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Original contribution

Semiquantitative assessment of cytomorphologic features can predict mutation status of thyroid nodules with indeterminate cytologic diagnosis☆,☆☆

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Summary Molecular diagnostics increasingly direct the management of thyroid nodules with an indeterminate cytologic diagnosis. This study was undertaken to correlate cytomorphologic features with the molecular profiles in an effort to identify features predictive of molecular aberrations. One hundred eighty-nine thyroid nodules with an indeterminate thyroid cytology diagnosis (atypia of undetermined significance, suspicious for follicular lesion, and suspicious for malignancy) with an adequate sample submitted for targeted mutation detection by polymerase chain reaction or next-generation sequencing were assessed semiquantitatively for the following cytomorphologic parameters: cellularity, Hurthle cell changes, microfollicles, nuclear elongation, nuclear grooves, nuclear enlargement, nuclear atypia, extent of atypia, and colloid. Based on this evaluation, a cumulative cytomorphologic score (CCS) and a more simplified overall atypia score (OAS) were assigned to each case. Associations among mutational status and each of the aforementioned parameters, CCS, and OAS were determined. Of the 189 nodules with indeterminate cytology, 63 (33.3%) harbored at least 1 mutation. *RAS* and *BRAF* were the most common mutations, found in 34 (18.0%) and 13 (6.9%) cases, respectively. Both CCS and OAS were highly associated with the presence

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of all mutations ($P < .0001$) and with the presence of *BRAF* and *RAS* mutations in particular (all $P < .01$). Semiquantitative assessment of various cytomorphologic features in indeterminate thyroid cytology cases showed a strong association of higher OAS and CCS and incidence of *BRAF* and *RAS* mutations. Using a more objective approach to thyroid cytology can potentially decrease the overall number of indeterminate diagnoses, leading to fewer repeat procedures and unnecessary surgical procedures.

1. Introduction

Thyroid nodules are common, with a reported prevalence as high as 68% when detected by ultrasonography [1,2]. Current guidelines recommend fine needle aspiration (FNA) cytology in clinically concerning nodules or nodules greater than or equal to 1 cm [1,3]. In the United States, reporting of thyroid cytology is primarily based on the Bethesda System for Reporting Thyroid Cytology (TBSRTC). TBSRTC includes 6 diagnostic categories. Each category is stratified by risk of malignancy and recommended clinical management [4]. The cytomorphologic features described in the TBSRTC have been used by the cytopathologists for more than a decade; however, wide variation in reporting of TBSRTC categories among institutions suggests inconsistent application of these criteria. The most marked discrepancies occur with indeterminate diagnostic categories: atypia of undetermined significance (AUS), suspicious for follicular neoplasm (FN), and suspicious for malignancy (SM). Indeterminate categories carry the highest degree of uncertainty in terms of diagnosis and risk of malignancy, thereby leading to repeat procedures and unwarranted surgical procedures. These categories constitute 9.9% to 38.2% of all cytologic TBSRTC categories in general practice [5-10]. To tackle the relatively high rate of indeterminate diagnosis, commercial, noncommercial, and institutional molecular testing is used to augment cytologic diagnosis [11]. Such testing provides an opportunity to further refine the conventional cytomorphologic criteria of thyroid lesions.

The purpose of this study was to semiquantitatively analyze the cytomorphologic features associated with indeterminate cytology using 2 different scoring schemes, looking for phenotypic patterns that might be predictive of specific molecular alterations. Associations between each of the scoring schemes and a mutation panel were used to assess scoring scheme performance. Such a system might provide more objective process in making indeterminate thyroid cytology diagnoses.

2. Materials and methods

2.1. Study cohort

After receiving institutional review board approval, a retrospective review of all thyroid FNA performed between 2013 and 2015 was conducted. During this period, 1172 nodules were sampled. Nodules with indeterminate TBSRTC categories (AUS, FN, and SM) which also had molecular results were selected for further study. The archived cytology slides from selected cases were retrieved for morphologic evaluation, and in cases where a subsequent resection was performed, histologic diagnosis was correlated to the cytology and molecular results.

