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Article

Comparative cyto-Histological Genetic Profile in a Series of Differentiated Thyroid Carcinomas

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Study conducted in Portugal.

Abstract: Introduction – Molecular tests can contribute to improve the preoperative diagnosis of thyroid nodules. Tests available are expensive and not adapted to different population. Aim – This study aimed to compare the cyto-histological genetic profile, and to evaluate the reliability of molecular tests on Ultrasound-guided Fine Needle Aspiration Cytology (US-FNAC) in accurately diagnosing differentiated thyroid carcinomas (DTCs) and predicting biologic behavior of papillary thyroid carcinomas (PTCs). Material and methods – The series included 259 patients with paired cyto-histological samples totalizing 518 samples. The genetic alterations were analyzed by PCR/Sanger sequencing. The association with clinicopathologic features was evaluated in PTCs. Results/Discussion – From the 259 patients included, histologies were 50 (19.3%) benign controls and 209 (80.7%) DTCs cases, from which 182 were PTCs; cytologies were 5.8% non-diagnostic, 18.2% benign, 39% indeterminate and 37.1% malignant. In histology, indeterminate nodules (n=101) were 22.8% benign and 77.2% malignant. Mutation frequencies in cytology and histology specimens were, respectively, *TERTp*: 3.7% vs. 7.9%; *BRAF*: 19.5% vs. 25.1%; *RAS*: 11% vs. 17.5%. The overall cyto-histological agreement of the genetic mutations was 94.9%, Cohen's k=0.67, and in indeterminate nodules 95.7%, k=0.64. The identified mutations exhibited a discriminative ability in diagnosing DTC with a specificity of 100% for *TERTp* and *BRAF*, and of 94% for *RAS*, albeit with low sensitivity. *TERTp* and *BRAF* mutations were associated with aggressive clinicopathological features and tumor progression in PTCs ($p<0.001$). The obtained good cyto-histological agreement suggests that molecular analysis in US-FNAC may anticipate the genetic profile and the behavior of thyroid tumors, confirming malignancy and contributing to refer patients towards surgery.

Keywords: ultrasound-guided fine needle aspiration cytology (US-FNAC); *TERT; BRAF; RAS*; genetics; differentiated thyroid carcinomas (DTCs); indeterminate nodules; papillary thyroid carcinomas (PTCs)

1. Introduction

Thyroid nodular disease is very common and distinguishing benign from malignant nodules is still a major challenge in clinical practice. Thyroid nodules are diagnosed by palpation in 5% of women and 1% of men in iodine-sufficient parts of the world [1]. By thyroid ultrasound, nodules can be identified in 19% to 68% of the individuals [2]. Thyroid cancers are diagnosed in 1% to 5% of nodules [3], with more than 90% being differentiated thyroid carcinoma (DTC) [4].

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The incidence of DTC, from which papillary thyroid carcinoma (PTC) is the most prevalent (over 95%) [5], has increased in the last years, mainly attributed to enhanced sensitivity of complementary diagnostic techniques and environmental factors, but not accompanied by a corresponding change in mortality rate [6–8]. Despite its favorable prognosis, it was advanced that clinicopathological features and the presence of some genetic alterations can influence their progression [9]. Pre-surgical accurate diagnosis is crucial to decide the optimal medical or surgical treatment.

Ultrasound-guided fine needle aspiration cytology (US-FNAC) is central in the diagnosis of nodular thyroid disease, but up to 30% of nodules remain without a definitive diagnosis [10]. Bethesda II and VI cytological results have excellent performance to assess the benign or malignant nature of a thyroid nodule. Diagnostic limitations are particularly significant in the non diagnosis (ND), and in indeterminate Bethesda categories III- atypia of undetermined significance (AUS), and IV: follicular neoplasm (FN) [11]. Each category harbors a different risk of malignancy and a different option for treatment and follow-up [12–14].

Numerous studies have been conducted to identify genetic or molecular biomarkers that facilitate the pre-surgical diagnosis (in particular in categories ND, III and IV) and prognosis of thyroid cancer, but current results remain inconclusive and without definitive evidence [15,16].

Thyroid surgery remains the only diagnostic (and usually curative) approach for suspicious and malignant nodules. However, the inability to differentiate, preoperatively, between benign and malignant nodules among those classified as Bethesda III or IV hampers the ability to prevent unnecessary surgeries and the reduction of its possible complications [17].

In 2017, the European Thyroid Association (ETA) presented guidelines for molecular diagnoses in US-FNAC of thyroid nodules after reviewing methodological aspects, and limitations of molecular diagnoses in thyroid cytology. Molecular tests have the potential in clinical practice for diagnosis (pre-surgical markers) and follow-up of thyroid nodules (post-surgical markers), if they are performed in specialized laboratories and with adequate calibration and analytical validation before being implemented in clinical practice [18].

