How Limited Molecular Testing Can Also Offer Diagnostic and Prognostic Evaluation of Thyroid Nodules Processed With Liquid-Based Cytology: Role of *TERT* Promoter and *BRAF* V600E Mutation Analysis

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BACKGROUND: Mutational analysis contributes to the diagnosis and prognosis of thyroid nodules analyzed with fine-needle aspiration cytology (FNAC). Although several advanced molecular tests based on multiple molecular markers are available for clinical use and have increased their impact on clinical management of patients, they are not widely available. Among them is *BRAF* V600E, one of the most studied mutations. Other genetic alterations, such as *TERT* promoter mutations, may coexist in thyroid carcinomas. Previous studies have demonstrated that this duet might be involved in the aggressiveness of thyroid cancer, although its prognostic value related to mortality remains undefined. The detection of such genetic alterations in thyroid liquid-based cytology (LBC) thus may assist with patient management. **METHODS:** From January 2013 to June 2014, 356 thyroid FNAC samples were processed by LBC, including 174 surgical follow-up samples. *BRAF* V600E and *TERT* mutation analyses were performed on both LBC and histopathology. **RESULTS:** The study included 119 samples categorized as atypia of undetermined significance, 42 categorized as follicular neoplasms, 61 categorized as suspicious for malignancy, and 34 categorized as positive for malignancy. *BRAF* V600E mutation was detected in 10.4% of all cases, whereas *TERT* promoter mutations were identified in 1.1%. *TERT*-mutated cases belonged to the positive for malignancy category, with a histologic diagnosis of tall cell variant of papillary thyroid carcinoma. These genetic alterations correlated with lymph node metastases ($P = .0349$) and higher disease stage. **CONCLUSIONS:** *BRAF* V600E and *TERT* analysis can be performed on LBC. *TERT* mutations are rarely identified in well differentiated thyroid carcinoma but are associated with higher stage. Although a larger molecular panel may offer more information, analyzing these few point mutations is still likely to be useful for managing potentially more aggressive thyroid carcinomas. ***Cancer Cytopathol* 2021;129:819-829**.

KEY WORDS: *BRAF* mutations; follicular neoplasms; follicular variant of papillary thyroid carcinoma; liquid-based cytology; papillary thyroid carcinoma; TERT promoter.

INTRODUCTION

Thyroid cancer is the most common type of endocrine tumor, of which papillary thyroid carcinoma (PTC) is the most frequent, with rising incidence in the last decades.[1-3] Although 90% of PTCs and PTC variants have an excellent prognosis, from 5% to 10% of these malignancies have an aggressive disease evolution,

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Received: January 6, 2021; **Revised:** April 17, 2021; **Accepted:** April 19, 2021

Published online June 2, 2021 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cncy.22454, wileyonlinelibrary.com

including mortality. The underlying genetics responsible for the significant difference between indolent and aggressive cases remains unknown. Hence, overtreatment of these cancers is frequently justified, driven by fear of potentially aggressive behavior.[1-5] Currently, the only effective risk stratification for PTC is based on clinical and morphologic features. It was demonstrated in the 1990s that thyroid cytologic material collected from a thyroid nodule by fine-needle aspiration (FNA) was capable of providing a sufficient amount of cells to detect individual point mutations or gene fusions. However, in the 2000s, it was recognized that *BRAF* mutations had significant diagnostic and prognostic utility. Specifically, it was the last decade in which researchers emphasized the role in thyroid cancer oncogenesis played by activation of the *MAPK* and *PI3K-AKT* signaling pathways,[5-11] with the *BRAF* V600E mutation frequently found in PTCs and involved in neoplasia initiation and progression to de-differentiation.[5-22] Several authors confirmed that the sensitivity of molecular testing was improved through the introduction of gene panels and next-generation sequencing (NGS), which can detect multiple types of genetic alterations in 1 assay using a very small number of cells obtained from thyroid FNA samples. The benefits are mostly linked to the finding that NGS has high sensitivity and is able to quantitatively assess the proportion of cells carrying a given mutation. Conversely, these advantages may be counteracted by the need for having centralized molecular laboratories and increased cost. Because molecular tests still are generally expensive and restricted to only a few specialized and/or centralized laboratories, the adoption of liquid based cytology (LBC), coupled with testing for only a few molecular somatic mutations, can offer valid diagnostic and even prognostic support for reaching a conclusive cytologic diagnosis in smaller laboratories without access to NGS.

