

## ORIGINAL PAPER

**TERT PROMOTER MUTATION AND PROEX C ARE USEFUL AS A SCREENING TOOL TO PREDICT THE MALIGNANT BEHAVIOR OF THYROID TUMORS OF UNCERTAIN MALIGNANT POTENTIAL**

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Gray zone thyroid tumors for which benignity or malignancy cannot be assessed are constantly increasing and accurate prediction of their malignant behavior is vital to avoid both violent surgical intervention for benign tumors and lax treatment of malignant tumors.

The retrospective study included 120 blocks of thyroid lesions; 60 patients underwent partial thyroidectomy for query atypical adenoma and 60 blocks of complementary thyroidectomy of the same patients. Complementary thyroidectomy blocks included 21 cases of thyroid cancer and 39 cases were multinodular goiter, while partial thyroidectomy blocks included 15 cases of thyroid adenoma, 17 cases were colloid nodules and 28 cases were tumors of uncertain malignant potential (TUMP). The expression levels of both telomerase reverse transcriptase (TERT) promoter mutations and proEx C antibodies were assessed in all blocks; analysis and correlation of marker expression of both groups (complementary and partial thyroidectomy) were done.

20 patients out of 28 suffering from TUMP developed thyroid carcinoma. Ten of them had positive TERT promoter mutation, all of which turned into cancer thyroid in a mean time of  $8.2 \pm 2.61$  years, and seventeen who had high proEx C labeling index (LI) underwent a malignant transformation in  $7.52 \pm 1.17$  years in a shorter time than the group with negative TERT/proEx C expression which transformed within  $12.31 \pm 3.57$  years, whereas only one case diagnosed first as thyroid adenoma transform into thyroid carcinoma.

TERT promoter mutation and proEx C expression are promising markers for determining the malignant potential in tumors of uncertain malignant potential.

**Key words:** TERT, proEx C, TUMP, thyroid.

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## Introduction

Since clinical diagnosis and surgical treatment of thyroid nodules are performed at their earliest detectable stage, lesions with borderline characteristics for which benignity or malignancy cannot be as-

essed are more frequently observed. The Chernobyl Pathologists Group suggests labeling tumors with “borderline” features as well-differentiated tumors of uncertain malignant potential (WDT-UWP) and follicular tumors of uncertain malignant potential (FT-UWP) [1].

Well-differentiated tumors of uncertain malignant potential: Diagnostic terminology in thyroid pathology proposed by Williams [2]. Tumors with (diffuse, equivocal) or (focal obvious) nuclear feature of PTC for the capsulated follicular pattern without invasion. It represents a true “gray zone” of “follicular patterned” thyroid lesions that need to be characterized to confirm the diagnosis of carcinoma and avoid unnecessary aggressive treatment [3].

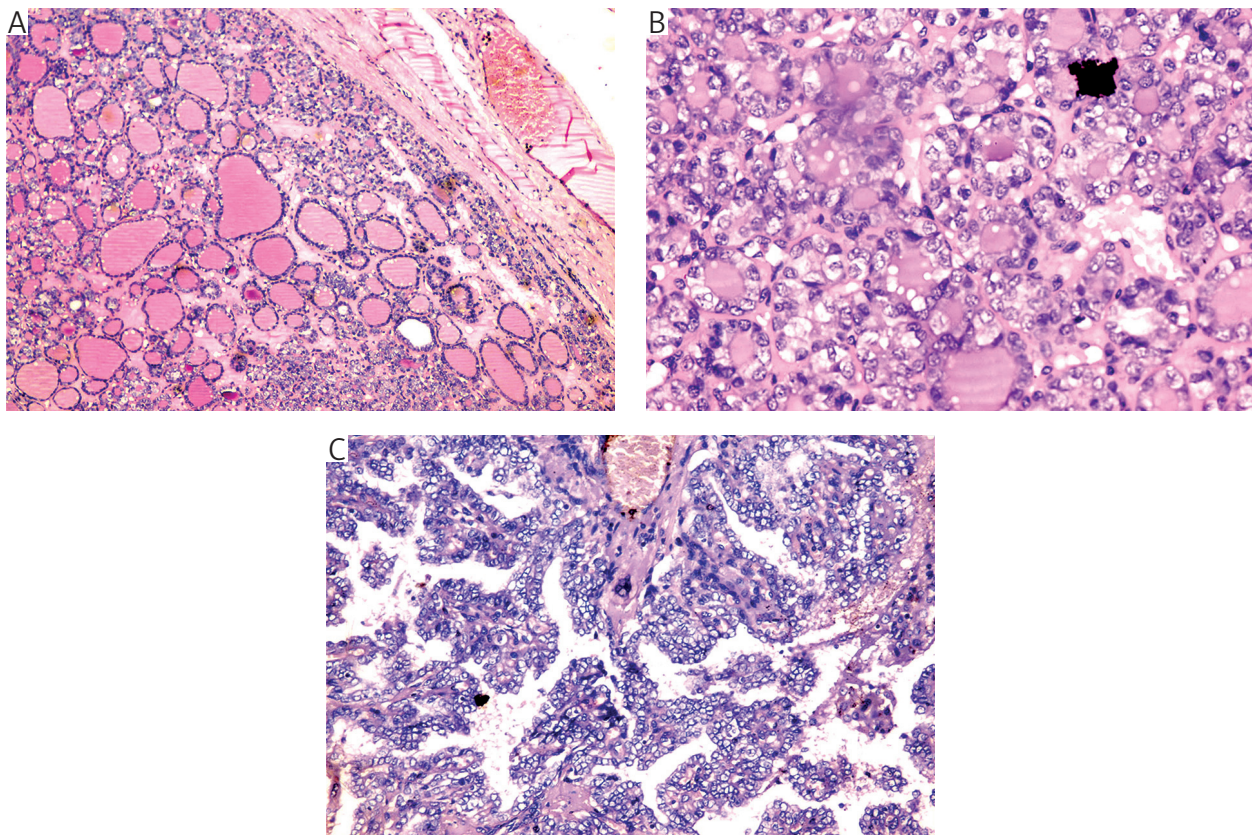
The World Health Organization (WHO) 2017 guidelines subclassify the thyroid borderline follicular tumors into 3 entities, namely, follicular tumors of uncertain malignant potential (FT-UMP), WDT-UMP, and non-invasive follicular tumor with a nuclear papillary function (NIFTP). The important histologic criterion for the first 2 entities is the “questionable capsular or vascular invasion” If the invasion is definite and not questionable, FT-UMP will be labeled as follicular carcinoma, whereas WDT-UMP will be a papillary thyroid carcinoma [4].