Although American and European Thyroid Associations [19,20] consider the inclusion of molecular tests in US-FNAC, no consensus is yet achieved on the best molecular panel to use in the diagnosis of thyroid nodules. Available molecular tests have been developed in United States of America, and it is important to assess whether such tests can be used in the European population [21,22]. The most significant disadvantage of commercially available tests is related to their cost, not supported by National Health Systems in Europe where, in many countries, its price is equal to or greater than a thyroidectomy; on the other hand, to perform these tests, highly specialized reference laboratories are required [23,24].

Studies have emerged on the use of artificial intelligence (AI) in the diagnosis and classification of thyroid nodules, constituting a new area of interest in scientific research, but still with no practical applicability [25,26].

The high prevalence of thyroid nodules [27,28], coupled with the rising incidence of thyroid neoplasm, urges the exploration of strategies to prevent unnecessary surgeries while effectively treating patients. This study aims to compare the cyto-histological genetic profile in matching cytology and histology samples, and to evaluate the reliability of molecular tests in preoperative diagnosis in order to improve the diagnostic accuracy of US-FNAC in indeterminate nodules.

2. Materials and Methods

2.1. Study design

This retrospective study was conducted in a series of patients with thyroid nodular disease that underwent surgery based in clinic, imageology and cytological criteria for suspicion of malignancy, in a single non oncologic hospital between 2013 and 2020.

Inclusion criteria: patients diagnosed, after surgery, with differentiated thyroid carcinoma (209 cases) or benign thyroid nodules (50 controls), of both gender, and over 18 years old with available representative histological and cytological sample.

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Exclusion criteria: patients with toxic goiter, papillary carcinoma smaller than 1 cm unless metastatic, and patients with other than thyroid neoplasm at the time of thyroid surgery.

A total of 518 samples (259 histology samples and corresponding 259 US-FNAC), representing 259 patients, along with clinical information, were included in the study. Epidemiological and clinicopathological features of patients and tumors were gathered based on the information available in the clinical and histopathological reports from the reference hospital.

All the samples were reviewed by two independent pathologists based on the 4th edition of the World Health Organization (WHO) classification of tumors of endocrine organs [29]. Two subgroups were separately analyzed, the indeterminate nodules corresponding to Bethesda categories III and IV, and the cases diagnosed as PTCs, to obtain homogeneous group.

2.2. Methodology

2.2.1. DNA extraction

To extract genomic DNA, 10 μm sections of formalin-fixed-embedded (FFPE) tissues were manually micro dissected using H&E guidance, and the GRS Genomic DNA BroadRange Kit (GRiSP Research Solutions, Portugal) was used. The smear of US-FNAC slides were scraped to a tube and the QIAmp® DNA Investigator Kit (Qiagen, USA) was used for DNA extraction of the US-FNACs according to the manufacturer's instructions. NanoDropTM One UV185 Vis Spectrophotometer (Thermo Fisher Scientific Inc) was used for quantification of isolated DNA.

2.2.2. Mutational screening

PCR and Sanger sequencing were used for genetic characterization of tumors regarding *TERTp*, *BRAF* and *RAS* (*NRAS*, *HRAS* and *KRAS*) mutations, with primers design accounting for the most frequent regions mutated on thyroid carcinoma, namely *TERTp* -124 and -146 promoter regions, *BRAF* exon 15 (codon 600), *NRAS* exon 2 (codon 61), *HRAS* and *KRAS* exons 1 and 2) (codons 12, 13 and 61) [30,31].

Multiplex PCR kit (QIAGEN, USA) and Bioline PCR kit (MyTaq HS Mix 2X, USA) were used for DNA amplification following manufacturer´s instructions. Sanger sequencing using the ABI Prism Big Dye Terminator kit v3.1 Cycle Sequencing and capillary electrophoresis using the Applied Biosystems 3130/3130xl Genetic Analyzers was performed. All detected mutations were validated by performing a new independent analysis.

2.2.3. Clinicopathological features

All primary lesions were evaluated, both concerning patient´s characteristics (age and gender), as well as tumor's clinicopathological features: tumor size, extra thyroidal invasion, capsule invasion, vascular and lymphatic invasion, presence of fibrosis, inflammatory infiltrate, tall cell, oncocytic component, psammoma bodies, calcification, necrosis, and presence of lymph nodes metastases, focality and laterality. Due to the reduced number of some histological subtypes of DTC, only PTCs were considered in the statistical analysis of the clinicopathological features of the patients.

