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Original Article

The real-world performance of ThyroSeqV.2 to diagnose thyroid "neoplasm requiring surgery"

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Abstract: Fine-needle biopsy (FNB) predicts benign or malignant thyroid nodules. For indeterminate (ITN) FNBs, commercial molecular tests may improve the diagnostic accuracy and reduce the number of operations. These tests have had limited independent implementation studies in routine clinical practice. This is a prospective observational study. At Boston Medical Center, the 1,316 consecutive FNBs were classified to one of the six categories in the Bethesda classification system. Those ITN samples were submitted for ThyroSeqV.2 next generation sequencing panel analysis. The performance of ThyroSeqV.2 to predict "neoplasm requiring surgery" (NRS) was evaluated. ThyroSeqV.2 assay was performed in 398 FNBs on 384 cytologically ITN nodules (308 Bethesda III, 47 Bethesda IV and 29 Bethesda V). The first evaluable ThyroSeq result for each nodule was used for final analysis. Seventy-seven (72.0%) of 107 patients with a high risk molecular test underwent thyroid surgery resulting in 41 NRS (53.2%) and 36 benign nodules (46.8%). Of the 249 patients with a low risk or negative molecular analysis, 51 (20.5%) had surgery revealing 47 benign nodules (92.2%) and 4 NRS (7.8%). Based on surgical outcome of 128 ITN with evaluable ThyroSeq results, this molecular test had a sensitivity of 91% (95% CI: 79%-98%), specificity of 56% (45%-67%), positive predictive value (PPV) of 53% (42%-65%), negative predictive value (NPV) of 92% (81%-98%), and an overall accuracy of 69% (55%-85%) with a prevalence of NRS of 35% (27%-44%). ThyroSeqV.2 in this clinical use study in ITN nodules provided a similar NPV but a lower PPV than expected compared to published studies due to the detection of an array of mutations in benign nodules. The NPV of 92.0% for ITN cytology confirmed its utility as a "rule-out" test to exclude NRS.

Keywords: Thyroid cancer, indeterminate nodule, neoplasm requiring surgery, molecular markers, thyroid biopsy

Introduction

Thyroid nodules are a common clinical finding especially after the increased usage of high resolution imaging such as ultrasound (US) technology, computed tomography and magnetic resonance imaging. Most nodules are often asymptomatic, and as a result, the main challenge in their management is to rule out thyroid cancer.

Fine needle biopsy (FNB) with cytology evaluation plays an important role in differentiating thyroid malignant nodules from benign ones [1]. The majority of thyroid nodule FNBs are benign, but 5% are malignant and 10%-35% will have an indeterminate (ITN) cytology, and incur costs to the health care system [2].

Several studies have documented that molecular markers can improve the diagnostic accuracy of FNB and reduce the number of unnecessary operations for benign thyroid nodules [3-7]. The current 2015 American Thyroid Association (ATA) thyroid nodule guideline does not recommend the routine use of molecular markers with thyroid FNB and notes the lack of outcome data on the use of molecular markers with ITN cytology [8].

Indeed, some of these tests have not had large independent validation or implementation studies in clinical practice, but are commercially available, aggressively marketed, and changing the management of ITN. One molecular test, ThyroSeqV.2 multigene next generation sequencing panel (ThyroSeqV.2), has emerged according to the initial investigator studies [4, 9], as both "rule-in" and "rule-out" test with a reported high sensitivity and specificity for malignancy in Bethesda III and IV cytology. This test is based on the detection of specific molecular markers including DNA mutations, chromosomal rearrangements and gene overexpression, and has been marketed for all ITN.

In the current large independent prospective implementation study, we evaluated the performance of the ThyroSeqV.2 analysis to detect "neoplasm requiring surgery" (NRS) in sequential thyroid nodule FNBs with an ITN (Bethesda category III-IV-V) cytology. We hope this study leads to more understanding with clinical significance of molecular tools, in specific, ThyroSeqV.2.

Materials and methods

Subjects

Between February 11, 2015 and October 24, 2017, a total of 1,316 consecutive FNBs on thyroid nodules were conducted under US guidance by endocrinologists in the Section of Endocrinology, Diabetes and Nutrition, Boston Medical Center (Boston, MA, USA). The American Thyroid Association (ATA) guidelines were followed to select nodule(s) appropriate for biopsy [8]. All nodules were >1 cm in size. In this study, FNB samples from both sexes were used without regard to sex.

The content of the first FNB was reserved for molecular analysis using the ThyroSeqV.2 system (CBL Path, Rye Brook, NY), according to the manufacturer's instructions. The remainder of the FNB sample was submitted to be interpreted by academic, board certified cytopathologists and categorized by the Bethesda classification [10]. At the time of assignment to a Bethesda classification, cytopathologists were blinded to the results of the ThyroSeqV.2 assay.