FT-UMP is a well-circumscribed tumor, or an encapsulated tumor composed of well-differentiated

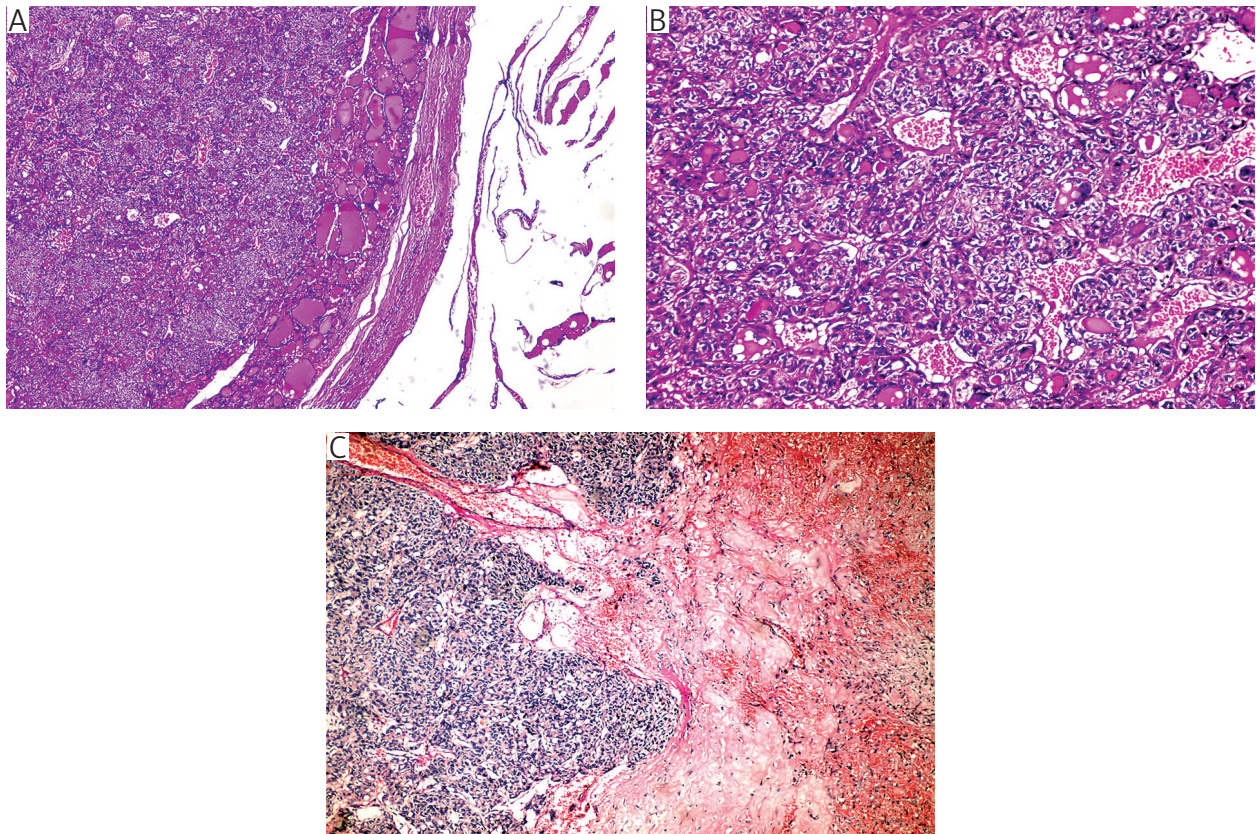
follicular cells with no nuclear changes of PTC and showing uncertain capsular or vascular invasion. This is a tumor indeterminate between follicular adenoma and follicular carcinoma (Fig. 2). WDT-UMP is an encapsulated or well-circumscribed tumor composed of well-differentiated follicular cells with well- or partially developed nuclear changes of PTC and showing uncertain capsular or vascular invasion [4] (Fig. 1).