2.2. Molecular testing

In most of the cases, FNA was performed with 4 passes and rapid onsite adequacy evaluation. Direct smears were made from passes 1, 2, and 4, whereas needle rinses from these passes and all of pass 3 were placed in RNA/DNA stabilization reagent (Roche Indianapolis, Indiana, USA) and stored at 4°C. Samples with indeterminate diagnosis were submitted to the University of Pittsburgh Medical Center molecular diagnostic laboratory for targeted mutation detection by polymerase chain reaction or by next-generation sequencing as part of ongoing clinical care, either as a result of reflex testing or by specific clinician request. From January of 2013 to September of 2013 (63 nodules), 7-gene mutational testing was performed that used real-time LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) polymerase chain reaction and fluorescence melting curve analysis to detect possible mutations (*BRAF*, *NRAS* codon 61, *HRAS* codon 61, *KRAS* codons 12 and 13) and single-step real-time reverse transcription real-time polymerase chain reaction to amplify the fusion points of the rearrangements (*RET/PTC1*, *RET/PTC3*, and *PAX8/PPARG*). Subsequently, ThyroSeq (UPMC and CBLPath, Pittsburgh, PA, USA) next-generation sequencing (NGS)–based versions 1 and 2 were used as they were made available from the reference laboratory [13-14]. Thyroseq v1 included NGS for 284 mutations in 12 key thyroid cancer–related genes including *BRAF*, *AKT1*, *CTNNB1*, *GNAS*, *NRAS*, *HRAS*, *KRAS*, *PIK3CA*, *PTEN*, *RET*, *TP53*, and *TSHR*, and detection of chromosomal rearrangements *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARG*. Thyroseq v2 included the above point mutations plus *EIF1AX*, *TERT*, and 42 gene fusions involving the following genes: *RET*, *PPARG*, *NTRK1*, *NTRK3*, *ALK*, *BRAF*, and *IGF2BP3*.

2.3. Semiquantitative evaluation of cytomorphologic features

The FNA material was reviewed simultaneously by 2 pathologists on a double-headed microscope. Pathologists were

blinded to the mutational status, cytologic diagnosis, or histologic diagnosis of any subsequent resection. The minimal cellularity criteria used for adequacy were presence of 6 groups of follicular cells each with at least 10 cells as defined in the TBSRTC. Nine cytomorphologic parameters were semiquantitatively scored for each case as follows: cellularity (1 = hypocellular; 2 = moderately cellular; 3 = hypercellular); Hurthle cell changes (0 = no Hurthle cells; 1 = focal Hurthle cells; 2 = abundant/diffuse Hurthle cells); microfollicles (0 = no microfollicles; 1 = occasional; 2=predominant); nuclear atypia which is a constellation of features including nuclear membrane irregularity, nuclear overlapping, fine chromatin, and conspicuous nucleoli (0 = no atypia; 1 = mild; 2 = moderate; 3:marked); nuclear enlargement (3-4 times of RBC) (0 = none; 1 = focal; 2 = multifocal); nuclear elongation (0 = none; 1 = focal; 2 = multifocal); nuclear grooves (0 = none;1 = focal; 2 = multifocal); extent of atypia (0 = no atypia; 1 = focal; 2 = multifocal; 3 = diffuse); and colloid (0 = no colloid; −1 = scant; −2 = moderate; −3 = abundant) [15].

The sum of all these scores generated a cumulative cytomorphologic score (CCS) with a range from −3 to 18 (Table 1). In addition to individual cytomorphologic feature evaluation, each case was assigned a separate more simplified simplified overall atypia score (OAS) based on pathologist's level of suspicion (1 = less likely neoplastic; 2 = moderate; 3 = most likely neoplastic). OAS can be best described as cytopathologists' level of suspicion, taking into consideration all cytomorphological features, for the likelihood of neoplasia. We used this simplified score which is more representative and easier to implement in routine practice of reporting thyroid cytology and compared its performance to more objective and somewhat complex assessment by CCS.

Table 1 Scoring scheme of the cytomorphologic parameters determining the CCS

	0	1	2	3
Cellularity		Hypocellular	Moderately cellular	Hypercellular
Hurthle cell changes	None	Focal	Abundant/ diffuse	
Microfollicle	None	Occasional	Predominant	
Nuclear atypia	None	Mild	Moderate	Marked
Extent of atypia	None	Focal	Multifocal	
Nuclear enlargement	None	Focal	Multifocal	
Nuclear elongation	None	Focal	Multifocal	
Nuclear grooves	None	Focal	Multifocal	
	0	−1	−2	−3
Colloid	None	Scant	Moderate	Abundant

NOTE. The sum of the scores on each row determines the CCS, which ranges from −3 to 18.

