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ORIGINAL ARTICLE

Study of the C228T mutation of the *TERT* promoter in thyroid aspirative punctures of IV category of the Bethesda classification

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KEYWORDS
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Abstract
Introduction: Since the C228T mutation in the *TERT* promoter (*TERTp*) has been identified almost exclusively in thyroid malignancies, our objective was to study the usefulness of its determination in thyroid fine needle aspirations (FNA) of the IV category of the Bethesda classification (B.IV).
Methodology: From the FNAs performed between 1993 and 2015, we selected those with a diagnosis of B.IV or equivalent and subsequent thyroidectomy. A retrospective study of the C228T mutation in *TERTp* was performed by pyrosequencing in neoplastic cases (adenomas, low risk neoplasms and carcinomas), both from the surgical specimen and from the FNA material if feasible.

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Results: 79 cases with a diagnosis of B.IV were identified, and mutational study was performed in the 61 cases corresponding to neoplasms, identifying 10 cases with the mutation (12.6% of the series), with a higher presence in Poorly Differentiated Carcinomas (PDC) or with a minor PDC component (45%), in cases with death attributable to thyroid carcinoma (50%) and in patients alive but with persistence of thyroid carcinoma (50%). The mutation was confirmed in 7 of 8 cases with the mutation and satisfactory cytological material. In 4 cases, preoperative knowledge of the mutation could have avoided a two-stage thyroidectomy.
Conclusion: The study of the C228T mutation of*TERTp* can be useful to detect malignancy and establish the best surgical approach in patients with thyroid FNA with a diagnosis of B.IV.

PALABRAS CLAVE
Telomerasa;
TERT;
Tiroides;
Carcinoma;
Punción aspirativa

Estudio de la mutación C228T del promotor de *TERT* en punciones aspirativas tiroideas de la categoría IV de la clasificación de Bethesda

Resumen
Introducción: Dado que se ha identificado la mutación C228T del promotor de *TERT* (*TERTp*) prácticamente de forma exclusiva en neoplasias malignas tiroideas, nuestro objetivo ha sido estudiar la utilidad de su determinación en punciones aspirativas con aguja fina (PAAF) tiroideas de la categoría IV de la clasificación de Bethesda (B.IV).
Metodología: De las PAAF realizadas entre 1993 y 2015, se han seleccionado los casos con diagnóstico B.IV o equivalente y posterior tiroidectomía, realizándose estudio retrospectivo de la mutación C228T de*TERT*p por pirosecuenciación en los casos neoplásicos (adenomas, neoplasias de bajo grado y carcinomas) tanto a partir de material de la pieza quirúrgica como de la PAAF si factible.
Resultados: Se han identificado 79 casos con diagnóstico B.IV, realizándose el estudio mutacional en los 61 correspondientes a neoplasias, identificándose la mutación en 10 casos (12,6% de la serie), con mayor presencia en carcinomas pobremente diferenciados (CPD) o con componente menor de CPD (45%), en los casos con exitus atribuible al carcinoma tiroideo (50%) y en los pacientes vivos pero con persistencia del carcinoma tiroideo (50%). Se ha confirmado la mutación en el material de la PAAF en 7 de los 8 casos con mutación y material citológico satisfactorio. En 4 casos el conocimiento prequirúrgico de la mutación podría haber evitado una tiroidectomía en dos tiempos.
Conclusión: El estudio de la mutación C228T de *TERTp* puede ser útil para detectar malignidad y establecer el mejor abordaje quirúrgico en los pacientes con PAAF tiroidea con diagnóstico B.IV.

Introduction

Thyroid nodules are common in the general population[1], identified by self-palpation or cervical examination, ultrasound, or other diagnostic techniques, such as PET-CT.[1,2] However, approximately 5% of them will correspond to a malignant neoplastic disease.[3]

Fine-needle aspiration (FNA) is considered one of the fundamental techniques to guide the diagnosis and management of patients with thyroid nodules, performed according to ultrasound findings, following the guidelines of ultrasound risk classification systems.[4] However, it has limitations in the case of nodules or neoplasms of follicular pattern, since the criterion of malignancy is located in the periphery of the lesion, in the form of capsular or vascular invasion,[5] findings that can only be assessed in the histological study of the thyroidectomy specimen.