ThyroSeqV.2 test

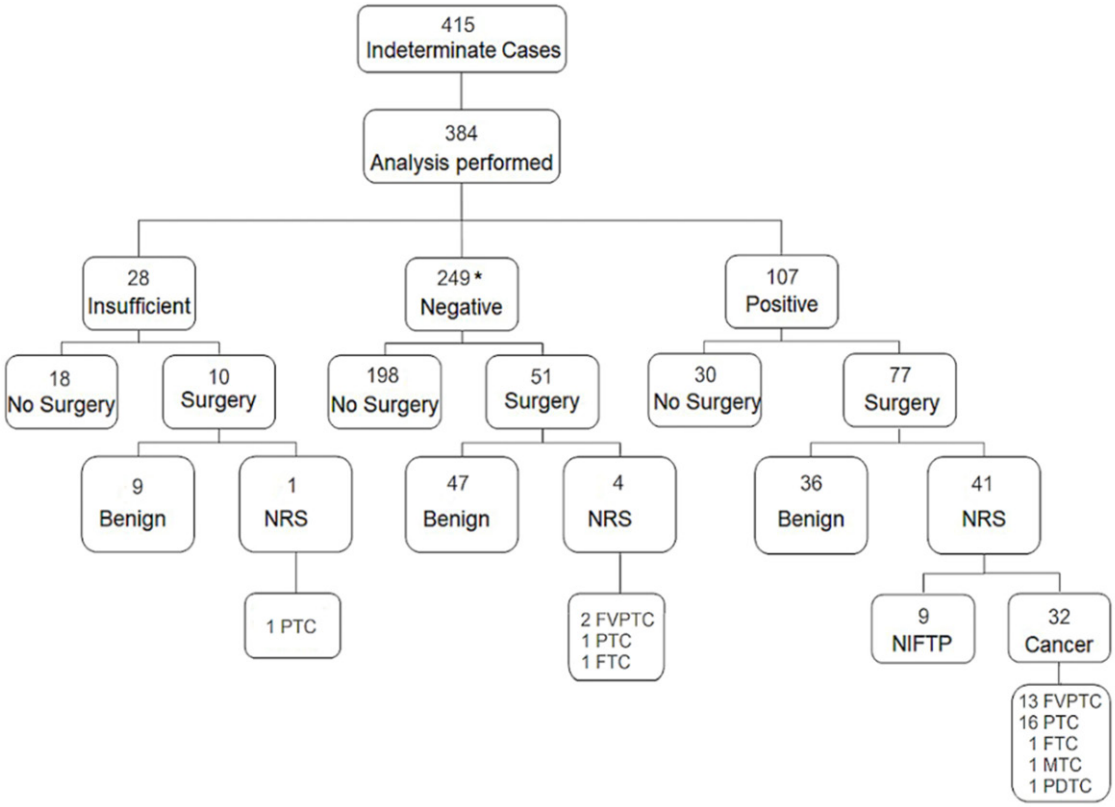
FNBs with an ITN (Bethesda category III-IV-V) cytology were submitted for molecular analysis. ThyroSeqV.2 next generation sequencing (NGS) panel performs simultaneous sequencing and detection in >1,000 hotspots of 14 thyroid cancer-related genes and 42 gene fusions known to occur in thyroid cancer [4, 9]. Some gene mutations [TSH receptor *(TSHR)* and *PTEN*] and gene over-expression [sodium-iodine symporter *(NIS)*] are associated with benign thyroid nodules and are considered to be associated with a low risk for malignancy [11]. For the purpose of this analysis, these molecular signatures are assigned to the category of a low risk or negative molecular marker result.

Patient management

Patients were selected for operation based upon clinical evaluations including the ThyroSeqV.2 results and patient preferences. At Boston Medical Center, during the study period, patients with a benign FNB were not recommended thyroid surgery unless there were clinical indications of size or obstructive symptoms. Every case with an ITN and positive high risk ThyroSeqV.2 test was advised to remove the nodule by surgery. All patients except one with an ITN cytology and a low risk or negative ThyroSeqV.2 analysis were advised that surgery was not necessary, however, surgery remained optional in some cases based on their preference or anxiety about an intermediate or high risk appearance on US, or Bethesda V cytology. Patients with ITN nodules who did not undergo thyroid surgery were monitored clinically for sonographic evidence of growth or development of suspicious US characteristics. This study was conducted in compliance with a protocol that was approved by the Boston University Institutional Review Board. Written informed consent was given by all patients before performing the procedure.

Pathology evaluation

The surgical pathology of the thyroid nodule was used as the gold standard for diagnosis. Cases in which the FNB specimens could not be matched to a specific nodule in the pathology report were reviewed with a pathologist. After review, one ITN FNB sample with a *BRAF*^{V600E} detected by ThyroseqV.2 was excluded from the analysis because the target nodule was benign on pathology with an adjacent 8 mm PTC. The post-operative *BRAF*^{V600E} testing of the benign nodule that was manually microdissected from paraffin sections turned out to



Measures	All Indeterminate
	Proportions and 95% Confidence Intervals (Exact) (%)
Sensitivity	91 (79 - 98)
Specificity	56 (45 - 67)
PPV	53 (42 - 65)
NPV	92 (81 - 98)
Accuracy	69 (55 - 85)
Prevalence	35 (27 - 44)

Figure 1. Performance of ThyroSeqV.2 NGS panel in all ITN nodules. ITN: indeterminate; NRS: neoplasm requiring surgery; NIFTP: non-invasive follicular thyroid neoplasms with papillary-like nuclear features; FVPTC: follicular variant of papillary thyroid carcinoma; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma; PPV: positive predictive value; NPV: negative predictive value. *: Included 219 negative results and 30 "low risk" alterations.

be negative, while the microdissected 8 mm PTC was confirmed harboring the *BRAF*^{V600E} mutation. We concluded that the adjacent 8 mm nodule identified on ultrasound was inadvertently sampled during FNB.

Definition of neoplasm requiring surgery (NRS)

After histopathology evaluation, nodules that were confirmed to be malignancies and non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) were classified as "neoplasm requiring surgery", i.e. NRS. NIFTP was included in NRS because this neoplasm must be resected for the necessary histopathology examinations required to distinguish it from the encapsulated FVPTC, despite its indolent clinical course and new classification as nonmalignant diagnosis by the WHO pathological category in 2017 [12, 13].

Statistics

Based on the NRS diagnosis after histopathology evaluation, the sensitivity, specificity, positive predictive value (PPV), negative predictive

value (NPV), accuracy and prevalence, and 95% exact binomial confidence intervals (CI), were computed for the entire sample of ITN and each of the three cytological sub-groups that comprise ITN. The PPV and NPV were then compared to those values determined in prior studies for ThyroSeqV.2 by Nikiforov et al. [4, 9], using a two-sided Z-test for proportions with pooled variance. All analyses were carried out in SAS 9.2 (Cary, NC) and a two-sided *P*-value <0.05 was considered to be statistically significant.