Equivocal nuclear changes (PTC-Nc) were observed when incomplete nuclear characteristics are detected as partial nuclear clearing or nuclear grooves without nuclear pseudo inclusions; thyroid lesions will be termed as WDT-UMP in this case. When PTC-Nc has been detected clearly in a diffuse pattern throughout the whole part of the capsulated tumor, it will be NIFTP. If obvious PTC-Nc has been found only in a part of the tumor, it is also termed as WDT-UMP [5] (Fig. 1).

According to [3], the main diagnostic criteria of WDT-UMP are: The major criteria include the absence of nuclear pseudo inclusions, nuclear clearing,



**Fig. 1.** Histopathological description of a well-differentiated tumor of uncertain malignant potential, WDT-UMP at the first diagnosis, and PTC at second diagnosis. A) A capsulated thyroid mass composed of variable-sized follicles (HE, 200 $\times$ ). B) Illustrating the high power of the previous picture with diagnostic nuclear features of WDT-UMP showing diffuse equivocal PTC-Ns, thin incomplete nuclear grooves, nuclear clearing, enlarged ovoid nuclei, mild nuclear crowdedness and nuclear pleomorphism (HE, 200 $\times$ ). C) Illustrating histopathological description of classic PTC at the second diagnosis with papillary architecture showing obvious diffuse PTC-Ns; nuclear clearing, enlarged ovoid nuclei, pleomorphism, and overlapping (HE, 400 $\times$ )



**Fig. 2.** Histopathological description of follicular tumor of uncertain malignant potential (FT-UMP) at first diagnosis and poorly differentiated thyroid carcinoma at second diagnosis. A) A capsulated mass with questionable capsular invasion (HE, 100 $\times$ ). B) Illustrating the high power of the previous picture with a dense, irregular microfollicular architecture and no nuclear changes of PTC (HE, 200 $\times$ ). C) Illustrating histopathological description of poorly differentiated thyroid carcinoma from the second diagnosis with a large area of tumor necrosis (HE, 100 $\times$ )

characteristic nuclear groove, nuclear crowd, ovoid nuclei, nuclear pleomorphism, and nuclear enlargement. Minor criteria include abortive papillae, distorted follicles, fibrosis/sclerosis, dense colloid, and necrosis (Fig. 1).

Mutations in the promoters of telomerase reverse transcriptase (TERT) were related to adverse clinical parameters in thyroid cancer and are rarely triggered in normal cells; however, the most commonly found mutational variants of the TERT gene promoter are C228 T and C250 T, are observable in 90 percent of human malignancies and have recently also been detected in thyroid-derived follicular-cell malignancies. Such mutations were closely associated with aggressive properties in thyroid cancer and were also proposed as a possible biomarker to help with prognosis [6, 7, 8, 9].

ProEx C is a newly developed immunocytochemical assay targeting the expression of two proteins, topoisomerase II- $\alpha$  and minichromosome maintenance. Topoisomerase II (Topo II) is necessary for many aspects of DNA metabolism including replication, chromosome transcription, segregation, and cell proliferation. Two Topo II iso-enzymes have been

found, Topo IIa and Topo IIb; the expression of Topo IIa was correlated with the rate of proliferation of tumor cells and was commonly expressed in thyroid tumors correlated with poor clinical prognosis [10, 11, 12]

Minichromosome maintenance protein (MCM2) is a member of the six MCM protein family and acts as a key factor for the progression of the cell cycle, playing a direct role in replication initiation of DNA synthesis and limiting replication after completing one cycle. MCMs remain clustered throughout the entire interphase in the nucleus, and throughout the entire process of cell proliferation. MCM2 has previously been examined in various tumors and premalignant lesions [13, 14, 15, 16].

This work aims to evaluate the relation between TERT promoter mutation and proEX C expression and malignant transformation among tumors of uncertain malignant potential.

## Material and methods

The retrospective study includes 120 blocks of thyroid lesions. 60 patients with recurrent thyroid

nodules were admitted to the General Surgery Department and the Department of Otorhinolaryngology for complementary thyroidectomy from January 2013 to December 2019. We searched the archives of the pathology department and collected 60 blocks of their previously partial thyroidectomy; the clinic-pathological data, including age, sex and time between the two operations, were collected from the archives of the Pathology Department, General Surgery Department and the Department of Otorhinolaryngology, Faculty of Medicine, Zagazig University.

Re-evaluation of all blocks according to WHO 2017 criteria [4] by two pathologists blindly revealed:

- partial thyroidectomy blocks included 28 cases of tumors of uncertain malignant potential (TUMP), 15 cases of thyroid adenoma, and 17 cases were colloid nodules,
- complementary thyroidectomy blocks included 21 cases of thyroid cancer and 39 cases were multinodular goiter.

TERT promoter mutation and ProEx C expression were assessed in all 120 blocks, analysis, and correlation of the markers of both groups (partial thyroidectomy and complementary) was done.

Immunohistochemical expression of human TERT reactivity was confirmed by the detection of TERT promoter mutation genes using PCR.