2.2.4. Statistical analysis

The analysis was performed with categorical variables presented with frequencies (percentages), and quantitative variables with mean, and standard deviation (SD) and range (minimum-maximum). Regarding quantitative variables, Student's t and non-parametric Mann Whitney tests were used to compare groups, as appropriate. In the case of categorical variables, the Chi-square or Fisher's exact tests were used, as required.

To assess the strength of cyto-histological genetic profile agreement, Cohen's Kappa was estimated and interpreted according to Altman (1999) [32]. The discriminative ability of genetic biomarkers regarding malignancy was performed by estimating sensitivity, specificity, positive and negative predictive values, with corresponding 95% confidence intervals. A level of significance

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α=0.05 was considered. Data analysis was performed using the SPSS software version 27.0 (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp).

3. Results

3.1. Series description

3.1.1. Epidemiologic data

From the 259 patients, 209 (80.7%) were female, with mean age of 53 (15.8) years (range 18-84), and 50 (19.3%) were male, with mean age of 54 (15.9) years (range 23-81). The distribution of age at diagnosis was the similar across categories of gender (p =0.890), and across categories of histological diagnosis (p=0.186).

Mean tumor size was 33.5 mm (10.79) for benign lesions and 27.6 mm (15.57) for malignant lesions; the distribution of tumor size was not the same across categories of histologic diagnosis, being malignant tumors smaller than benign lesions (p<0.001).

The 259 histology samples were composed by 50 (19.3%) benign lesions (control), and 209 (80.7%) malignant lesions (cases). PTC represented the larger group in malignant histology, 182 (87.1%) cases.

The matched 259 cytology samples were distributed according to the Bethesda classification, as follows: I. non diagnostic (ND), 15 samples (5.8%); II. benign (B), 47 samples (18.1%); III. atypia of undetermined significance (AUS) 43 samples (16.6%); IV. Follicular Neoplasm (FN) 58 samples (22.4%); V. suspicious for malignancy (SM) 42 (16.2%); VI. malignant 54 samples (20.9%).

The distribution of the cytology samples within each histological subtype is presented in Table 1.

The 39% of nodules whose cytological result was indeterminate corresponded on histology to 23 benign (22.8%) and to 78 malignant lesions (77.2%).

Table 1. Distribution of the cytology samples within histological subtypes in all series.

Cytology diagnosis n=259	**Histology diagnosis n=259**						
	Benign	**WDT-UMP**	**NIFT**	**PTC**	**FTC**	HCC	Total
1.ND	0 (0%)	2 (0.8%)	2 (0.8%)	11 (4.2%)	0 (0%)	0 (0%)	15 (5.8%)
2.Benign	25 (9.7%)	1 (0.4%)	3 (1.2%)	15 (5.8%)	2 (0.8%)	1(0.4%)	47 (18.1%)
3.AUS	12 (4.6%)	1 (0.4%)	0 (0%)	30 (11.6%)	0 (0%)	0 (0%)	43 (16.6%)
4.FN	11 (4.2%)	5 (1.9%)	1 (0.4%)	36 (13.9%)	2 (0.8%)	3 (1.2%)	58 (22.4%)
5.SM	2 (0.8%)	1 (0.4%)	0 (0%)	35 (13.5%)	3 (1.2%)	1 (0.4%)	42 (16.2%)
6.Malignant	0 (0%)	1 (0.4%)	0 (0%)	53 (20.5%)	0 (0%)	0 (0%)	54 (20.9%)
Total	50 (19.3%)	11 (4.2%)	6 (2.3%)	180(69.5%)	7 (2.7%)	5 (1.9%)	259 (100%)

Legend: ND: Non-diagnostic, B: Benign, AUS: Atypia of Undetermined Significance, FN: Follicular Neoplasm,.

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SM: Suspicious for malignancy and M: Malignant. WDT-UMP: Well differentiated thyroid tumor of uncertain malignant potential; NIFT: noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; HCC: Hürthle cell carcinoma.

3.1.2. Cyto-histological genetic profile

The molecular status of *TERTp*, *BRAF*, and *RAS* (*NRAS*, *HRAS* and *KRAS*) genes of the cytologies and histologies in all series is summarized in Table 2. Mutations were present in 85 cytologies (32.8%), and in 130 histologies (50.2%).

The mutation frequencies observed in cytology and histology specimens within our series were, respectively, *TERTp*: 3.7% vs. 7.9%; *BRAF*: 19.5% vs. 25.1%; *NRAS*: 4.4% vs. 7.9%; *HRAS*: 4.8% vs. 7.6%; *KRAS*: 1.6% vs. 2.8%.

Table 2. Genetic mutations in cytology and histology in all series.