Different authors, including our group, have extensively studied the role of somatic mutations, including the *BRAF* V600E mutation, on cytologic samples processed with LBC. Therefore, the use of LBC to test a few pertinent somatic mutations might be useful for many laboratories around the world.

Of note, it is relevant to underline that the majority of thyroid carcinomas have an indolent course, and even the recent reclassification of the noninvasive, encapsulated follicular variant of PTC has suggested that very few genetic alterations, such as *BRAF* V600E and *TERT* promoter mutations, are likely to be associated with a worse course.[20]

Additional events, such as *TP53* and *TERT* promoter mutations, occur late in oncogenesis with increasing frequency in thyroid cancers that lose differentiation and are associated with an unfavorable outcome and prognosis.[12-22] In fact, the *TERT* gene, which is repressed in most differentiated human cells, can be reactivated by somatic *TERT* alterations, which is also induced by transcription factors. Specifically, reactivated *TERT* contributes to the development and progression of cancer and to a poor prognosis in several cancers.[14]

Some authors have documented that *TERT* promoter mutations are commonly found in poorly differentiated thyroid carcinoma and anaplastic thyroid carcinoma (eg, in up to 70% of cases), although such *TERT* mutations (namely, C228T and C250T) have also been identified in PTC and its variants as well as Hurtle cell carcinoma.[15,23-31] Nevertheless, when a *TERT* promoter mutation coexists with a *BRAF* V600E mutation, thyroid cancers, including PTCs, are likely to have a synergic role in a poorer prognosis and a negative effect on clinical outcome. Liu et al studied the molecular mechanism of their interaction using cancer cell lines from thyroid cancers and melanoma.[13]

Liu and colleagues reported that a simple 4-genotype classification of PTC, especially the classic variant (cPTC), linked the risk of mortality to the genetic duet of *BRAF* V600E and *TERT* mutations.[1] According to this molecular prognostic stratification system, patients with coexisting mutations have a higher mortality risk.[1] The possibility of recognizing these potential prognostic molecular findings in thyroid lesions sampled by FNA cytology (FNAC) might help tailor patient management.[32] Therefore, the detection of *BRAF* V600E and/or *TERT* promoter mutations may delineate more aggressive surgery for concerning thyroid FNAC cases.

The objective of this study was to support the use of small molecular panels, including *BRAF* V600E plus *TERT* promoter mutations, on thyroid lesions sampled by FNAC and processed using LBC and to determine whether this limited molecular panel supports a worse outcome.

MATERIALS AND METHODS

A retrospective search was performed for all thyroid FNACs that were diagnosed as suspicious for malignancy (SFM) and positive for malignancy (PM) over a 1.5 year period (from January 2013 to June 2014) at the Fondazione

Policlinico Universitario "Agostino Gemelli" in Rome, Italy. In addition, to determine the genetic alterations in benign versus malignant thyroid entities, we also included all indeterminate lesions, such as atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) and follicular neoplasm/suspicious for follicular neoplasm (FN/SFN), that were diagnosed during the same study period. A further total of 100 benign thyroid lesions diagnosed as goiter in the same reference period were used as a negative molecular case-control group. The institution's electronic medical record system (Armonia-Metafora) was searched for thyroidectomy and lobectomy specimens during the same study period. All information on each patient's age, sex, FNAC diagnoses, and follow-up surgical pathology was recorded. All available pathology slides were reviewed. Most of the thyroid nodules were evaluated and biopsied under ultrasound guidance by clinicians and radiologists. We received internal institutional ethical approval for this study.

Thyroid FNAC Specimens

All aspirations (usually with 2 passes performed for each thyroid lesion) were performed with 25-gauge to 27-gauge needles. No rapid on-site assessment for adequacy of material was performed. All patients consented to their procedure. All FNAC specimens were processed using a ThinPrep 5000 processor (Hologic). Prepared slides were fixed in 95% methanol and stained with a Papanicolaou stain. Any remaining material was stored in PreservCyt solution for potential ancillary studies.

Specimen adequacy was determined according to the Bethesda and British Royal College of Pathologists RCPath classification schemes.[33-35] The cytology cases were classified and diagnosed according to the new Italian Working Group (Italian Society of Pathology and Cytodiagnostics-Italian Division of International Academy of Pathology [SIAPEC-IAP]) classification.[36,37] All cases were re-evaluated and then reclassified according to The Bethesda System for Reporting Thyroid Cytology II, 2017 (TBSRTC).[35] For this retrospective study, analyses were conducted using TBSRTC terminology. This case series included the following distribution of diagnoses: 5.9% nondiagnostic, including cystic cases; 77.8% benign lesions; 3% AUS/FLUS; 6.1% FN; 2.2% SFM; and 5% PM. All cytology and histology cases were reviewed by 2 cytopathologists, and reclassification according to TBSRTC was undertaken by 1 cytopathologist (E.D.R.). Cases with an equivocal interpretation were subjected to consensus review. The concordance between SIAPEC-IAP and TBSRTC classification systems was 95.9%.

