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Preoperative detection of *RAS* mutation may guide extent of thyroidectomy

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Abstract

Background—Preoperative detection of *RAS* mutations can contribute to cancer risk assessment in indeterminate thyroid nodules, although *RAS* is not always associated with malignancy.

Methods—Fine-needle aspiration samples classified in 1 of 3 indeterminate cytology categories were prospectively tested for *N*-, *H*-, and *K*-*RAS* mutations using next-generation sequencing assay.

Results—In the study, 93 patients with 94 nodules had preoperative *RAS* detected, of whom 86 patients had an operation (69% total thyroidectomy, 29% lobectomy). In total, 76% of *RAS*-positive nodules were malignant and follicular variant papillary thyroid cancer was the most common cancer type (83%). *HRAS* mutations had the greatest risk of cancer (92%) followed by *NRAS* (74%) and *KRAS* (64%; $P = .05$). No preoperative variables were associated with malignancy including age ($P = .07$), sex ($P = .49$), *RAS* isoform ($P = .05$), mutational allelic frequency ($P = .49$), nodule size ($P = .14$), cytology category ($P = .63$), or ultrasound bilaterality ($P = .24$), multifocality ($P = .23$), or presence of 1 suspicious feature ($P = .86$). Only 60% of patients with a unifocal nodule on ultrasound had single focus low-risk encapsulated follicular variant papillary thyroid cancer or benign disease.

Conclusion—Preoperative *RAS* mutation detection in thyroid nodules carries a substantial risk of cancer with a greater risk associated with *HRAS* and *NRAS*. Most *RAS* malignancies are follicular variant papillary thyroid cancer, which may inform the extent of operation.

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Thyroid cancer is the most common endocrine malignancy, and its reported incidence is on the rise.¹ Although thyroid nodules can be detected in up to 50% of otherwise healthy patients by age 60 years, only 5–15% of thyroid nodules are malignant.² Since its introduction in the 1970s, initial evaluation of thyroid nodules using ultrasound (US) and fine-needle aspiration (FNA) biopsy is well established and has decreased the number of patients requiring operation while increasing the likelihood of thyroid malignancy in patients who undergo operation.³ However, cytology remains unable to diagnose all thyroid malignancies preoperatively and for the ~20–25% of biopsy results that are indeterminate, operation is still often needed for histologic evaluation.

Activating mutations of *RAS* in thyroid epithelial cells were first reported in the 1980s, but only recently have been implicated directly as early and frequent events in transformation and proliferation of thyroid carcinoma.⁴ *RAS* represents the second most common genetic mutation identified in thyroid cancer and includes a family of 3 highly homologous isoforms: *NRAS*, *KRAS*, and *HRAS*.⁵ Point mutations in codons 12, 13, and 61 produce oncogenic alleles and cause loss of intrinsic GTPase activity, leading to constitutive activation of *RAS*-mediated MAPK and phosphatidylinositol 3-kinase/Akt (or protein kinase B) pathway signaling.⁶

The diagnostic and prognostic importance of *RAS* mutations is not fully defined because these mutations have been reported not only in thyroid cancers, but also in benign follicular adenomas (FA).⁷ In tumors of follicular cell origin, *RAS* mutations essentially are restricted to FA, follicular thyroid cancer (FTC), and follicular-variant papillary thyroid cancer (FVPTC), which are difficult to differentiate as benign or malignant based on cytology alone, and therefore often carry an indeterminate cytologic diagnosis on preoperative FNA biopsy.⁸ In a series of 67 prospectively identified *RAS*-positive thyroid nodules, cytology was malignant in 3%, benign in 3%, and indeterminate in 94%.⁹ Another large series inclusive only of nodules with indeterminate cytology found that using the 7-gene panel *RAS* was the most common mutation detected (72%), and when *RAS* was detected preoperatively, the probability of malignancy was 85%.¹⁰ Most *RAS* mutations have correlated to histologically low-grade FVPTC, but they also occur in other cancer types including FTC, poorly differentiated, and anaplastic thyroid cancer, and even medullary thyroid cancer.^{9,11,12}

Recently, next-generation sequencing (NGS) has been introduced as a sensitive and reliable method for detecting *RAS* and other mutations in thyroid FNA samples.^{13,14} In this study, we evaluated a large series of cytologically indeterminate thyroid nodules, which tested positive for *RAS* mutations preoperatively by NGS with the aim of identifying variables associated with histologic outcome that may be used help guide the extent of initial thyroidectomy.

