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Relevance of *BRAF*^{V600E} Mutation Testing Versus *RAS* Point Mutations and *RET/PTC* Rearrangements Evaluation in the Diagnosis of Thyroid Cancer

Martina Rossi,[1,2] Mattia Buratto,[1] Federico Tagliati,[1] Roberta Rossi,[1,2] Sabrina Lupo,[1,2] Giorgio Trasforini,[2] Giovanni Lanza,[3] Paola Franceschetti,[2] Stefania Bruni,[2] Ettore degli Uberti,[1,2] and Maria Chiara Zatelli[1,2]

Background: A molecular profile including *BRAF* and *RAS* mutations as well as *RET/PTC* rearrangement evaluation has been proposed to provide an accurate presurgical assessment of thyroid nodules and to reduce the number of unnecessary diagnostic surgeries, sparing patients' health and saving healthcare resources. However, the application of such molecular analyses may provide different results among different centers and populations in real-life settings. Our aims were to evaluate the diagnostic utility of assessing the presence of *BRAF* and *RAS* mutations and *RET/PTC1* and *RET/PTC3* rearrangements in all cytological categories in an Italian group of thyroid nodule patients assessed prospectively, and to understand whether and which mutation testing might be helpful in cytologically indeterminate nodules.
Methods: A total of 911 patients were submitted to ultrasound and fine-needle aspiration biopsy examination. Cytological evaluation was performed in parallel with molecular testing and compared to pathological results in 940 thyroid nodules, including 140 indeterminate lesions.
Results: *BRAF* mutation testing provided the best contribution to cancer diagnosis, allowing the disease to be detected at an early stage, and identifying indeterminate nodules in which diagnostic lobectomy could be spared. On the contrary, *RAS* and *RET/PTC* analysis did not further increase diagnostic sensitivity for thyroid cancer. In addition, we found *RET/PTC* rearrangements in benign lesions, indicating that this molecular marker might not be useful for the detection of thyroid cancer.
Conclusion: *BRAF*^{V600E} mutation analysis is superior to *RAS* point mutations and evaluation of *RET/PTC* rearrangements in the diagnosis of thyroid cancer, even in indeterminate lesions.

Introduction

The diagnostic and therapeutic approach to thyroid cancer has been highly debated in recent years. Ultrasound (US), cytology, and molecular profiling (by mRNA gene expression platforms, protein immunocytochemistry, miRNA panels, and screening for somatic mutations. including *BRAF*^{V600E} and *RAS* mutations, as well as *RET/PTC1*, *RET/PTC3*, *PAX8/PPARγ*, *TK*, and *ALK* rearrangements) have been employed in order to provide the most accurate presurgical assessment of thyroid nodules with the aim of increasing the sensitivity for cancer detection and avoiding surgery for lesions erroneously identified as malignant (1–3). The availability of presurgical information can improve preoperative risk stratification and often influences the extent of surgery (4–7). The revised American Thyroid Association (ATA) guidelines indicate that thyroid cancer should be treated according to risk stratification, assessed based on disease stage (8). The provided evidence indicates that treatment needs to be tailored according to the risk of recurrence, suggesting that a more conservative attitude, avoiding radioiodine ablation, may be indicated for patients with very low risk of recurrence (9,10). As a consequence, early diagnosis is crucial in order to detect the disease at an early stage and to guide the patient to a less aggressive treatment, thereby avoiding unnecessary risks for the patient's health and saving healthcare resources (11,12). The main diagnostic tool is fine-needle aspiration biopsy (FNAB). However, FNAB cannot provide a definitive diagnosis in cases with nondiagnostic (ND) or indeterminate cytology. The latter may represent a malignant lesion in ~20% of the cases, which are not accurately predictable by US risk factors

[1]Section of Endocrinology and Internal Medicine, Department of Medical Sciences, University of Ferrara, Ferrara, Italy.
[2]Endocrinology Unit, Azienda Ospedaliero-Universitaria di Ferrara, Ferrara, Italy.
[3]Section of Pathology and Biomolecular Diagnostics, Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy.

