, 0.8 μl of FAM dye-labeled TaqMan MicroRNA Assay (miR-146b-5p, 20×), 0.8 μl of VIC dye-labeled TaqMan MicroRNA Assay (U6, 20×), 8 μl of QuantStudio 3D Digital PCR Master Mix v2, and 3.4 μl of nuclease-free water (Thermo Fisher Scientific). Each dPCR reaction was loaded into the QuantStudio 3D Digital PCR Chip v2 and incubated in the ProFlex 2x Flat PCR System at 96 °C for 10 min, followed by 44 cycles of 60 °C for 2 min, 98 °C for 30 s, with a final extension at 60 °C for 2 min, and then held at 10 °C.

Image capture and primary chip analysis were performed with a QuantStudio 3D Digital PCR Instrument (Thermo Fisher Scientific). Secondary chip analysis was performed using the QuantStudio 3D Analysis Suite (Thermo Fisher Scientific) to obtain the absolute number of copies per μl for the target and endogenous control. For each sample, the copy number per μl of miR-146b-5p was normalized to that of the endogenous control U6. Each experiment included non-template controls for the target and endogenous control.

Receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were used to assess the diagnostic value of miR-146b-5p expression levels and to find the optimal cut-off point for discriminating between histologically benign and malignant nodules. The molecular test was positive when miRNA expression levels in cytological specimen exceeded the calculated cut-off value.

Data analysis

Samples with single-assay positivity (mutation detection or miR-146b-5p expression) or combined-assay positivity

(mutation detection and miR-146b-5p expression) in the molecular assay were considered positive for thyroid malignancy; those with negative findings in both assays were classified as negative for thyroid malignancy. We analyzed the results of single- and combined-assay molecular testing against surgical histology findings and evaluated their ability to correctly classify the FNA samples as benign or malignant (sensitivity, negative predictive value (NPV), specificity, positive predicted value (PPV)).

All statistical analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software Inc., San Diego, CA). *p* Values <0.05 were considered statistically significant. Continuous variables were reported as means ± standard deviation and compared using the Mann–Whitney U test. Categorical variables were compared using the Fisher's exact test. ROC curves were constructed for miR-146b-5p FNA levels, and AUC values with 95% confidence intervals were calculated to evaluate their ability to discriminate between benign and malignant thyroid nodules.

Results

Nodule characteristics

FNA samples from a total of 156 thyroid nodules (139 patients) were subjected to molecular assay. Thirty-nine nodules (25%) were excluded from the final analyses because of technical issues (assay failure due to the low nucleic acid yield of the FNA sample in 37 cases, NGS library problems in two cases). Molecular test data were thus available for 117 thyroid nodules. Aspirates from 40 (34%) of the 117 nodules (from 39 patients) had been classified as Bethesda III or IV on cytology (indeterminate subset) (Fig. 1).

Table 2 shows the outcomes of FNA cytology and surgical histology for the 117 thyroid nodules represented in the final sample set. Over half ($n = 63$, 53.8%) of the nodules were histologically diagnosed as malignant, including 57 papillary thyroid cancers (45 classical, 6 tall

cell, 3 follicular, 1 diffuse sclerosing, 1 hobnail, 1 oncocytic), 2 NIFTP (noninvasive follicular thyroid neoplasm with papillary-like nuclear features), 1 oncocytic thyroid cancer, 1 poorly differentiated thyroid cancer, 1 anaplastic cancer, 1 medullary thyroid cancer. The prevalence of histologically documented malignancy in the indeterminate subset of nodules was far lower (9/40, 22.5%). A similar prevalence was found in the initial indeterminate sample set (which included cases without molecular test results), and both figures were in line with data reported in the literature (data not shown).

Molecular test performance in the whole sample set

Supplementary Table 2 shows diagnostic performance parameters for the molecular assay in the entire FNA sample set. NGS-based mutation analysis alone classified 71 (61%) of the 117 nodules as malignant, including 12 (16.9%) that were histologically benign (false positives). The 46 mutation-negative nodules included four (8.7%) that were false negatives.

NGS analysis alone detected a driver gene mutation in 59 of the 63 thyroid nodules that were pathologically malignant (sensitivity: 94%). Forty-two of the 54 histologically benign nodules were mutation negative (specificity: 78%). The probability that a mutation-positive nodule was actually malignant (PPV) was 83%; the odds that a mutation-negative nodule was truly benign (NPV) was 91% (42/46).

Mir-146b-5p expression levels in FNA samples were significantly higher for nodules histologically diagnosed as malignant (0.06922 ± 0.04940 vs 0.6059 ± 0.5915 in those that were benign; $p < 0.0001$; Supplementary Fig. 1a). ROC curve analysis revealed an AUC of 0.8895 (CI: 0.8295–0.9495) and an optimal cut-off of 0.1562, reflecting good sensitivity (71%) and excellent specificity (96%) (Supplementary Fig. 1b, Supplementary Table 2). Among the miRNA-positive nodules, the probability of malignancy (PPV) was 96% (45/47). The probability that a miRNA-negative nodule was benign (NPV) was 74% (52/70) (Supplementary Table 2).