Molecular Analysis for BRAF *V600E and* TERT *Mutations*

DNA was extracted from both LBC stored, aspirated material and paraffin-embedded tissues, according to our previous experiences with the performance of ancillary techniques on thyroid samples.[38-42] *BRAF* V600E mutational analysis was performed on DNA extracted from cytologic and surgical specimens containing ≥70% tumor. Details of the molecular protocol used have been previously published by our group.[40-42] For *TERT* analysis, genomic DNA was extracted from LBC samples stored in PreservCyt solution (Hologic) using the QIAamp DNA mini kit (Qiagen), according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed in 20-µL reactions containing genomic DNA (100 ng), 0.2 µmol/L of primers (forward 5′-CACCCGTCCTGCCCCTTCACCTT-3′ and reverse 5′-GGCTTCCCACGTGCGCAGCAGGA-3′) and 2x PCRBIO HS Taq Mix (PCR Biosystems Inc). PCR conditions were as follows: initial denaturation at 95 °C for 10 minutes followed by 35 cycles at 95 °C for 40 seconds, 62 °C for 40 seconds, and 72 °C for 40 seconds. *hTERT* promoter amplification was performed on an C1000 Touch Thermal Cycler (BioRad). The yielded fragment was separated by electrophoresis on a 2% agarose gel containing ethidium bromide and was observed by ultraviolet illumination. PCR product was treated with EXOSap (UBS, SIAL), following the manufacturer's protocol, and directly sequenced using a BigDye Terminator kit version 3.1 (Applied Biosystems) with forward and reverse primers in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Histopathology Specimens

All surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin, and 5-µm-thick sections were then stained with hematoxylin and eosin. Diagnoses of the classical variant of PTC (cPTC) and of the different PTC variants were classified according to the 2017 World Health Organization classification.[43] For the definition of the tall cell variant (TCV)

of PTC, we included cases of PTC with ≥30% TCV component. The histologic diagnosis of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) was rendered according to the criteria described by Nikiforov et al.[44] All malignant cases were staged according to the seventh edition of the tumor-node-metastasis (TNM)-based staging system recommended by the American Joint Commission on Cancer.[45]

Statistical Analysis

Statistical analysis was performed using GraphPad-Prism 5 software (Graph Pad Software) and MedCalc version 10.2.0.0 (MedCalc Software). Statistical comparison of continuous variables was performed using the Mann-Whitney *U* test or the paired *t* test, as appropriate. Comparison of categorical variables was performed using the χ^2 statistic and the Fisher exact test. *P* values < .05 were considered statistically significant.

RESULTS

Our study included 356 cytology samples that were examined during the 18-month study period. Patient demographics and clinicopathologic features are described in Table 1. We included all cytologic samples diagnosed as indeterminate thyroid lesions (AUS/FLUS and FN/SFN), SFM, and PM, with histologic follow-up in 174 cases. For negative controls, 100 benign cases (from the same study period) were included in this series, including 25 that had surgical follow-up. The series included 102 men and 254 women, the median age was 44.5 years (range, 18-79 years; mean, 46 years), and the thyroid neoplasms ranged in size from 0.4 to 7.0 cm (median, 3.2 cm; median value, 3.1 cm).

Our cytologic series (cases) included the following distribution of thyroid diagnoses: 100 benign, 119 AUS/FLUS, 42 FN/SFN, 61 SFM, and 34 PM (Table 2). All subcentimeter lesions were discovered incidentally during radiologic screening for causes unrelated to the thyroid gland. There was no significant difference in the size of lesions among the diagnostic entities. No statistical correlation was found with the clinicopathologic data (Table 1).

Table 2 depicts the histologic diagnoses rendered in 174 of 356 cases (48.8%), including 33 benign cases (18.9%), 141 malignant histologic cases (81%), and 2 NIFTP cases (0.1%). The surgical pathology follow-up findings for the different cytologic categories are described in Table 2. The surgical pathology series also included 25 benign cases diagnosed as 22 goiters and 3 follicular adenomas (FAs). Fifteen of 119 (12.6%) AUS/FLUS cases had surgical follow-up and were diagnosed as 2 goiters, 9 FAs (including 2 oxyphilic adenomas [OAs]), 1 invasive follicular variant of PTC (I-FVPTC), and 3 cPTCs. The 4 AUS/FLUS cases with a malignant diagnosis were revised and confirmed. The remaining AUS/FLUS cases were followed with repeat FNAC and molecular testing, resulting in a lack of any malignancy.