Genetic mutations	Cytology			Histology		
	n*	Mutated n (%)	Mutation type	n*	Mutated n (%)	Mutation type
TERTp	246	9 (3.7)	08 (-124 G>A) 01 (-146 G>A)	254	20 (7.9)	13 (-124G>A) 07 (-146 G>A)
BRAF	251	49 (19.5)	48 (p.V600E) 01 (p.K601E)	255	64 (25.1)	62 (p.V600E) 02 (p.K601E)
NRAS	250	11 (4.4)	11 (p.Q61R)	254	20 (7.9)	20 (p.Q61R)
HRAS	250	12 (4.8)	07 (p.Q61R) 05 (p.Q61K)	251	19 (7.6)	12 (p.Q61R) 06 (p.Q61K) 01 (p.G13A)
KRAS	250	04 (1.6)	04 (p.Q61R)	251	07 (2.8)	05 (p.Q61R) 01 (p.G12A) 01 (p.G12R)
Total		85 (32.8)			130 (50.2)	

*Molecular results were not conclusive in all samples due to technical issues. Legend: *TERTp* - telomerase reverse transcriptase promoter; *BRAF* - V-raf murine sarcoma viral oncogenes homolog B1; *RAS* - Rat sarcoma viral oncogenes homologue, *NRAS* - Neuroblastoma RAS Viral Oncogene Homolog, *HRAS* - Harvey Rat Sarcoma Viral Oncogene Homolog, and *KRAS* - Kirsten rat sarcoma viral oncogene homolog.

Mutation frequencies in cytology and histology samples by histology subtypes is show in Table 3, being detected in PTCs in 97.6% (83/85) of the cytology's and in 94.6% (122/129) of the histology specimens.

TERTp and *BRAF* mutation was present only in PTCs, both in cytology and in histology. *RAS* mutation was present in cytology and histology, respectively in 25 and 38 PTCs, in one and three benign cases, in two and one WDT-UMD cases, and only in histology samples of one Follicular thyroid carcinomas (FTC), and one Non-invasive follicular thyroid neoplasm with papillary like nuclear features (NIFTP). In Hürthle Cell carcinoma (HCC) no mutations were identified.

Table 3. Mutation frequencies in cytology and histology samples by histology subtypes.

Mutations	Histology subtypes n=259						
	Benign n=50	WT-UMD n=10	NIFT n=6	PTC n=176	FTC n=7	HCC n=5	total
TERT							
H	0	1	0	19	0	0	20 (7.9%)
n=254	0	0	0	9	0	0	9 (3.7%)

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C n=246							
BRAF							
H n=255	0	1	0	63	0	0	64 (25.1%)
C n=251	0	1	0	48	0	0	49 (19.5%)
RAS							
H n=251	3	2	1	38	1	0	45 (17.9%)
C n=250	1	1	0	25	0	0	27 (10.8%)
Total							
Histology	3	4	1	120	1	0	129 (100%)
Cytology	1	2	0	82	0	0	85 (100%)

Legend: H: histology; C: cytology; WDT-UMP: Well differentiated thyroid tumor of uncertain malignant potential;. NIFT: noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PTC: papillary thyroid carcinoma;. FTC: follicular thyroid carcinoma; HCC: Hürthle cell carcinoma. *TERTp*- telomerase reverse transcriptase promoter;. *BRAF*- V-raf murine sarcoma viral oncogenes homolog B1; *RAS*- Rat sarcoma viral oncogenes homologue.

Genetic mutations in cytology samples through Bethesda categories were presented in Supplementary Table 1.

BRAF mutations represented the majority of mutations, 43 cases, being five cases in Bethesda III and IV, eight cases in Bethesda V and 30 cases in Bethesda VI. *RAS* mutations were present in 26 cases, divided into the different Bethesda categories. Concomitant mutation of *TERTp*/*BRAF* (6 cases) or *TERT*/*RAS* (1 case) were identified always in cases with malignant histology, but with variable cytology: one Bethesda II, two Bethesda III and four Bethesda VI. No mutations were found in Bethesda ND category.

3.2. Genetic alterations in Indeterminate nodules

From the 259 cases 39% presented cytology of indeterminate nodules (101/259), with 43 AUS (42.6%) cases and 58 FN (57.4%). Among the indeterminate nodules, 23 (22.8%) were classified as benign lesions in histology, while 78 (77.2%) were classified as malignant in histology.

From the 78 malignant cases with indeterminate cytology, 66 were PTCs in histology. The remaining 12 indeterminate nodules were distributed among the other types of DTC cases (Table 1).