### Immunohistochemistry

Immunohistochemistry was performed on paraffin blocks and sectioned into 4- $\mu$ m slices and immunostaining was carried out using a Leica BOND-MAX autostainer (Leica GmbH, Nussloch, Germany), according to the manufacturer's protocol. Slides were dewaxed in Bond Dewax Solution (Leica Microsystems) and rehydrated in Bond Wash Solution (Leica Microsystems). Antigen retrieval was performed at pH 6 using Bond Epitope Retrieval 1 Solution (Leica Microsystems) for 30 min at 100°C. Slides were incubated for 20 min at room temperature with Anti-Telomerase catalytic subunit (RABBIT) Antibody – 600-401-252S (Rockland Immunochemicals, Inc., Limerick, PA, USA) and proEx C (clone MCM2 26H6.19, MCM2 27C5.6, TOP2A SWT3D1 – 005-11000-40 – TriPath Imaging, Inc 780 Plantation Drive Burlington, NC 27215, USA). Primary antibody binding to tissue section was visualized using biotin-free Bond Polymer Refine Detection (Leica Microsystems). After post-primary amplification (8 min; Leica Microsystems) and detection with the Novolink Polymer Detection System (15 min; Leica Microsystems) using 3,3'-diaminobenzidine (Novocastra Laboratories; 1:50), slides were counterstained with hematoxylin (Leica Microsystems). Positive IHC reactions were defined for TERT expression as brown cytoplasmic

or nuclear pattern immunostaining and proEx C was the exclusive nuclear pattern.

### Immunohistochemical evaluation (Scoring)

#### *TERT* [17]

The immunostaining of all samples was evaluated independently by two surgical pathologists; each pathologist assessed the intensity and extent of immunoreactivity for each case, the expression of TERT protein was evaluated in tissue samples which exhibited a nuclear, cytoplasmic pattern or both immunoreactivity.

The extent of the positive reaction was classified into three grades (1, 2, or 3) as follows:

- grade 1, a positive reaction was detected in < 20%
- grade 2, a positive reaction between 21 and 50%;
- grade 3, a positive reaction was detected in > 50%.

The staining intensity was also classified into three grades, as follows:

- grade 1, weak intensity;
- grade 2, moderate intensity;
- grade 3, strong intensity.

Subsequently, a total score ranging from 2 to 6 was obtained by adding the scores of the two categories analyzed:

- score 2 weak,
- score 3-4 moderate,
- score 5-6 strong.

#### *proEx C*

The percentage of positive cells for proEx C (proEx C index) was calculated by counting 3000-4000 tumor cell nuclei in the most positive areas, at least 10 high-power fields (HPFs; 0.16 mm<sup>2</sup>) in each case, the brown coloration of epithelial cell nuclei was regarded as positive staining while any non-epithelial cells were disregarded. The percentage of labeled cells was determined according to the following equation: labeling index (LI) = number of labeled nuclei/total counted nuclei  $\times$  100. During the evaluation, all the cases were randomized and the positive slides were scored as LI, which is defined as the percentage of epithelial cells that were immunoreactive [13].

### Molecular study

TERT promoter mutation evaluation in tissue using real-time qPCR.

*DNA purification;* Serial 10  $\mu$ m-thick tissue sections were obtained from paraffin blocks for DNA extraction from the primary tumor tissue. Hematoxylin and eosin reference slides were marked to identify the area of interest in macro-dissection. For the extraction of the DNA, embedded sections were

deparaffinized in xylene, dehydrated through a graded series of alcohols, and processed using a diaminobenzidine detection system (Ventana Medical Systems, Inc., Tucson, AZ, USA), following the manufacturer's protocol. DNA was purified using the Qiagen RNeasy FFPE kit (Qiagen GmbH, Hilden, Germany). DNA was quantified using a Nanodrop spectrophotometer. The DNA was evaluated for the two most frequently identified variations of the promoter of the TERT gene for C228T and C250T. Consequently, the TERT promoter was amplified by PCR on genomic DNA using primers 5'-AGTGGATTCGCGGGCACAGA-3' (sense) and 5'-CAGC-GCTGCCTGAACTC-3' (antisense). This resulted in a PCR product of 235 bp, containing the sites where mutations C228T and C250T occur [18] and GAPDH as a housekeeping gene according to [19]. Forward: 5'-TGGCCTCCAAGGAGTAAGAAAC-3' Reverse: 5'-GGCCTCTCTCTTGCTCTCAGTATC-3'. The presence of either mutation C228T or C250T or both, in any case, is considered TERT promoter mutation.

### Statistical analysis

Continuous variables were expressed as mean  $\pm$  SD and median (range), and categorical variables were expressed as number (percentage). Continuous variables were checked for normality by using the Shapiro-Wilk test. The Mann-Whitney U test was used to compare two groups of non-normally distributed variables. Percent of categorical variables was compared using Pearson's chi-square test or Fisher's exact test when was appropriate. All tests were two-sided. A p-value  $< 0.05$  was considered significant. All statistics were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

## Results

### Clinicopathological features of all studied patients (n = 60) and thyroid carcinoma patients (n = 21)

This study included 60 patients; 68.3% of patients were female. The first diagnosis was TUMP in twenty-eight, 46.7% of patients, follicular adenoma in fifteen, 25% of patients, a colloid nodule in seventeen, 28.3% of patients.