TABLE 1. Summary of Clinicopathologic Data, n = 356

Clinicopathologic Features	No. of Patients (%)
Patient age, y	
Mean	46
Median [range]	44 [18-79]
Patient sex	
Male	102 (28.6)
Female	254 (71.4)
Cytology diagnosis	
Benign	100 (29.0)
AUS/FLUS	119 (33.4)
FN/SFN	42 (11.0)
SFM	61 (17.0)
PM	34 (9.6)
Histopathology diagnosis[a]	
Benign[b]	33 (18.9)
Malignant	141 (81.1)

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; PM, positive for malignancy; SFM, suspicious for malignancy.
[a]Histology was available in only 174 cases.
[b]Benign diagnoses include follicular adenomas and noninvasive follicular thyroid neoplasms with papillary-like nuclear features.

Our 42 FN/SFN cases had subsequent surgical resections that were diagnosed as 27 FAs (including 6 OAs), 1 NIFTP, 8 PTCs (including 1 TCV-PTC), 5 I-FVPTCs, and 1 follicular carcinoma. The 61 SFM cases included 1 NIFTP, 40 cPTCs, 14 PTC variants (including 9 TCV-PTCs, 2 hobnail variants of PTC, 1 Warthin-like variant of PTC, 1 solid variant of PTC, and 1 sclerosing variant of PTC), and 5 I-FVPTCs. The 34 PM cases were diagnosed as 17 cPTCs, 14 PTC variants (including 6 TCV-PTCs, 1 hobnail variant of PTC, 1 Warthin-like variant of PTC, 1 solid variant of PTC, and 5 I-FVPTCs), 2 medullary thyroid carcinomas, and 1 anaplastic thyroid carcinoma. Molecular analysis was performed on the entire series, including our 100 benign cases.

Table 3 highlights the distribution of cPTC and its variants in the different cytologic categories. The

TABLE 2. Cytologic-Histologic Correlation in 174 Cases

Pathology Diagnosis	No. of Cases								
	Goiter	FA	NIFTP	PTC	I-FVPTC	FC	OFC	MTC	ATC
Benign (25 cases)[a]	22	3	—	—	—	—	—	—	—
AUS/FLUS (15 cases)	2	9[b]	—	3	1	—	—	—	—
FN/SFN (42 cases)	—	27[c]	1	8	5	1	—	—	—
SFM (61 cases)	—	—	1	54	5	—	—	—	—
PM (34 cases)	—	—	0	26	5	—	—	2	1

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; ATC, anaplastic thyroid carcinoma; FA, follicular adenoma; FC, follicular carcinoma; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; I-FVPTC, invasive follicular variant of papillary thyroid carcinoma; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; MTC, medullary thyroid carcinoma; OFC, oncocytic follicular carcinoma; PM, positive for malignancy; PTC, papillary thyroid carcinoma; SFM, suspicious for malignancy.
[a]Twenty-five of 100 benign cases had histologic follow-up.
[b]These included 2 oxyphilic adenomas.
[c]These included 6 oxyphilic adenomas.

TABLE 3. Distribution of Histopathologic Diagnoses for Classic Papillary Thyroid Carcinoma and Variants

Diagnosis	No. of Cases						
	cPTC	TCV	I-FVPTC	Hobnail-PTC	SV-PTC	DSV-PTC	Warthin-PTC
AUS/FLUS	3	1	—	—	—	—	—
FN/SFN	7	1	5	—	—	—	—
SFM	40	9	5	2	1	1	1
PM	17	6	5	1	1	—	1

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; cPTC, classic papillary thyroid carcinoma; DSV-PTC, diffuse sclerosing variant of papillary thyroid carcinoma; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; I-FVPTC, invasive follicular variant of papillary thyroid carcinoma; PM, positive for malignancy; SFM, suspicious for malignancy; SV-PTC: solid variant of papillary thyroid carcinoma; TCV, tall cell variant of papillary thyroid carcinoma.

evaluation of genetic alterations for *BRAF* V600E and *TERT* promoter mutations resulted in a total of 40 of 356 (11.3%) *BRAF* V600E-mutated and 4 (1.1%) *TERT*-mutated cases. The latter 4 *TERT*-mutated cases had simultaneous expression of *BRAF* V600E mutation. Morphologic features associated with *BRAF* V600E mutations included the presence of plump follicular cells with sickle-shaped nuclei in all of the mutated cases (Fig. 1). No specific morphologic features were specifically linked to the *TERT* promoter mutations (Fig. 2).