MATERIALS AND METHODS

Study cohort

FNA biopsy results classified in 1 of 3 indeterminate cytology categories: atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS),

follicular neoplasm (FN), and suspicious for malignancy (SUSP) were tested prospectively for *N*-, *H*-, and *K-RAS* mutations as part of routine clinical practice since 2008.¹⁵ After University of Pittsburgh Medical Center Quality Improvement Institutional Review Board approval (QIIRB#441), we retrieved and examined the records associated with all nodules with indeterminate cytology and with a positive *RAS* mutation from September 2013 to June 2015. Clinicopathologic data were retrospectively assessed for each case. Radiology reports and images were reviewed. US bilaterality was defined as the presence of 1 nodule of any size in each lobe, while US multifocality was defined as the presence of >1 nodule of any size. The presence of 1 of the following established sonographic features was considered suspicious: marked hypoechogenicity, loss of echogenic halo, irregular border, microcalcifications, and taller-than-wide shape. All patients in the present study have not been previously described.

Mutational analysis using next-generation sequencing

At the time of initial ultrasound-guided FNA biopsy and after preparation of cytology slides, a portion of the specimen was collected for potential molecular analysis and stored at -20°C . For cytologically indeterminate specimens, nucleic acids were isolated using the Compact MagNA Pure Instrument (Roche Molecular Biochemicals, Indianapolis, IN). Samples were tested using NGS on the Ion Torrent PGM or Ion Proton (Life Technologies, Carlsbad, CA) and the ThyroSeq panel, as previously described.¹⁴ The analytic sensitivity of the assay was ~3% mutant alleles, but the clinical sensitivity was set at mutational allelic frequency (AF) 10% (eg, 10% of tested cells contain a mutant allele).¹⁶ Mutation detection was defined as “low level” when mutational AF was <10%.

Statistical analysis

Statistical analysis was performed at a 95% confidence interval by using χ^2 for categorical data, unpaired *t* test for comparison between means, Mann-Whitney *U* test for unmatched, nonparametric continuous data. All analyses were performed using SPSS statistical software (version 22; SPSS Inc, Chicago, IL).

RESULTS

During the study interval, 93 patients with available clinical records had 94 *RAS*-positive nodules. There were approximately 2,590 patients with indeterminate cytology that had molecular testing performed during the study period and the rate of *RAS*-positive nodules was 3.6%. The *RAS* mutation was identified at a low AF of <10% in 23/94 nodules (24%) and at a high AF of 10% in 71 (76%) nodules. Overall, *NRAS* mutations were most common and were found in 46 (49%) nodules with the majority occurring in codon 61 ($n = 45$). *HRAS* mutations were identified in 28 (30%) nodules of which 24 were in codon 61. *KRAS* mutations were detected in 20 (21%) nodules and 12 were in codon 12/13. Histology was unavailable for 7 patients who elected for active surveillance (mean follow-up 0.9 months, range 0–24 months). These patients either declined operation or had medical comorbidities that precluded operation. Of the remaining 86 patients with 87 nodules, 59 (69%) had total thyroidectomy, 25 (29%) had thyroid lobectomy, and 2 had completion thyroidectomy after initial lobectomy performed 15 and 1.5 years prior (Fig).

Malignancy was diagnosed in 66/87 (76%) of *RAS*-positive resected nodules. The greatest risk of malignancy was associated with *HRAS*-positive nodules (92%) followed by *NRAS* (74%) and *KRAS* (64%, $P = .05$). In nodules with a low mutational AF, the risk of malignancy was equivalent to nodules with a high mutational AF (67% vs 78%, $P = .49$). Additional codons tested with NGS that were not previously tested were *HRAS* codon 12/13, *KRAS* codon 61, and *NRAS* codon 12/13. In total, 11/87 (12.6%) nodules had a detected mutation in one of these additional *RAS* codons including 3 nodules with *HRAS* codon 13, 7 with *KRAS* codon 61, and 1 with an *NRAS* codon 12 mutation. In these 11 nodules, 8 were malignant and the majority of these were *KRAS* codon 61-positive (6, 75%).