Compared with single-component analysis, combined assessment of driver mutations and miR-146b-5p expression was associated with higher sensitivity (97%) and NPV (95%) (Supplementary Table 2). The combined approach yielded 14 false-positive results: the nodules involved had final histological diagnoses of adenomatous nodule ($n = 6$), oncocytic adenoma ($n = 5$), and follicular adenoma ($n = 3$). Two nodules were mutation-negative/miR-146b-5p-positive; the other 12 were miR-146b-5p-negative, but harbored mutations involving *EIF1AX* ($n = 3$), *HRAS* ($n = 2$), *NRAS* ($n = 2$), *BRAF* ($n = 1$), *KRAS* ($n = 1$), *PTEN* ($n = 1$), *CHEK2* ($n = 1$), or *RET* ($n = 1$) at AFs ranging from 6% (*BRAF*) to 51% (*RET*). (The presence of the *BRAF* p.V600E

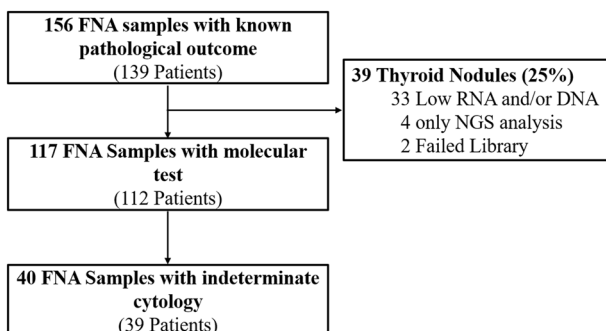


Fig. 1 Archival FNA sample set selection. FNA fine-needle aspiration, NGS next-generation sequencing

Table 2 Clinical and pathological characteristics of the 117 nodules with molecular test results

Nodule characteristics	Samples <i>n</i> = 117 (100%)	Cancer at histology <i>n</i> = 63 (54.2%)
Size in mm—mean (range)	19.62 (4–64) ^a	
Cytology class— <i>n</i> (%)		
TIR1/Beth Class I (non-diagnostic)	1 (0.8%)	0 (0%)
TIR2/Beth Class II (benign)	20 (17.1%)	0 (0%)
TIR3A/Beth Class III (AUS/FLUS) ^b	9 (7.7%)	1 (11%)
TIR3B/Beth Class IV (FN/SFN) ^b	31 (26.5%)	8 (26%)
TIR4/Beth Class V (suspicious for malignancy)	24 (20.5%)	22 (92%)
TIR5/Beth Class VI (malignant)	32 (27.3%)	32 (100%)

AUS atypia of undetermined significance, FLUS follicular lesion of undetermined significance, FN follicular neoplasia, SFN suspicious for follicular neoplasm

^aData unavailable for 5/118 nodules

^bNodules in the indeterminate subset

Table 3 Molecular test performance in the indeterminate nodule subset (*n* = 40)

Test component	Test outcome <i>n</i> (%)	Histological outcome— <i>n</i> (%)		Predictive values % (95% CI)
		Malignant 9 (22.5)	Benign 31 (77.5)	
Mutation detection	Positive 14 (35)	True positive <i>n</i> = 8	False positive <i>n</i> = 6	PPV 57% (28.9–82.3%)
	Negative 26 (65)	False negative <i>n</i> = 1	True negative <i>n</i> = 25	NPV 96% (75.9–96.3%)
	Performance	Sens. 89% (51.7–99.7)	Spec. 81% (62.5–92.5%)	
MIR-146b-5p expression	Positive 9 (22.5)	True positive <i>n</i> = 7	False positive <i>n</i> = 2	PPV 78% (40.0–97.2%)
	Negative 31 (77.5)	False negative <i>n</i> = 2	True negative <i>n</i> = 29	NPV 93% (78.6–99.2%)
	Performance	Sens. 78% (40.0–97.2%)	Spec. 93% (78.6–99.2%)	
Both components	Positive 17 (42.5)	True positive <i>n</i> = 9	False positive <i>n</i> = 8	PPV 53% (27.8–77.0%)
	Negative 23 (57.5)	False negative <i>n</i> = 0	True negative <i>n</i> = 23	NPV 100% (85.2–100.0%)
	Performance	Sens. 100% (66.4–100.0%)	Spec. 74% (55.4–88.1%)	

CI confidence intervals, FP false positive, FN false negative, TP true positive, TN true negative, NPV negative predictive value—TN/(TN + FN), PPV positive predictive value—TP/(TP + FN), Sens. sensitivity—TP/(TP + FN), Spec. specificity—TN/(TN + FN)

mutation in case 3 (follicular adenoma) at an AF of 6% suggests the presence of a cellular subclone within the nodule.) Histological review of the slides confirmed the original diagnosis (Supplementary Table 3).

False-negative molecular assay results included two PTCs, which had been cytologically flagged as suspicious for malignancy (Supplementary Table 4).

