P53 expression in cytology samples may represent a marker of early stage cancer

Federica Policardo MD[1°], Pietro Tralongo MD[1°], Damiano Arciuolo MD[1] Vincenzo Fiorentino MD[1] Lina Cardasciani MD[1], Francesco Pierconti MD[1], Angela Carlino MD[1], Mariangela Curatolo MD[1], Alfredo Pontecorvi MD[2], Guido Fadda MD[1], Carmela De Crea MD[3], Celestino Pio Lombardi MD[3], Marco Raffaelli MD[3], Luigi Maria Larocca MD[1§], Liron Pantanowitz MD[4§], Esther Diana Rossi MD[1§]

[1]Division of Anatomic Pathology and Histology; [2]Division of Endocrinology; [3]Division of Endocrine-Surgery-Fondazione Policlinico Universitario "Agostino Gemelli"-IRCCS, Rome Italy; [4]Department of Pathology, USA

§These authors share seniorship; ° These authors share first authorship

RUNNING TITLE: P53 and thyroid cytology

KEY WORDS: Fine needle aspiration cytology, thyroid cancer, p53, thyroid neoplasms, follicular lesions, personalized medicine

Number of pages:22 Figures:4; Tables: 4

***Corresponding author**: Esther Rossi, MD, PhD, MIAC- Division of Anatomic Pathology and Histology – Fondazione Policlinico "Agostino Gemelli"-IRCCS, Università Cattolica del Sacro Cuore,– Largo Francesco Vito, 1 – 00168 Rome (Italy) Phone: +3906-3015-4433; e-mail: esther.rossi@policlinicogemelli.it

BACKGROUND: TP53 gene plays a major role in the negative control of cell proliferation and in the regulation of signaling cascades. TP53 mutation may have a relevant role in the malignant transformation of thyroid cells as well as thyroid tumor progression. TP53 mutation has been detected only in few well differentiated thyroid carcinomas and is absent in benign conditions.

MATERIAL AND METHODS: One hundred and sixty-two prospective thyroid cytology and corresponding histological samples diagnosed from atypia of undeterminate significance-AUS to malignant, were studied via immunocytotechemistry (ICC) for p53. Hence, 50 Benign lesions (B) were used as negative control. Molecular analysis for p53 only was performed.

RESULTS: The cytology resulted in 50B, 48AUS, 40FN (folllicular neoplasms), 23SFM (suspicious for malignancy), and 1M (malignant) case. We reported 102negative and 60positive p53 cases. The 60 positive included 27cases with weak/focal cytoplasmic positivity (+1), and 33cases moderate(2+) to strong(3+) cytoplasmic/nuclear expression. Overall, 71 cases had histology (2B, 11AUS, 37FN, 20SFM, 1M) including 61.7%benign and 38.2%malignant diagnoses. Only 16/71 (5FN, 10 SFM, 1M) were p53 positive. Furthermore, 100%AUS and 86.5%FN cases were p53negative, none of which had malignant histology. All p53 positive cases were associated with a larger nodule size, TCV subtype, multifocality, extrathyroidal infiltration, nodal metastases. NIFTP were negative for p53. Few discrepancies in p53 intensity were observed on histology; there were no differences with the molecular testing

CONCLUSIONS: P53 might be useful in discriminating thyroid follicular lesions. P53 is likely to be a useful diagnostic marker in recognizing indeterminate lesions that are well-differentiated thyroid cancers

Introduction

Papillary thyroid carcinoma (PTC) is one of the most common malignancies and the most common malignant tumor in the thyroid gland (1-3). Despite the fact that the majority of PTCs have a good prognosis, a few exhibit recurrences and a poor prognosis. Numerous publications have taught us that several factors have prognostic implications for PTC including tumor size, histological type, patient age, and lymph node metastases (1-3). Several genetic alterations have been identified in thyroid cancers and they offer a promising role in personalized patient management (4-9).

In these last few decades, a series of molecular markers have been described in thyroid cancers, supporting diagnostic and prognostic scoring systems (4-9). Among them, mutations in the p53 tumor suppressor gene are present in approximately 50% of all human malignancies, including thyroid cancers and mostly undifferentiated/anaplastic (ATC) thyroid carcinomas (10). Nonetheless, very few studies have discussed the diagnostic and/or prognostic role of p53 mutation in well-differentiated thyroid cancers (WDTC) including PTC (10-12). In fact, it seems that p53 protein is overexpressed in larger thyroid nodules and in lymph node metastases. Thus, its evaluation may likely contribute to more personalized treatment for PTC or help target lymph node recurrences (10-12).

The correlation of p53 with a more aggressive behavior is confirmed by its expression in up to 50% of ATC cases and only a minority of WDTC. For this reason, some authors hypothesized that p53 is likely a precursor and initial step for tumors that might undergo de-differentiation (10-13). Additionally, analogous with the gastrointestinal tract, where the expression of p53 is assessed in highly dysplastic intestinal cells, we followed the hypothesis of a possible multi-step process including a dysplastic phase for thyroid cells of indeterminate lesions that may in turn progress into malignant cells. The finding of p53 might accordingly help in discriminating indeterminate thyroid nodules that are benign versus malignant.

Although p53 can be assessed by molecular detection, the possibility of immunohistochemical (IHC) evaluation is an alternative testing modality, especially in those laboratories where molecular testing is limited (11-20). To date, a few papers have found a discrepancy between IHC and p53 gene mutations, with a lack of mutations in cases that demonstrated positive p53 staining by IHC (21-22). The majority of papers evaluated p53 immunostaining on histological thyroid samples (10-20), and, to the best of our knowledge, no publications have been reported about its role in cytological samples, including liquid based cytology (LBC).