The rates of malignancy in the *RAS*-positive nodules were similar in all 3 indeterminate cytology categories (Table I, $P = .63$). The most common cancer type was FVPTC ($n = 55$, 83%), of which 49/55 (89%) were encapsulated. Other *RAS* cancer types included classic variant PTC ($n = 8$, 12%) and poorly differentiated/anaplastic thyroid cancer ($n = 3$, 5%; Table II). Of the 3 patients with poorly differentiated/anaplastic thyroid carcinoma, 1 had preoperative computed tomography with evidence of tracheal invasion, and 1 presented with a rapidly enlarging thyroid and cervical lymphadenopathy. The third patient had a mildly hypoechoic nodule without suspicious features on US. The 2 poorly differentiated thyroid cancers were *NRAS*- and *HRAS*-positive with mutational AF >40%, and 1 had a coexistent *TERT* promoter mutation. The anaplastic thyroid cancer was *NRAS*-positive, had a mutational allelic frequency of 15%, and mutations in *TERT*, *EIF1AX*, and *p53* also were identified.

The demographic and preoperative factors for the 86 patients with 87 *RAS*-positive nodules are shown in Table III. On univariable analysis, age ($P = .07$), sex ($P = .49$), *RAS* isoform ($P = .05$), allelic frequency ($P = .49$), nodule size ($P = .14$), or cytology category ($P = .63$) were not predictive of malignancy. No US feature was predictive of *RAS* malignancy including bilaterality ($P = .24$), multifocality ($P = .23$), or presence of 1 suspicious US feature ($P = .86$; Table III).

We examined additional histologic outcomes to assess appropriate extent of initial operation. In 59 patients who had total thyroidectomy, bilateral cancers were diagnosed in 17 (29%) patients. A single focus of *RAS*-positive low-risk encapsulated FVPTC or benign disease on final operative pathology was present histologically in the remaining 28/59 (47%) patients. Among the 25 patients who had lobectomy, no additional operation was needed for 15 (60%) who had either benign disease or low-risk encapsulated FVPTC (Fig). Overall, when considering the 30 patients with a unilateral nodule on preoperative US who ideally would have been a candidate for initial lobectomy, 18 (60%) had histologic single focus low-risk encapsulated FVPTC ($n = 12$, 67%) or benign disease ($n = 6$, 33%), and lobectomy alone would have been sufficient extent of operation.

One patient with *NRAS*-positive anaplastic thyroid carcinoma developed multisystem organ failure and was transitioned to comfort care on postoperative day 3. Of the remaining 65 patients with *RAS*-associated malignancy, 14 (22%) received radioiodine ablation, including 11 who had total thyroidectomy and 3 who had lobectomy followed by completion

thyroidectomy. On short-term follow-up of 6–24 months, there have been no recurrences to date in these 65 patients.

The present series is not large, and we were interested in the cumulative malignancy rate of *RAS* lesions since 2008.¹⁵ The compiled histology for all consecutive patients ($n = 278$) from September 2008 to June 2015 who had both preoperative indeterminate FNA biopsy results and a *RAS* mutation was calculated and may be seen in Table IV.¹⁵ *RAS* testing was performed using 2 different techniques including real-time polymerase chain reaction and fluorescence melting curve analysis ($n = 191$ nodules) and next-generation sequencing as discussed above ($n = 87$ nodules). Overall, during an 8-year period of observation, any *RAS* mutation with indeterminate cytology had an 81% risk of malignancy. In this cumulative series, the risk of malignancy differs significantly by isoform and was greatest in *HRAS*-positive nodules (94%), followed by *NRAS*-positive nodules (83%), and *KRAS*-positive nodules (50%, $P < .001$). Although FVPTC was again the most common histologic variant, poorly differentiated/anaplastic cancers were seen with each of the 3 *RAS* isoforms. Indeterminate nodules with *NRAS* and *HRAS* mutation were associated with similar types of histologic cancer ($P = .3$), but both differed from *KRAS*-associated cancers ($P = .002$). In addition, *KRAS*-associated cancers were more likely to be classic PTC (15%) and FTC (15%). Poorly differentiated/anaplastic cancers were rare as expected ($n = 4$), and were seen with *NRAS* and *HRAS* mutations while 2 medullary thyroid cancers were associated only with *KRAS* and *HRAS* mutations (Table IV).