and thus lead to the need for diagnostic surgery (13). The preoperative use of molecular markers is still highly debated because, among other reason, the incidence of mutations in the different categories outlined in the Bethesda System for Reporting Thyroid Cytopathology (BSRTC) (14) is still unknown. To date, the ATA guidelines suggest considering molecular testing only to refine a cytological indeterminate result (8). Moreover, genetic, environmental, and clinical background may profoundly impact the incidence of mutations, and hence there is a need to explore the applicability of molecular testing of thyroid nodules in different populations in the clinical setting. The aim of our study was to evaluate the diagnostic utility of assessing the presence of three previously employed thyroid cancer molecular markers—*BRAF* and *RAS* mutations, and *RET/PTC1* and *RET/PTC3* rearrangements—in FNAB material from all cytological categories in a real-life context involving a group of Italian thyroid nodule patients in order to improve patient management and surgical treatment. In addition, we aimed to assess mutation incidence in each Bethesda category and to understand whether and which mutation testing might be helpful in indeterminate nodules.

We therefore assessed the feasibility of obtaining reliable results from FNAB material for the search for these molecular markers (*BRAF*^{V600E}, *RAS* mutations, and *RET/PTC* rearrangements) in daily clinical practice employing previously reported methods with slight modifications.

Materials and Methods

Subjects

From January 2007 to July 2013, 6500 thyroid nodules from 5800 patients underwent FNAB at the Section of Endocrinology of the University of Ferrara. Among these, 940 FNAB specimens from 911 consecutive patients, displaying at least two clinical and/or US characteristics of suspected malignancy, prospectively underwent evaluation for somatic mutations, including *BRAF*^{V600E} and *RAS* point mutations and *RET/PTC1* and *RET/PTC3* rearrangements, partially overlapping the approach described previously by Nikiforov *et al*. (15). Patients gave written informed consent for molecular analysis and data collection.

Medical and US examination

All 911 patients recruited in this study were submitted to a careful US examination by a single operator (S.L.) during routine medical care. The collected US features included nodule size ($<$ or $>$ 1 cm), structure (solid, mixed, or cystic), echogenicity (iso-, hypo-, or hyperechoic), presence or absence of micro calcifications, and margins. In addition, the patients' clinical information regarding age, sex, family history of thyroid cancer, or history of previous external beam radiation exposure was collected.

FNAB procedures

All 940 US-guided FNAB procedures were performed by two experienced endocrinologists (G.T and P.F.) using a standardized protocol, as previously described (16). Cytological evaluation was performed in parallel with molecular testing. All FNAB results were categorized according to the BSRTC (14), including class III (atypia of undetermined significance/follicular lesion of undetermined significance [AUS/FLUS]), IV (follicular neoplasm or suspicious for a follicular neoplasm [FN]), and V (suspicious of malignancy [SM]) categories in the group of indeterminate lesions.

DNA and RNA isolation

FNAB material from a needle pass through the nodule was used for cytology, and a second pass was collected in 5 mL of RNA Later solution (Resnova) for molecular analysis. Genomic DNA for *BRAF* and *RAS* somatic mutation analysis was obtained as previously described (16,17). Total RNA isolation for *RET/PTC1* and *RET/PTC3* rearrangements evaluation was performed by centrifuging 2 mL of FNAB sample for 5 minutes at 5000*g*, and the pellet was then suspended in 350 μL of RLT Lysis Buffer (Qiagen). Later, the samples were processed in the QIAcube instrument (Qiagen) using the RNeasy micro kit (Qiagen) according to manufacturer's protocol, obtaining 30 μL of purified total RNA. Samples were then processed as described below. All samples displaying a genetic variation were tested in a second assay by a different technician.

BRAF *and* RAS *mutation analysis*

BRAF^{V600E} mutation analysis was performed as previously described (16,17), employing a well-established methodology.