Results

Cytology distribution of the 1,316 consecutive FNBs

The 1,316 consecutive FNBs were classified to one of the six categories in the Bethesda classification system [10]. A poor cell yield was found in 140 cases (10.6%) and regarded to be insufficient for diagnosis (Bethesda I). 704 (53.5%) of the FNBs were categorized as benign (Bethesda II), and 57 (4.3%) as malignant (Bethesda VI). Indeterminate results (Bethesda III-IV-V) were identified in 415 (31.5%) of the FNBs, specifically, Bethesda III (atypia or follicular lesion of undetermined significance; AUS/FLUS) cytology was present in 336 cases (25.5%), Bethesda IV (follicular neoplasm or suspicious for follicular neoplasm; FN/SFN) in 47 cases (3.6%), and Bethesda V (suspicious for malignancy; SM) in 32 cases (2.4%).

The ratio of Bethesda III/Bethesda IV was high at 7.1 (336/47) which might cause concerns about over-diagnosis of AUS/FLUS (Bethesda III) or under-diagnosis of malignancy [14]. Nonetheless, all ITN FNBs except 7 (Bethesda III) were sent to a second facility (CBL Path, Inc.) for cytopathology review. Among the 408 FNBs, only 6 (all Bethesda III) were reassigned to Bethesda II and 3 (all Bethesda V) were reassessed as Bethesda VI.

Molecular analysis of the ITN FNBs

After confirmation of the Bethesda classification at Boston Medical Center on 408 ITN samples, 9 were not submitted for molecular analysis as they were reassigned to Bethesda II or VI by CBL Path. In addition, one case (Bethesda III) was excluded as mentioned in the Subjects and Methods. Hence, the remaining 398 ITN FNBs (322 Bethesda III, 47 Bethesda IV and 29 Bethesda V) had molecular analysis preformed with the ThyroSeqV.2 assay. Among them, 14 nodules were biopsied twice and got identical Bethesda III category. Their first evaluable ThyroSeq results were used for final analysis, which consisted of 384 ITN (308 Bethesda III, 47 Bethesda IV and 29 Bethesda V).

The total number of evaluable ThyroSeqV.2 cases was 356 (92.7%) of the submitted ITN (**Figure 1**). The panel detected a variety of genetic alterations in 35.7% (137/384). "Low risk" alterations included *TSHR* mutations (*n* = 15), *PTEN* mutation (*n* = 8), *NIS* over-expression (*n* = 5), and PTH over-expression (*n* = 2). "High risk" alterations for thyroid malignancy were found in 27.8% (107/384) of all ITN, specifically, 23.7% (73/308) of Bethesda III, 34.0% (16/47) of Bethesda IV, and 62.1% (18/29) of Bethesda V cytologies.

Pathology results of the surgically removed ITN nodules

An operation was performed in 35.9% (138/384) of all ITN nodules: 72.0% (77/107) with a positive high risk ThyroSeqV.2 molecular test, 20.5% (51/249) with a low risk or negative molecular test, and 35.7% (10/28) with a failed molecular test. Of 138 surgically confirmed nodules, 46 (33.3%) were NRS, including 37 cancers and 9 NIFTPs (**Figure 1**).

Surgical resection of the 77 ITN with a high risk molecular assay revealed benign pathology in 36 cases (46.8%) and NRS results in 41 cases (53.2%). The risk of NRS in these cases increased by Bethesda class with 41.2% (21/51) in Bethesda III, 57.1% (8/14) in Bethesda IV and 100% (12/12) in Bethesda V cytologies. Of the 51 ITN cases with a low risk or negative molecular test and surgical resection, 47 were benign (92.2%) and 4 were NRS. Surgical resection of 10 of 28 (35.7%) ITN with an insufficient material for molecular analysis revealed 1 cancer.

Genetic alterations in pathologically confirmed ITN nodules

The most frequent genetic abnormality in resected ITN nodules was *RAS* mutation (**Table 1**). The risk of malignancy (ROM) in ITN varied with the genetic alterations (**Table 2**).

Table 1. Genetic abnormalities in resected ITN nodules based on histopathology

	Patient Number with Positive Analysis	% Among Mutations in the Category
BENIGN (n = 80)	43	100
BRAF (p.K601E, c.1801A>G)	3	7.0
EIF1AX (p.A113_splice, c.338-1G>A)	1	2.3
EIF1AX (p.R13delinsGKNRR, c.36_37insGGTAAAAACAGA)	1	2.3
EIF1AX (p.G6D, c.17g>a)	1	2.3
EIF1AX (p.R13P, c.38G>C) + *GNAS* (p.Q227H c.681G>T) + *NIS* (SLC5A5) overexpression	1	2.3
HRAS (p.Q61R, c.182A>G)	4	9.3
HRAS (p.Q61K, c.181C>A)	1	2.3
KRAS (p.G12D, c.35G>A)	1	2.3
KRAS (p.G12V, c.35G>T)	1	2.3
KRAS (p.Q61R, c.182A>G)	1	2.3
NRAS (p.Q61K, c.181C>A)	4	9.3
NRAS (p.Q61R, c.182_183AA>GC)	1	2.3
NRAS (p.Q61R, c.182A>G)	14	32.6
PTEN (p.P248fs, c.741dupA and p.Q110fs, c.330delA)	1	2.3
PTEN (p.T319fs*6, c.955_956insA)	1	2.3
PTEN (p.K128N, c.384G>C and p.P96L, c.287C>T)	1	2.3
TSHR (p.M453T, c.1358T>C)	2	4.7
TSHR (p.I486M, c.1458C>G)	1	2.3
NIS Overexpression	1	2.3
PAX8/PPARG fusion	1	2.3
NRAS (p.Q61R, c.182A>G) + *EIF1AX* (p.A113_splice, c.338-2A>T)	1	2.3
NIFTP (n = 9)	9	100
PAX8/PPARG fusion	1	11.1
KRAS (p.G12R, c.34G>C)	1	11.1
KRAS (p.Q61R, c.182A>G)	1	11.1
NRAS (p.Q61R, c.182A>G)	5	55.6
THADA/IGF2BP3 fusion	1	11.1
FVPTC (n = 15)	13	100
BRAF (p.K601E, c.1801A>G)	1	7.7
EIF1AX (p.A113_splice, c.338-1G>C)	1	7.7
NRAS (p.Q61R, c.182A>G)	5	38.5
NRAS (p.Q61R, c.182A>G) + TERT (p.C250T, c.1-146C>T)	1	7.7
KRAS (p.Q61R, c.182A>G) + TERT (p.C228T, c.1-124C>T)	1	7.7
HRAS (p.Q61R, c.182A>G 8%)	1	7.7
PAX8/PPARG fusion	1	7.7
THADA/IGF2BP3 fusion	2	15.3
PTC (n = 17)	16	100
BRAF (p.V600E, c.1799T>A)	10	62.5
BRAF (p.K601E, c.1801A>G)	1	6.3
BRAF (p.T599del, c.1794_1796del)	1	6.3
NRAS (p.Q61R, c.182A>G)	2	12.5
HRAS (p.Q61K, c.181C>A)	1	6.3
ETV6/NTRK3 fusion	1	6.3
FTC (n = 2)	1	100
TERT (p.C228T, c.1-124C>T)*	1	100