Twenty five (24.8%) indeterminate nodules presented mutations in cytology samples, one *RAS* mutation in a benign lesion and 24 in malignant tumors, being 13 (17.3%) for *RAS* mutation, seven (9.3%) for *BRAF* and four (5.6%) for *TERTp* mutation.

In the histology samples of the indeterminate nodules, 48 (47.5%) were mutated, being two benign lesions with *RAS* mutation and in 46 malignant tumors, of which 11 (14.5%) for *TERTp* mutation, 13 (16.9%) for *BRAF* and 22 (29.3%) for *RAS* mutation. Concomitant *TERTp* and *BRAF* mutations were identified in three cases.

Mutation frequencies in cytology and histology samples of indeterminate nodules were, respectively: *TERTp*: 4.3% vs. 11.1%; *BRAF*: 7.2% vs. 13%; *RAS*: 14.4% vs. 24.5% (Table 4).

The molecular status of *TERTp*, *BRAF*, and *RAS* (*NRAS*, *HRAS* and *KRAS*) genes, including mutations type in cytologies and histologies for indeterminate nodules is summarized in Supplementary Table 2.

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Table 4. Genetic mutation in cytology and histology of indeterminate nodules.

Genetic mutations		**Cytology (mutated) n=25 (24.8%)**			Histology (mutated) n=48 (47.5%)			
Histology	**n**	**Benign n=23**	**Malignant n=78**	**total n=101**	n	Benign n=23	Malignant n=78	total n=101
TERTp	94	0 (0%)	4 (5.6%)	4 (4.3%)	99	0 (0%)	11 (14.5%)	11 (11.1%)
BRAF	97	0 (0%)	7 (9.3%)	7 (7.2%)	100	0 (0%)	13 (16.9%)	13 (13%)
RAS	97	1 (1%)	13 (17.3%)	14 (14.4%)	98	2 (8.7%)	22 (29.3%)	24 (24.5%)

Legend: *TERTp* - telomerase reverse transcriptase promoter; *BRAF* - V-raf murine sarcoma viral homolog B1;. *RAS* - Rat sarcoma viral oncogenes homologue oncogenes.

3.3. Papillary thyroid carcinomas

3.3.1. Clinicopathological features of PTCs

For statistical analysis of the clinicopathological features in our series, only PTCs were considered due to the limited number of the other malignant subtypes of DTC. From the 209 patients with malignant histology of DTCs, 182 (87.1%) were PTCs, and 27 (12.9%) were other subtypes of DTC (Table 3).

Of the 182 PTC tumors, 150 (82.4%) were females; the mean age at diagnosis was 52 (15.9) years (range 18 - 84); mean tumor size was 26.7 mm (15.38) ($p< 0.001$).

The histological cases of PTCs encompassed different cytological diagnoses. The distribution by Bethesda categories was as follows: I. (ND) 12 (6.6%); II. (B) 16 (8.8%); III. (AUS) 30 (16.5%); IV. (FN) 36 (19.8%); V. (SM) 35 (19.2%); VI. (M) 53 (29.1%) samples.

The histological distribution of PTC variants and the corresponding clinicopathological features is presented in Supplementary Table 3.

3.3.2. Cyto-histological genetic profile in PTCs

In PTCs, mutation frequencies in cytology and histology were, respectively: *TERTp*: 5.3% (9) vs. 10.8% (19); *BRAF*: 27.4% (48) vs. 35.6% (63); *RAS*: 14.3% (25) vs. 22.1% (38).

Concomitant *TERTp* and *BRAF* mutations were identified in 10 PTCs, in cytology and/or histology samples.

The mutations in cytology and histology samples through histology subtypes of PTCs are showed in Supplementary Table 4.

3.3.3. Relationship between the clinicopathological features and the genetic profile in PTCs

The associations between the clinicopathological features and the genetic alterations of PTCs are presented in Supplementary Table 5 (A, B and C). No statistical significant diference was achieved for gender or age. The distribution of tumor size was different across types of mutations at diagnosis: tumors with *BRAF* mutations were smaller ($p<0.001$), than tumors without mutation.

The presence of *TERTp* mutations was significantly associated with vascular invasion ($p=0.004$) and with oncocytic component ($p=0.017$). *BRAF* mutations showed a significant association with the presence of extra thyroidal invasion, capsule invasion, vascular invasion, lymphatic invasion, oncocytic component, fibrosis, inflammatory infiltrate, tall cell component, psammoma bodies, calcification, focality, and LNM ($p<0.001$ for all). Concomitant *TERTp* and *BRAF* mutations were significantly associated with extra thyroidal invasion, capsular invasion, vascular and lymphatic invasion, tall cells, and oncocytic component ($p<0.001$ for all).