A first evaluation of *RAS* mutations was performed by applying real-time polymerase chain reaction (PCR) amplification followed by high resolution melting (HRM) analysis. Amplification of *RAS* gene targets (codon 12, 13, and 61 of *N-RAS*, *H-RAS*, and *K-RAS* gene isoforms) was performed by using the MeltDoctor HRM Mastermix (Life Technologies) and specific primers (*N-RAS* exon 2 FOR 5′-TTG CTG GTG TGA AAT GAC TGA GT-3′ and REV 5′-TAG CTG GAT TGT CAG TGC GC-3′; *N-RAS* exon 3 FOR: 5′-CAG AAA ACA AGT GGT TAT AGA TGG TGA-3′ and REV 5′-CAA ATA CAC AGA GGA AGC CTT CG-3′; *H-RAS* exon 2 FOR: 5′-GGA GCG ATG ACG GAA TAT AAG C-3′ and REV 5′-GTA TTC GTC CAC AAA ATG GTT CTG-3′; *H-RAS* exon 3 FOR 5′-GGA AGC AGG TGG TCA TTG ATG-3′ and REV 5′-GCA TGT ACT GGT CCC GCA T-3′; *K-RAS* exon 2: FOR 5′-TCA CAT TTT CAT TAT TTT TAT TAT AAG GC-3′ and REV 5′-GAT TCT GAA TTA GCT GTA TCG TCA AG-3′; *K-RAS* exon 3: FOR 5′-TCC AGA CTG TGT TTC TCC CTT C-3′ and REV 5′-TAC ACA AAG AAA GCC CTC CC-3′). Mutated samples were then genotyped by direct sequencing using the same primers on the 3130 Genetic Analyzer (Life Technologies) employing the Ready Reaction Cycle Sequencing 1.1 mix (Life Technologies). This approach, which is very similar to that previously employed (18,19), allowed reliable results to be obtained from FNAB material with a turnaround time of 72 hours.

RET/PTC *rearrangement analysis*

For the evaluation of *RET/PTC1* and *RET/PTC3* rearrangements, total RNA from FNAB samples was analyzed by One Step Real Time RT-PCR, performed on a 7900 HT Real Time System (Life Technologies), employing a modified method compared to Nikiforov *et al*. (15). The presence of *RET/PTC1* and *RET/PTC3* rearrangements has been

assessed using two different custom Taqman Gene Expression assays (Life Technologies), each represented by a rearrangement specific primer-probe set; probes have been designed centered on the rearrangement site, in order to avoid false positive results. Sequences of primers and probes for *RET/PTC1* were: FOR: 5′-CGC GAC CTG CGC AAA-3′, REV 5-CAA GTT CTT CCG AGG GAA TTC C-3′, and PROBE: 5′-FAM-CCA GCG TGA CCA TCG AGG ATC CAA AGT-NFQ-3′. Sequences of primers and probes for RET/PTC3 were: FOR: 5′-CCC CAG GAC TGG CTT ACC C-3′, REV 5′-CAA GTT CTT CCG AGG GAA TTC C-3′ and PROBE: 5′-FAM-AAA GCA GAC CTT GGA GAA CAG TCA GGA GG-NFQ-3′. All runs were multiplexed with Eukaryotic 18S rRNA Endogenous Control (Life Technologies). The reaction mix included iScript One-Step RT-PCR Kit for probes (Bio-Rad) and the appropriate Taqman assays, described above. To test the method sensitivity, each target sequence assay was diluted 1:10, 1:100, 1:1000, and 1:10,000 in not-rearranged cDNA. Both rearrangements were correctly identified up to a 1:1000 dilution by the employed method. To exclude the possibility of cross-reactions, *RET/PTC1* and *RET/PTC3* assays were employed to amplify *RET/PTC3* and *RET/PTC1* targets respectively, and no signal was obtained. RNA from one or more tumors or cell lines known to carry a particular rearrangement was used as a positive control. This approach allowed obtaining reliable results from FNAB material with a turnaround time of 24 hours.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each detection method and for combined methods, considering histology as the gold standard. Statistical analysis was carried out using the R Software package v3.0.2 (R Foundation for Statistical Computing). The chi-square test (with Yates continuity correction) was employed to compare the diagnostic sensitivity of cytology with that observed performing both cytology and genetic analysis and to assess the presence of a significant association between the presence of each mutation and US features. A p-value of < 0.05 was considered significant in all tests.