MTC (n = 1)	1	100
Calcitonin overexpression*	1	100
PDTC (n = 1)	1	100
NRAS (p.Q61R, c.182A>G)	1	100

ITN: indeterminate; NIFTP: non-invasive follicular thyroid neoplasms with papillary-like nuclear features; FVPTC: follicular variant of papillary thyroid carcinoma; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma. *No other genetic abnormalities detected.

Performance of ThyroSeqV.2 NGS panel in ITN (Bethesda III-IV-V)

In the group of 128 ITN nodules with known histopathology and an evaluable ThyroSeqV.2 test, the molecular assay correctly identified the nodules as benign (*n* = 47) and NRS (*n* = 41), with 4 false negative and 36 false positive tests. Thus, the performance of the ThyroSeqV.2 test in our series showed a sensitivity of 91% (95% CI: 79%-98%), specificity of 56% (45%-67%), PPV of 53% (42%-65%), NPV of 92% (81%-98%), and an overall accuracy of 69% (55%-85%) with a prevalence of disease of 35% (27%-44%) (**Figure 1**).

Individual performance of the ThyroSeqV.2 NGS panel in nodules with Bethesda III, Bethesda IV and Bethesda V cytology is presented in **Figure 2**.

The NPV and PPV values for Bethesda III or IV nodules in this series were compared to the investigator's data [4, 9] (**Figure 3**). There was no statistically significant difference in the NPV between our experience (for Bethesda III, 91% vs. 96%, $P = 0.44$; for Bethesda IV, 100% vs. 96%, $P = 0.51$). However, the PPV of 41% in this series was worse for detection of NRS compared to the investigator's data (for Bethesda III, 41% vs. 82%, $P < 0.01$; for Bethesda IV, 57% vs. 83%, $P = 0.05$). It was due to the detection of "high risk" ThyroSeqV.2 results in benign nodules. The majority of these false positive assays were *RAS* mutations.

The performance of ThyroSeqV.2 NGS panel in Bethesda V cytology has been rarely reported. In this study, evaluable ThyroSeqV.2 test was available in 27 of 29 Bethesda V nodules, among them 18 had high risk genetic alteration. Surgical pathology was obtained on 19 nodules, including 12 with high risk result (4 $BRAF^{V600E}$ mutation, 5 *NRAS* mutation, 1 *HRAS* mutation, 1 *KRAS* mutation and 1 *THADA/IGFBP2* fusion) and 7 with low risk or negative result. The ThyroSeqV.2 molecular assay correctly identified the nodules as benign (n = 6) and NRS (n = 12), with 1 false negative and 0 false positive tests. The NRS that were misclassified as benign by the molecular assay was an ATA low risk FVPTC. Hence, in Bethesda V nodules, the ThyroSeqV.2 test demonstrated good PPV of 100% (74%-100%) and overall accuracy of 95% (56%-100%) with a prevalence of disease of 68% (43%-87%) to detect NRS. Unfortunately, there were insufficient numbers of specimens in the Bethesda V category for an accurate comparison analysis with prior studies.

Follow-up of ITN without surgery

Review of medical records demonstrated that every case with an ITN and positive high risk ThyroSeqV.2 test was advised to remove the nodule by surgery. By May 31, 2018, 30 cases did not have surgery. Of them 17 cases had been followed by clinical evaluation by US and/or repeat FNB over the median 12 months (range 1.5 m-27 m). Fifteen of 17 patients had an US to assess for subsequent growth. Of these 80.0% (12/15) showed no significant growth by US (defined as >20% increase in 2 dimensions) or acquired new high risk US characteristics, and 3 patients (20%) had a significant nodule growth and were suggested again for surgery. Two patients had a second FNB during the follow-up yielded identical results as their initial Bethesda III cytology and mutated *NRAS* in the ThyroseqV.2 molecular assay, and refused surgery again (**Figure 4A**).