2.4. Statistical methods

Demographic and pathological characteristics were summarized and compared by mutation status (any, *BRAF*, *RAS*) compared to no mutation (or no *BRAF* or *RAS*, respectively) using χ^2 and Fisher exact tests for categorical data and *t* tests and Wilcoxon rank sum tests for continuous data as appropriate. Potential cutoffs for the CCS were determined using receiver operating characteristic (ROC) curves and the corresponding area under the curve (AUC). Cutoffs were chosen to maximize sensitivity while maintaining specificity of 50% (CCS <7/7+) and to maximize AUC (both sensitivity and specificity; CCS <10/10+) for comparisons by mutation status (any versus none). Point estimates and exact 95% confidence intervals (CIs) for the sensitivity and specificity of these cutoff scores are presented comparing the presence of any mutation, *BRAF*, and *RAS* to no mutation, no *BRAF*, or no *RAS* mutations, respectively. Because the SM category nodules were associated with higher cytomorphologic feature scores, a sensitivity analysis was conducted excluding the 17 SM category nodules from the analysis. Data were analyzed using SAS 9.3 (Cary, NC), and $P < .05$ was considered statistically significant.

3. Results

The 1172 distinct thyroid nodules aspirated during this study period were categorized as follows according to TBSRTC: Indeterminate cytologic diagnosis was rendered in 250 (21.3%) cases, 184 (15.6%) were AUS, 36 (3.1%) were SFN/FN, and 30 (2.6%) were SM. Of these, 228 (19.5%) were submitted for molecular studies. Reportable molecular results were available on 189 aspirates from 169 patients who were selected for this study. All the 189 aspirates also met the criteria for adequate cellularity. One hundred thirty-four of the patients were female and 35 were male. The FNA diagnosis was AUS in 146 cases, SFN in 26 cases, and SM in 17 cases. Mutations were identified in 63 cases, with *RAS* identified as the most common (n = 34, 18.0%) and *BRAF* the second most common mutation (n =13; 6.9%). Three nodules harbored more than 1 mutation (Table 2). Seventy-eight nodules were surgically excised.

3.1. Correlation of individual cytomorphologic features with mutational status

Table 3 shows the prevalence of each of 9 individual cytomorphologic features score by presence or absence of mutation. Extent of atypia, that is, focal, multifocal, or diffuse ($P < .0001$); nuclear enlargement ($P < .0001$); nuclear elongation ($P < .0001$); nuclear atypia ($P = .0007$); nuclear grooves ($P = .0005$); and cellularity ($P = .01$) were significantly different between mutation-positive and mutation-negative nodules. No differences by mutation status were observed for Hurthle cell change, microfollicles, or absence of colloid.

Table 2 Cytology, mutation status, and type of mutation (N = 189)

	n	%
Cytology		
AUS	146	77.3
SFN/FN	26	13.7
SM	17	9.0
Mutation		
No	126	66.7
Yes	63	33.3
Mutation type		
ALK	1	1.6
BRAF	12	19.1
BRAF, TERT, PIK3CA, AKT1	1	1.6
EIFIAX	1	1.6
GNAS	2	3.2
HRAS	6	9.5
HRAS, RET	1	1.6
KRAS	9	14.3
NRAS	17	27.0
NRAS, TSHR	1	1.6
P53	4	6.4
PAX8/PPARg	2	3.2
PTEN	2	3.2
TSHR	4	6.4

Further analysis of the individual cytomorphologic features by specific mutation type was performed for *RAS* and *BRAF*. *BRAF* mutation was associated with the same general pattern of cytopathologic feature score significance as the overall group, except that cellularity score was no longer significant ($P = .44$). In addition to the pattern shown for the overall group, the presence of *RAS* mutations also correlated significantly with microfollicles ($P = .002$).

Because the SM category nodules were associated with higher cytomorphologic feature scores, statistical analysis was also performed after excluding the 17 SM category nodules from the analysis. This did not impact cytomorphologic score statistical significance by mutation status except for "cellularity" feature, which did not retain its significance ($P = .09$).