Of the 28 TUMP cases, 21 patients (75%) had WDT-UMP character while seven patients, 25% were FT-UMP. All patients underwent partial thyroidectomy and after a mean time of  $12.31 \pm 3.57$  years since the first diagnosis, they developed a recurrent thyroid nodule and underwent complementary thyroidectomy. The mean age at the second diagnosis

was 55.80 years. The second diagnosis was 21 cases (35%) of patients with thyroid carcinoma and thirty-nine cases (65%) of patients with multinodular goiter. Twelve patients (20%) had papillary thyroid carcinoma at second diagnosis, 2 cases (3.3%) had follicular carcinoma, three cases (5%) had poorly differentiated carcinoma and four cases (6.7%) had anaplastic carcinoma (Table I).

### TERT promoter mutation and proEx C expression among all studied patients (n = 60)

Ten patients (16.7%) had positive TERT promoter mutations in the first and second diagnosis by both immunohistochemistry and PCR. All cases expressed TERT with immunohistochemistry, detected with PCR. Ten patients (16.7%) who had both (TERT mutation and high proEx C LI) in the first diagnosis

Table I. Clinicopathological features

CLINICOPATHOLOGICAL FEATURES	ALL PATIENTS (N = 60)	
	No.	%
Sex		
Male	19	31.7
Female	41	68.3
First diagnosis		
TUMP	28	46.7
Follicular adenoma	15	25
Colloid nodule	17	28.3
WDT-UMP	21	35
FT-UMP	7	11.7
Follicular adenoma	15	25
Colloid nodule	17	28.3
Age 2 <sup>nd</sup> diagnosis (years)		
Mean $\pm$ SD	55.80 $\pm$ 11.24	
Median (range)	51 (40-83)	
Time between diagnoses (years)		
Mean $\pm$ SD	12.31 $\pm$ 3.57	
Median (range)	13 (6-19)	
Second diagnosis		
Thyroid carcinoma	21	35
Multinodular goiter	39	65
Papillary thyroid carcinoma	12	20
Follicular carcinoma	2	3.3
Poorly differentiated carcinoma	3	5
Anaplastic carcinoma	4	6.7
Multinodular goiter	39	65

Categorical variables are expressed as number (percentage); Continuous variables are expressed as mean  $\pm$ SD and median (range)

**Table II.** Immunohistochemical staining and PCR for TERT promoter mutation and proEx C

IMMUNOHISTOCHEMICAL STAINING AND PCR	FIRST DIAGNOSIS (N = 60)		SECOND DIAGNOSIS (N = 60)	
	No.	%	No.	%
<b>TERT IHC</b>				
Negative	50	83.3	50	83.3
Positive	10	16.7	10	16.7
Negative	50	83.3	50	83.3
Weak positive	7	11.7	0	0
Moderate positive	3	5	7	11.7
Strong positive	0	0	3	5
<b>TERT PCR</b>				
Negative	50	83.3	50	83.3
Positive	10	16.7	10	16.7
<b>proEx C LI</b>				
Mean $\pm$ SD	13.81 $\pm$ 10.51		22.63 $\pm$ 16.59	
Median (range)	9.40 (3.20-43.90)		14.70 (3.20-59.70)	
<b>proEx C LI IHC</b>				
Negative	40	66.7	31	51.7
Positive	20	33.3	29	48.3
<b>+ve TERT/High proEx C LI</b>				
Absent	50	83.3	50	83.3
Present	10	16.7	10	16.7

*Categorical variables are expressed as number (percentage)*

*Continuous variables are expressed as mean  $\pm$ SD & median (range)*

also had TERT mutation and a high proEx C LI in the second diagnosis (Table II).

#### **Relationship between TERT promoter mutation at first diagnosis and parameters at second diagnosis (including neoplastic transformation) among TUMP patients (n = 28; Figs. 3, 4)**