DISCUSSION

Use of *RAS* testing for preoperative risk stratification of nodules with indeterminate FNA biopsy results is controversial. Because *RAS* is also found in benign follicular adenomas, its ability to predict malignancy preoperatively is sometimes inappropriately discounted. In the present series, preoperative detection of any *RAS* mutation with indeterminate cytology was associated with a high (76%) risk of histologic cancer, which is equivalent to the risk of cancer associated with a SUSP cytology result.

As previously reported, we also observed that *RAS*-positive follicular tumors represent a continuum from benign FA to poorly differentiated/ATC.⁹ *RAS* is present at all stages of tumor differentiation, which is not a characteristic feature of other molecular alterations.^{17,18} Prior studies have supported the role of *RAS* mutation as an early transformative event associated with step-wise tumorigenesis leading from adenoma to carcinoma.¹⁸ This histologic continuum also suggests that *RAS* mutations may be critical for the development of invasive histology and, rarely, further progression to ATC.^{19,20} Thus, although 24% of *RAS*-positive nodules in this report were histologically benign FA, the presence of a known oncogenic mutation may have predisposed these nodules to progress to invasive tumors and, in some rare instances, to dedifferentiated, aggressive tumors.

As the present data demonstrate, although all *RAS*-positive indeterminate nodules should be considered for operative resection, surveillance of low-risk nodules could potentially be considered, given identifiable predictors. We thus sought to identify preoperative risk factors that may be used to differentiate benign from malignant *RAS*-positive indeterminate

nodules. Younger age was associated with malignancy, although this did not reach statistical significance ($P = .07$). Currently, no preoperative variable seems to be associated with *RAS* malignancy including male sex, mutational allelic frequency, nodule size, US features, or cytology category. The sonographic characteristics that have been described to be suspicious typically are identified in PTC and rarely seen in FVPTC.²¹ We previously reported that the negative predictive value of having at least 1 suspicious US feature, including intranodular hypervascularity, was low⁹ and again observed here that US is limited at differentiating malignant from benign *RAS*-positive nodules.

Interestingly, *RAS*-positive nodules with SUSP cytology had an 83% risk of cancer, which was equivalent to the risk seen in the AUS/FLUS cytology category (78%). Low cellular yield on FNA biopsy is a common clinical concern and in the absence of molecular testing, a repeated AUS/FLUS cytology result is often an indication for operation. We previously had set the clinically significant mutational AF threshold at 10% (ie, 10% of cells contain a mutant allele).²² However, for *RAS* detected at <10% mutational AF, we observed a malignancy rate that was equivalent to detection at >10% mutational AF. It is possible that the significance of detecting a *RAS* mutation at a low allelic frequency depends also on tumor cellularity, and additional study is needed to determine if low level *RAS* mutation has a reliably equivalent high risk of cancer in the different categories of indeterminate cytology.

We previously found that the likelihood of malignancy was greatest with *HRAS* (95.5%), compared with *NRAS* (86.8%) and *KRAS* (41.7%) (15). In the current study, we confirm that high-risk pattern (*HRAS* 92%, *NRAS* 74%, *KRAS* 61%) (15). With NGS, we have been able to expand the number of *RAS* codons being tested, which here allowed detection of an additional 8/66 (12%) thyroid cancers that would otherwise have been missed (thus potentially altering the relative rates for *RAS* isoforms). With a cumulative experience using the 2 different testing methodologies, but now inclusive of 278 nodules with preoperative *RAS* and indeterminate cytology, we can conclude that different *RAS* isoforms are indeed associated with varying rates of malignancy (*HRAS* > *NRAS* > *KRAS*). Furthermore, although *NRAS* and *HRAS*-positive nodules have a high (86–88%) likelihood of being associated with FVPTC, *KRAS*-positive nodules have a lower rate of association with FVPTC (65%) and also can be seen with other cancer types including classic PTC (15%) and FTC (15%; Table IV).