The distribution of *BRAF* V600E-mutated and *TERT*-mutated cases is reported in Table 4. Specifically, we identified 5 *BRAF* V600E-mutated indeterminate neoplasms (4 AUS/FLUS and 1 FN/SFN), 21 mutated SFM cases, and 15 mutated PM cases. The 4 *TERT*-mutated cases belonged to the malignant category. Histologic correlation revealed 14 *BRAF* V600E-mutated cPTCs, 10 cPTCs with multifocal patterns, 13 TCV-PTCs, 2 hobnail variants of PTC, and 2 I-FVPTCs. Specifically, 4 of the 13 TCV-PTCs also had *TERT* promoter mutations with tumors that exhibited ≥50% TCV component and lymph node metastases.

We correlated genetic alterations with tumor size, multifocality, and lymph node metastases. Analyzing the prognostic role of *BRAF* V600E and *TERT* genetic alterations, we found a higher correlation with these mutations and lymph node metastases ($P = .0349$; 95% CI, .06882; range, .4758-.9954). Nevertheless, no significant correlation was found with tumor size ($P = .2149$; 95% CI, .8693; range, .7056-1.071) or multifocality ($P = .6538$; 95% CI, .9064; range, .6380-1.288).

A correlation was detected between *BRAF* V600E and thyroid disease stage, indicating a significant *P* value of .0009 for stages II and III. Although the limited number of *TERT* promoter-mutated cases showed no statistically significant correlation, these *TERT*-mutated cases were associated with aggressive PTC variants (ie, TCV-PTC), local invasiveness, and nodal metastases.

DISCUSSION

PTC usually behaves as a well differentiated tumor with a very high 10-year survival rate of approximately 95%.[1-4] Nonetheless, a small percentage of PTC cases show aggressive

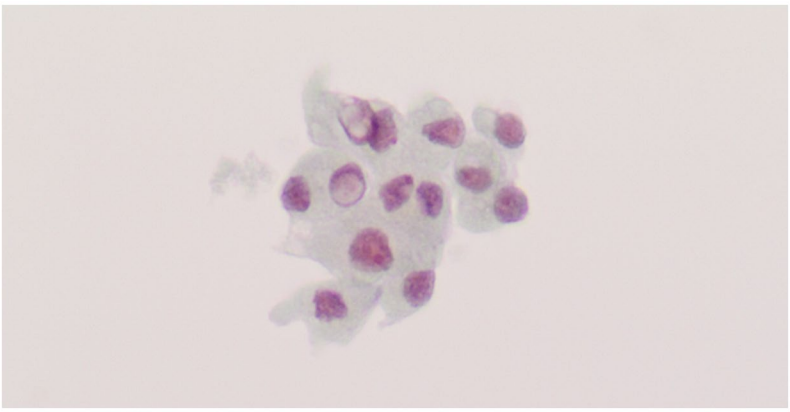

Figure 1. Morphologic features of a malignant thyroid lesion diagnosed as positive for malignancy favor papillary thyroid carcinoma. The lesion harbored a *BRAF* V600E mutation and had moderate eosinophilic cytoplasm and sickle-shaped nuclei (Papanicolaou stain; original magnification ×40).

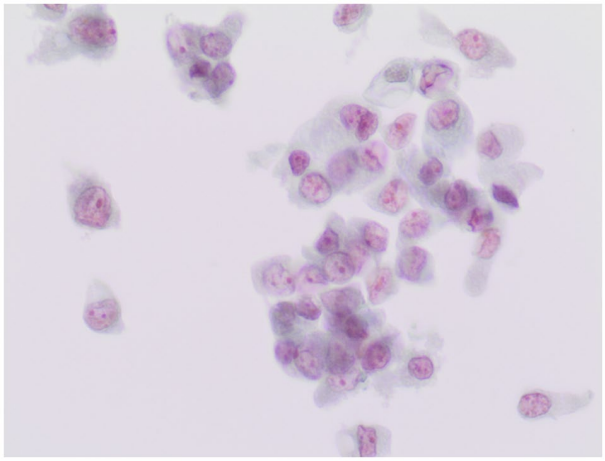

Figure 2. A fine-needle aspiration cytology sample that was diagnosed as positive for malignancy harbored *TERT* and *BRAF* V600E mutations. The cells have significant pleomorphic and irregular nuclei with some clearing, which are not pathognomonic of the classic features seen in papillary thyroid carcinoma on cytology (Papanicolaou stain; original magnification ×40).

