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HOW LIMITED MOLECULAR TESTING CAN ALSO OFFER DIAGNOSTIC AND PROGNOSTIC EVALUATION OF THYROID NODULES PROCESSED WITH LIQUID BASED CYTOLOGY (LBC). ROLE OF TERT PROMOTER AND BRAF^{V600E} MUTATION ANALYSIS

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ABSTRACT

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 found that this duet might be involved in the aggressiveness of thyroid cancer, even though its prognostic value related to mortality remains undefined. The detection of such genetic alterations in thyroid liquid based cytology (LBC) may thus assist with patient management.

Method: During January 2013 and June 2014,356 thyroid FNAC samples were processed by LBC including 174 surgical follow-up.$BRAF^{V600E}$ and *TERT* mutations were performed on both LBC and histopathology.

Results: We included 119 atypia of undetermined significance (AUS), 42 follicular neoplasms (FN), 61 suspicious for malignancy (SFM) and 34 positive for malignancy (PM). $BRAF^{V600E}$ mutation was detected in 10.4% whilst *TERT* promoter mutations were found in 1.1% of all cases. *TERT* mutated cases belonged to the PM category, with a histologic diagnosis of tall cell variant of PTC. These genetic alterations correlated with lymph node metastases (p=0,0349) and higher 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.

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 PTC and its variants have an excellent prognosis, 5-10% of these malignancies have an aggressive disease evolution 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 morphological features. It was in the 90s that thyroid cytological material collected from a thyroid nodule by FNA showed to be able to provide a sufficient amount of cells to detect individual point mutations or gene fusions. However, in the 2000s, it was the recognition that *BRAF* mutations has a significant diagnostic and prognostic utility. Specifically, it was in the last decade that researchers have emphasized the role in thyroid cancer oncogenesis played by the activation of *MAPK* and *PI3K-AKT* signaling pathways (5-11) with $BRAF^{V600E}$ mutation frequently found in PTC 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 one assay using a very small number of cells obtained from thyroid FNA samples. The benefits are mostly linked to the fact that NGS has high sensitivity and is able to quantitatively assess the proportion of cells carrying a given mutation. On the other hand, these advantages may be counteracted by the need for having centralized molecular laboratories and increased cost. Since molecular tests are generally still expensive and restricted to only a few specialized and/or centralized laboratories, the adoption of liquid based cytology (LBC) coupled with performing only a few molecular somatic mutations can offer valid diagnostic and even prognostic support for reaching a conclusive cytological diagnosis in smaller laboratories without access to NGS.

Different authors, including our group, have extensively studied the role of somatic mutations including $BRAF^{V600E}$ mutation on cytological 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 re-classification of non-invasive, encapsulated follicular variant of papillary thyroid carcinoma 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, *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, to poor prognosis in several cancers **(14).**

Some authors have documented that *TERT* promoter mutations are commonly found in poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) (e.g. in up to 70% of cases), even though such *TERT* mutations (namely *C228T* and *C250T*) have also been identified in PTC and its variants as well as Hurtle cell carcinoma (HCC) (15, 24-32). Nevertheless, when a *TERT* promoter mutation coexist with *BRAF^{V600E}* mutation thyroid cancers, including PTC are likely to have a synergic role on a poorer prognosis and negative effect on clinical outcome. Liu et al studied the molecular mechanism of their interaction using cancer cell lines of thyroid cancers and melanoma. (13).

Liu et al demonstrated that a simple 4-genotype classification of PTC, especially the classic variant (cPTC), linking mortality risk to the genetic duet of *BRAF^{V600E}* and *TERT* mutations (1). According to this molecular prognostic stratification system, patients with co-existing mutations have a higher mortality risk (1). The possibility of recognizing these potential prognostic molecular findings in thyroid lesions sampled by fine-needle aspiration cytology (FNAC) might help tailor patient management (23). Thus, the detection of *BRAF^{V600E}* and/or *TERT* promoter mutations may delineate more aggressive surgery for concerning thyroid FNAC cases.