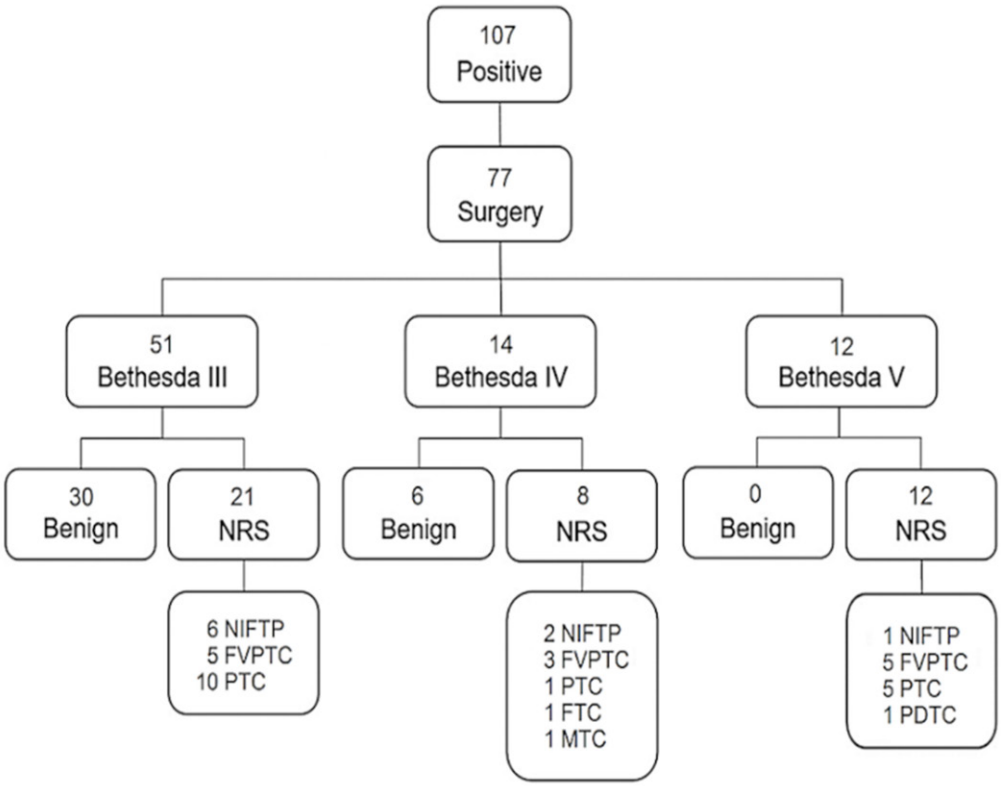
By May 31, 2018, of cases with an ITN and negative or low risk molecular result, 80% (198/249) did not undergo surgery. Clinical follow-up was conducted in 70.7% (140/198) over the median 13 months (range 2 m-39 m). Of these, 88.6% (124/140) had not grown significantly or developed high risk US characteristics in the clinical observation, while 4.3% (6/140) of the nodules increased in size. During the fol-

Table 2. Positive ThyroSeqV.2 test and risk of NRS in ITN thyroid nodules

	Positive Molecular Analysis	Positive Molecular Analysis with Surgery	Cancer at Surgery (%)	NIFTP at Surgery (%)	Benign at Surgery (%)
POINT MUTATIONS					
BRAF (p.V600E, c.1799T>A)	12	10	10 (100%) PTC 10	0 (0%)	0 (0%)
BRAF (p.K601E, c.1801A>G)	5	5	2 (40%) PTC 1 FVPTC 1	0 (0%)	3 (60%)
BRAF (p.T599del, c.1794_1796del)	1	1	1 (100%) PTC 1	0 (0%)	0 (0%)
EIF1AX	8	4	1 (25%) FVPTC 1	0 (0%)	3 (75%)
HRAS (p.Q61R, c.182A>G)	11	5	1 (20%) FVPTC 1	0 (0%)	4 (80%)
HRAS (p.Q61K, c.181C>A)	2	2	1 (50%) PTC 1	0 (0%)	1 (50%)
KRAS (p.G12D, c.35G>A)	1	1	0 (0%)	0 (0%)	1 (100%)
KRAS (p.G12C, c.34G>T)	1	0	0 (0%)	0 (0%)	0 (0%)
KRAS (p.G12R, c.34G>C)	1	1	0 (0%)	1 (100%)	0 (0%)
KRAS (p.G12V, c.35G>T)	1	1	0 (0%)	0 (0%)	1 (100%)
KRAS (p.Q61R, c.182A>G)	3	2	0 (0%)	1 (100%)	1 (0%)
NRAS (p.Q61K, c.181C>A)	6	4	0 (0%)	0 (0%)	4 (100%)
NRAS (p.Q61R, c.182_183AA>GC)	1	1	0 (0%)	0 (0%)	1 (100%)
NRAS (p.Q61R, c.182A>G)	33	27	8 (30%) PTC 2 FVPTC 5 PDTC 1	5 (19%)	14 (51%)
PTEN	8	3	0 (0%)	0 (0%)	3 (100%)
TERT (p.C228T, c.1-124C>T)	2	1	1 (100%) FTC 1	0 (0%)	0 (0%)
TP53	2	0	0 (0%)	0 (0%)	0 (0%)
TSHR	15	3	0 (0%)	0 (0%)	3 (100%)
EIF1AX (p.R13P, c.38G>C 55%) + *GNAS* (p.Q227H, c.681G>T) + *NIS* (SLC5A5) gene overexprssion	1	1	0 (0%)	0 (0%)	1 (100%)
KRAS (p.Q61R c.182A>G) + *TERT* (p.C228T c.1-124C>T)	1	1	1 (100%)	0 (0%)	0 (0%)
NRAS (p.Q61R, c.182A>G) + *EIF1AX* (p.A113_splice, c.338-2A>T)	1	1	0 (0%) FVPTC 1	0 (0%)	1 (100%)
NRAS (p.Q61R, c.182A>G) + *TERT* (p.C228T, c.1-124C>T)	1	0	0 (0%)	0 (0%)	0 (0%)

NRAS (p.Q61R, c.182A>G) + *TERT* (p.C250T, c.1-146C>T)	1	1	1 (100%) FVPTC 1	0 (0%)	0 (0%)
NRAS (p.Q61R, c182A>G) + *TP53* (p.T170M, c.509C>T)	1	0	0 (0%)	0 (0%)	0 (0%)
GENE OVEREXPRESSION*					
Calcitonin	1	1	1 (100%) MTC 1	0 (0%)	0 (0%)
NIS	5	1	0 (0%)	0 (0%)	1 (100%)
MET	1	0	0 (0%)	0 (0%)	0 (0%)
GENE FUSIONS					
ETV6/NTRK3 fusion	1	1	1 (100%) PTC 1	0 (0%)	0 (0%)
PAX8/PPARG fusion	3	3	1 (33%) FVPTC 1	1 (33%)	1 (33%)
THADA/IGF2BP3 fusion	5	3	2 (66.7%) FVPTC 2	1 (33.3%)	0 (0%)

NRS: neoplasm requiring surgery; ITN: indeterminate; NIFTP: non-invasive follicular thyroid neoplasms with papillary-like nuclear features; FVPTC: follicular variant of papillary thyroid carcinoma; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma. *PTH over-expression (n = 2) was not included.