In the present study, we examined the immunocytochemical (ICC) expression of p53 in a prospective series of thyroid lesions, including indeterminate categories, suspicious for malignant and malignant diagnoses with cases that had surgical follow-up. These cases were analyzed for possible correlation between p53 expression via ICC and genetic analysis, combined with clinical-pathological features.

MATERIALS AND METHODS

A prospective evaluation of p53 ICC was performed of all consecutive thyroid cytological cases diagnosed in the cytological categories ranging from indeterminate neoplasms, suspicious for malignancy to malignant categories including those with subsequent surgical pathology follow-up. For our purposes we use also 50 benign lesions, as negative control on cytology. The cases were diagnosed and recorded in the archives of the Division of Anatomic Pathology and Histology of the "Fondazione Policlinico Universitario Agostino Gemelli" – IRCCS during the period between September 2021 and May 2022. The institution's electronic medical record system (Armonia-Metafora, Italy) documented those cases with surgical procedures (thyroidectomy specimens). The patient's age, gender, diagnosis, previous fine needle aspiration cytology (FNAC) diagnosis, and follow-up information were tabulated. All cytological material and thyroidectomy slides were reviewed for the current study.

These patients all underwent thyroid ultrasound performed in the “Centre for Thyroid Diseases” at our hospital. All aspirations (usually two passes performed for each lesion) were performed with 25 to 27G needles. No rapid on-site assessment for adequacy of material was done. All patients consented to their procedure. We received institutional (Catholic University of the Sacred Heart) ethics approval for this study (CE ID-3832). Cytology samples were processed using ThinPrep (Hologic Co., Marlborough, MA). Prepared slides were fixed in 95% methanol and stained with the Papanicolaou stain. Any remaining material was stored in Preservcyt solution for possible preparation of additional slides and ancillary investigations (e.g. immunostains and molecular analysis) if needed.

The lower limit for cytological adequacy of each sample was established according to the Bethesda and British RCPath classification schemes; specifically, the minimum number of adequate cells in each sample was six groups of thyroid follicular epithelial cells per submitted slide where each of these groups contained at least 10 well-visualized epithelial cells (23-25). The cytology cases were classified and diagnosed according to the New Italian Working Group SIAPEC-IAP classification (26). These categories are defined as follows: TIR1: inadequate, TIR1C: cystic-hemorrhagic lesions, TIR2: benign nodules, TIR3A, follicular neoplasm (low-risk indeterminate lesions), TIR3B: follicular neoplasm (high-risk indeterminate lesions), TIR4: suspicious for malignancy, and TIR5: positive for malignant neoplasm. All of cases were re-evaluated and then re-classified according to The Bethesda System for Reporting Thyroid Cytology (TBSRTC) (25). For the purpose of this study, analyses were conducted using TBSRTC terminology. The entire FNAC series from the reference period included the following distribution of cases: 2% non-diagnostic including cystic cases, 71.3% benign; 8% atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS); 11.7% follicular neoplasm/suspicious for follicular neoplasm (FN/SFN); 4.5% suspicious for malignancy (SFM) and 2.5% as malignant (M). All cytology and histology

cases were reviewed by two cytopathologists. Equivocal cases were reviewed by additional pathologists in order to achieve final consensus agreement.

Immunocytochemistry (ICC) analysis

p53 immunostaining was performed using the FDA-approved Dako p53 pharmDx (clone 22C3; Dako/Agilent Technologies, Carpinteria, California) on a Dako Autostainer Link 48 (Dako/Agilent Technologies). Antigen retrieval was performed using PT Link with Target Retrieval Solution (low pH; Dako/Agilent Technologies) as specified by the manufacturer without any modification to adjust for differences between cytology and histology. We adopted the same LBC criteria for performing ICC and the % of positivity/negativity was similar as undertaken with the validation and long-standing performance of HBME-1 and Galectin-3 immunocytochemistry previous described by our group (27-30). FNA specimens were reviewed to identify cases with adequate material for the study. The minimal percentage of adequate lesional cells for the performance of p53 evaluation was defined at 100 tumor cells in LBC samples. The p53 levels were compared with the internal and external controls represented by stromal fibroblasts and endothelial cells. Furthermore specific external control as endometrial tissue was compared with our series. According to the evaluation and remembering also our previous experiences in the adoption of ICC on LBC from HBME-1 and Galectin-3 papers, we put a cutoff for the expression of p53 in our study was defined at 30% staining of lesional cells (27-31). The p53 ICC positivity was defined as nuclear and/or cytoplasmic staining, and staining intensity was defined as 0 = negative expression; 1 = weak expression when visible with a 40x microscope objective lens; 2 = moderate when visible with a 10x and/or 20x microscope objective lens, and 3 = strong when visible with a 4x microscope objective lens. For statistical purposes only, the scores 0 and 1 were considered as negative, whilst score 2 and 3 represented positive p53 expression. According to these combined scoring systems, we considered as positive, only cases with intensity score 2 and/or 3 in more than 30% of the neoplastic/lesional cells. For the evaluation of histological samples, the same staining cutoff values were used as for

cytological samples; we noticed that histological cases with p53 expression were uniformly positive in up to 50% of lesional cells. P53 control**s** were run concurrently; they included the Dako provided positive and negative cell-line controls represented by stromal fibroblasts and endothelial cells (internal) and endometrial cells (external). For the histological evaluation we selected sections including the neoplastic/ cancrinomatous cells and benign thyroid tissue. Our decision to apply ICC to LBC samples was based on three reasons: 1) our cytology team has had long-standing success with validated ICC protocols on LBC, 2) our previous personal experience with ICC (Galectin-3 and HBME-1) resulted in contradictory results when LBC results were compared to formalin fixed, paraffin-embedded cell blocks, and 3) to demonstrate the feasibility of LBC for p53 analysis. P53 expression was evaluated independently by two different cytopathologists who scored these cytology slides, being blinded to the scoring results of concomitant histology specimens. Each case was evaluated for inter-observer agreement; any case with more than 10% discordance was reviewed together for a final consensus opinion. The assessment of p53 was prospective so that the pathologists were blinded to p53 staining when assessing diagnoses. The patients were not triaged to surgery based on the presence/absence of p53 staining but based on the guidelines for the cytological diagnostic categories.