features at presentation, and they are likely to develop early distant metastases or relapse, which are associated with adverse outcomes.[2] Determining which PTCs will behave badly early on in the management of afflicted patients may greatly improve clinical outcomes. The last few decades have witnessed significant progress in understanding the molecular pathology and pathogenesis of thyroid cancer, especially PTC. Among several molecular markers, the diagnostic and prognostic value of the *BRAF* V600E mutation and *TERT* promoter mutations have been well studied in PTCs and other thyroid malignancies. However, the role of these and other somatic mutations and rearrangements, such as *RET*/*PTC* and *RAS* mutations, in PTC still require further elucidation.[1-10] Fugazzola et al, Puxeddu et al, and Xu et al have pointed out the lack of a significant association of the *BRAF* V600E mutation with high-risk pathologic characteristics or disease-free survival.[46-48] However, in some of those studies, there was a limited number of cases, mostly ranging from 50 to 60 lesional samples, without subtype stratification of PTC and its variants. This may represent an important shortcoming because some genetic alterations, including *BRAF* V600E mutations, occur mostly in cPTC and TCV-PTC and occur uncommonly in invasive FVPTC, which represents a more favorable variant.[49,50]

Other studies have reported a correlation between *BRAF* V600E and more aggressive clinicopathologic outcomes of cPTC, especially with respect to bilateral disease, extrathyroidal extension, and nodal involvement.[5-15] Although data from the current series confirm the correlation of *BRAF* V600E with nodal involvement ($P = .0349$), no correlation was noted with disease multifocality.[42] Similar findings were documented by Ahmad et al, who concluded that the association of *BRAF* V600E mutation with extrathyroidal extension indicates its aggressive nature and thus can provide insights into the potential progression of thyroid tumors.[31] Yan et al found that a *BRAF* V600E mutation was present in 83.7% of their 2048 patients and demonstrated significant correlation with bilateral multifocal disease, but was less significantly correlated with lymph node metastases.[31] Chakraborty et al also found a significant correlation between *BRAF* V600E

TABLE 4. Cytologic-Histopathologic Correlation Combined With *BRAF* V600E and *TERT* Promoter Mutations

Cytology Diagnosis	Histopathology Diagnosis	*BRAF* V600E, N = 40	*TERT* Promoter Mutation, N = 4
Benign (25 cases)[a]	Goiter	WT	WT
	FA	WT	WT
AUS/FLUS (15 cases)	Goiter	WT	WT
	FA[a]	WT	WT
	I-FVPTC	WT	WT^
	PTC	4 Mutated	WT
FN/SFN (42 cases)	FA	WT	WT
	OA	WT	WT
	NIFTP	WT	WT
	PTC	1 Mutated	WT
	I-FVPTC	WT	
	OFC	WT	WT
	FC	WT	WT
SFM (61 cases)	FA	WT	WT
	NIFTP	WT	WT
	PTC	17 Mutated	WT
	I-FVPTC	WT	WT
PM (34 cases)	NIFTP	WT	WT
	PTC	11 Mutated	4 Mutated
	I-FVPTC	2 Mutated	WT
	MTC	WT	WT
	ATC	1 Mutated	WT
Total		40	4

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; ATC; anaplastic thyroid carcinoma; FA, follicular adenoma; FC, follicular carcinoma; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; I-FVPTC, invasive follicular variant of papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; OA, oncocytic adenoma; OFC, oncocytic follicular carcinoma; PM, positive for malignancy; PTC: papillary thyroid carcinoma; SFM, suspicious for malignancy; WT, wild type.
[a]These included 75 additional benign lesions without histology that were wild type for both *BRAF* V600E and *TERT* promoter mutations.

mutation status and extrathyroidal extension, lymph node metastasis, and tumor stage.[51] Hence, the detection of *BRAF* V600E mutation may be a helpful biomarker for prognostication.