The aim of this study was to support the use of small molecular panels, including the performance of *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 diagnosed as suspicious for malignancy (SFM) and positive for malignancy (PM) over a 1.5 year period (January 2013 to June 2014) at the Fondazione Policlinico Universitario "Agostino Gemelli" in Rome, Italy. Furthermore, in order 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) 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, Italy (CU) was searched for thyroidectomy and lobectomy specimens during the same study period. All patient's ages, gender, FNAC diagnoses and follow-up surgical pathology information were recorded. All available pathology slides were reviewed. The majority 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 (with usually two passes performed for each thyroid lesion) were performed with 25 to 27G 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 5000TM processor (Hologic Co., Marlborough, MA, USA). 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 RCPath classification schemes (33-35). The cytology cases were classified and diagnosed according to the new Italian Working Group SIAPEC-IAP classification (36-37) All of the cases were re-evaluated and then re-classified according to The Bethesda System for Reporting Thyroid Cytology II (TBSRTC, 2017) (35). For this retrospective study, analyses were conducted using TBSRTC terminology. This case series included the following distribution of

diagnoses: 5.9% non-diagnostic (ND) including cystic cases; 77.8% benign lesions (BL); 3% AUS/FLUS; 6.1% FN; 2.2% SFM and 5% PM cases. All cytology and histology cases were reviewed by two cytopathologists whilst the re-classification, according to TBSRTC, was undertaken by one cytopathologist (EDR). 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* mutation

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 cytological and surgical specimens containing at least 70% tumor. Details of the molecular protocol employed have been previously published by our group (40-42). For *TERT*, genomic DNA was extracted from LBC samples stored in PreservCyt solution (Hologic, Marlborough, MA, USA) with QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. 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., Wayne, Pennsylvania USA). 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, Hercules, CA, USA). The yielded fragment was separated by electrophoresis on 2% agarose gel containing ethidium bromide and visualized by UV illumination. PCR product was treated with EXOSap (UBS, Sial, Rome, Italy), following the manufacturer's protocol, and directly sequenced using a BigDye Terminator kit v3.1 (Applied Biosystem, Foster City, CA, USA) with forward and reverse primers in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

Histopathology Specimens
All surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin and 5 micron-thick sections then stained with hematoxylin-eosin (H&E). The diagnosis of classical variant of papillary thyroid carcinoma (cPTC) and the different PTC variants were classified according to the WHO 2017 (43). For the definition of tall cell variant (TCV) of PTC, we included cases of PTC with equal or more than 30% TCV component. The histological

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 (AJCC) (45).

Statistical Analysis

Statistical analysis was performed using GraphPad-Prism 5 software (Graph Pad Software, San Diego, CA) and MedCalc version 10.2.0.0 (MedCalc Software, Mariakerke, Belgium). Statistical comparison of continuous variables was performed using the Mann-Whitney U-test or Paired t-test, as appropriate. Comparison of categorical variables was performed using the chi-square statistic, and the Fisher's exact test. P-values less than 0.05 were considered as statistically significant.

RESULTS

Our study included 356 cytology samples examined during the 18-month study time period. The patient demographics and clinical-pathologic features are described in Table 1. We included all cytological samples diagnosed as indeterminate thyroid lesions (AUS/FLUS and FN/SFN), SFM and PM with histological follow-up in 174 cases. For negative control cases, 100 benign cases (from the same study time period) were included in this series, including 25 of which had surgical follow-up. The series included 102 male and 254 female patients with a median age of 44.5 years (range 18-79 years and mean: 46 years) with thyroid neoplasms ranging in size from 0.4 to 7 cm (median 3.2 and median value 3.1).

Our cytological 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 sub-centimeter 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 clinical-pathological data (Table 1).

Table 2 depicts the histological diagnoses rendered in a total of 174 out of 356 cases (48.8%) including 33 benign cases (18.9%) and 141 malignant histological cases (81%), and 2 NIFTP cases (0.1%). The surgical pathology follow-up findings for the different cytological categories

is described in Table 2. The surgical pathology series also included 25 benign cases diagnosed as 22 goiters and three follicular adenomas (FA). Fifteen out of 119 (12.6%) AUS/FLUS cases had surgical follow-up and were diagnosed as two having goiter, 9 follicular adenomas (FA) including two oxyphilic adenoma (OA), one I-FVPTC and three cPTC. The 4 AUS/FLUS cases with a malignant diagnosis were revised and confirmed. The remaining AUS/FLUS cases were followed-up with repeat FNAC and molecular testing resulting in a lack of any malignancy.