Results

Patient findings

Among the 911 patients who participated in the study, 51 had a family history of thyroid cancer, 712 were female, and the mean age was 59 ± 0.46 years (range 25–81 years). Patients with BSRTC class V and VI lesions, or with a nodule displaying $BRAF^{V600E}$ mutation (independently of cytology results), or with large goiters underwent total thyroidectomy (TT). Patients with repeatedly class I cytology and patients with BSRTC class IV lesions or with a nodule displaying either *RAS* mutations or *RET/PTC* rearrangements underwent lobectomy (LT), independently of US nodule features, in line with the previously demonstrated increased cancer risk associated with these mutations (18). Patients with class III lesions without a genetic variation in the studied genes underwent a second FNAB and then underwent lobectomy if the cytological diagnosis was confirmed. Otherwise, the patients were managed according to the new BSRTC class. Finally, patients with class II lesions underwent clinical follow-up.

Cytology, molecular testing, US, and pathology findings

Cytological results and genetic alteration frequencies are displayed according to BSRTC classes in Table 1. Among 940 FNAB, 134 displayed at least one mutation (14.2%), specifically a $BRAF^{V600E}$ mutation in 4.2% of all nodules, *RAS* mutations in 3.4% (25 at *N-RAS* codon 61, one at *H-RAS* codon 13, one at *H-RAS* codon 61, two at *K-RAS* codon 12, one at *K-RAS* codon 13, two at *K-RAS* codon 61), and *RET/PTC* rearrangements in 7.3% (3.9% *RET/PTC1* and 3.4% *RET/PTC3*). The highest incidence of *RAS* mutations was found within BSRTC class III and class VI samples, while the highest incidence of *RET/PTC* rearrangements was found among BSRTC class I samples (of which about 30% was operated on and had a benign histology) and among BSRTC class III and VI samples (Table 1).

The presence of a $BRAF^{V600E}$ mutation was significantly associated ($p < 0.01$) with hypoechogenicity, microcalcifications, and a diameter < 1 cm. *RAS* mutations were significantly ($p < 0.01$) associated with isoechogenicity and a diameter > 1 cm. *RET/PTC3* rearrangements were significantly ($p < 0.01$) associated with isoechogenicity on US.

Overall, 72 patients underwent TT, and 45 patients underwent LT, which was completed in five patients (11.1% of LT), for a total of 117 operated patients. Among these, 62 patients (52.1%) had an indeterminate lesion on cytology: 23 AUS/FLUS (class III), 17 FN (class IV), and 22 SM (class V).

Table 1. Genetic Alterations and Their Frequencies in Each Bethesda System for Reporting Thyroid Cytopathology Class Nodules

Genetic alteration (n)	*BSRTC classes*						
	I	*II*	*III*	*IV*	*V*	*VI*	*Total*
$BRAF^{V600E}$	4	3	7	4	6	10	34
BRAF and *RET/PTC 1*	0	0	2	0	1	1	4
BRAF and *RET/PTC 3*	0	0	0	0	1	1	2
RAS	1	21	4	2	1	2	31
RAS and *RET/PTC 3*	0	1	0	0	0	0	1
RET/PTC-1	2	25	3	0	1	1	32
RET/PTC-3	4	19	3	1	1	1	29
RET/PTC-1 and *-3*	0	0	0	0	0	1	1
Total samples with genetic alteration(s)	11	69	19	7	11	17	134
None	22	699	33	30	11	11	806
All samples	33	768	52	37	22	28	940
Genetic alteration frequency (%)							
$BRAF^{V600E}$	12.1	0.4	17.3	10.8	36.3	42.8	4.2
RAS	3	2.8	7.7	5.4	4.5	7.1	3.4
RET/PTC-1 and *RET/PTC-3*	18.2	5.8	15.4	2.7	18.2	17.8	7.3
Total(s)	33.3	9	36.5	18.9	50.0	60.7	14.2

BSRTC, Bethesda System for Reporting Thyroid Cytopathology.