3.2. Correlation of CCS with mutational status

The CCSs were widely distributed, ranging from −2 to 18 (Table 4). The 63 mutation-positive nodules had a median CCS of 10 compared to a median score of 6 in the 126 mutation-negative nodules ($P < .0001$). *BRAF*-positive nodules exhibited a higher median CCS compared to *RAS*-positive nodules, 12 versus 10.5.

CCS cutoffs of 7 and 10 were calculated based on ROC analysis defining maximum sensitivity and specificity, respectively. Eighty nodules (16 with mutations and 64 without mutations) fell below a CCS cutoff of 7. A total of 43.1% (47 of 109) of nodules with CCS of 7 or more were positive for mutation compared to 20% (16 of 80) with CCS <7 ($P = .0009$) (Fig. 1). At this cutoff, the sensitivity and specificity for detecting mutation were 74.6% (95% CI: 62.1-84.7) and 50.8% (95% CI: 41.7-59.8), respectively; Using a cutoff score of 10, 22.5% (31 of 138) cases with CCS <10 were positive for mutations, and 62.7% (32 of the 51) with a CCS score of 10 or more were positive for mutation ($P \leq .0001$). At this cutoff, the sensitivity and specificity for detecting mutation were 50.8% (95% CI: 37.9-63.6) and 84.9% (95% CI: 77.5-90.7), respectively. CCS cutoff >10 or higher performed slightly better than CCS cutoff >7 in accurately predicting mutation (positive likelihood ratio 3.36 versus 1.5).

For detection of *BRAF* mutation only, the sensitivity and specificity of scores equal to or above 7 were 100% (95% CI: 75.3-100.0) and 45.5% (95% CI: 38.0-53.1), respectively, and for scores equal to and above 10, the sensitivity and specificity were 84.6% (95% CI: 54.6-98.1) and 77.3% (95% CI: 70.4-83.2), respectively. For *RAS* mutations, the sensitivity and specificity of scores equal to or above 7 were 79.4% (95% CI: 62.1-91.3) and 47.1% (95% CI: 39.0-55.3), respectively, and for scores equal to and above 10, the sensitivity and specificity were 58.8% (95% CI: 40.7-75.4) and 80.0% (95% CI: 72.8-86.0), respectively.

3.3. Correlation of OAS with mutational status

The OAS was also highly associated with the mutation status ($P < .0001$, Table 3). In the nodules where mutations were detected, 49.2% of the overall population had an atypia score of 3, whereas 84.6% of *BRAF* mutation–positive and 55.9% of *RAS* mutation–positive nodules had an atypia score of 3. Conversely, the majority of cases (53.2%) without mutation (s) had atypia score of 1.

The correlation of mutation status and the OAS was still statistically significant after excluding nodules in the SM category (data not shown).

If the status of mutation is considered as the criterion standard and OAS of 3 to define a positive test, sensitivity and specificity of OAS for predicting mutational status were 49.2% (95% CI: 36.4-62.1) and 82.5% (95% CI: 74.8-88.7), respectively.

3.4. Correlation of histologic diagnosis with CCS and OAS

Follow-up resection data were available for 78 of the 189 nodules. Twenty were non-neoplastic (adenomatoid nodule, nodular hyperplasia, Hashimoto thyroiditis) (median CCS score = 6, range 2-15), 24 were categorized as benign neoplasm (follicular adenoma) (median CCS score = 9.5, range 4-13), and 34 were categorized malignant neoplasm (4 follicular carcinomas, 28 papillary thyroid carcinomas, 2 medullary thyroid carcinomas) (median CCS score = 11, range: 4-18). CCS was significantly higher in malignant neoplasm group compared to the non-neoplastic ($P = .0004$) and benign ($P = .02$) groups. Similarly, the OAS showed a statistically significant difference among the 3 groups ($P = .0002$).

To assess the impact of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), a new

Table 3 Correlation of individual cytomorphologic parameters and OAS with mutational status