TERT promoter mutations in thyroid cancer are also strongly associated with aggressive and metastatic behavior.[12-21,52] A high prevalence of *TERT* C228T mutation has been observed in aggressive thyroid cancers, such as anaplastic and poorly differentiated thyroid carcinomas.[23,25,27-29] Only a few studies, including that by Wang et al, reported that a C228T mutation was identified in 2% of FAs and 17% of atypical adenomas.[53] Melo et al documented that distant metastases show an enrichment in *TERT* promoter mutations and a decrease in *BRAF* V600E mutations and that this is likely important in the development of metastatic disease.[29] Trybek et al studied 568 PTCs with known *BRAF* V600E and *TERT* status and concluded that coexisting *BRAF* V600E and *TERT* mutations in patients with PTC (in 10% of their cases) are associated with poor initial prognostic factors and clinical course.[54] As such, Trybek and colleagues advocated that these genetic alterations may be useful for predicting a poor response to therapy, recurrence, and poor outcomes in patients who have these mutations.[54] Vuong et al, in a meta-analysis that included 42 studies with 11,109 PTC samples, demonstrated that PTCs with concurrent *BRAF* V600E and *TERT* promoter mutations were associated with increased tumor aggressiveness, especially distant metastases, compared with PTCs harboring *BRAF* V600E or *TERT* promoter mutations alone.[27,55]

Additional published data have suggested that *BRAF* V600E activates the mutated *TERT* promoter through the fos proto-oncogene, which is a downstream effector of MAPK signaling and the GA-binding protein complex.[14] In fact, Song et al found that *TERT* mRNA was increased by the coexistence of *BRAF* V600E in a series of 331 PTCs.[15] They demonstrated that the synergistic effect between *BRAF* V600E and *TERT* promoter mutations on cancer invasiveness and progression in PTC may be explained by increased TERT expression, which may result from the BRAF-induced upregulation of several transcription factors.[15]

Rusinek et al analyzed 54 PTCs performing the molecular profile for *BRAF* V600E, *RAS*, and *TERT* promoter mutations. Their yields confirmed that *TERT*-mutated PTCs were more aggressive, as indicated by larger greatest dimensions, invasion of surrounding tissues,

cervical nodal metastases, locally persistent disease after the first surgery, and distant metastases in tumors that had coexpression of *BRAF* V600E and *TERT* promoter mutations.[16]

Lee et al studied 242 cytohistologic thyroid nodules, including 207 PTCs examined for *BRAF* V600E and *TERT* promoter mutations. The *TERT* mutation was associated with recurrence ($P = .03$), whereas the coexistence of both mutations was significantly associated with older age and advanced stage.[56]

Furthermore, the results from Liu et al suggested that patients with either a *BRAF* V600E mutation (2.4%), a *TERT* promoter mutation (6.3%), or both (22.7%) had increased PTC-specific mortality.[1] This highlights a key role for *BRAF* V600E mutation in thyroid tumorigenesis and as a genetic driver for a higher mortality risk working in synergy with other genetic alterations, such as *TERT* promoter mutations. Our study corroborates these findings from the literature.

The case series published in the literature were conducted using histologic samples and focused on the postsurgical management of thyroid cancers.[52] To date, these studies have not been replicated using cytology specimens.

This is important because the diagnosis of thyroid cancer usually begins with a cytologic diagnosis based on an FNAC sample. Advances with new sequencing techniques, such as NGS, have propelled the role of molecular testing in thyroid cytologic samples, especially in helping resolve indeterminate lesions after FNAC. The use of NGS is currently limited to highly specialized reference or academic laboratories, mostly because of the high cost and the availability of skilled staff in many countries. On the basis of these limitations, different authors, including Bellevicine et al, have studied the opportunity to adopt a 7-gene in-house assay covering the 7 most frequent genomic alterations occurring during thyroid oncogenesis (ie, *BRAF* V600E, *HRAS*, *NRAS*, *KRAS*, *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARg*).[57] Specifically, this assay exhibits sensitivity and specificity ranging from 18% to 100% and from 82% to 100%, respectively. The authors concluded that a 7-gene test may represent a valid adjunct technique to provide risk-stratification analyses based solely on microscopic criteria. We further restricted our molecular panel by combining only the detection of *BRAF* V600E with *TERT* promoter mutations in an attempt to identify malignant lesions with a worse outcome. The decisions was based on the finding that *RAS* mutations, which mostly are found in follicular thyroid lesions, cannot univocally discriminate between adenomas and carcinomas. Furthermore, the introduction of NIFTP has confirmed that certain genetic alterations can be documented in noninvasive and encapsulated follicular variants of PTC, emphasizing that *BRAF* V600E and *TERT* mutations are likely to be linked with more aggressive thyroid cancers.[44]