The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) is used worldwide for the cytological diagnosis of thyroid FNAs, reaching its 3rd edition in 2023.[6] FNAs performed on follicular patterned neoplasms, especially those that do not show nuclear features of papillary carcinoma, are usually categorized in the indeterminate categories III and IV of this classification.[7] For this reason, there are multiple studies focused on the search for aids to improve the accuracy regarding malignancy (or absence thereof) in these diseases, which include encapsulated malignant neoplasms of follicular pattern (follicular carcinoma and follicular

variant of papillary carcinoma) and poorly differentiated carcinoma, for which it is difficult to make the diagnosis of malignancy by FNA).[8,9] Confirmation of malignancy could contribute to a better decision regarding the management and type of elective surgery in these patients.[9,10]

Mutations in the promoter of telomerase reverse transcriptase (TERTp) confer telomerase activity to the cells that present them, that is, the ability of the cell during cell division to conserve the length of telomeres in chromosomes, and therefore absence of chromosomal senescence, such as stem cells and germ cells.[11] The presence of TERTp mutations, mostly C228T, has been observed in thyroid malignant neoplasms, including follicular carcinoma, papillary carcinoma, poorly differentiated carcinoma, and anaplastic carcinoma, with different frequencies.[9,12,13] Since its presence in benign thyroid neoplasms is extremely rare,[14] its utility has been postulated to identify malignant neoplasms in thyroid FNAs.

Often, the study of TERTp mutations, in isolation or together with other molecular alterations, has been evaluated by grouping categories III and IV of the TBSRTC.[15,16] However, this classification has always associated a higher risk of malignancy with category IV, although with a relatively wide range due to the multiplicity of studies.[6] Therefore, it seems likely that the performance of the study of TERTp mutations will be much better if only Bethesda category IV is considered. The objective of our study has been to identify the presence of the majority mutation C228T in TERTp, given that it is the most prevalent, in a retrospective series of cases with cytological diagnosis of TBSRTC category IV (consistent with follicular neoplasm or oncocytic neoplasm) with subsequent thyroidectomy, performing the mutational study both in the surgical specimen and in the previous cytology in cases with the presence of the mutation and in a selection of cases without the presence of the mutation in the histological material, estimating its diagnostic value and clinical impact if it had been determined in the preoperative cytology.

Methodology

We conducted a retrospective observational cohort, following the STROBE guideline. The study was approved by *Corporación Sanitaria Parc Taulí de Sabadell* Health Clinical Research Ethics Committee (reference: 2017558), and informed consent was requested from the patients.

Case inclusion and data collection

From the computerized archive of the Pathology Anatomy service, all thyroid FNAs diagnosed from January 1st, 1993, through December 31st, 2015, were identified. Cases prior to the 1st edition of the TBSRTC were reviewed based on the diagnosis, microscopic description, or review of the cytological preparations, and their category has been established based on the TBSRTC. The cases of each category were quantified and their percentage established, including category I cases attributable to cyst diagnosis, and excluding punctures of category I and the insufficient/non-assessable subcategory. In the presence of >1 diagnosis in the same puncture act because >1 lesion was punctured, the highest category in the TBSRTC was assigned to the case.

Cases with a diagnosis of TBSRTC category IV (consistent with follicular neoplasm or oncocytic neoplasm) with subsequent thyroidectomy were considered for the study.

The following clinical and histopathological data were collected: sex, age at intervention, size of the lesion, type of intervention, oncocytic appearance, histological diagnosis, pathological TNM stage in the case of malignant neoplasms, vital status, and years of follow-up.

Determination of C228T mutation in TERTp

The study for the C228T mutation in TERTp was conducted on the material from the thyroidectomy specimens in all cases with histological diagnosis of neoplasm (benign, low-grade, or malignant) and on the material from the corresponding previous FNAs in all cases with the presence of the mutation in the histological material, and in a selection of cases without the presence of the mutation in the histological material. The study has been conducted in cases with non-neoplastic histological diagnosis or follicular nodular disease (nodular hyperplasia).