	No mutation (n = 126)		Any mutation (n = 63)			No *BRAF* (n = 176)		*BRAF* (n = 13)			No *RAS* (n = 155)		*RAS* (n = 34)		
	n	%	n	%	*P*	n	%	n	%	*P*	n	%	n	%	*P*
0	29	23.0	14	22.2		38	21.6	5	38.5		36	23.2	7	20.6	
1	68	54.0	27	42.9		92	52.3	3	23.1		80	51.6	15	44.1	
2	29	23.0	22	34.9		46	26.1	5	38.5		39	25.2	12	35.3	
Cellularity (1-3)					.01					.44					.001
1	21	16.7	4	6.4		25	14.2	0	0.0		24	15.5	1	2.9	
2	82	65.1	37	58.7		109	61.9	10	76.9		102	65.8	17	50.0	
3	23	18.3	22	34.9		42	23.9	3	23.1		29	18.7	16	47.1	
Microfollicles (0-3)					.24					.86					.002
0	42	33.3	20	31.8		57	32.4	5	38.5		56	36.1	6	17.7	
1	59	46.8	23	36.5		77	43.8	5	38.5		69	44.5	13	38.2	
2	20	15.9	14	22.2		31	17.6	3	23.1		25	16.1	9	26.5	
3	5	4.0	6	9.5		11	6.3	0	0.0		5	3.2	6	17.7	
Nuclear atypia (0-3)					.0007					<.0001					.26
0	5	4.0	0	0.0		5	2.8	0	0.0		5	3.2	0	0.0	
1	95	75.4	35	55.6		128	72.7	2	15.4		110	71.0	20	58.8	
2	23	18.3	19	30.2		37	21.0	5	38.5		31	20.0	11	32.4	
3	3	2.4	9	14.3		6	3.4	6	46.2		9	5.8	3	8.8	
Nuclear enlargement					<.0001					<.0001					.09
0	16	12.7	0	0.0		16	9.1	0	0.0		16	10.3	0	0.0	
1	103	81.8	46	73.0		146	83.0	3	23.1		121	78.1	28	82.4	
2	7	5.6	17	27.0		14	8.0	10	76.9		18	11.6	6	17.7	
Nuclear elongation					<.0001					<.0001					.01
0	90	71.4	28	44.4		116	65.9	2	15.4		104	67.1	14	41.2	
1	36	28.6	29	46.0		58	33.0	7	53.9		47	30.3	18	52.9	
2	0	0.0	6	9.5		2	1.1	4	30.8		4	2.6	2	5.9	
Nuclear grooves					.0005					<.0001					.26
0	108	85.7	42	66.7		148	84.1	2	15.4		126	81.3	24	70.6	
1	18	14.3	16	25.4		27	15.3	7	53.9		25	16.1	9	26.5	
2	0	0.0	5	7.9		1	0.6	4	30.8		4	2.6	1	2.9	
Focal/diffuse (extent of) atypia					<.0001					<.0001					.003
0	8	6.4	2	3.2		10	5.7	0	0.0		8	5.2	2	5.9	
1	88	69.8	24	38.1		110	62.5	2	15.4		101	65.2	11	32.4	
2	25	19.8	23	36.5		44	25.0	4	30.8		33	21.3	15	44.1	
3	5	4.0	14	22.2		12	6.8	7	53.9		13	8.4	6	17.7	
Colloid					.19					.13					.85
0	51	40.5	31	49.2		73	41.5	9	69.2		66	42.6	16	47.1	
−1	43	34.1	13	20.6		52	29.6	4	30.8		48	31.0	8	23.5	
−2	21	16.7	15	23.8		36	20.5	0	0.0		29	18.7	7	20.6	
−3	11	8.7	4	6.4		15	8.5	0	0.0		12	7.7	3	8.8	
OAS (0-3)					<.0001					<.0001					.0002
1	67	53.2	15	23.8		82	46.6	0	0.0		75	48.4	7	20.6	
2	37	29.4	17	27.0		52	29.6	2	15.4		46	29.7	8	23.5	
3	22	17.5	31	49.2		42	23.9	11	84.6		34	21.9	19	55.9	

entity introduced in 2016 for borderline tumors [16], all surgical specimens in this cohort were re-reviewed, and 11 cases were reclassified as NIFTP. Previous histologic diagnosis of these cases was as follows: 6 PTCs, 4 adenomas, and 1 nodular hyperplasia. However, the rate of malignancy in this study is determined by the original histologic classification. By cytology, the NIFTP cases were categorized as follows: 6 AUS, 4 SFN and 1 SM. Eight of the 11 cases (73%) were positive for mutations with 5 *RAS* mutations, 1 *P53*, 1 *BRAF K601E* and 1 *PAX8/PPARg* translocation. The median CSS of NIFTP cases was 9 (mean: 9.6, range 5-13); OAS was 3 in 5 cases, 2 in 5 cases, and 1 in 1 case. These scores are closer to malignant than benign neoplasms.