Although some genetic alterations, such as *BRAF* V600E, have been extensively studied on cytologic samples, investigations of *TERT* promoter mutations on thyroid FNAC specimens have been limited.[22,26,32,40-42,50] To demonstrate the feasibility of a 4-genotype classification for PTC, we analyzed *BRAF* V600E and *TERT* promoter mutations on our cytology samples, specifically using LBC specimens from indeterminate, SFM, and PM cases. Whereas the accuracy and feasibility of LBC versus conventional cytology for molecular testing has been extensively confirmed by several groups, including ours,[38-42,49-50,58-61] to the best of our knowledge, this is the second study, after a publication by Decaussin-Petrucci and colleagues, documenting the evaluation of *TERT* promoter mutations on LBC stored material.[62] Although Decaussin-Petrucci et al found that *TERT* promoter mutations were rare, but very specific for malignancy (5.5%) in indeterminate cytology, the current series confirmed a scant number of *TERT* mutated cases (4 of 356 cases; 1.1%) only among the malignant category and, despite the limited number of *TERT*-positive cases, all of them exhibited pleomorphic nuclei and tall cell features. Nonetheless, as previously demonstrated by our group, *BRAF* V600E-mutated cases are likely to show some peculiar morphologic features (ie, sickle-shaped nuclei, eosinophilic cytoplasm[58]), and none of the limited (only 4) *TERT*-mutated cases had distinctive findings apart from tall cell appearance with more severe pleomorphic nuclei that need to be confirmed in a larger series of *TERT*-mutated cases. Our data confirmed that *TERT* promoter mutations are found only in malignant thyroid carcinomas with some aggressive features and that they represent a rare genetic alteration in thyroid lesions. Furthermore, we confirmed that our 4 cases with concomitant mutations were stage III or IV PTCs. These results helped identify those patients who are a minimal proportion of PTC series but have the highest risk of aggressive disease, demonstrating the utility of this genetic duet to provide a powerful molecular prognostic system. In fact, according to several publications, although some

genetic alterations are unable to discriminate between benign and malignant lesions or between indolent and more aggressive thyroid carcinomas, the detection of positive preoperative *BRAF* and *TERT* promoter mutations would justify more hardline treatment, such as more aggressive initial thyroid surgery followed by more intense monitoring for disease recurrence.

In conclusion, these data indicate that *TERT* promoter mutations in our Italian patient population are rarely seen in well differentiated thyroid carcinomas but, when present, were mostly linked with aggressive features. This study further illustrates that *TERT* promoter mutation analysis can be easily performed on LBC samples. The use of isolated genetic alterations thus is likely to contribute to diagnosis in those laboratories where more expensive molecular evaluation is unavailable. Nonetheless, apart from the cost analysis, which might be relevant especially for laboratories in developing countries, an additional issue is represented by the lack of high technological infrastructure and supplies necessary for the adoption of an NGS system, which are linked to departments with high-volume samples. The analysis of a single genetic alterations is likely to be easily accessible even by small laboratories, following an algorithm approach starting with the morphologic evaluation of lesions and, for difficult cases, with a preliminary analysis of *BRAF* and *TERT* mutations to exclude a potentially more aggressive thyroid cancer. The combined evaluation of morphologic features and a lack of these mutations, in agreement with data from the literature, is in favor of less aggressive surgical treatment, supported in some cases by frozen-section analysis. Nevertheless, studies involving more patients are necessary to help further unravel the importance of *TERT* promoter mutations and how exposing their combination with *BRAF* V600E mutation can be leveraged to better tailor the therapeutic approach and long-term surveillance of patients with thyroid cancer.

FUNDING SUPPORT

No specific funding was disclosed.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Marco Dell'Aquila: Worked on the data, tables, and revised the initial draft and statistics. **Vincenzo Fiorentino:** Worked on the data, tables, and revised the initial draft and statistics. **Maurizio Martini:** Performed the molecular and immunohistochemical analyses. **Sara Capodimonti:** Performed the molecular and immunohistochemical analyses. **Tonia Cenci:** Performed the molecular and immunohistochemical analyses. **Celestino Pio Lombardi:** Contributed revisions of the draft. **Marco Raffaelli:** Contributed revisions of the draft. **Alfredo Pontecorvi:** Worked on the data, tables, revised the initial draft and statistics, and contributed revisions of the draft. **Guido Fadda:** Contributed revisions of the draft. **Liron Pantanowitz:** Planned and organized the study. **Luigi Maria Larocca:** Planned and organized the study and performed the molecular and immunohistochemical analyses. **Esther Diana Rossi:** Planned and organized the study.

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