DNA extraction

The material for DNA extraction from thyroidectomy specimens was obtained from selected areas of formalin-fixed paraffin-embedded (FFPE) tissue; genomic DNA was extracted from 5 sections of 10 μm each. The material for DNA extraction from FNA samples was obtained from selected areas of the cytological smears (previously marked with a diamond pen), regardless of whether they were air-dried or alcohol-fixed for different stains, or from selected areas in the FFPE cell block, depending on the amount of tissue with suspicious cellularity and its preservation. In the case of smears, genomic DNA was extracted from selected areas scraped with the tip of a scalpel after decolorization with HCl-ethanol (3:97).

DNA was extracted using the QIAmp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). Sterile tubes with FFPE sections or scraped cells were washed 2 times with 1 mL of xylene; after adding xylene, the sample was briefly vortexed and centrifuged at maximum speed. The supernatant was removed and the precipitate washed with 1 mL of 100% ethanol. The samples were air-dried, and 20 μL of proteinase K and 180 μL of buffer were added for overnight digestion.

DNA columns were used to isolate genomic DNA, with elution volumes of 50 μl. DNA was quantified by fluorometry with the QUBIT dsDNA BR Assay (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts, USA).

Polymerase chain reaction (PCR)

PCR was performed with internally designed primers (amplified region TERT (NM_198253.2):c.1-124C > T) and Pyromark Gold reagents (QIAGEN). Each PCR mixture was prepared with 25 ng (for DNA quantified by spectrophotometry) or 5 ng of genomic DNA (for DNA quantified by fluorometry). Briefly, 12.5 μL of Master Mix, 2.5 μL of Coral Load, 0.5 μL of each of the 2 primers (one of which was biotinylated), 4 μL of RNase-free water, and 5 μL of sample were mixed to form

Table 1 Distribution and percentage of informative punctures by Bethesda System categories in the 1993–2015 interval.

Bethesda category	N	%
I (C) - Cyst	457	9.99
II	3,359	73.45
III	466	10.19
IV	92	2.01
V	10	0.22
VI	189	4.13
Total	4,573	

Table 2 Summary of clinical data of cases with cytological diagnosis of Bethesda System category IV.

Variable	Value
N	79
Sex	53 female / 26 male
Age	Range: 7–83 years (median: 50 years)
Type of surgery	27 lobectomies 43 total thyroidectomies 9 two-stage total thyroidectomies
Lesion diameter	Range: 12–90 mm (median: 35 mm)
Years of follow-up	Range: 3–29 years (median: 18 years)

a total volume of 25 μl. After an initial denaturation step (95 °C, 15 min), 42 cycles of 20 s at 95 °C, 30 s at 53 °C, and 20 s at 72 °C were continued, with a final extension step at 72 °C for 5 min. Subsequently, the PCR products were immobilized on beads (Streptavidin Sepharose High Performance, GE Healthcare, Buckinghamshire, UK), and the strands were separated, allowing purification and subsequent processing of the biotinylated single-stranded DNA.

Pyrosequencing analysis

A total of 1 μL of streptavidin beads, 39 μL of PyroMark Binding Buffer (QIAGEN, Hilden, Germany), 10 μL of sample, and 20 μL of RNase-free water were mixed, followed by incubation for 15 min on a vibrating table at 1,400 rpm. Amplicons were then separated, denatured, and washed, being added to 25 μL of hybridization buffer containing the sequencing primer using the PyroMark Q24MDx Vacuum Workstation (QIAGEN, Hilden, Germany).

The sequencing primer was hybridized by incubating the samples for 2 min at 80 °C, then cooling to room temperature. Subsequently, PyroMark Gold reagents were used to analyze the sequences, with pyrosequencing performed on the PyroMark Q24 MDx (QIAGEN, Hilden, Germany).

The final pyrograms were analyzed with PyroMark Q24 2.0.6.20 software. The limit of detection (LOD) for the TERTp C228 T mutation was defined as 10%. Peaks between LOD and LOD + 3 units were considered borderline values and were confirmed as mutations only after re-analysis in a duplicate run with a wild-type DNA sample).