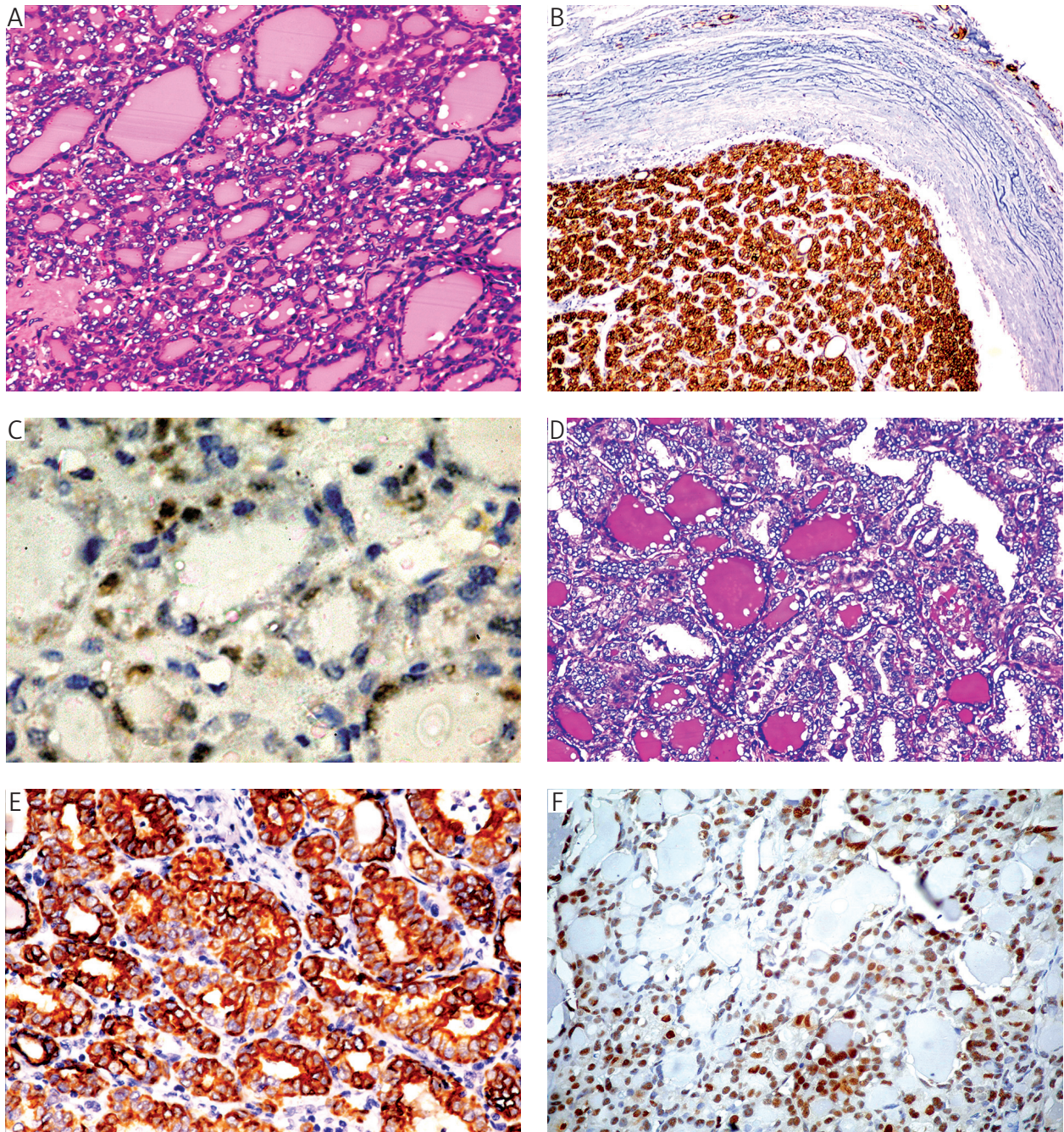
There was a significant association between the presence of TERT mutation at the first diagnosis and cancer detection at the second diagnosis, where ten patients (100%) with TERT mutation and diagnosed as TUMP at the first diagnosis developed thyroid carcinoma (p-value < 0.05).

There was a significant association between the presence of TERT mutation at the first diagnosis and the type of thyroid carcinoma at the second diagnosis where 10% who had positive TERT mutation at the first diagnosis developed papillary thyroid carcinoma, and 40% who had positive TERT mutation at the first diagnosis developed anaplastic thyroid carcinoma (p-value < 0.001), 20% developed follicular carcinoma and 30% developed poorly differentiated carcinoma. Patients with TERT mutation at the first

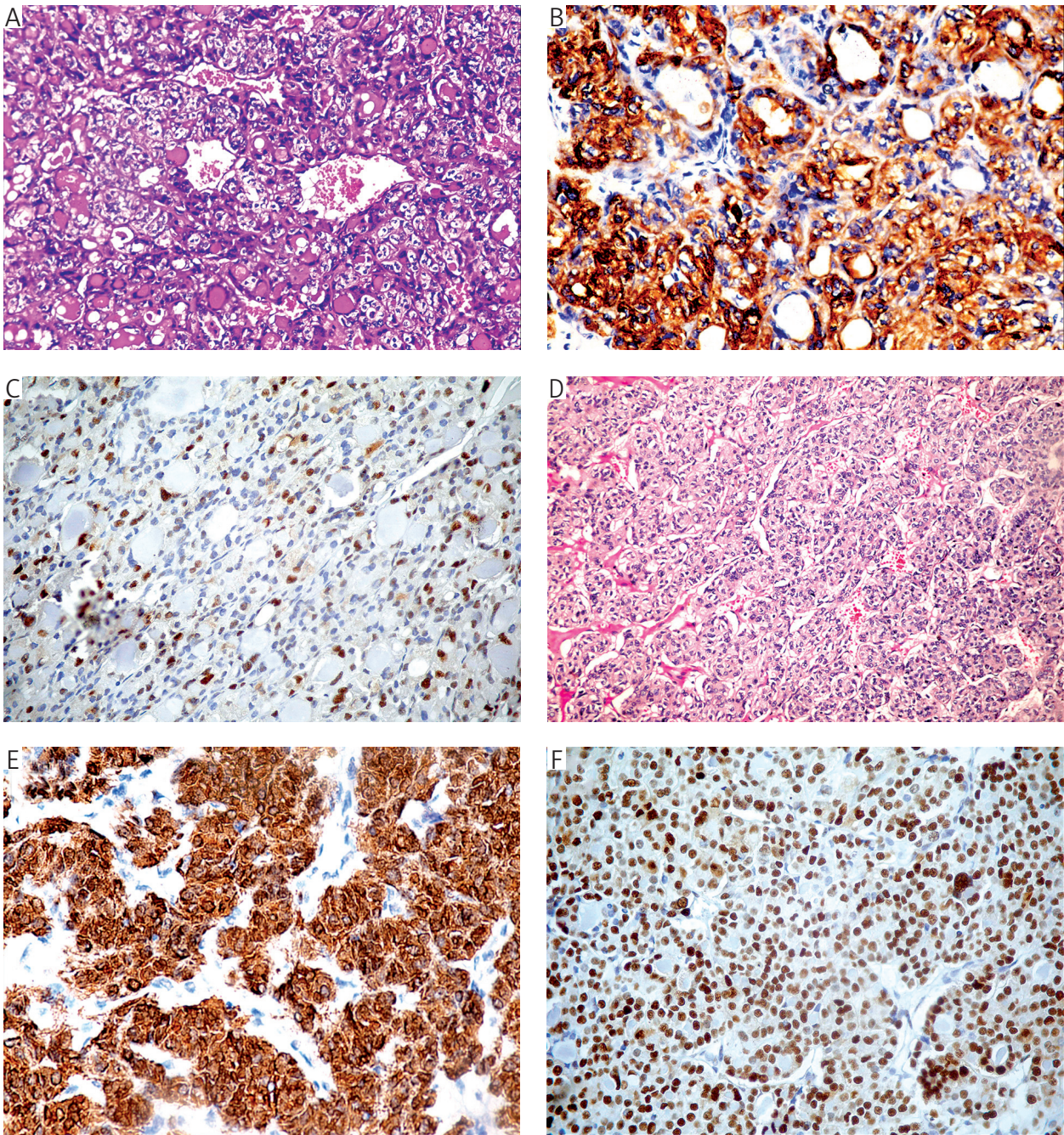
diagnosis were significantly older at the second diagnosis than patients without TERT mutation at the first diagnosis, where the mean age at the second diagnosis was 66.80 years versus 55.38 years respectively (p-value < 0.05).