Our 42 FN/SFN cases had subsequent surgical resections that were diagnosed as 27 FAs (including 6 OA), one NIFTP, eight PTCs (including one TCV of PTC), five I-FVPTCs and one follicular carcinoma (FC). The 61 SFM cases included one NIFTP, 40 cPTCs and 14 PTC variants (including 9 TCVPTC, 2 hobnail variant of PTC, one Warthin-like variant of PTC, one solid variant of PTC and one sclerosing variant of PTC) and 5 I-FVPTCs. The 34 PM cases were diagnosed as 17 cPTCs, 14 PTC variants (including 6 TCVPTC, one hobnail variant of PTC, one Warthin-like variant of PTC, one solid variant of PTC, 5 I-FVPTCs), 2 medullary thyroid carcinomas (MTC) and one anaplastic thyroid carcinoma (ATC). 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 evaluation of genetic alterations for $BRAF^{V600E}$ and $TERT$ promoter mutations resulted in a total of 40 out 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. Morphological features associated with $BRAF^{V600E}$ mutations included the presence of plump follicular cells with sickle shaped nuclei in all of the mutated cases (Figure 1). No specific morphological features were specifically linked to the $TERT$ promoter mutations (Figure 2).

The distribution of $BRAF^{V600E}$ and $TERT$ mutated cases is reported in table 4. Specifically, we found five $BRAF^{V600E}$ mutated indeterminate neoplasms (4 AUS/FLUS and 1 FN/SFN), 21 mutated SFM and 15 mutated PM cases. The four $TERT$ mutated cases belonged to the malignant category. Histological correlation revealed 14 $BRAF^{V600E}$ mutated cPTC, 10 cPTC with multifocal pattern, 13 TCVPTC, 2 hobnail variant of PTC, and 2 I-FVPTC. Specifically, four out of the 13 TCVPTC cases also had $TERT$ promoter mutations with tumors that exhibited at least 50% amount of 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 alteration we found a higher correlation with these mutations and lymph node metastases (p=0,0349; CI 95% 0,06882 range 0,4758-0,9954). Nevertheless, no significant correlation was found with tumor size (p=0,2149;

CI 95% 0,8693 range 0,7056-1,071) and multifocality (p= 0,6538, CI 95% 0,9064 range 0,6380-1,288.

A correlation was detected between $BRAF^{V600E}$ and thyroid stage, showing a significant p value=0,0009 for stages II and III. While the limited number of $TERT$ promoter mutated cases showed not statistically significant correlation, these $TERT$ mutated cases were associated with aggressive PTC variants (i.e. 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 features at presentation and they are likely to develop early distant metastases or relapse associated with adverse outcome (2). Figuring out which PTCs will behave badly early on 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 $BRAF^{V600E}$ mutation and $TERT$ promoter mutations have been well studied in

PTC 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 pathological 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 since some genetic alterations including *BRAF^V600E* mutation occur mostly in cPTC and TCV-PTC and uncommonly in invasive FVPTC, which represents a more favorable variant (49-50).

Other studies have reported a correlation between *BRAF^V600E* and more aggressive clinicopathological outcomes of cPTC, especially with respect to bilateral disease, extrathyroidal extension and nodal involvement (5-15). While the data from the current series confirms the correlation of *BRAF^V600E* with nodal involvement (p=0.0349), no correlation was noted with disease multifocality (42). Similar findings were documented by Ahmad et al, concluding 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 (32). 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* 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, 55). A high prevalence of *TERT* C228T mutation has been found in aggressive thyroid cancers, such as ATC and PDTC (24, 26, 28-30). Only a few studies, including Wang et al, reported that C228T mutation was identified in 2% of FA and 17% of atypical adenomas (56). 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 (30). Trybek et al studied 568 PTC cases with known *BRAF^V600E* and *TERT* status, concluding 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 (52). As such, Trybek and colleagues advocated that these genetic alterations may be useful for predicting a poor response to therapy, recurrence, and poor outcome in mutated patients (52).

Vuong et al in their meta-analysis that included 42 studies with 11.109 PTC samples demonstrated that PTC with concurrent $BRAF^{V600E}$ and $TERT$ promoter mutations were associated with increased tumor aggressiveness, especially distant metastases, in comparison with PTCs harboring $BRAF^{V600E}$ or $TERT$ promoter mutations alone (28, 53).

Further published data has suggested that $BRAF^{V600E}$ activates the mutated $TERT$ promoter by fos proto-oncogene (FOS), which is a downstream effector of MAPK signaling and the GA-binding protein (GABP) complex (14). In fact, Song et al found that $TERT$ mRNA was increased by the coexistence of $BRAF^{V600E}$ in a series of 331 PTC (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 shown by larger diameters, invasion of surrounding tissues, cervical nodal metastases and locally persistent disease after the 1st surgery and distant metastases in those case with co-expression of $BRAF^{V600E}$ and $TERT$ promoter mutations. (16)

Lee et al studied 242 cyto-histological thyroid nodules including 207 PTCs examined for $BRAF^{V600E}$ and $TERT$ promoter mutations. The $TERT$ mutation was associated with recurrence (p=0.03), whilst a coexistence of those mutations were significantly associated with older age and advanced stage (54).