With a better understanding of what cancer phenotypes are associated with the different *RAS* isoforms, preoperative nodule risk stratification could be further improved. In the 2015 American Thyroid Association Guidelines, the recommendations for extent of initial thyroidectomy for a known thyroid cancer 1–4 cm include options for both lobectomy or total thyroidectomy, with or without central compartment lymph node dissection.²³ As observed here, because *NRAS* and *HRAS* indeterminate nodules are likely to be FVPTC with no observed instances of central compartment lymph node metastasis, lobectomy alone may be sufficient in the absence of other indications for total thyroidectomy. For the 30 study patients with unilateral *RAS*-positive nodules who potentially would have been candidates for lobectomy by preoperative features, lobectomy was indeed adequate operation based on histologic findings in most (60%). Initial lobectomy will likely be adequate for an expanding subset of *RAS*-positive thyroid cancer patients in the future.

Initial total thyroidectomy followed by radioactive iodine ablation is still considered the optimal treatment for patients with a high likelihood of recurrence.²³ In a study by Gupta et al,⁹ bilateral cancer was diagnosed in 43% of patients with *RAS*-positive malignancy. In this subsequent series, we observed a lower rate of 29% for patients who had total thyroidectomy. Although nodules that were *KRAS*-positive had a lower rate of malignancy overall, we observed that the *KRAS* cancers also seemed to be associated with clinically significant cancers, ie, to be FTC and/or to have extrathyroidal extension or lymph node involvement. The number of *KRAS*-positive malignancies is still low and ongoing prospective analysis of the *KRAS* subset is needed. *RAS* mutations associated with poorly differentiated, anaplastic, and medullary thyroid cancers are uncommon, but this diagnosis should still be considered regardless of which *RAS* isoform is detected if the clinical history, cytology, and imaging features are consistent with an aggressive subtype.

The limitations of this study include its nature as a single-center experience that cannot account for geographic variations in incidences of thyroid cancer or thyroid cancer subtypes. In addition, our anatomic pathologists were not formally blinded to cytologic or molecular results, which could introduce bias; similarly, the reporting of imaging features can be subject to interobserver variability, and we did not examine if the presence of suspicious sonographic features was another preoperative factor that may have influenced the decision for biopsy or operation. In addition, this series was limited to nodules with operative treatment, which may have contributed to referral bias. The long-term outcome of *RAS*-positive nodules that are being managed nonoperatively remains unknown.

In summary, detection of a *RAS* mutation in a cytologically indeterminate thyroid nodule carries a high (76–81%) risk of malignancy. The risk of cancer remains high even if *RAS* is detected at a low level. Histologic malignancies associated with preoperative *RAS* usually are encapsulated FVPTC without central compartment lymph node involvement or extrathyroidal extension (74%), but can also be classic or poor-prognosis PTC. The majority (60%) of patients with *RAS*-positive unifocal nodules on preoperative imaging may be adequately treated by initial lobectomy.

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Fig.
Extent of operation and treatment for the patient cohort.