Molecular test performance in the subset of samples from indeterminate nodules

Table 3 shows the performance parameters for each component of the molecular test—used separately or combined—in aspirates from the 40 cytologically indeterminate nodules. Used alone, the NGS-based mutation analysis displayed higher sensitivity (89%) and a higher NPV (96%) than the dPCR-based assessment of miR-146b-5p levels. In contrast, the miR-146b-5p assay offered higher specificity (93%) and a

higher PPV (93%). Combined use of both analyses eliminated false-negative results, but only 23 of the 31 histologically benign nodules were negative in both assays (specificity 74%). The probability that a molecular test-positive nodule was actually malignant (PPV) was 53% (9/17).

Discussion

Deciding whether patients with a cytologically indeterminate thyroid nodule should be referred for surgery or for active surveillance is an important challenge for clinicians. Several approaches have been proposed to improve their diagnostic workup, including sonographic risk stratification systems [18, 19], elastosonography [20], their combination [21], or other clinical scoring systems [22]. In a significant number of cases of this type, the decision is made to schedule diagnostic surgery, based on a probability of

malignancy ranging from 15 to 40%. However, only the 25% of these resected nodules are diagnosed cancer [1, 23]. This means that—aside from the impact of this practice in terms of healthcare spending—most indeterminate thyroid nodules are currently being over-treated and the patients harboring them are being unnecessarily exposed to surgical risks (i.e., recurrent laryngeal nerve injury, hypocalcemia, hemorrhage/hematoma, hypothyroidism) [24–28]. A pre-operative test that could reliably exclude malignancy would be a valuable aid for choosing an optimal management strategy, in particular for supporting a recommendation for deferring surgery (and possibly avoiding it altogether).

The results of the present study demonstrate that the diagnostic yield of FNA cytology can be increased by adjunct analysis of the aspirate with a dual-component molecular test targeting thyroid cancer-related somatic gene mutations and a single thyroid cancer-relevant microRNA. More specifically, this approach can reduce the number of nodules that will be classified as indeterminate and increase the number of those that can be reliably classified as benign. In our indeterminate FNA sample set, where the prevalence of cancer was 22.5%, the test displayed high sensitivity and NPV, making it a powerful tool for ruling-out the possibility of malignancy in this subset of thyroid lesions: it would have avoided 23 (57.5%) unnecessary surgeries.

In the last decade, a variety of molecular tests have been marketed as reliable aids for increasing the accuracy of preoperative diagnosis of such lesions. Four molecular tests have been developed in United States and are now commercially available there for evaluation of thyroid FNA samples: the Afirma Genomic Sequencing Classifiers [29], the ThyroSeq v.3 Genomic Classifier [30, 31], the Thy-GeNEXT[®]/ThyraMIR[™] [32, 33], and Rosetta GX Reveal [34] (Supplementary Table 5). Although their goals are the same, these tests vary substantially from one another in terms of the molecular markers they target (mutations, mRNA, or microRNA expression), the underlying methodology (NGS, castPCR, RT-qPCR), the type of samples (same as those initially used for cytological diagnosis or additional samples), and, last but not least, their diagnostic performance. Our dual-component assay offers some advantages, including a relatively low number of molecular markers, which translates into lower overall testing costs. Like the Rosetta Gx Reveal, our test can be carried out on the same material used for the cytologic diagnosis. Moreover, it employs high-sensitivity methods for both mutation detection (NGS, which is also used in the ThyroSeq test) and assessment of miRNA expression levels (dPCR), ensuring reliable results as well as the detection of low-frequency, multiple, or unknown variants [12], and precise quantification of miRNA levels. And last but not least, it offers high performance, with sensitivity and NPV higher than those of the currently available tests mentioned above.

As demonstrated in the TCGA study focusing on papillary thyroid cancer [6], multi-level approaches involving mutational analysis and microRNA/mRNA expression profiling may help to better define thyroid tumors, by offering multiple markers for cancer diagnosis in FNA specimens. Combined methods have been already tested in indeterminate thyroid lesions (i.e., ThyGeNEXT/ThyraMIR) with good results. In our series, the combined assessment of driver mutations and miR-146b-5p levels appreciably increased the NPV of the overall test, eliminating all three of the false-negative findings that had emerged with single-component analysis (one for mutation detection, two for miR-146b-5p expression).

The cohort we studied was certainly small and included only a few malignant cases other than PTC (i.e., two NIFTP, one oncocytic thyroid cancer, one poorly differentiated thyroid cancer, one anaplastic thyroid cancer, 1 medullary thyroid cancer). However, the test correctly classifies all these poorly represented cancers. Validation of these results in a prospective, multicenter study cohort is already being planned. If the results are consistent with those of the present study, the test we have developed can be expected to reduce the frequency of diagnostic surgery for cytologically indeterminate thyroid nodules, thereby providing significant benefits in terms of lower health care and improved quality of life for patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures involving humans were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent Written informed consent was obtained from all subjects included in the study.

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