Molecular analysis for *p53* mutation

DNA was extracted from both LBC stored material and paraffin embedded tissue. The *p53* mutational analysis was performed on DNA extracted from cytological and surgical specimens containing at least 70% tumor. Details of the protocol employed have been previously published by our group (27-32).

Histopathology

All surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin and then subsequent 5 micron-thick sections were stained with hematoxylin-eosin (H&E). The peri-thyroid

adipose tissue, if present, was submitted and examined for lymph nodes. The diagnosis of PTC was based on the presence of true papillary structures and distinctive nuclear features, whereas the diagnosis of follicular variant of PTC (FVPTC) relied upon the detection of entirely follicular architecture and nuclear features of PTC in multiple foci. Encapsulated tumors with either lympho-vascular invasion (within the capsule or beyond) or capsular penetration were diagnosed as invasive FVPTC (33). All the cases were classified according to the eighth edition of the tumor-node-metastasis (TNM)-based staging system recommended by the American Joint Commission on Cancer (AJCC) (34). The histological diagnosis of non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) was rendered according to the criteria described in the paper by Nikiforov et al (35). In fact, in our institution NIFTP terminology was used for follicular neoplasms without any overt papillary structures. All suspected NIFTP tumors were submitted entirely for microscopic examination. The diagnosis of follicular adenoma (FA) was based on the evidence of an encapsulated nodular lesion with typical follicular cells. The follow-up period for thyroidectomy ranged between 2 and 9 months.

Statistical Analysis

Statistical analysis was performed using GraphPad-Prism 6 software (Graph Pad Software, San Diego, CA) and MedCalc version 10.2.0.0 (MedCalc Software, Mariakerke, Belgium). Comparison of categorical variables was performed using the chi-squared test and the Fisher's exact test, with a 95% CI.P-values that were lesser than 0.05 were considered as statistically significant.

RESULTS

Our study included 162 cytology samples examined during the 10 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 (including both AUS/FLUS and FN/SFN), SFM and M with histological follow-up. For the negative control cases, 50 benign cases (from the same study period) were included in this series. The total number of 162 cases is obtained by the exclusion of 20 cases because 10 of these cases did not have adequate tumor cells so that p53 was not performed, and 10 additional samples that resulted in fewer than 100 tumor cells after having performed the p53 stain. The series included 50 male and 112 female patients with a median age of 49 years (range 15-85 years and mean: 49.2 years), and their thyroid neoplasms ranged in size from 0.5 to 7.3 cm (Table 1).

For the study period, our cytological series (162 cases) included the following distribution of thyroid diagnoses: 50 benign; 48 AUS/FLUS; 40 FN/SFN; 23 SFM and 1 M (Table 2)**.** All sub-centimeter lesions were discovered incidentally during radiologic screening for causes unrelated to the thyroid gland and according to the radiologic criteria of suspicious nodules undergone FNAC directed to those nodules with diagnoses ranging from indeterminate to malignant categories. There was no significant difference in the size of lesions among the diagnostic entities. No statistical correlation was found with different parameters related to the clinical-pathological data.

Seventy-one cases had histological follow-up with benign histology in 63.38% (45 cases), malignant in 36.62% (26 cases), and the inclusion of three NIFTP cases in the benign cohort. The correlation with the surgical pathology follow-up of the different cytological categories is described in Table 2. The 50 benign cases included only two histological cases diagnosed as goiters. The 11 AUS/FLUS cases were diagnosed as three nodular goiters, seven follicular adenomas (FAs) and one invasive follicular variant of papillary thyroid carcinoma (I-FVPTC). The 37 FN/SFN cases were confirmed to be: 1 goiter, 29 FAs including two oncocytic adenomas (OAs), three NIFTP, and four classic PTCs. The 20 SFM cases included 15 classic PTCs and five I-FVPTCs. The one M case was diagnosed as classic PTC.

There were a total 26 PTC cases based on histology, including 20 classical variant of PTC, with TWO of them showing a minor tall cell subtype of 20% of cells, and 6 I-FVPTC. Among these 26

cases, histologically 9 had multifocal PTC (5 classic PTC and 4 I-FVPTC), and 7 had lymph node metastases. Fourteen of these tumors were larger than 1 cm and 10 were larger than 1.5 cm.

In Table 3 we analyzed the expression of ICC p53 in our series of 162 cytological cases (Table 3). Among the cases**,** none of the benign lesions and NIFTP had p53 expression (Figure 1). Our series included 60 (37%) cases with p53 expression distributed according to the following categories of TBSRTC: 10 AUS/FLUS cases (16.9%); 32 FN/SFN cases (54.2%); 17 SFM cases (28.8%); and 1 M case (1.6%). All of these positive cases were characterized by positive cytoplasmic and membranous stain expression (Figures 1 and 2a-b). As previously noted, we evaluated p53 expression using the cut-off value of 30%. When analyzing stain intensity, the majority of these cases (26 cases) had moderate expression in more than 30% of lesional cells; five cases demonstrated strong cytoplasmic expression of p53 in the majority of cells (more than 50% of them), whilst three cases had strong cytoplasmic and nuclear positivity, including the 2 PTC with a minor TCV (tall cell variant) subtype component. Nevertheless, 27 cases showed weak cytoplasmic positivity**.** Table 4 shows the correlation and intensity of p53 expression only in the cyto-histological series (71 cases). Only 16 out of these 71 cases had a positive p53 stain, distributed among the categories of FN, SFM and M. Only one FA and one OA had cytoplasmic positivity, whilst three out of four PTC were strongly positive. Of note, NIFTP cases were p53 negative. In the SFM group, 10 out of 20 PTC had moderate and strong cytoplasmic positivity, as well as the malignant case. To confirm our cytology results we evaluated p53 staining in corresponding histological samples. P53 staining in LBC samples showed high concordance with matched histological specimens. All of the positive cases had diffuse and homogeneous positivity for p53. All of the negatively stained samples also showed 100% concordance with histology . Only a few discrepancies were identified, related mostly to differences in the intensity of staining between corresponding cytological and histological specimens, but there wa**s** no statistically significant difference and no apparent clinical implications. We attributed those discrepancies to different