Patients with TERT mutation at the first diagnosis had a significantly shorter time before the second diagnosis than patients without TERT mutation at the first diagnosis, where the mean time before the second diagnosis was 8.2  $\pm$ 2.61 years versus 12.33  $\pm$ 4.57 years respectively (p-value = 0.026).

There was a significant association between the presence of TERT mutation at the first diagnosis and TERT expression at the second diagnosis, where 100% of patients with positive TERT mutation at the first diagnosis were also positive for TERT mutation at the second diagnosis (p-value < 0.001). Patients with TERT mutation at the first diagnosis had a significantly higher proEx C LI at the second diagnosis than patients with negative TERT mutation at the first diagnosis where the mean proEx C LI at the second diagnosis was 42.84 versus 32.15 respectively (p-value = 0.023) (Table III).



**Fig. 3.** A well-differentiated tumor of uncertain malignant potential, WDT-UMP at the first diagnosis, and follicular variant of PTC at the second diagnosis. A) Histopathological description of WDT-UMP at the first diagnosis with diffuse equivocal PTC-Ns, thin incomplete nuclear grooves, nuclear clearing, enlarged ovoid nuclei, mild to moderate nuclear crowdedness (HE, 200 $\times$ ). B) TERT immunohistochemical staining of WDT-UMP cells at the first diagnosis shows strong diffuse immunoreactivity (100 $\times$ ). C) ProEx C immunohistochemical staining of WDT-UMP cells at the first diagnosis shows moderate LI nuclear immunoreactivity (400 $\times$ ). D) Histopathological description of classic PTC, follicular variant at the second diagnosis with obvious PTC-Ns; nuclear clearing, enlarged ovoid nuclei, pleomorphism, and overlapping (HE, 400 $\times$ ). E) TERT immunohistochemical staining of PTC (follicular variant) at the second diagnosis shows strong diffuse cytoplasmic immunoreactivity (400 $\times$ ). F) ProEx C immunohistochemical staining of PTC (follicular variant) cells from the second diagnosis shows high LI nuclear immunoreactivity (200 $\times$ )