Measures	Bethesda III	Bethesda IV	Bethesda V
	Proportions and 95% Confidence Intervals (Exact) (%)		
Sensitivity	88 (68 - 97)	100 (63 - 100)	92 (64 - 100)
Specificity	50 (37 - 63)	65 (38 - 86)	100 (54 - 100)
PPV	41 (28 - 56)	57 (29 - 82)	100 (74 - 100)
NPV	91 (76 - 98)	100 (72 - 100)	86 (42 - 100)
Accuracy	61 (45 - 80)	76 (46 - 100)	95 (56 - 100)
Prevalence	29 (19 - 39)	32 (15 - 54)	68 (43 - 87)

Figure 2. Individual performance of ThyroSeqV.2 NGS panel in Bethesda III, Bethesda IV and Bethesda V nodules. NRS: neoplasm requiring surgery; NIFTP: non-invasive follicular thyroid neoplasms with papillary-like nuclear features; FVPTC: follicular variant of papillary thyroid carcinoma; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma; PPV: positive predictive value; NPV: negative predictive value.

low-up, 8 ITN had a repeat FNB, by which showed Bethesda II cytology in 3 and Bethesda III cytology in 5 cases. ThyroseqV.2 panel in 2 Bethesda III nodules had a second negative result, while another 1 was detected to have both *NRAS* and *TERT* mutations. The patient, who had mutations newly identified but suffering from severe COPD, was continuing with surveillance US exams (**Figure 4B**).

Discussion

This study is to date the largest independent single-institution prospective implementation study to evaluate the performance of ThyroSeqV.2. Previous studies focused on its abilities to identify malignancies, while we assessed the value of ThyroSeq in recognizing NRS, including both malignant thyroid nodule and NIFTP. Though NIFTP has been removed from thyroid malignancy by the World Health Organization (WHO) in 2017 [12], we considered it to be a true positive in the test because NIFTP must be surgically resected for complete pathological examination to distinguish it from a FVPTC. The two ThyroSeqV.2 studies by the investigators [4, 9] were completed before the introduction of the NIFTP nomenclature; therefore NIFTP was included in their "malignan-

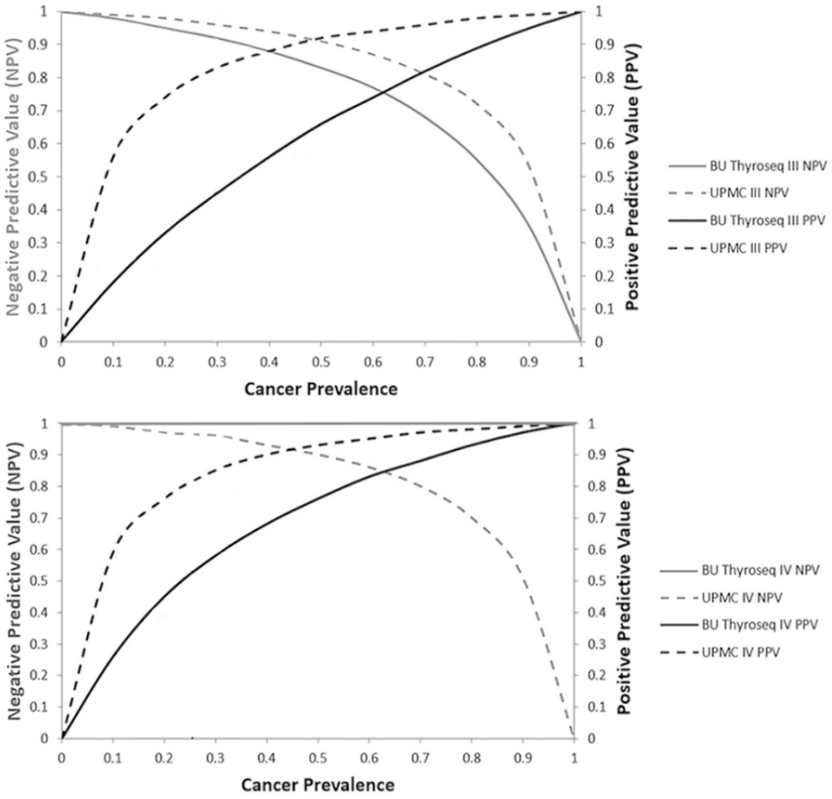


Figure 3. Comparison of the NPV and PPV values in this series to the published data. Upper, Bethesda III; Lower, Bethesda IV. BU: Boston University; UPMC: University of Pittsburgh Medical Center; PPV: positive predictive value; NPV: negative predictive value.

cies", which was in fact equivalent to the "NRS" in our study.

In this series, the prevalence of NRS in ITN biopsies was within the range reported in the literature (15%-60%) [1, 15-17]. According to the analysis performed by investigators of ThyroSeqV.2, with a cancer prevalence in AUS/FLUS nodules ranging between 6% and 48%, the NPV of this test would range between 99% and 92%, and PPV between 42% and 91%; with a cancer probability ranging between 14% and 34% in FN/SFN nodules, the NPV of the test would range between 98% and 95%, and the PPV between 68% and 87% [4, 9]. Therefore, our study demonstrated a similar NPV, but much lower PPV than expected, which could not be attributed to unequal prevalence of NRS in our cohort versus others. Our results are comparable to an observational study in Bethesda III and IV thyroid cytology by Valderrabano [18], which demonstrated a NPV of 91% (82-92) and a PPV of 42% (25-61). Taken together, in "real life" clinical use of ThyroSeqV.2 for ITN cytology, the high NPV confirmed its utility as a "rule-out" test to exclude NRS, whereas the insufficient PPV limited its power as a "rule-in" test to identify NRS.