The presence of a cancer was histologically confirmed in 72 patients (61.5% of operated patients), including 70 papillary thyroid cancers (PTC; 96.05%), one follicular thyroid cancer (FTC), and one anaplastic thyroid cancer (ATC). Among the patients with a final malignant histology, more than half carried one or more somatic genetic alteration and displayed stage I disease (Table 2).

In particular, 40 patients who displayed a somatic $BRAF^{V600E}$ mutation (including six who also displayed a *RET/PTC* rearrangement) underwent TT and had a PTC on final histology.

Among the 31 patients who displayed an isolated somatic *RAS* mutation, 10 were submitted to LT and one to TT. Histology revealed the presence of a cancer in two cases, including one ATC and one FTC (the latter initially submitted to LT and then to completion thyroidectomy). The remaining nine patients who were operated on showed a follicular adenoma (FA) in six cases and hyperplastic nodules (HN) in three cases. Moreover, one patient with a malignant cytology, displaying a somatic *RAS* mutation, was not operated on due to several comorbidities. The remaining 19 patients refused surgery, mostly because of the finding of a benign cytology.

The presence of a *RET/PTC* rearrangement was found in 69 FNAB, six of which also harbored a $BRAF^{V600E}$ mutation and were therefore submitted to TT. One patient also carried a *RAS* mutation and was submitted to LT with final histology of a FA. One patient was to have both *RET/PTC* rearrangements and was submitted to TT with a final histology of PTC. Among the 62 patients displaying an isolated *RET/PTC* rearrangement, five underwent TT (in the presence of a BSRTC class V in two patients and class VI in three patients) and 19 underwent LT. Histology revealed the presence of a cancer in five cases (all PTC), while 11 lesions were FA and eight HN. The remaining 38 patients refused surgery, mostly because of the finding of a benign cytology. No correlation was found between the presence of a malignant lesion and the amount of *RET/PTC* rearranged mRNA, preventing the identification of a threshold value that discriminates benign from malignant lesions.

Indeterminate lesions

We then evaluated cytology, molecular testing, and pathology findings in the group of indeterminate nodules, which were included in the whole group described above.

We found that 37 (26.4%) of the 140 cytologically indeterminate lesions (corresponding to 14.8% of all FNAB), including 19 class III, seven class IV, and 11 class V lesions, displayed at least one genetic alteration. Among these patients, two refused LT (class III cytology) and 35 underwent TT. Final histology showed 24 thyroid cancers (23 PTC and 1 FTC), eight FA, and three HN. Among the 23 identified PTCs, 21 carried a somatic $BRAF^{V600E}$ mutation.

Among the 103 patients with a cytologically indeterminate lesion not displaying a genetic alteration, all the 11 patients with a class V lesion underwent TT, with a final histology of 10 PTC and one HN. Ten out of 30 patients with class IV lesions agreed to undergo LT, with a final histology of three PTC (then submitted to completion thyroidectomy) and seven FA. All 62 patients with a class III lesion underwent a second FNAB that confirmed an indeterminate lesion in 33 cases; six of these patients agreed to undergo LT, and the final histology showed one FTC, four FA, and one HN. Cytology showed a benign lesion in the other 29 patients who were then reclassified as BSRTC class II and subsequently followed with US. The management of these patients was chosen according to the ATA guidelines (8), in order to avoid unnecessary surgery in keeping with the low cancer risk of BSRTC class III nodules (in contrast with the higher cancer risk of BSRTC class IV and V nodules).

Taken together, in our series, malignancy rates in each BSRTC class overlap those described by Cibas *et al.* (14). The cancer risk in thyroid nodules with indeterminate cytology according to BSRTC classification and genetic alterations is shown in Table 3.