Concerning *RAS* mutations, cases exhibiting *NRAS* mutations demonstrated a significant association with the presence of capsule ($p=0.030$) and unifocality ($p=0.030$). No significant associations were observed between the presence of *HRAS* or *KRAS* mutations and any clinicopathological features.

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Table 5. The cyto-histological agreement in DTCs, PTCs, and indeterminate nodules.

	All series n=259		PTCs n=182		Indeterminate nodules n=101	
	Concordance (%)	Cohen's k	Concordance (%)	Cohen's k	Concordance (%)	Cohen's k
Genes (Total)	94.9%	0.670	94.6%	0.659	95.6%	0.643
TERTp	94.6%	0.493	94.5%	0.512	94.6%	0.591
BRAF	92.7%	0.790	91.9%	0.781	94.8%	0.710
NRAS	95%	0.576	95.5%	0.620	95.8%	0.579
HRAS	97%	0.744	96.8%	0.724	93.7%	0.695

Legend: DTCs- Differentiated Thyroid Carcinomas, PTCs- Papillary Thyroid Carcinomas. *TERTp* - telomerase reverse transcriptase promoter; *BRAF* - V-raf murine sarcoma viral oncogenes homolog B1; *RAS* - Rat sarcoma viral oncogenes homologue, *NRAS* - Neuroblastoma RAS Viral Oncogene Homolog, *HRAS* - Harvey Rat Sarcoma Viral Oncogene Homolog. Cohen's k - Cohen´s Kappa value: to interpret the strength of the agreement (Altman, 1999).

3.4. Statistical analysis of cyto-histological profile

The cyto-histological agreement achieved for molecular alterations in our series is summarized in Table 5. The comparison between cytology and histology for molecular alterations was possible in 244 cases. The cyto-histological concordance was observed in 94.9% of cases, with a Cohen´s k=0.67, which is considered substantial agreement (0.60-0.80). The concordance obtained in PTCs was 94.6% with a substantial cyto-histological agreement (k=0.659).

The agreement of the molecular testing in cytological and histological samples was evaluated in 95 indeterminate nodules, with concordance in 95.6% of cases, and a k=0.643 also considered as substantial agreement.

When analyzing the genes independently, the genes showing cyto-histological molecular agreement considered substantial were *BRAF* and *HRAS* in DTC, in PTCs, and in indeterminate nodules. *NRAS* and *TERTp* genes presented a cyto-histological molecular agreement considered moderate (0.40-0.59). *KRAS* mutation was excluded from this analysis due the small number of cases.

The mutations' discriminative ability for the diagnosis of malignancy in DTCs, PTCs and indeterminate nodules is shown in Table 6 for all series and in Supplementary Table 6 for each group.

The *TERTp* and *BRAF* mutations exhibited a specificity of 100% both in cytology and in histology, with a Positive Predictive Value (PPV) of 100% in all the series. *RAS* genes mutations demonstrated a specificity of 98% in cytology and 94% in histology for DTCs and PTCs, with a PPV in cytology and histology, respectively of 96.3% and 93.3% in DTCs, and 96.2% and 92.7% in PTCs; in indeterminate nodules, *RAS* presented a specificity of 95.5% in cytology (PPV 92.9%) and 91.3% in histology (PPV 91.7%). However, the sensitivities and the Negative Predictive Value (NPV) of all mutations were very low in our series for DTCs, PTCs and indeterminate nodules.

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Table 6. The discriminative ability of mutations in DTCs, PTCs, and indeterminate nodules.

Mutations	DTCs n=209			PTCs n=180			Indeterminate nodules n=101		
	Se (%)	Sp (%)	PPV (%)	Se (%)	Sp (%)	PPV (%)	Se (%)	Sp (%)	PPV (%)
TERTp									
histology	9.8	100	100	10.8	100	100	14.5	100	100
cytology	4.6	100	100	5.26	100	100	5.56	100	100
BRAF									
histology	31.2	100	100	35.6	100	100	16.9	100	100
cytology	24.3	100	100	27.4	100	100	9.33	100	100
RAS									
histology	21	94	93.3	22.1	94	92.7	29.3	91.3	91.7
cytology	12.9	98	96.3	14.3	98	96.2	17.3	95.5	92.9

Legend: DTCs- Differentiated thyroid carcinomas; PTCs- papillary thyroid carcinomas; Se- Sensitivity; Sp- Specificity;. PPV- Positive Predictive Value. *TERTp* - telomerase reverse transcriptase promoter. *BRAF* - V-raf murine sarcoma viral oncogenes homolog B1; *RAS* - Rat sarcoma viral oncogenes homologue.