The unsatisfactory PPV performance underscores a key message that not all conventionally expected "high risk" genetic alterations equal malignancy, and the ROM varies for the molecular markers detected by ThyroSeqV2. Some markers are oncogenes with a high specificity for malignancy such as *BRAF*^{V600E} mutation and *RET/PTC* fusions but some somatic genetic changes such as *RAS* mutations and *PAX8/PPARG* fusions are present often in benign nodules [19-25]. Along with earlier literature [26] and a separate analysis on our own data [27], it is suggested that a considerable overlap among *RAS* mutations in benign nodules and NRS urges cautious interpretation of their significance in finding NRS.

It is more difficult to assess the ROM with other mutations that were detected such as *EIF1AX*. Of the 33 *EIF1AX* mutations only the A113_ splice mutation was detected in thyroid malignancy, FVPTC, especially when it coexisted with *RAS* mutations [28]. We concluded that although *EIF1AX* has a low prevalence in benign and malignant nodules, the possible 20% risk of FVPTC in ITN FNB could not allow us to classify this mutation as a "low risk" ThyroSeqV.2 result.

Based on our findings, we proposed a clinical decision-making flowchart with the use of the ThyroSeqV.2 panel in the management of a thyroid nodule (**Figure 5**). Thyroid nodules with a Bethesda III-IV but not V cytology would be assessed with the ThyroSeqV.2 assay. Nodules

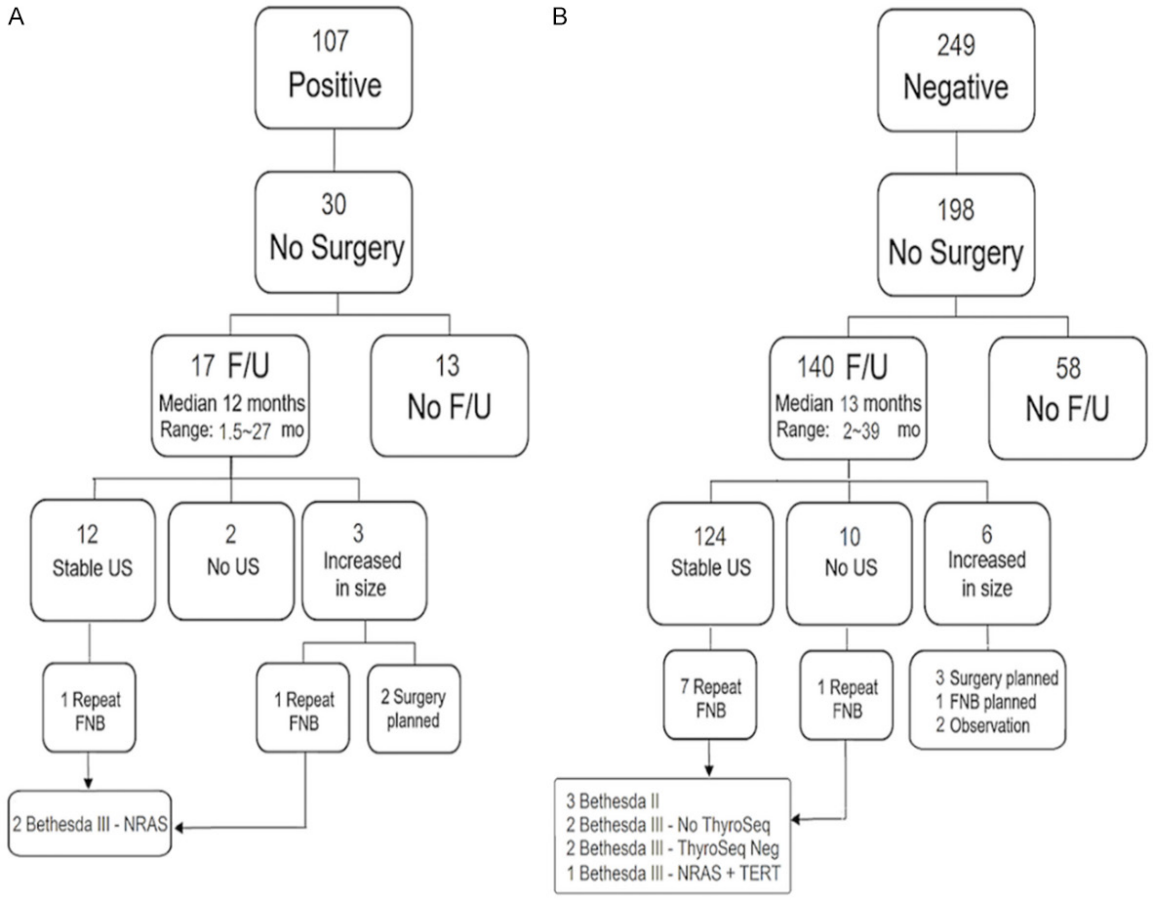


Figure 4. Follow-up of ITN nodules without surgery. F/U: follow-up; US: ultrasound; FNB: fine needle biopsy.

with a low risk or negative test can be managed as benign nodules, whereas those with a high risk genetic alteration (not including isolated *RAS* mutation) are expected to have NRS and then undergo thyroid surgery. Particular mention should be given to the *RAS* mutations. Despite its oncogene nature and conventional classification into the high risk category, isolated *RAS* mutation represents benign or low risk NRS, suggesting more conservative strategies such as watchful waiting or lobectomy for ITN nodules of this type [27]. We await future studies including larger numbers of subjects with long term clinical observation and incorporating sonographic appearance or elastography. They may help determine which nodules can be followed rather than surgically removed.

This current report is the only published data on the performance of the ThyroSeqV.2 test in the Bethesda V cytology subgroup. The performance of this test seemed excellent in PPV (100%) compared to Bethesda III and IV cytologies. Though the high pre-test probability of cancer making this test less informative for the decision of surgery, the mutational analysis could be used for surgical planning and prognosis predicting. For example, the presence of a high risk mutations such as *BRAF*^{V600E} plus *TERT* [29] or multiple genetic mutations [30] would suggest a total thyroidectomy rather than a lobectomy for small tumors because of the high risk of metastatic disease and increased probability of tumor recurrence.