Diagnostic value of cytology and molecular analyses

The diagnostic value of cytology and of the studied mutational analyses is reported in Table 4a, which also shows the results obtained by performing the three available genetic analyses in combination. Our data show that cytology displays optimal PPV and specificity, while sensitivity for thyroid cancer is low. When performed alone, $BRAF^{V600E}$ analysis shows, as compared to cytology, a significantly higher diagnostic sensitivity ($p < 0.05$), which increases by 20.8% ($p < 0.01$) when the two evaluations are performed together (Table 4b). On the other hand, the presence of *RAS* mutations and *RET/PTC* rearrangements shows a very low sensitivity for thyroid cancer when evaluated alone (Table 4a) and does not significantly increase the diagnostic sensitivity of cytology (Table 4b). In addition, the increased sensitivity recorded when all three genetic analyses are performed in combination is not significantly higher compared to the sensitivity obtained by

Table 2. Distribution According to TNM Stages and the Presence/Absence of a Genetic Alteration

TNM staging (AJCC/UICC)	*Genetic alteration*		
	Positive	*Negative*	*Total*
I	28	19	47
II	0	0	0
III	13	6	19
IV	6	0	6
Total	47	25	72

Table 3. Cancer Risk in Thyroid Nodules with Indeterminate Cytology According to BSTRC Classification and Genetic Alteration

%	*Class III*	*Class IV*	*Class V*	*Indeterminate cytology*
Cytology alone	19.2	21.6	90.9	27.1
Any mutation	47.3	71.4	90.9	63.1
BRAF	100	100	100	100
RAS	0	50	0	14.2
RET/PTC-1	40	—	100*	57.1
RET/PTC-3	0	0	100*	33.3
No mutations	3	10	90.9	13.5

*The patients with a PTC displaying *RET/PTC* rearrangements also had a $BRAF^{V600E}$ mutation, or a class V or a class VI BSTRC cytology.

Table 4. (A) Diagnostic Value of Cytology and of Genetic Analyses in All 940 Samples

	Cytology	BRAF	RAS	RET/PTC	*All genetic analyses*
PPV	100	100	25	34.4	63.3
NPV	50	58.4	34.3	28.2	34.2
Sensitivity	37.5	55.6	4.2	15.3	66.7
Specificity	100	100	80	53.3	31
Accuracy	61.5	72.6	33.3	29.9	53.8

Table 4. (B) Diagnostic Value of Cytology Combined with Genetic Analyses in All 940 Samples

	Cytology combined with			
	BRAF	RAS	RET/PTC	*All genetic analyses*
PPV	100	76.3	61.1	66.7
NPV	72.6	45.6	38.1	51.9
Sensitivity	76.4	40.3	45.8	82.2
Specificity	100	80	53.3	31.8
Accuracy	85.5	55.6	48.7	63.2

Table 4. (C) Diagnostic Value of Genetic Analyses in the 140 Indeterminate Lesions According to BSRTC Classification

	Class III		*Class IV*		*Class V*	
	BRAF	*All genetic analyses*	BRAF	*All genetic analyses*	BRAF	*All genetic analyses*
PPV	100	52.9	100	71.4	100	90.9
NPV	92.9	83.3	69.2	70	14.3	9.1
Sensitivity	90	90	50	62.5	40	50
Specificity	100	38.5	100	77.8	100	50
Accuracy	95.7	60.9	76.5	70.6	45.5	50

PPV, positive predictive value; NPV, negative predictive value.

performing $BRAF^{V600E}$ analysis alone, even when combined with cytology. These data indicate that, in our setting, $BRAF^{V600E}$ analysis suffices to increase the diagnostic sensitivity of cytology for thyroid cancer.

We then evaluated the diagnostic sensitivity of the genetic analysis panel in the subset of the indeterminate lesions in order to understand whether and which mutation testing might be helpful in this group. We found that the diagnostic sensitivity for thyroid cancer of the three genetic analyses in the indeterminate group, performed alone or in combination, overlaps that identified in the whole group. We then analyzed each BSRTC class included in the indeterminate group (Table 4c) and found that the diagnostic sensitivity for thyroid cancer reaches 90% in class III when $BRAF^{V600E}$ analysis is performed. This value does not change when *RAS* mutations and *RET/PTC* rearrangements are simultaneously included. In class IV and V samples, when all three genetic abnormalities are analyzed in combination, the diagnostic sensitivity for cancer is greater compared to $BRAF^{V600E}$ alone, but the difference is not statistically significant. In addition, the analysis of *RAS* mutations and *RET/PTC* rearrangements does not seem to be important to increase further the high NPV of $BRAF^{V600E}$ analysis in class III and IV samples.