technical procedures and material preparation (cytology versus histology); even if none of our negative case on cytology was positive on histology and vice versa.

Those differences could be also attributed to insufficient numbers of adequate tumoral cells in cytological slides and the overestimation of p53 expression in cytology cases. In particular, while weak and moderate expression maintained the same staining intensity on corresponding cytology and histology cases, four cases with strong p53 cytological expression exhibited only moderate p53 staining on matching histology specimens (expressed in more than 50% of the neoplastic cells).

P53 mutation, evaluated by direct DNA sequencing of thyroid LBC samples, correlated with p53 expression in our series of cases (Table 3). None of the AUS/FLUS and SM resulted in a p53 mutation, whilst only five malignant cases harbored *p53* mutation including five PTC specimens. When we compare ICC and molecular results, we found that these 5 mutated cases corresponded to three cases with more intense cytoplasm positivity and only two with also nuclear positivity. To note, none of the FA and NIFTP cases was *p53* mutated. All the five PTC cases with p53 expression had a more aggressive behavior in terms of multifocality, nodal metastases and larger size (> 1.5 cm). Furthermore, two out of these five PTC had a minor tall cell subtype component of at least 20%. Larger series are necessary in order to confirm these data.

We compared p53 (cytological and histological) expression with matched thyroid staging, according to the 8th edition of AJCC in the 26 malignant cases of our series. The data showed that the majority (23 cases; 88.4%) of our patients had stage I disease, three patients had stage II (5.3%), and none of them had stage III and/or IV (data not tabled). The comparison between p53 expression and AJCC stages (I-IV) was not found to be statistically significant (p value = 0.4842; Chi-square=2.451; 3 degrees of freedom).

The comparative analysis of stage and p53 staining did not show any significant correlation confirming the lack of any correlation between p53 expression and thyroid lesion aggressiveness. In fact, our results showed that the majority of cases, regardless of p53 expression, were stage I (41.5% p53 positive versus 51.3% p53 negative cases in Stage I). Nonetheless, the three cases with

more advanced stage (stage III) had p53 strong expression. The evaluation of p53 ICC and the three patients in stage II found that they belonged to the group of cytoplasm expression, without any nuclear positivity. Due to the lack of any pure PTC subtypes, a possible correlation of p53 with more aggressive subtypes of PTC needs larger investigative series.

DISCUSSION

Currently, the first and most valid diagnostic tool for the diagnosis of thyroid nodules is FNA (36-38). Although FNA is able to correctly identify the majority of benign and malignant thyroid lesions, the category of indeterminate nodules cannot be further specified by morphology alone (39-40). The different classification systems, including TBSRTC, has subclassified indeterminate lesions in order to provide a more accurate morphological evaluation linked to specific risk of malignancy (25). Despite this sub-classification, in some cases, using the morphological criteria alone, a conclusive discrimination between benign and malignant entities is not always possible.

Over the last few decades, we have witnessed increased attempts to personalize the management of thyroid lesions by combining morphological criteria with the support of ancillary techniques including ICC and molecular testing (10-20; 36-40). ICC represents a simple and relatively inexpensive test, which can be performed in most laboratories for the routine evaluation of thyroid nodules. Many different antibodies have been developed and tested in order to demonstrate their diagnostic role in thyroid lesions. As documented in prior studies, including some from our group, among the antibodies investigated are HBME-1 and Galectin-3, which have shown high diagnostic accuracy in discriminating benign versus malignant thyroid nodules (27). As previously suggested, the positive expression of a panel made up of HBME-1 and Galectin-3, in cases classified as either AUS/FLUs or FN, can effectively distinguish those thyroid lesions that need immediate surgery (high risk) from those which can be followed-up (low risk) (27). However, this immunopanel and some other markers studied on FNA material have not been able to offer 100% conclusive results. Additionally, the support of genetic alterations, including somatic mutations and rearrangements,

has shown high specificity for thyroid carcinoma, especially PTC, and that these molecular findings can be used feasibly and yield reliable results on cytological material. Among them, *BRAF*^{V600E} and *TERT* promoter mutations seem to be linked with a more aggressive neoplastic behavior in terms of multifocality, local recurrences and nodal metastases (41-46). Although the diagnostic and prognostic role of the latter gene mutations have been extensively studied, controversial and scant data are available concerning the possible role of *TP53* determined by genetic evaluation and p53 as an immunomarker in WDTC.