Table 3 Clinicopathological data of cases with histological diagnosis of malignancy.

Variable	Value
N	33
Sex	23 female / 10 male
Age at diagnosis	Range: 7–83 years (median: 54 years)
Maximum diameter	Range: 17–90 mm (median: 45 mm)
Type of surgery	Lobectomy: 3 Two-stage total thyroidectomy: 9 One-stage total thyroidectomy: 21
pT (8th ed. AJCC)	pT1b: 4 pT2: 10 pT3a: 19
pN (8th ed. AJCC)	pNX: 22 pN0: 7 pN1a: 3 pN1b: 1
M (8th ed. AJCC)	M1 at diagnosis: 2 M0 at diagnosis: 31
Stage (8th ed. AJCC)	I: 21 II: 10 IVB: 2
Follow-up available	32/33
Years of follow-up	Range: 3–27 years (median: 14 years and 6 months)
Vital status	4 deaths attributable to thyroid carcinoma 4 alive with thyroid carcinoma 4 deaths from other causes, no evidence of carcinoma 20 alive with no evidence of carcinoma

Data analysis

The obtained data and their percentages were quantified, both for the global data of the cytologies of the studied interval, and for the clinicopathological data and the mutational study of the selected group, identifying separately the cases with a diagnosis of malignancy. The validity of the TERTp mutation as a diagnostic test indicative of malignancy was evaluated based on its specificity, sensitivity, positive predictive value, and negative predictive value.

Results

Between 1993 and 2015 inclusive, a total of 5980 thyroid FNAs were received in the Pathology Anatomy service. Of these, 1407 were insufficient or unsatisfactory, so the final number of informative punctures was 4573, including cyst diagnoses. Table 1 illustrates the cases of each category and their percentage. The ratio between categories III and VI was 2.46.

A total of 92 cases corresponded to TBSRTC category IV (B.IV), representing 2.01% of the total informative punc-