Discussion

This prospective study confirms the diagnostic utility of assessing the presence of a $BRAF^{V600E}$ mutation (16). On the other hand, the investigation of two additional genetic abnormalities (*RAS* mutations and *RET/PTC* rearrangements) did not significantly increase the diagnostic sensitivity of cytology toward thyroid cancer in this cohort, even in the category with indeterminate lesions. Despite the fact that the techniques employed in our study are very similar to those employed by others (5,15,18), the results do not overlap. It should be noted that the method employed here to assess *RET/PTC* rearrangements displayed a 10-fold higher sensitivity compared to that employed by Nikiforov *et al*. (15,18), but provided low sensitivity and specificity in detecting malignant lesions. Therefore, the identification of *RET/PTC* rearrangements by a very sensitive method may not be useful to increase FNAB diagnostic sensitivity for thyroid cancer. These data suggest that the contribution of this genetic marker to presurgical diagnosis of thyroid nodules may not be so relevant, since we also found a very high incidence of *RET/PTC* rearrangements in benign lesions.

US characteristics provide the basis of performing FNAB (8), and often accurately predict the presence of a $BRAF^{V600E}$ mutation (20). In our hands, the presence of a $BRAF^{V600E}$ mutation was significantly associated with hypoechogenicity, microcalcifications, and a diameter < 1 cm, strengthening the evidence that nodules displaying these US characteristics very likely reflect the presence of a cancer. Our study highlights, for the first time, that *RAS* mutations and *RET/PTC* rearrangements correlate with specific US findings (i.e., isoechogenicity and diameter > 1 cm). However, these genetic

abnormalities do not indicate the presence of a cancer with high accuracy in our population, and therefore the related US characteristics cannot be taken into account as predictive of cancer.

The distribution of our samples among BSRTC classes is in line with literature data, indicating that the investigated nodules had been selected according to the indications of the ATA guidelines (8). In particular, >80% of FNAB cytologies turned out to be a benign lesion, and ~12% of the samples displayed an indeterminate cytology. The latter result is very similar to the percentage of indeterminate lesions that were retrieved in our previous study (17), which included an unselected nodule population, indicating that the application of strict selection criteria for FNAB does not influence the number of indeterminate lesions. While the percentage of malignant lesions identified by cytology in our series (2.9%) is comparable to the literature data, the incidence of ND reports is quite high (3.5%). This may be because the retrieved FNAB material was used for several diagnostic procedures, which may have reduced the sample quantity dedicated to cytology.