The present study deals with the evaluation of p53 immunoexpression in differentiating benign from malignant thyroid lesions, comparing its results with the molecular evaluation of p53 in order to assess if p53 ICC is as reliable as p53 molecular testing. The purpose is to verify the possible beneficial use of p53 ICC instead of the molecular testing, mostly because the latter is expensive, it is not available in every laboratory and is likely to delay the management. According to the literature, p53 is an important tumor suppressor gene regulating cell response to DNA damage by inhibiting cell proliferation and malignant transformation (47-53). For this reason, we decided to study the possible role of p53 in a prospective series of thyroid lesions classified according to TBSRTC. Different papers, including ours, have described and validated the diagnostic role of some panels of immunomarkers (i.e. HBME-1, Galectin-3, CK-19 and CD56), even though none of them, as single marker or in an immunopanel, is likely to answer all diagnostic questions when dealing with an indeterminate thyroid proliferation (27-29, 36-38). Although many authors emphasized that p53 is more likely to be associated with high-grade thyroid cancers, including PDTC and ATC, others pointed out that p53 expression was assessed only in the early stages of thyroid cancer and in WDTC (46-50). This latter concept is in line with published evidence that a p53 positive gastrointestinal tract biopsy is synonymous with a dysplastic lesion (19). Nonetheless, right now, similar dysplastic morphology has not been defined in thyroid lesions.

To date, in the majority of series, the evaluation of p53 has been performed on histological specimens. In our study, we used both LBC stored material and histological samples. Our approach

was focused on two major points: 1) feasibility of performing p53 ICC on LBC material and 2) to demonstrate that a combination of morphology and p53 ICC can be helpful in discriminating between low and high-risk thyroid indeterminate nodules. Furthermore, we also used molecular testing to evaluate p53 on the same lesions.

According to the literature, Shin et al demonstrated an overexpression of p53 protein in 47.3% of their PTC cases without any significant correlation with clinical-pathologic features (11). On the other hand, Hosal et al documented significant p53 overexpression in PTC with extrathyroidal infiltration and/or metastases (16). Similar results were also reported by Morita et al (21) and Horie et al. (22), reporting a significant correlation with large tumor size and lymph node metastases. In our series, including 71 cases with histological follow-up, only 16 cases had p53 positive expression and this was observed only in malignant lesions diagnosed as classic PTC and I-FVPTC. We did not find any statistical correlation with aggressive behavior, even for the two cases with strong positivity that had a minor TCV subtype of 20%, and 5 cases with strong positivity that showed multifocality and lymph node metastases. We further compared p53 expression with thyroid staging of cancers according to the 8th edition of the AJCC (34). This correlation was not statistically significant with the majority of cancers in Stage I (88.4%), and only three patients in stage II with none of them in stage III or IV. Those three cases expressed only cytoplasm positivity without any nuclear expression. We need to underline that our large number of stage I tumors may limit the ability to test whether p53 is associated with thyroid aggressiveness. Perhaps larger series, including different stages and a different design, are necessary to better clarify if there is indeed a correlation with p53 and more aggressive subtypes of PTC.

Despite the fact that a cytological diagnosis of PTC is based on specific architectural and nuclear features, there are still some difficult cases to interpret characterized by subtle findings, in which the differential diagnosis includes hyperplastic nodules, FA, NIFTP or follicular carcinoma (FTC). Tan et al reported the lack of p53 expression in FA and FTC (48), whilst Nasir found 90% p53 positivity in FTC and only 15% positivity in FA (49). Shin et al calculated 85% sensitivity and

72.7% specificity of p53 in discriminating PTC from other benign thyroid lesions, showing that it can be added as an additional diagnostic immunomarker in a panel (11). Marcello et al found that p53 expression was more frequently seen in malignant than benign thyroid lesions, thereby representing a useful marker in discriminating follicular indeterminate lesions (49). Hence, they found a correlation of p53 with smaller nodules (< 2 cm) and solitary nodules. Our series did not find any statistical correlation with nodule size, even though strong p53 expression was found in nodules larger than 1.5 cm.

Considering the Bethesda categories, we found that among the FN category, the majority of FN cases with a malignant histology (75%) had positive expression of p53, whilst only two FNs out of 26 histological FAs resulted in a positive finding (76.9%). This might suggest that p53 positivity is likely to identify high-risk FNs, akin to a precursor like dysplasia. We further classified the expression of p53 immunostaining as nuclear and cytoplasmic, paying attention to differences with stain intensity. A common finding was the detection of cytoplasmic or membranous expression in several cases, mostly in the AUS/FLUS and FN cases, whilst combined nuclear and cytoplasmic expression was seen in malignant cases. In our surgical follow-up series, none of the AUS/FLUS cases has p53 positive staining, confirming that the majority of AUS/FLUS lesions are histologically benign. For the FN category, only a minority of them (5 cases) had p53 expression including 3 PTC and only 2 adenomas. Despite the limited number of cases, it seems that p53 expression is likely correlated with thyroid malignancy.

Several papers compared p53 with the *TP53* gene mutation in the clinical context of follicular thyroid lesions. Whilst mutations in the p53 tumor suppressor gene are described in 50% of human cancers and frequently seen in ATC, they have been reported in only 0-25% of WDTC (47-53). Zafon et al confirmed that p53 ICC detection seemed to be linked only with mutated p53 (51), whilst currently it has been confirmed that p53 overexpression is not always due to p53 mutations, which can be associated with the expression of abnormal proteins or complete absence of p53 expression (52). In fact, a series of papers, especially regarding the early stages of gastrointestinal,

liver and lung cancers, contradicted the opinion that the p53 gene is involved in advanced cancers. Park et al assessed an increased expression of p53 protein by immunohistochemistry (IHC) in WDTC in the absence of any p53 mutation. (53). Marcello et al confirmed this, showing that five out of eight alterations were intronic without protein alterations; the remaining three exonic alterations were not linked to an increase of p53 protein, perhaps because of compensation from the wild-type allele toward the alterated one (49). Specifically in our series, none of the indeterminate lesions with a benign histology had a p53 mutation, whilst only two cases, resulted in a histological diagnosis of PTC, had a p53 mutation and p53 nuclear and cytoplasmic ICC expression. Specifically, these two cases had a larger size (larger than 2 cm), nodal metastases and a minor TCV subtype of 20%. The number of cases is limited to assess any definitive conclusion, with only 2/5 mutated cases with a nuclear p53 expression and stage II. This observation, adapted to large and/or multi-institutional series might be linked to the idea of a more aggressive malignancy when there is a p53 nuclear positivity.