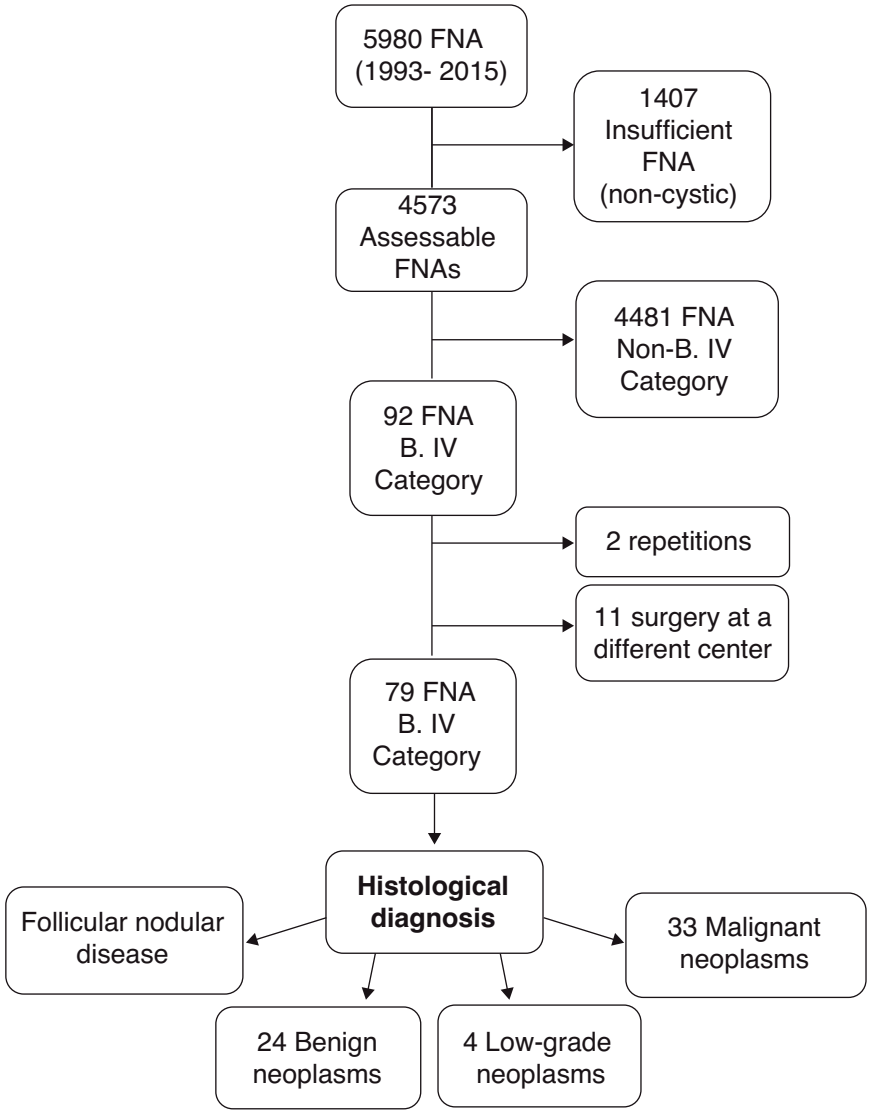

Figure 1 Flow chart of the extraction of histological cases for the mutational study.

tures. In 2 cases, the same patient had undergone 2 FNAs at 2 different times with the same diagnosis, and in 11 cases, no subsequent intervention was performed at our center, so the cases included in the study were finally those corresponding to 79 patients. The extraction of cases is illustrated with a flow chart in Fig. 1. The summary of clinicopathological data is shown in Table 2. A total of 61 cases corresponded to neoplastic lesions (excluding follicular nodular disease, currently included in benign neoplasms according to the World Health Organization[17]), 33 of which were diagnosed as malignant (41.7% overall). The clinicopathological data of the malignant cases are shown in Table 3.

The c228T mutation in TERTp was identified in 10 of the 61 studied cases corresponding to neoplasms, all of them corresponding to malignant neoplasms, which represents 12.6% of the total cases in the series (estimating that the cases corresponding to follicular nodular disease would not have the mutation). The percentage of positivity in malignant cases was 30.33%. This percentage is higher in cases corresponding to poorly differentiated carcinomas (PDC) and differentiated carcinomas with a minor component of poorly differentiated carcinoma, reaching 45.4% (5 of 11 cases). Table 4 shows the distribution of TERTp mutational status based on histological diagnoses. The specificity rate for malignancy was 100%, and the sensitivity, 30%. The positive predictive value was 100%, and the negative predictive value, 67%.

The mutational study was successfully conducted on the cytological material in 26 cases (6 adenomas, 1 non-invasive non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), and 19 carcinomas). Of the 10 cases with mutation in the histological material, the presence of the mutation was identified in 7 of them (1 papillary carcinoma, 1 follicular carcinoma, 1 oncocytic carcinoma, 1 well-differentiated carcinoma NOS, 1 follicular carcinoma with a component of poorly differentiated carcinoma, and 2 PDCs); in 1 case of papillary carcinoma with a component of poorly differentiated carcinoma, the mutation was not identified, and in 2 cases (1 oncocytic carcinoma and 1 poorly differentiated carcinoma), sufficient viable DNA for

Table 4 Distribution of histological diagnoses and the presence of TERTp C228T mutation in histological and cytological material.