The present series shows that 14.2% of the investigated nodules harbored at least one mutation, a higher incidence than the previously reported (~9%) (18), probably due to the different inclusion criteria. In addition, 6% of mutated FNAB samples displayed more than one genetic alteration, confirming that *BRAF* and *RAS* mutations, as well as *RET/PTC* rearrangements, are not mutually exclusive, as previously indicated (21). Our data also show that the applied FNAB criteria allowed diagnosing thyroid cancers at an early stage of disease, since 65.3% of the diagnosed cancers were Stage I. In addition, nearly 50% of Stage I cancers had a negative cytology but displayed at least one genetic alteration, most commonly a $BRAF^{V600E}$ mutation, which allowed a correct diagnosis to be established. These data indicate that $BRAF^{V600E}$ mutation analysis helps PTC to be identified at an earlier stage, possibly resulting in a more conservative treatment with potential consequences on patient health and healthcare resources. Moreover, 76% of Stage III and IV cancers displayed a genetic alteration, in line with the hypothesis that the latter may characterize a more aggressive behavior (22,23), as previously indicated (24). Last, the applied protocol allowed 31 out of 46 false negative lesions to be diagnosed correctly as cancers, corresponding to 43% of the diagnosed malignant lesions. Among these 31 patients, 21 harbored a $BRAF^{V600E}$ mutation and an indeterminate cytology, and were therefore submitted to TT rather than to a diagnostic LT. Moreover, seven patients were submitted to TT only based on positivity for a $BRAF^{V600E}$ mutation and turned out to have a PTC (six Stage I and one Stage III). The latter finding strengthens the evidence that $BRAF^{V600E}$ mutation analysis facilitates early diagnosis. On the other hand, in our setting, *RAS* mutations have a poor diagnostic value, in keeping with their rarity, and are predominantly associated with follicular lesions, mainly represented by FA that may, in part, be considered as precursors of malignant lesions (25). In keeping with the latter hypothesis, *RAS* mutated cancers were characterized by an aggressive histology and a high disease stage. In our patients, each *RET/PTC* rearrangement was nearly as frequent as $BRAF^{V600E}$ mutations, but had a poor diagnostic value, since the rearranged lesions were mostly found in benign nodules (64.5% of the cases), contrary to what was observed by Cantara *et al*. (5) and Nikiforov *et al*. (18), but in line with Marotta *et al*. (26), even if a prognostic significance cannot be ruled out (27). These differences may be due to different genetic backgrounds and to geographic factors, but may also be due to the applied selection criteria. Among the samples harboring *RET/PTC* rearrangements, the 11 PTC cases had a $BRAF^{V600E}$ mutation and/or a suspicious or malignant cytology, and were therefore submitted to TT independently of the presence of a *RET/PTC* rearrangement.

A previous report (18) showed an increased diagnostic sensitivity for thyroid cancer in a large group of indeterminate nodules submitted to multiple genetic analyses (including $BRAF^{V600E}$ and *RAS* mutations as well as *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARγ* rearrangements). The study showed a high NPV for this panel of molecular markers, indicating that the absence of a genetic mutation very likely excludes the presence of a malignant lesion. On the contrary, we did not obtain high NPV values in the indeterminate group when performing the three analyses together ($BRAF^{V600E}$ and *RAS* mutations, as well as *RET/PTC1* and *RET/PTC3* rearrangements), but we found a high NPV for $BRAF^{V600E}$ mutation analysis alone, which is even higher in class III nodules. The latter finding, together with the low cancer risk, suggests that in the absence of a $BRAF^{V600E}$ mutation, diagnostic LT may not be necessary in class III nodules. In class IV nodules without mutations, we found a slightly higher cancer risk, which importantly increased when a *RAS* mutation was present. These data, together with a suboptimal NPV of $BRAF^{V600E}$ analysis in class IV lesions, do not support a conservative management in these settings (i.e., avoiding a LT). On the other hand, cancer risk is high in class V nodules, indicating that an aggressive surgical management (i.e., TT) is justified in these patients, independently of the presence of a mutation, such as in class VI lesions. Taken together, these data demonstrate that, among the investigated molecular markers, only $BRAF^{V600E}$ mutation may modify patient management and has an impact on the surgical approach. Therefore, our data concerning indeterminate lesions are only partially in keeping with previous findings (18), probably due to the different inclusion criteria, which may play an important role in molecular studies.

In conclusion, our results confirm that $BRAF^{V600E}$ analysis performed in all BSRTC classes increases the diagnostic sensitivity of cytology for thyroid cancer, which is not further enhanced by investigating the presence of *RAS* mutations or *RET/PTC* rearrangements, even among indeterminate nodules. In addition, our data demonstrate that $BRAF^{V600E}$ analysis, when negative, may be useful for identifying class III nodules at very low risk of being cancerous, suggesting that these cases may be treated more conservatively and do not need to be submitted to a LT. Moreover, we conclude that $BRAF^{V600E}$ analysis is useful for the diagnosis of thyroid cancer at an early stage, possibly reducing the clinical impact of a delayed diagnosis, which may result in higher costs for the patient and the healthcare system.

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Address correspondence to:
Maria Chiara Zatelli, MD, PhD
Section of Endocrinology and Internal Medicine
Department of Medical Sciences
University of Ferrara
Via Savonarola 9
44121 Ferrara
Italy

E-mail: ztlmch@unife.it