Furthermore, the results from Liu et al suggested that patients with either a $BRAF^{V600E}$ mutation (2.4%), $TERT$ 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 histological samples, and focused on the post-surgical management of thyroid cancers (55). To date, these studies have not been replicated using cytology specimens.

This is important because the diagnosis of thyroid cancer usually begins with a cytological diagnosis based on a FNAC sample. Advances with new sequencing techniques such as next generation sequencing (NGS) have propelled the role of molecular testing in thyroid cytological samples, especially in helping resolve indeterminate lesions following FNAC. The use of NGS is currently limited to highly specialized reference or academic laboratories, mostly due to their high cost and availability of skilled staff in many countries. Based on 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 (i.e., *BRAF^V600E, HRAS, NRAS, KRAS, RET/PTC1, RET/PTC3,* and *PAX8/PPARg*) (58). Specifically, this assay exhibits a 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 fact that *RAS* mutations, mostly found in follicular thyroid lesions, cannot univocally discriminate between adenoma and carcinomas. Furthermore, the introduction of NIFTP has confirmed that certain genetic alterations can be documented in non-invasive and encapsulated follicular variant of PTC, emphasizing that *BRAF^V600E* and *TERT* mutations are likely to be linked with more aggressive thyroid cancers (44).

Whilst some genetic alterations such as *BRAF^V600E* have been extensively studied on cytological samples, investigations of *TERT* promoter mutations on thyroid FNAC specimens have been limited (22, 23, 27, 40-42, 50). In order to demonstrate the feasibility of a 4-genotype classification for PTC, we performed *BRAF^V600E* and *TERT* promoter mutations on our cytology samples, and specifically using LBC specimens from indeterminate, SFM and PM cases. While 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, 59-62), to the best of our knowledge, this is the second study, following a paper by Decaussin-Petrucci, documenting the evaluation of *TERT* promoter mutations on LBC stored material (57). While Decaussin-Petrucci et al found that *TERT* promoter mutation was rare, but very specific for malignancy (5.5%) in indeterminate cytology, the current series confirmed a scant number of *TERT* mutated cases (4/356 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 morphological features (i.e sickle shaped nuclei, eosinophilic cytoplasm, 59), none of the limited (only 4) *TERT* mutated cases had distinctive findings apart from tall cell appearance with more severe pleomorphic nuclei, which needs 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 four cases with concomitant mutations were PTC with a stage III or IV. These results helped identify those patients, who are a minimal proportion of PTC series but with the

highest risk of aggressive disease, demonstrating the utility of this genetic duet to provide a powerful a molecular prognostic system. In fact, according to several publications, whilst some genetic alterations are unable to discriminate between benign and malignant lesions, or between indolent and more aggressive thyroid carcinoma, the detection of positive pre-operative *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 is thus 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 a NGS system, which are linked to departments with high volume samples. The performance of single genetic alterations is likely to be easily accessible even by small laboratories, following an algorithm approach starting with the morphological evaluation of lesions, and for difficult cases with the preliminary analysis of *BRAF* and *TERT* mutations to exclude a potentially more aggressive thyroid cancer. The combined evaluation of morphological features and 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 its combination with *BRAF^{V600E}* mutation can be leveraged to better tailor the therapeutic approach and long-term surveillance of patients with thyroid cancer.

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Table 1. Summary of clinical-pathologic data

Clinical-pathological features	Proportion (n=356 cases)
Patient age	
Mean	46 years
Median	44 years
Range	18-79 years
Patient gender	
Male	102 (28.6%)
Female	254 (71.4%)
Cytology diagnosis	
Benign	100 (29%)
AUS/FLUS	119 (33.4%)
FN/SFN	42 (11%)
SFM	61 (17%)
PM	34 (9.6%)
Histopathlogy diagnosis§	
Benign*	33 (18.9%)
Malignant	141 (81.1%)

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; *Includes follicular adenomas and NIFTP; § histology available in only 174 cases