In conclusion, these data about ICC evaluation of p53 proposes an overexpression of p53 in high risk and malignant lesions, so that p53 seems to be a feasible and reliable additional immunomarker in distinguishing malignant from other benign thyroid lesions. All of the immunoexpression identified in our study was confirmed with molecular evaluation. In our limited series, we did not find a correlation with aggressive findings including larger size, nodal metastases, but only two cases with a minor TCV subtype component, which needs further evaluation in a larger series. In this regard, for those centers, where the performance of molecular testings is difficult, the initial use of p53 ICC, may be followed by the performance of p53 molecular analysis only to those cases that have negative p53 by ICC.

Contributorship statement: **EDR, LP, LML, conceptualization, Investigation, validation, writing-original draft and writing review-editing, data curation, supervision, PT, FP, DA, VF, LC, MC, VF resources, formal analysis, methodology, data curation, software, investigation, AP, GF, MR, CPL, CDC writing-review**

Acknowledgment:

"This research was supported by grant from Ministry of Health RF-2019-12370266 to Alfredo Pontecorvi."

References

1) Pellegriti G, Frasca F, Regalbuto C et al. Worldwide increasing incidence of thyroid cancer: Update on epidemiology and risk factor. J Cancer Epidemiol 2013; 2013: 965212

2) Haugen BR, Alexander E, Bible KC, et al. American Thyroid Association (ATA) Guidelines Taskforce on Thyroid Nodules and Differentiated Thyroid Cancer. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer. Thyroid 2016; 26: 1-133.

3) Xing M, Haugen BR, Schlumberger M et al. Progress in molecular based management of differentiated thyroid cancer. Lancet 2013; 381: 1058-69

4)Nikiforov YE, Nikiforova MN. Molecular genetics and diagnosis of thyroid cancer. Nat Rev Endocrinol 2011; 7: 569-80

5) Xing M. BRAF mutation in thyroid cancer. Endocr Relat Cancer 2005; 12: 245-62

6) Tufano RP. Teixera GV, Bishop J et al. BRAF mutation in papillary thyroid cancer and its value in tailoring initial treatment: A systematic review and meta-analysis. Medicine (Baltimore) 2012; 91: 274-86

7) Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases and clinical implications. Endocr Rev 2007; 28: 742-62

8) Xing M. Prognostic utility of BRAF mutation in papillary thyroid cancer. Mol Cell Endocrinol 2010; 32: 86-93

9) Liu R, Xing M. TERT promoter mutations in thyroid cancer. Endocr Relat Cancer 2016; 23: R143-R155

10)) Dobashi Y, Sakamoto A, Sugimura H et al. Overexpression of p53 as a possible prognostic factor in human thyroid carcinoma. Am J surg Pathol 1993; 17: 375-81

11) Shin MK, Kim JW. Clinicopathologic and diagnostic significance of p53 protein expressionin papillary thyroid carcinoma. Asian Pac J cancer Prev 2014; 15: 2341-2344

12) Marcello MA, Morari EC, Cunha LL et al. P53 and expression of immunological markers may identify early stage thyroid tumors. Clin and Develop Immunol 2013; 846584

13) Morita N, Ikeda Y, Takami H. Clinical significance f p53 protein expression in papillary thyroid carcinoma. World J Surg 2008; 32: 2617-22

14) Choudhury M, Singh S, Agarwal S. Diagnostic utility of ki-67 and p53 immunostaining on solitary thyroid nodule-a cytohistological and radionuclide scintigraphic study. Indian J Pathol Microb 2011; 54: 472-75

15) Dwidevi SS, Khandeparkar SGS, Joshi AR et al. Study of immunohistochemical markers (CK-19, CD56, KI-67, P53) in differentiating benign and malignant solitary thyroid nodules with special reference to papillary thyroid carcinomas. J Clin Diagn Research 2016; 10-EC14-19

16) Pollina L, Pacini F, Fontanini G et al. Bcl-2, p53 and proliferatingg cell nuclear antigen expression is related to the degree of differentiation in thryoid carcinoma. British J Cancer 1996; 73: 139-43

17) Puglisi F, Cesselli D, Damante G et al. Expression of pax-8, p53, and bcl-2 in human benign and malignant thyroid diseases. Anticancer Res 2000; 20: 311-16

18) Soares P, Camoselle-Teijeiro, Sobrinho-Simoes M. Immunohistochemical detection of p53 in differentiated, poorly differentiated and undifferentiated carcinomas of the thyroid. Histopathol 1994; 24: 205-10

19) Zheng Y, Wang L, Zhang JP, Yang JY, Zhao ZM, Zhang XY. Expression of p53, c-erbB-2 and Ki67 in intestinal metaplasia and gastric carcinoma. World J Gastroenterol. 2010 Jan 21;16(3):339-44.

20) Othman NH, Omar E, Mahmood MH , Madhavan M. Ret and p53 expression in thyroid follicular adenoma: A study of 52 cases with 14 years follow-up. Malays J Pathol 2005; 27: 91-8.