4. Discussion

Molecular tests have been proposed to be used in clinical practice for diagnosis (pre-surgical markers) and follow-up of thyroid nodules. The 2017 ETA guidelines review methodological aspects and limitations of molecular diagnoses of US-FNAC of thyroid nodules (18). In recent years, panels of somatic genetic alterations have been proposed as a potential approach. However, due to the current limitations in accurately predicting the malignancy of thyroid nodules, most authors consider that further studies with larger sample numbers, rigorous normalization techniques and results validation, are required before their widespread adoption in clinical practice [33,34].

The present study was designed to contribute to clarify some of these challenges. A series of 259 surgical resected thyroid nodules, 209 with a histologic diagnosis of malignancy and 50 with benign diagnosis (as control), was select and the matched pre surgical cytologic diagnostic slides collected. Then we evaluated the genetic alterations in the 518 matched histological/cytological samples. Strength from our study is the relatively large number of matched cytology/histology samples included. This less usual design of the study also implies that our series will have a significant number of malignant tumors in the Bethesda category III and IV (please see bellow limitations of the study), resulting from the series selection criteria. But that design has also advantages by allowing more accurately evaluate at what extent genetic markers can improve cytology pre-surgical results.

BRAF mutations were predominant in our series, either in cytology as well as in histology samples, followed by *RAS* mutations, reflecting the composition of the series that was enriched in PTC and in low-grade tumors. In accordance with other published studies [36,37], including a study from Whitney S. Goldner (2019) [38], the majority of *BRAF* mutations were p.V600E. *TERTp* mutations were assessed in two different hotspots and most mutations in cytologies and histologies were found in the -124 position, as reported previous in thyroid tumors [39]. Tumors in the malignant category (Bethesda VI) presented a high frequency of *BRAF* mutations, followed by concomitant *TERT* and *BRAF*, and *TERT* and *RAS* mutations.

Our first approach was to evaluate cyto-histological genetic profile agreement for molecular alterations with a special attention to the nodules that have been diagnosed in indeterminate cytological diagnostic, category that raise important diagnostic doubts.

An acceptable cyto-histological genetic profile agreement for molecular alterations was attained in the overall series, indicating a good level of consistency between the two types of samples in the genetic analysis. *BRAF* and *HRAS* were the genes showing a substantial cyto-histological molecular agreement, whereas a moderated agreement was found for *NRAS* and *TERTp* genes. In each case the divergent molecular results between cytology and histology can reflect tumor heterogeneity, multifocality and/or low allelic frequencies, being the repercussion of these factors more marked on cytology samples that present a lower percentage of (neoplastic) cells that could increase the number of false negative results.

Then we went to evaluate if the mutational status in cytology, particularly for lesions categorized as indeterminate (AUS and FN), can enhance the diagnostic ability, and improve patient management [35]. In cytology the frequency of the mutations increased from lower Bethesda category (B and AUS) to higher Bethesda category (SM and M). Of note, in the control cases (50 selected histological benign lesions), only one case in cytology, and 3 in histology, present mutations and all in the *RAS* genes. If we consider *TERTp*, *BRAF* and *RAS* mutations in indeterminate categories (n=101 cases), the cytologic result taken together with clinical, imageology and molecular information, could be improved in 25% of the cases (24 cases with mutations in any gene). Being more cautious, since *RAS* mutations are relatively frequent, and were found in a few, benign nodules of our series, and considering only the predictive value for malignancy of *TERTp* and *BRAF* mutations (both with a 100% specificity, 100% PPV), then 10% of the indeterminate nodules of our series (n=10) could be diagnosed as malignant in a pre-surgical phase, without performing more FNACs and allowing a better surgical option and health costs reduction.

Indeterminate nodules represented a heterogeneous group. The high number of malignant cases in our series result, as referred before, from two reasons: in one hand because the case selection was done based in a histologic diagnosis of malignancy (or benignity in the 50 control cases), and in the other hand these patients underwent surgery due to clinical reasons besides FNACs results (US features p. ex.). A good cyto-histological genetic profile agreement for molecular alterations was achieved between the two types of samples in indeterminate nodules, reinforcing the role of molecular analyses before surgery in those cases. *RAS* mutations were the most frequent mutated gene in indeterminate nodules, in accordance with Censi S. (2017), who evaluated a large cohort of indeterminate thyroid nodules and detected *RAS* mutations in 18% of all series.

Our series was particularly enriched in PTCs, which allow us to evaluate the clinicopathological implications of the genetic background of the tumors, as evaluated by the molecular analysis of the histologies. Regarding the molecular results of the PTC's, we found a prevalence of mutations in *BRAF* and *TERT* genes similar to those reported by Liu R. (2014) [41] and Insilla A. (2017) [42], but lower than in some studies (DOI: 10.3390/cancers13092048).