Histological Diagnoses		N = 79	WT-h	C228T-h	WT-c	C228T-c	NI-c	NR-c
Benign disease	42 (53.16%)							
Nodular disease			16[1]	0[1]	0	0	0	16
Nodular Hashimoto's thyroiditis			2[1]	0[1]	0	0	0	2
Follicular adenoma			16	0	4	0	0	12
Oncocytic adenoma			8	0	2	0	0	6
Low-grade neoplasms	4 (5.06%)							
Hyalinizing trabecular adenoma			2	0	0	0	0	2
NIFTP		2	0	1	0	0	1	
Malignant neoplasms	33 (41.7%)		23 (69.7%)	10 (30.3%)	17 (51.5%)	7 (21.2%)	2 (6.1%)	7 (21.2%)
Papillary carcinoma								
Classical subtype			0	1	0	1	0	0
Oncocytic subtype			1	1	0	0	1	1
Follicular subtype								
Infiltrative			1	0	1	0	0	0
Encapsulated, minimally invasive			1	0	0	0	0	1
Encapsulated, angioinvasive			1	0	0	0	0	1
Follicular carcinoma								
Minimally invasive			3	0	0	0	0	3
Angioinvasive			5	1	5	1	0	0
Widely invasive			1	0	1	0	0	0
Oncocytic carcinoma								
Minimally invasive			1	0	1	0	0	0
Angioinvasive			3	1	3	1	0	0
Minimally invasive WD NOS		1	0	1	0	1	0	0
Minor component of PDC								
WD + PDC			1	0	1	0	0	0
FC + PDC			1	1	1	1	0	0
FVPC + PDC			1	1	2	0	0	0
PDC			3	3	2	2	1	1

-c: study in cytological material; WD: well-differentiated carcinoma NOS; FC: follicular carcinoma; PDC: poorly differentiated carcinoma; FVPC: follicular variant papillary carcinoma; -h: study in histological material; NI: not informative; NOS: not otherwise specified; NR: not performed; WT: wild type (no mutation).

[a] In the case of non-neoplastic histological diagnoses, the mutation study was not performed, but it is estimated that all cases would be WT.

the mutational study was not obtained. The mutation was not identified in any of the cases that did not present it in the surgical specimen either.

In 4 of the cases with the mutation, total thyroidectomy was performed in 2 stages, completing the total thyroidectomy after the histological diagnosis in the hemithyroidectomy specimen.

At the clinical follow-up, a total of 4 cases died due to thyroid carcinoma, all of them with tumors carrying the mutation (100%), and 4 remain alive with the presence of carcinoma, 2 of them also carrying the mutation (50%).

Discussion

In our series, the mutational study for TERTp demonstrated the presence of the C228T mutation in 10 cases out of a total of 79 cases operated on at our center after a cytological diagnosis of TBSRTC category IV, which represents 12.6% of the series. This rate is higher than that published in other series.[18–20] A factor that may explain this difference is the low percentage of category IV diagnoses made at our center, by applying very restrictive criteria for making this cytological diagnosis, which also entails a considerable risk of malignancy in this category, of 41.7%. In contrast, in our historical series of cytohistological correlation, cases with a diagnosis of category III have a risk of malignancy of 27% in operated cases and 10% if follow-up cases are included, which represent 60% of all cases with a cytological diagnosis of category III (unpublished data). Similarly, our center ratio between categories III and VI is 2.46, lower than 3, as recommended to avoid an excessive degree of indeterminacy in cytological diagnoses.[21] In conclusion, with a higher risk of malignancy in the group of cases with a diagnosis of category IV, the probability of including cases with the presence of the mutation is also higher, and consequently, the study of the mutation may be more efficient. In contrast, the low risk of malignancy in our series of Bethesda III cases suggests that in this group, the determination would not be cost-effective. On the other hand, the risks of malignancy for categories V and VI have been 90% and 99%, respectively, so we do not consider mutational study necessary to favor the diagnosis of malignancy. We agree with other authors,[22,23]

on the need to know the risks of malignancy by category in each center and in the published series to be able to assess the cost-effectiveness of molecular studies in both cases.

We consider the study of the TERTp C228T mutation important because it is a mutation practically exclusive to malignancy.[11] The occasions in which this mutation has been observed in benign cases or in cases of low risk of malignancy are extremely rare, some of which have shown in their evolution to be malignant.[24]

It seems particularly useful in category IV (follicular neoplasm) since many cases correspond to encapsulated neoplasms in which the diagnosis of malignancy cannot be established by cellular morphology and it is necessary to confirm the diagnosis of malignancy in the thyroidectomy specimen.[5] In our series, 67.22% of the cases effectively corresponded to neoplastic processes (adenomas, low-grade neoplasms, and carcinomas), so we consider the cost-effectiveness to be considerable, and more so considering the small number of cases it represents.