Table 2. Cytolocial-histological correlation in 174 cases

Pathology Diagnosis	Goiter	FA	NIFTP	PTC	I-FVPTC	FC	OFC	MTC	ATC
Benign (25 cases)^	22	3	/	/	/	/	/	/	/
AUS/FLUS (15 cases)	2	9§	/	3	1	/	/	/	/
FN/SFN (42cases)	/	27*	1	8	5	1	/	/	/
SFM (61 cases)	/	/	1	54	5	/	/	/	/
PM (34 cases)	/	/	0	26	5	/	/	2	1

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 PTC, NIFTP: "noninvasive follicular thyroid neoplasm with papillary-like

nuclear features: MTC: medullary thyroid carcinoma; OFC: Onococytic follicular carcinoma; PM: positive for maliganncy; PTC: Papillary thyroid carcinoma, .SFM: Suspicious for Malignancy; §includes 2 oxyphilic adenomas; *includes 6 oxyphilic adenomas; ^25 benign cases out of 100 had histological follow-up

Table 3. Distribution of histopathological diagnoses for classic PTC and variants

	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

AUS/FLUS: Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance; cPTC: classic papillary thyroid carcinoma; DSV-PTC: diffuse sclerosing variant of PTC; FN/SFN: Follicular Neoplasm/Suspicious for Follicular Neoplasm; I-FVPTC: invasive follicular variant of PTC; PM: positive for maliganncy; SFM: Suspicious for Malignancy; SV-PTC: solid variant of PTC;TCV: tall cell variant PTC;

Table 4. Cytological-histopathological 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)	Goiter	wt	wt
	FA	wt	wt
AUS/FLUS (15 cases)	Goiter	wt	wt
	FA*	wt	wt
	I-FVPTC	wt	wt
	PTC	4mutated	wt
FN/SFN (42 cases)	FA	wt	wt
	OA	wt	wt
	NIFTP	wt	wt
	PTC	1 mutated	wt
	I-FVPTC	wt	wt
	OFC	wt	wt
	FC	wt	wt
SFM (61 cases)	FA	wt	wt
	NIFTP	wt	wt
	PTC	17 mutated	wt
	I-FVPTC	4wt	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

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 PTC, MTC: Medullary thyroid carcinoma; NIFTP: ; OA; Oncocytic Adenoma;

OFC: oncocytic follicular carcinoma; PM: positive for malignancy;   PTC: Papillary thyroid carcinoma, SFM: Suspicious for Malignancy; wt: wild type
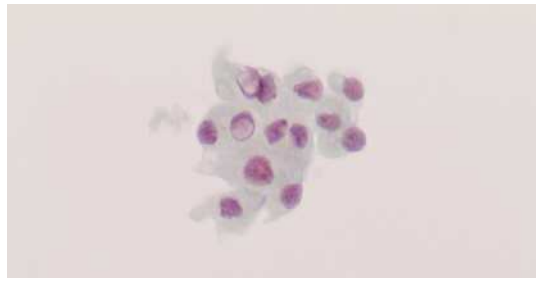
*Includes 75 additional benign lesions without histology and wild type for both *BRAF^{V600E}* and *TERT* promoter mutations

Figure Legends

Figure 1. Morphological features of a malignant thyroid lesion diagnosed as positive for malignancy favoring a papillary thryoid carcinoma. The lesion harbored *BRAFV600E* mutation and the moderate eosinophilic cytoplasm and sickle-shaped nuclei (Pap stain 40x)

Figure 2. A FNAC from a malignant case diagnosed as positive for malignancy, harboring *TERT* and BRAFV600E mutation. The cells showed significant pleomorphic and irregular nuclear, with some clearing, which were not patognomonic of the classic features seen in papillary thyroid carcinoma on cytology (Pap stain 40x).
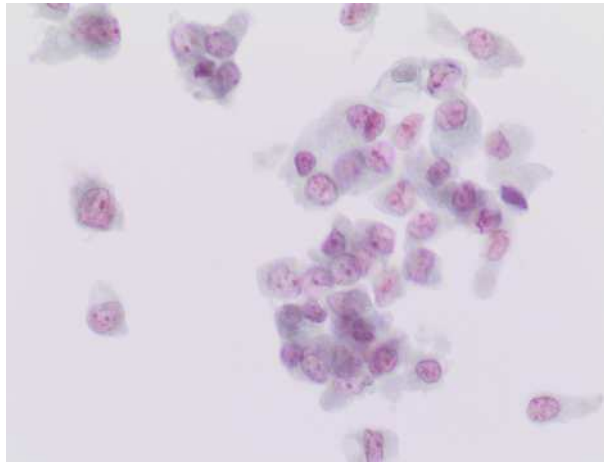
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