The strengths of this implementation study include the facts that it was independent, prospective, and sequential FNBs were treated in a uniform manner. The cytology interpretations fell within an expected distribution of the Bethesda classification (I-VI) [8, 15]. The introduction of terminology (i.e. NRS) reflected what we expect molecular markers to identify, as both NIFTP and thyroid cancer needs surgical intervention. This terminology would be easy to adopt. In addition, we presented individual

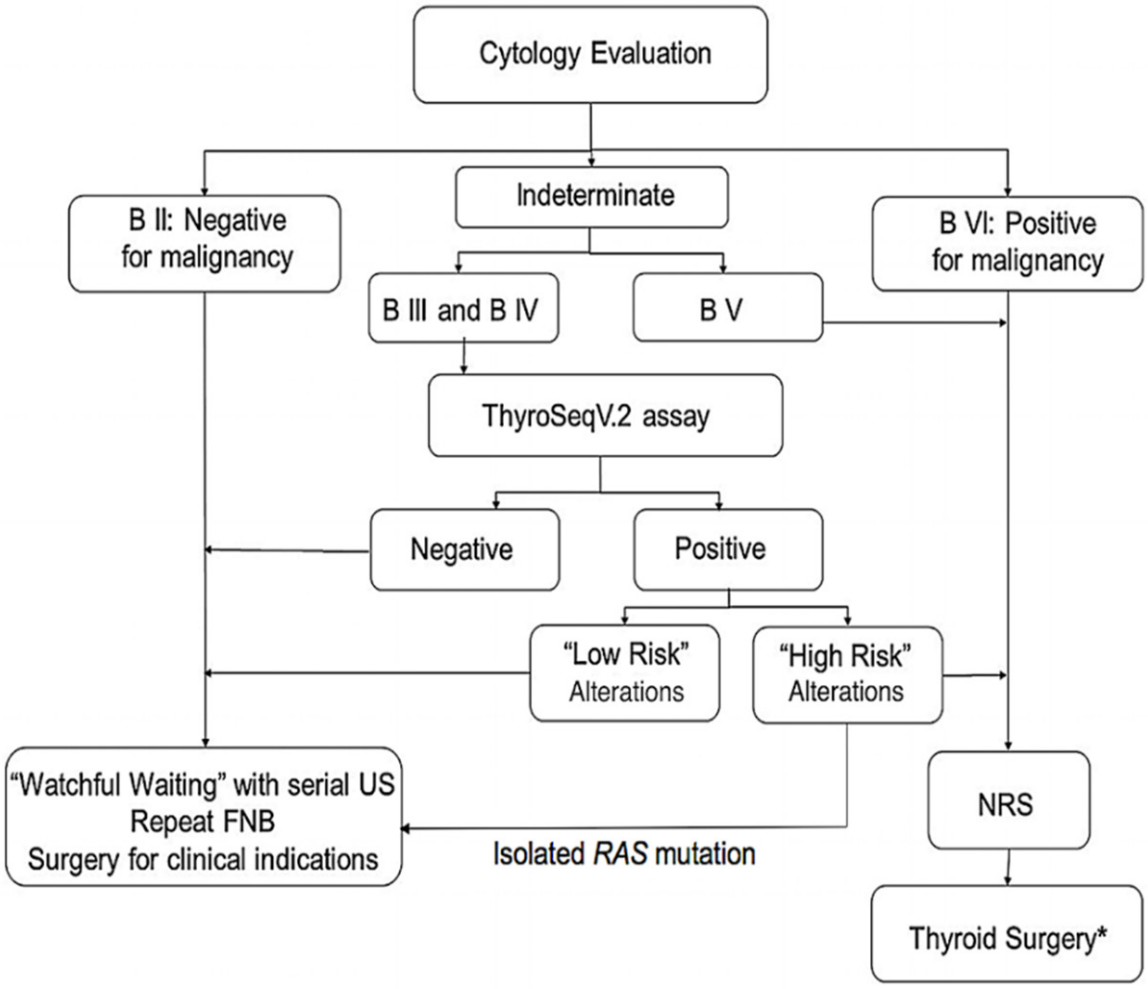


Figure 5. Clinical decision-making flowchart with applying ThyroSeqV.2 panel to the management of a thyroid nodule. "Low risk" alterations include mutations for *PTEN*, *TSHR* and *NIS* overexpression, the rest of genetic alterations are categorized as "high risk" alterations. *The extent of thyroid surgery should be based on clinical evaluation and the genetic alterations detected. B: Bethesda; FNB: fine needle biopsy; NRS: neoplasm requiring surgery.

genetic events (mutations, gene fusions, expression) found in this cohort, which is a good reference, and highlights the potential values of the data derived from ThyroSeq testing. Finally, the investigators intended to conduct long-term clinical surveillance of the entire cohort.

Limitations of the current study include the fact that it was from a single institution. The data was only collected in a single institution. With the announcement of ThyroSeqV.3 [31], one would anticipate that ThyroSeqV.2 will diminish in use, limiting the impact of this study. However, it is still fundamentally instructive to have iterative clinical data about this test generation to inform how clinicians would view subsequent versions. Most importantly, the number of patients predicted to be low-risk and undergoing surgery is not enough, thus it may threaten the validity of the test performance (sensitivity, specificity, NPV, PPV). But as a "real life" study, our study can be helpful to implement the use of molecular markers in clinical settings, and reiterate the need for larger, independent, multicenter, clinical validation studies with long term clinical observation.

In conclusion, ThyroSeqV.2 in this clinical use study in ITN nodules provided a similar NPV but a lower PPV than expected compared to published studies due to the detection of an array of mutations in benign nodules. It has a good performance to exclude a new category of NRS

that includes NIFTP and malignancy, thus can be used as a "rule-out" test to avoid thyroid surgery. It also provides detailed information on genetic alterations that would be helpful for predicting tumor risk and prognosis. Future studies will determine the risk of malignancy associated with each genetic alteration based upon long-term clinical surveillance of patients, and they will hopefully establish whether the combination of imaging studies, cytology, and specific genetic alterations better define the best strategy for managing patients with ITN in a cost-effective way.

Disclosure of conflict of interest

None.

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