21) Morita N, Ikeda Y, Takami H. Clinical signifcance of p53 protein expression in papillary thyroid carcinoma. World J Surg 2008; 32: 2617-22

22) Horie S, Maeta H, Endo K et al. Overexpression of p53 protein and MDM2 in papillary carcinomas of the thyroid: Correlation with clinicopathologic features. Pathol Int 2001; 51:11-15

23) Gharib H, Goellner RJ, Johnson DA. Fine needle aspiration of the thyroid: a 12 years experience with 11.000 biopsies. Clin Lab Med 1993; 13: 699-709

24) Perros P, Boelaert K, Colley S, Evans C, Evans RM, Gerrard Ba G et al. Guidelines for the management of thyroid cancer. Report of the Thyroid Cancer Guidelines Update Group. British Thyroid Association, Royal College of Physicians. London. 2007, 3rd edition

25) Ali S and Cibas ES. The Bethesda system for reporting thyroid cytopathology. 2nd Edition. Springer, Berlin 2018.

26) Nardi F, Basolo F, Crescenzi A, Fadda G, Frasoldati A, Orlandi F, et al.Italian consensus for the classification and reporting of thyroid cytology. J Endocrinol Invest 2014; 37:593–9.

27) Fadda G, Rossi ED, Raffaelli M, Pontecorvi A, Sioletic S, Morassi Fet al. Follicular thyroid neoplasms can be classified as low and high risk according to HBME-1 and Galectin 3 expression on liquid based fine needle cytology. Eur J Endocrinol 2011; 165: 447-453.

28) Rossi ED, Martini M, Capodimonti S, Cenci T, Bilotta M, Pierconti F et al.Morphology combined with ancillary techniques: An algorithm approach for thyroid nodules. Cytopathol 2018; 29: 418-27.

29) Rossi ED, Martini M, Capodimonti S, Cenci T, Straccia P, Angrisani B et al.Analysis of immunocytochemical and molecular BRAF expression in thyroid carcinomas: a cyto-histological institutional experience. Cancer Cytopathol 2014; 122: 52-56

30) Fadda G, Rossi ED.Liquid based cytology in fine needle aspiration biopsies of the thyroid gland. Acta Cytol 2011; 55: 389-400.

31) Kobel M, Ronnett BM, Singh N et all. Interpretation of P53 Immunohistochemistry in Endometrial Carcinomas: Toward Increased Reproducibility. Int J Gynecol Pathol 2019; 1:S123-31

32) Rossi ED, Martini M, Capodimonti S, Lombardi CP, Pontecorvi A, Vellone VG, et al. BRAF (v600e) mutation analysis on LBC-processed aspiration biopsies predicts bilaterality and nodal involvement in papillary thyroid microcarcinoma. Cancer Cytopathol 2013; 121: 291-7

33) Lloyd R. V., Osamura R. Y., Kloppel G.,Rosai J. (Eds): WHO Classification of Tumours of Endocrine Organs (4th edition). IARC: Lyon 2017

34) American Joint Commission on Cancer (AJCC) Cancer Staging Atlas, 2017 8th Edition.

35) Nikiforov YE, Seethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LD et al. Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. JAMA Oncol 2016; 2 (8): 1023-9

36) Oertel YC. Fine needle aspiration in the evaluation of thyroid neoplasms. Endocr Pathol 1997; 8: 215-24

37)Fadda G, Rossi ED.Liquid based cytology in fine needle aspiration biopsies of the thyroid gland. Acta Cytol 2011; 55: 389-400.

38) Decaussin-Petrucci M, Descotes F, Depaepe L et al. Molecular testing of BRAF, RAS and TERT on thyroid FNAs with indeterminate cytology improves diagnostic accuracy. Cytopathology 2017; 28: 482-87

39) Bellevicine C, Migliatico I, Spariglia R et al. Evaluation of BRAF, RAS, RET/PTC, and PAX8/PPARg alterations in different Bethesda diagnostic categories: A multicentric prospective

study on the validity of the 7-gene panel test in 1172 thyroid FNAs deriving from different hospitals in South Italy. Cancer Cytopathology 2020; 128: 107-118

40) Rossi ED, Faquin WC, Pantanowitz L. Cytologic Features of Aggressive Variants of Follicular-Derived Thyroid Carcinoma. Cancer Cytopathol 2019; 127(7):432-446

41)Rossi ED, Martini M, Bizzarro T et al. Uncommon BRAF mutations in the follicular variant of thyroid papillary carcinoma: New insightsCancer Cytopathol. 2015;123(10):593-602.

42) Chakraborty A, Narkar A, Mukhopadhyaya R, Kane S, D'Cruz A, Rajan MG. BRAFV600E mutation in papillary thyroid carcinoma: significant association with node metastases and extra thyroidal invasion. Endocr Pathol 2012 Jun;23(2):83-93

43) Trybek T, Walczyk A, Gąsior-Perczak D, et al. Impact of BRAF V600E and TERT Promoter Mutations on Response to Therapy in Papillary Thyroid Cancer. Endocrinology 2019; 160(10):2328-2338

44)Dell'Aquila M, Fiorentino V, Martini M, Capodimonti S, Cenci T, Lombardi CP, Raffaelli M, 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. Cancer Cytopath 2021; 129: 819-29

45) Melo M, Gaspar da Rocha A, Batista R et al. *TERT, BRAF, and NRAS* in Primary Thyroid Cancer and Metastatic Disease J Clin Endocrinol Metab . 2017; 102(6):1898-1907.

46) Yan S, Huang M, Li X, Wang T, Ling R. Relationship between *BRAFV600E* and clinical features in papillary thyroid carcinoma. Endocr Connect. 2019 Jul;8(7):988-996.