Table I*RAS* malignancy rates within the indeterminate cytology categories

	AUS/FLUS <i>N</i> = 51	FN <i>N</i> = 30	SUSP <i>N</i> = 6	Overall <i>N</i> = 87
<i>HRAS</i>	16/17 (94%)	7/8 (88%)	0	23/25 (92%)
<i>KRAS</i>	8/12 (67%)	2/4 (50%)	1/2 (50%)	11/18 (61%)
<i>NRAS</i>	16/22 (73%)	12/18 (67%)	4/4 (100%)	32/44 (74%)
Malignancy rate	40 (78%)	21 (70%)	5 (83%)	66 (76%)

Table IIHistologic features of *RAS* thyroid malignancies

	NRAS <i>N</i> = 32	HRAS <i>N</i> = 23	KRAS <i>N</i> = 11
Thyroid cancer type			
FV-PTC: encapsulated	24 (75%)	18 (78%)	7 (64%)
FV-PTC: nonencapsulated/infiltration	1 (3%)	3 (13%)	2 (18%)
Classic PTC	5 (16%)	1 (4%)	2 (18%)
Poorly differentiated	1 (3%)	1 (4%)	0
Anaplastic	1 (3%)	0	0
Multifocality	13 (43%)	6 (26%)	4 (37%)
Bilateral cancer, no./no. who had TT (%) [*]	9/29 (31%)	5/19 (26%)	3/11 (27%)
No. with CCLN assessed	21	12	8
No. CCLN positive	0	0	1
Extrathyroidal extension	0	0	2

^{*} Includes only 59 patients who had total thyroidectomy.

CCLN, Central compartment lymph nodes; *FV-PTC*, follicular variant papillary thyroid carcinoma; *PTC*, papillary thyroid carcinoma; *TT*, total thyroidectomy.

Table IIIComparison of features in benign and malignant *RAS*-positive nodules

	Benign	Malignant	<i>P</i> value
Patients, <i>n</i> [*]	21	65	
Nodules, <i>n</i>	21	66	
Age, y [*]			
Mean (range)	57 ± 24	49 ± 13.6	.07
Men, <i>n</i> [*]	3 (14%)	13 (21%)	.49
Ultrasound characteristics			
Nodule size, cm			
Mean (range)	2.0 ± 0.84	2.6 ± 1.9	.14
Bilaterality, <i>n</i>	10 (48%)	40 (62%)	.24
Multifocality	10 (48%)	49 (75%)	.23
(>1 nodule), <i>n</i>			
1 Suspicious feature, <i>n</i> [†]	6 (29%)	18 (28%)	.86
Indeterminate cytology			.63
AUS/FLUS	11 (52%)	40 (61%)	
FN	9 (43%)	21 (30%)	
SUSP	1 (5%)	5 (7.6%)	
<i>RAS</i> isoform			.05
<i>NRAS</i>	12 (57%)	32 (48%)	
<i>HRAS</i>	2 (9.5%)	23 (35%)	
<i>KRAS</i>	7 (33%)	11 (17%)	
Mutational allelic frequency			
<10%	6 (29%)	14 (21%)	.49
10%	15 (71%)	52 (79%)	

^{*} Denominator includes the number of patients.

[†] Suspicious ultrasound features include marked hypoechogenicity, loss of echogenic halo, irregular border, microcalcifications, and taller-than-wide shape.

Table IV

Cumulative experience^{*} with preoperative *RAS* testing in nodules with indeterminate cytology ($N=278$ nodules)¹⁵

	NRAS <i>N</i> = 172	HRAS <i>N</i> = 66	KRAS <i>N</i> = 40	<i>P</i> value
Overall malignancy rate	142 (83%)	62 (94%)	20 (50%)	<.001
Cytology category				NS
AUS/FLUS	57 (77%)	34 (96%)	8 (44%)	
FN	65 (84%)	27 (90%)	11 (41%)	
SUSP	20 (95%)	1 (100%)	1 (50%)	
Cancer type				.008
FVPTC	125 (86%)	56 (88%)	13 (65%)	
Classic PTC	12 (8.2%)	2 (3%)	3 (15%)	
FTC	2 (1.4%)	2 (3%)	3 (15%)	
Poorly differentiated/anaplastic	3 (2%)	1 (1.6%)	0	
MTC	0	1 (1.5%)	1 (5%)	

* Testing modalities included real-time polymerase chain reaction and fluorescence melting curve analysis ($n=191$ nodules) and next-generation sequencing ($n=87$ nodules).

AC, Anaplastic carcinoma; FC, follicular carcinoma; FV-PTC, follicular variant papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; NS, not significant; PTC, papillary thyroid carcinoma.