4. Discussion

TBSRTC has standardized classification and management of thyroid nodules; however, cytomorphologic evaluation and interpretation remain subjective [6]. Reviewer bias of

Table 4 Performance of CCS and atypia score in determining the mutation status

	No mutation (n = 126)		Any mutation (n = 63)			No *BRAF* (n = 176)		*BRAF* (n = 13)			No *RAS* (n = 155)		*RAS* (n = 34)		
	n	Median (range)	n	Median (range)	*P*	n	Median (range)	n	Median (range)	*P*	n	Median (range)	n	Median (range)	*P*
CCS	126	6 (–2 to 16)	63	10 (2-18)	<.0001	176	7 (–2 to 16)	13	12 (8-18)	<.0001	155	7 (–2 to 18)	34	10.5 (3-15)	.0003
CCS cutoff 1 %					.0009					.0007					.005
<7	64	50.8	16	25.4		80	45.5	0	0.0		73	47.1	7	20.6	
7+	62	49.2	47	74.6		96	54.6	13	100.0		82	52.9	27	79.4	
CCS cutoff 2 %					<.0001					<.0001					<.0001
<10	107	84.9	31	49.2		136	77.3	2	15.4		124	80.0	14	41.2	
10+	19	15.1	32	50.8		40	22.7	11	84.6		31	20.0	20	58.8	
Atypia score %					<.0001					<.0001					<.0001
1 or 2	104	82.5	32	50.8		134	76.1	2	15.4		121	78.1	15	44.1	
3	22	17.5	31	49.2		42	23.9	11	84.6		34	21.9	19	55.9	

indeterminate nodules (AUS and FN) is concerning. Molecular testing offers an objective single result for determining malignancy risk and triage for diagnostic surgery for these intermediate groups [11-15,17]. At the same time, successful molecular testing often requires additional sampling and has added cost. Subclassification of the indeterminate cytologic categories by cytologic features provides another potential tool to determine malignancy risk.

This study semiquantitatively evaluated 9 cytomorphologic parameters. Among the individual cytomorphologic features analyzed, cellularity, nuclear atypia, nuclear enlargement, elongation, grooving, and extent of atypia showed statistically significant differences but could not individually distinguish across mutation/rearrangement status. A CCS cutoff of 10 provided the greatest discrimination for mutation detection based on ROC analysis. CCS >10 had high sensitivity and specificity for detecting mutations in general and even higher sensitivity (84.6%) and specificity (77.3%) for *BRAF* mutation in particular (Fig. 2). A high degree of correlation was also found for atypia score 3 and mutation presence (49.2%) versus atypia score 1 and absence of mutation (53.2%). Assessing the sensitivity and specificity of OAS along with CSS for detection of molecular aberrations enabled us to compare the performance of both subjective and objective approaches in parallel. OAS appears to be more sensitive (82.5% versus 50.8%), whereas CCS is more specific (84.9% versus 49.2%) in predicting molecular aberration status. If we hypothetically consider CCS of 10 or OAS of 3 as cutoff for recommendation