As is well known, there are various tests and platforms that combine the study of multiple molecular alterations (mutations, fusions, and rearrangements) to try to determine malignancy (or its absence) in the material from thyroid FNAs, with a considerable economic cost, but without reaching 100% specificity.[25] In these studies, the most frequently identified mutations are found in the RAS and BRAF genes. In the case of RAS, these mutations can also be found in benign neoplasms,[26] while BRAF will rarely be found in a Bethesda IV case, since the nuclear features in papillary carcinomas with BRAF mutation are usually evident, and consequently, these cases will correspond to Bethesda categories V or VI. Therefore, the mutation in TERTp would be the most exclusive and frequent in carcinomas with a follicular pattern without nuclear features of papillary carcinoma, so we consider its determination to be of particular interest in cases with a cytological diagnosis of category IV.

The main contribution of this determination would be its possible role in deciding the type of thyroidectomy to perform in the face of a cytological diagnosis of category IV. In this series, knowledge of the mutation could have suggested a different surgical approach in 4 cases, initially treated with hemithyroidectomy, in which total thyroidectomy had to be completed to be able to subsequently perform radioiodine ablation. Evidently, after a cytological diagnosis of Bethesda IV, other factors must be taken into account (related to the patient's condition and wishes, ultrasound data, underlying thyroid gland disease, etc.) which, evaluated in a multidisciplinary committee, may influence the decision on the type of thyroidectomy that should be performed,[27] which will sometimes be the same, regardless of the result of the mutation study. However, in some cases, its identification in the cytological material, practically equivalent to malignancy, can condition the performance of total thyroidectomy from the outset.[9] In any case, prospective studies are necessary to assess the real contribution of TERTp determination in the surgical management of patients.[25]

The higher incidence rate of the mutation in high-grade or more aggressive carcinomas is well documented.[28] In our series, we have identified a higher frequency of the mutation in cases of poorly differentiated carcinoma or in differentiated carcinomas with a minor poorly differentiated component, which considered as a group have a mutation presence of 45%. This higher risk of aggressive behavior may also be a determining factor for the decision of a total thyroidectomy as the initial surgical technique.[9]

We consider as limitations of our study the reduced number of cases studied, as it is a single-center study, and not having considered conducting the mutational study on cases with a histological diagnosis of follicular nodular disease, estimating that its yield would be zero. However, of note, the change in nomenclature in nodular thyroid disease (from nodular hyperplasia to follicular nodular thyroid disease[17] is due to the observation that some of the nodules considered hyperplastic are actually clonal[29] and, therefore, could correspond to neoplastic processes.

The study of mutations in TERTp can be conducted from paraffin-embedded material, but also from cytological smears or liquid cytology.[19,30] Each center, depending on its system for obtaining cytological material, can establish protocols for performing the technique, feasible from all types of cytological material, with the possibility of performing the mutational study from the cytological material already studied. This allows selecting the cases in which the determination can really provide decisive information, avoiding the expenditure of time and reagents in cases where its determination does not modify the surgical strategy already decided by other factors. However, in our series, the presence of the mutation in cytological material could only be confirmed in 7 of the 10 cases with mutation in the histological material. In 2 of the remaining cases, satisfactory material was not obtained from the cytological material in the archive, and in one case, the mutation was not identified. The long interval between obtaining the material and carrying out our study, or the use of cell blocks for immunohistochemical studies at the time of diagnosis, may have made it difficult to obtain well-preserved material for the study. The application of more modern techniques, such as digital PCR, as well as the immediate performance of the mutational study on cytological material in clinical practice, can very likely achieve more satisfactory results, without ruling out the possibility of performing a new FNA dedicated fundamentally to the mutational study if necessary. On the other hand, tumor heterogeneity, observed in some studies,[11,24] could justify the case without evidence of mutation in the cytological material, so it seems advisable to direct the puncture needle in several directions during the FNA to perform a broad sampling of the lesion.

In conclusion, we consider that the TERTp mutation appears in a non-negligible number of cases with a cytological diagnosis of TBSRTC category IV and that its determination in the material obtained by FNA can be an important factor in some cases to decide the type of surgical approach.

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Declaration of competing interest

None declared.

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