47) Hosal SA, Apel RL, Freeman JL et al. Immunohistochemical localiation of p53 in human thyroid neoplams: correlations with biological behaviour. Endocr Pathol 1997; 18: 21-28

48) Tan A, Etit D, Bayol U et al. Comparison of proliferating cell nuclear antigen, thyroid transcription factor-1, Ki-67, p63, p53, and high-molecular weight cytokeratin expressions in papillary thyroid carcinoma, follicular carcinoma and follicular adenoma. Ann Diagn Pathol 2011; 15: 108-116

49) Marcello MA, Morari EC, Cunha LL et al. P53 and expression of immunological markers may identify early stage thyroid tumors. Clin Developm Immunol 2013; 846584

50) Nasir A, Catalano E, Calafati S et al. Role of p53, CD44V6 and CD57 in differentiating between benign and malignant follicular neoplasms of the thyroid. In vivo; 2004; 18: 189-95

51) Zafon C, Obiols G, Castellvi J et al. Clinical significance of RET/PTC and p53 protein expression in sporadic papillary thyroid carcinoma. Histopathology 2007; 50: 225-31

52) Liu MC, Gelmann EP. P53 gene mutations: case study of a clinical marker for solid tumors. Semin Oncol 2002; 29: 246-57

53) Park KY, Koh JM, Kim YI et al. Prevalence of Gsx, RAS, P53 mutations and RET/PTC rearrangement in differentiated thyroid tumors in a Korean population. Clin Endocrinol 1998; 49: 317-23

Legends of the pictures

Figure 1 a and b-The figures show details of the mild-light cytoplasmic positivity for p53 in a case of indeterminate lesion, classified as Follicular Neoplasm and resulted as a Follicular adenoma on histology (20x ThinPrep)

Figure 2 a and b- The figures show details of the moderate-focaly intense cytoplasmic/nuclear positivity for p53 in a case of suspicious for malignancy, resulted as an invasive follicular subtype of Papillary thyroid carcinoma on histology (20x ThinPrep)

Table 1. Summary of clinical-pathological data

Clinical-pathological features	Proportion (n= 162 cases)
Age	
Mean	**49.2 years**
Median	**49 years**
Range	**15-85 years**
Gender	
Male	**50 (30.86%)**
Female	**112 (69.13%)**
Cytology diagnosis	
Benign	**50 (30.86%)**
AUS/FLUS	**48 (29.62%)**
FN/SFN	**40 (24.69%)**
SFM	**23 (14.19%)**
Malignant	**1 (0.62%)**
Histopathology diagnosis	
Benign*	**45§ (63.38%)**
Malignant	**26 (36.62%)**

Legend: AUS/FLUS: Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance; FN/SFN: Follicular Neoplasm/Suspicious for Follicular Neoplasm; SFM: Suspicious for Malignancy; M: Malignant; * Includes follicular adenomas; § including 3 non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP)

Table 2. Cyto-histological correlation (71 cases) according to TBSRTC

Diagnosis	**Goiter**	**FA**	**OA**	**NIFTP**	**PTC °**
Benign (2case)^	2 (100%)	0	0	0	0
AUS/FLUS (11 cases)	3 (27.2%)	7 (63.6%	0	0	1 (0.9%)
FN/SFN (37 cases)	1 (2.7%)	27 (72.9%)	2 (5.4%)	3 (8.1%)	4 (10.8%)
SFM (20 cases)	0	0	0	0	20 (100%)
M (1 cases)	0	0	0	0	1 (100%)

Legend: AUS/FLUS: Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance; FN/SFN: Follicular Neoplasm/Suspicious for Follicular Neoplasm; SFM: Suspicious for Malignancy; M: Malignant; FA: Follicular adenoma, PTC: Papillary carcinoma; ° including PTC subtypes

Table 3. p53 expression and localization in different thyroid lesions (162 cases) for the cytological series only

Diagnosis	Cytoplasmic P53 expression	nuclear p53 expression	Cytoplasmic + nuclear p53 expression
Benign (50 case)	**0**	**0**	**0**
AUS/FLUS (48cases)	**10* (20.8%)** Weak=3 Moderate=7 Strong=0	**0**	**0**
FN/SFN (40cases)	**29 (72.5%)** Weak=17 Moderate=10 Strong=2	**0**	**3** (7.5%) Weak=0 Moderate=0 Strong=3
SFM (23 cases)	**15** (65.2%) Weak=7 Moderate=6 Strong=2	**0**	**2** (8.6%) Weak=0 Moderate=2 Strong=0
Malignant (1 cases)	**1** (100%) Weak=0 Moderate=1 Strong=0	**0**	**0**

Legend: AUS/FLUS: Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance; FN/SFN: Follicular Neoplasm/Suspicious for Follicular Neoplasm; SFM: Suspicious for Malignancy; M: Malignant; FA: Follicular adenoma, NIFTP; PTC: Papillary thyroid carcinoma

Table 4 Cyto-histological (71 cases) correlation related to P53 expression

Cytology diagnosis	Histology diagnosis	p53 negative	p53 positive
Benign° **(2 case)**	Goiter 2	2/2 (100%)	0
AUS/FLUS **(11 cases)**	Goiter 3 FA 7 PC 1	11 (100%)	0
FN/SFN **(37 cases)**	Goiter 1 FA 27 OA 2 NIFTP 3 PC 4	1 26 (96.29%) 1 (50%) 3 (100%) 1 (25%)	0 1 (3.7 %) (strong cytoplasmic) 1 (50%) (weak cytoplasmic) 0 3 (75%) (strong cytoplasmic plus nuclear)
SFM **(20 cases)**	PC 20	10 (50%)	8 (40%) (moderate cytoplasmic) 2(10%) (strong cytoplasmic)
Malignant **(1 cases)**	PC 1	0	1 (100%) (moderate cytoplasmic)

Legend: AUS/FLUS: Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance; FN/SFN: Follicular Neoplasm/Suspicious for Follicular Neoplasm; SFM: Suspicious for Malignancy; M: Malignant; FA: Follicular adenoma, PTC: Papillary carcinoma;