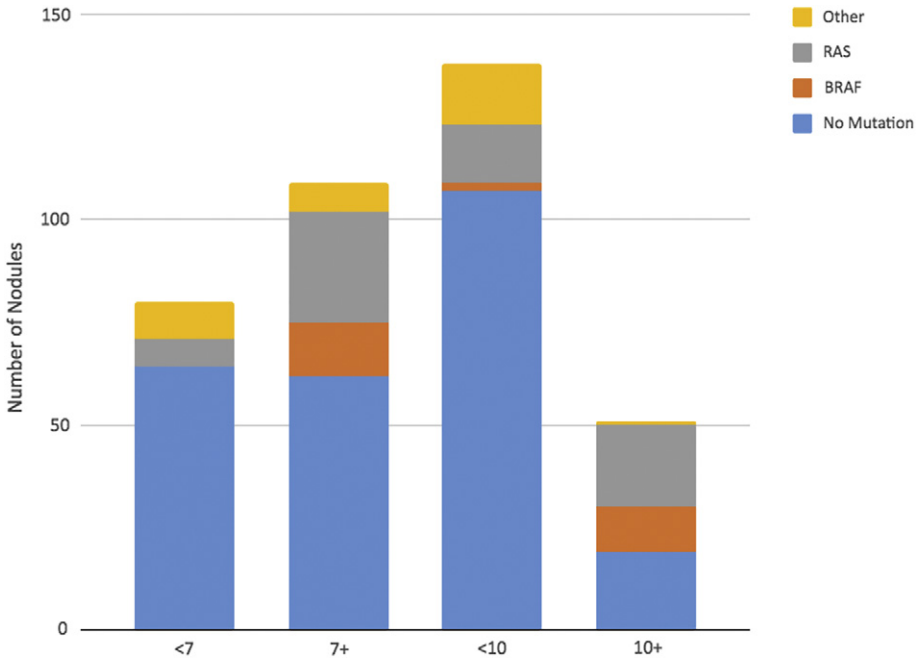

Fig. 1 Distribution of mutations using cumulative cytomorphologic score cutoffs of 7 and 10.

for surgery, equivalent to SFN or SM according to TBSRTC, it would result in 26 AUS cases to be upgraded with 2.2% reduction in AUS cases and 3.2% decrease in the rate of malignancy in the remaining cases (5% versus 8.2%) in this study cohort. A significantly higher rate of malignancy (23%) in the upgraded AUS cases reflects the usefulness of these scoring schemes in capturing high-risk nodules.

In addition to cytology-molecular correlation, histologic correlation was also made where histologic diagnosis was available and showed that both CCS and OAS were significantly higher in malignant neoplasm. A total of 5 nodules with final histologic diagnosis of malignancy failed to show any genetic aberration. Actually, 2 of these nodules were found to be medullary thyroid carcinoma, and *RET* gene was not included in the initial version of assay; therefore, part of this discrepancy can be attributed to tumors having mutations not targeted by the assay. Another reason could be inadequate sampling, a recognized pitfall in cytology, particularly the use of dedicated pass for molecular studies that is not subjected to rapid onsite cytologic evaluation for adequacy. More recent assays which retrieve material from a Diff Quik–stained slide known to contain diagnostic material improve the yield [18].

A few other studies have stratified malignancy risk using cytologic morphologic qualifiers. Renshaw found that the risk of malignancy was significantly different across subclasses of atypical follicular cell category (rule out papillary carcinoma versus rule out Hurthle cell neoplasm categories) [9]. Wu et al subclassified AUS as AUS-PTC and AUS-FN and found that it carried a higher risk of malignancy and neoplasm, respectively, than their other 2 described AUS subclasses [19]. Shrestha et al and Johnson et al used cytologic versus architectural atypia for risk stratification of AUS/FLUS group, showing higher malignancy rate in cases with cytologic atypia [20,21]. Correlation of cytologic findings with molecular results has also been reported by a few others [22,23]. Baca et al showed AUS subclassification on the basis of cytologic atypia alone or cytoarchitectural atypia correlated with suspicious Afirma GEC results [24]. Bellevicine et al demonstrated that *BRAF* and *RAS* mutations are associated with different AUS qualifiers (cytologic atypia, architectural atypia, and Hurthle cell changes) and therefore carry different risks of malignancy [25]. *RAS* mutations which are known to be associated with follicular pattern thyroid neoplasms [26] were also found to exhibit statistically significant association with microfollicle score in this study. A study by Rossi et al had shown high incidence of *BRAF* mutation in thyroid cytology specimens with at least focal plump cells with abundant eosinophilic cytoplasm [27]; in our study, 4 of 13 *BRAF*-positive cases had a high Hurthle cell score (although statistically not significant). *BRAF*-positive cases have been shown to be associated with higher incidence of tall cell variant, an aggressive type of PTC [28]; however, based on cytology-histology correlation, no case in our study was classified as tall cell variant.