Several statistically significant associations between the clinicopathological and molecular features of the PTCs were found in our series. *BRAF* mutations were significantly associated with several clinicopathological features, in accordance with Liu, X. (2018) [43]. *TERTp* mutations were significantly associated with features of worst prognosis, as mentioned by Bournaud et all [44]. On the contrary, *RAS* mutations were associated with the presence of capsule and a better outcome, as reported in previous study (Povoa et al) [45].

Xing, M. et al. (2014) reported that 6.9% of all PTCs have concomitant mutations in *TERTp* and *BRAF* genes, which were significantly associated with clinicopathological features of worst prognosis and tumor progression, namely LNM, extra thyroidal, and vascular invasion. Estrada Flórez A. P. et al. (2019) published similar results, with concomitant *TERTp* and *BRAF* mutations in 10% of their series and the same significant associations. In accordance with the above mentioned studies, we observed concomitant *TERTp* and *BRAF* mutations in 5.6% of PTCs, significantly associated with clinicopathological features related to tumor aggressiveness. On the other hand, Melo M. et al. (2014) have not found this association in their series. Ren H. et al. (2018) have found no associations between

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presences of both mutations and LNM, but they found a significant association with extra thyroidal invasion, large tumors, and older patients. No concomitant mutations were observed for *BRAF* and *RAS* as expected since these mutations are described as mutually exclusive events [46].

The *BRAF* and/or *TERTp* mutations were only present in malignant cases, in both cytology and histology samples, which we could consider as "rule in" tests, in accordance with American Thyroid Association (ATA) guidelines (PPV >95%) [47,48]. *RAS* mutations presented slightly lower specificity in our series due to its presence in some benign cases and low grade tumors. However, *RAS* mutation showed a higher specificity when compared with previous reports, resulting from the selection of cases in our series. The sensitivities and NPV of all mutations were much lower in both cytology and histology samples, not excluding malignancy within our series, as reported in other studies [49,50].

This study, conducted in a European population, presented limitations namely due to its retrospective nature, the only way to obtain a large series of operated thyroid nodules, and to perform a matching cyto-histological comparative analysis of the genetic profile in DTCs. The small number of genetic mutations analyzed, and the detection limit of Sanger sequencing used in this study, could be considered a potential limitation, especially when compared to the extensive mutation panels available [51,52] and other sequencing methods. However, our aim was to assess the reliability of molecular diagnoses obtained by US-FNAC in thyroid nodules using a laboratory method that offers practical replicability, is widely available, and at low costs. By focusing on a smaller set of mutations, we assessed the confidence level of our findings and provided valuable insights within the constraints of our study design.

Our results obtained by cyto-histological comparative analysis of the genetic profile in thyroid nodules reinforce the potential clinical utility of molecular testing in cytological assessments by US-FNAC, contributing to anticipate the genetic profile of the tumors and its biological behavior. By incorporating genetic information, clinicians achieve better informed decisions, optimize treatment strategies, including active surveillance and surgical options, and provide better care for patients with ambiguous cytological findings.

5. Conclusions

Our study yielded compelling results, demonstrating a good agreement between cytological and histological findings for molecular alterations in both DTCs and in indeterminate nodules. This minimally invasive and cost-effective approach has the potential to enhance diagnostic accuracy and streamline patient management by integrating molecular testing into routine clinical practice. While the genetic profile´s ability to exclude malignancy is limited, it effectively confirms malignancy in cytology samples, reducing repeated US-FNAC, and contributing to refer patients towards diagnostic, including surgical option. Moreover, the numerous associations identified between clinicopathological features and genetic profile of PTCs, suggest that molecular analysis in US-FNAC can provide early insights into tumor genetic profile and behavior. To gain a comprehensive understanding of tumor evolution and assess the applicability of molecular tests in the European population, further studies are needed that will shed new light on the dynamics of these tumors and their genetic characteristics.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: MLM and PS conceived the study and wrote the paper. MP and AG performed experiments and analyzed data. SC reviewed the cytology and histology samples. MLM, MA and AP performed statistical analyses. AP, MB and PS supervised the work, on the conception, analyses and reviewing the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Centro Hospitalar Universitário Lisboa Central (CES586, 19.07.2018) and Nova Medical School (nº 58/2018/CEFCM, 05.09.2018) and adhered to national and institutional ethical standards (Law n°12/2005).

Informed Consent Statement: Written informed consent has been obtained from each patient involved in the study, after full explanation of the purpose and nature of all procedures used, whenever possible, and we guaranteed data confidentiality and patient privacy through data anonymization.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

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