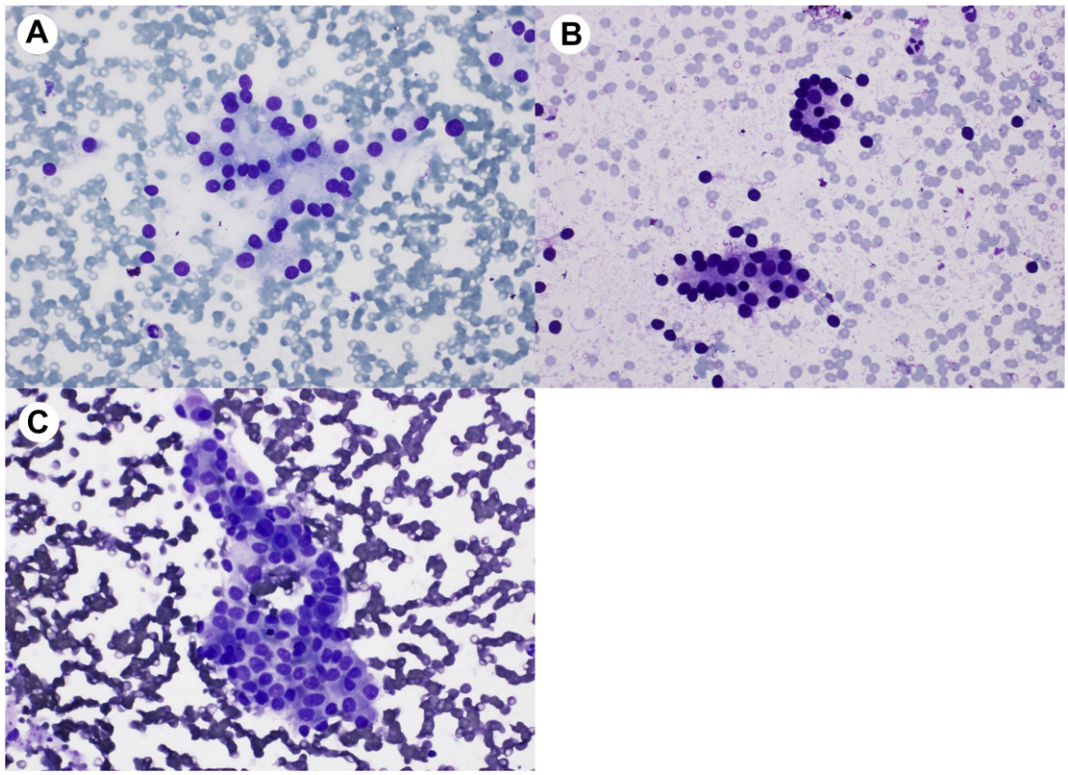

Fig. 2 Representative photomicrographs of thyroid FNA with AUS diagnosis and low and high CCS (Diff Quik, original magnification ×40). A, CCS of 6 with no mutation detected, nodular hyperplasia on histology. B, CCS of 10 with *NRAS* mutation, follicular adenoma on histology. C, CCS of 11 with BRAF mutation, papillary carcinoma on histology.

The rationale for using molecular data as a reference to compare with morphologic features is that, currently, it is probably the most objective method to access neoplastic nature of the lesion, which is also supported by strong evidence of association of molecular aberrations and malignant histologic diagnosis in this study. Furthermore, such an approach can also help optimally triage the samples, that is, whether the patient would benefit from ancillary mutational analysis that can provide crucial information for clinical decision making. This study does not advocate a laborious scoring system for routine use in thyroid cytology sign outs but emphasizes the importance of an objective approach backed by molecular data, which could reduce the number of indeterminate cases and avoid unnecessary workup and overtreatment.

This study included cases from a single academic center with a relatively modest number of indeterminate cytology cases that were subjected to molecular testing by 2 different test platforms (polymerase chain reaction–based mutational testing and NGS-based Thyroseq V1 and V2) which could be considered as potential limitations. Although the analytic performance of both platforms is comparable [13], Thyroseq V1 and V2 have higher clinical sensitivity because of its ability to detect more mutations [11-14]. However, it is interesting to note that most of the additional mutations (*TSHR, PTEN, ALK, GNAS*, and *P53*) detected by Thyroseq in our study were associated with benign nodules. Only 1 case in this study with *EIF1AX* mutation specific to Thyroseq V2 turned out to be malignant. Molecular testing in this study was only approved for cases with indeterminate cytologic diagnosis and therefore was not performed on benign TBSRTC category which could have provided further insight into the ability of CCS and OAS to differentiate between mutation-negative benign versus mutation-positive benign versus indeterminate benign cases with or without mutations.

In summary, the prevalence of mutation(s) in indeterminate TBSRTC category cases was associated with higher semiquantitatively determined cytomorphologic scores. These data suggest that the implementation of more objective scoring method may be used to refine and more precisely categorize indeterminate thyroid FNAs based on TBSRTC.

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