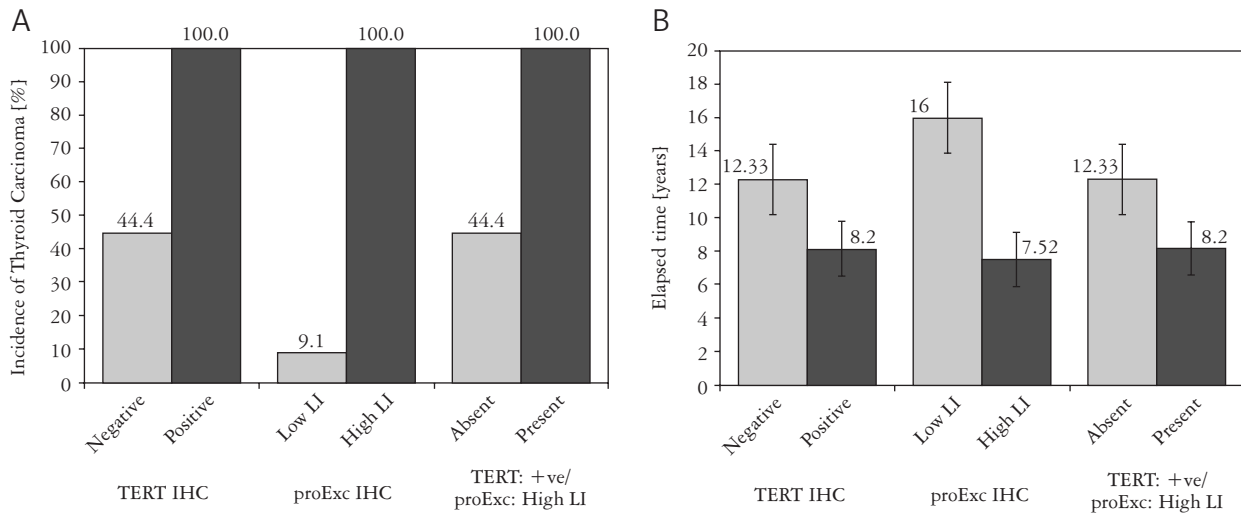


**Fig. 4.** A follicular tumor of uncertain malignant potential FT-UMP at the first diagnosis and follicular carcinoma FC at the second diagnosis. A) Histopathological description of FT-UMP at the first diagnosis with a dense, irregular micro-follicular architecture and no nuclear changes of PTC (HE, 200 $\times$ ). B) TERT immunohistochemical staining of follicular tumor of uncertain malignant potential at the first diagnosis shows moderate cytoplasmic immunoreactivity (400 $\times$ ). C) ProEx C immunohistochemical staining of follicular tumor of uncertain malignant potential cells from the first diagnosis shows moderate LI nuclear immunoreactivity (400 $\times$ ). D) Histopathological description of follicular carcinoma from the second diagnosis showing a trabecular pattern of small follicles (HE, 200 $\times$ ). E) TERT immunohistochemical staining of follicular carcinoma cells at the second diagnosis shows strong immunoreactivity in both the nucleus and the cytoplasm (400 $\times$ ). F) ProEx C immunohistochemical staining of follicular carcinoma cells at the second diagnosis shows high LI nuclear immunoreactivity (200 $\times$ )

**Table III.** Relation between immunohistochemical staining for TERT promoter mutation, proEx C at first diagnosis and second diagnosis clinicopathological parameters among patient with TUMP (n = 28)

PARAMETERS	TERT IHC AT 1 <sup>ST</sup> DIAGNOSIS				PROEXC IHC AT 1 <sup>ST</sup> DIAGNOSIS				
	NEGATIVE (N = 18)		POSITIVE (N = 10)		LOW LI (N = 11)		HIGH LI (N = 17)		P-VALUE
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
2 <sup>nd</sup> diagnosis									
Thyroid carcinoma	8	44.4	10	100	1	9.1	17	100	< 0.001‡
Multinodular goiter	10	55.6	0	0	10	90.9	0	0	
Papillary thyroid carcinoma	8	44.4	1	10	0	0	9	52.9	< 0.001‡
Follicular carcinoma	0	0	2	20	0	0	2	11.8	
Poorly differentiated carcinoma	0	0	3	30	1	9.1	2	11.8	
Anaplastic carcinoma	0	0	4	40	0	0	4	23.5	
Multinodular goiter	10	55.6	0	0	10	90.9	0	0	
Age 2 <sup>nd</sup> diagnosis (years)									
Mean ±SD	55.38 ±11.34		66.80 ±11.29		56.54 ±13.67		61.35 ±11.58		0.172•
Median (range)	51 (40-79)		63.50 (52-83)		51 (40-82)		57 (49-83)		
Time between diagnoses (years)									
Mean ±SD	12.33 ±4.57		8.20 ±2.61		16 ±1.61		7.52 ±1.17		< 0.001•
Median (range)	14 (6-19)		7.50 (6-15)		16 (14-19)		7 (6-9)		
TERT IHC at 2 <sup>nd</sup> diagnosis									
Negative	18	100	0	0					
Positive	0	0	10	100					
proExc LI at 2 <sup>nd</sup> diagnosis									
Mean ±SD	32.15 ±12.66		42.84 ±9.98		24.25 ±6.64		43.55 ±9.45		< 0.001•
Median (range)	30.20 (14.60-59.70)		39.40 (30.40-58.90)		24.70 (14.60-33.50)		40.30 (30.40-59.70)		
proEx C IHC at 2 <sup>nd</sup> diagnosis									
Low LI	2	11.1	0	0	2	18.2	0	0	0.146‡
High LI	16	88.9	10	100	9	81.8	17	100	

Categorical variables were expressed as number (percentage); • Mann-Whitney U test; ‡ Chi-square test; p < 0.05 is significant.



**Fig. 5.** A) Bar chart shows relationship between TERT, proExc, combined IHC staining at first diagnosis and incidence of thyroid carcinoma at second diagnosis. B) Error bar chart shows relationship between TERT, proEx C, combined IHC staining at first diagnosis and time between first and second diagnosis; bar represents mean, Y-error bar represents 95% CI (confidence interval) around mean

#### Relationship between proEx C immunohistochemistry at first diagnosis and parameters at second diagnosis (including neoplastic transformation) among TUMP patients (n = 28; Figs. 3, 4)

There was a significant association between proEx C immunohistochemical staining at the first diagnosis and second diagnosis where seventeen (100%) patients with high proEx C LI at the first diagnosis developed thyroid carcinoma (p-value < 0.001).

There was a significant association between proEx C immunohistochemical staining at the first diagnosis and type of thyroid carcinoma at the second diagnosis, where nine (52.9%) patients with high proEx C LI at the first diagnosis developed papillary thyroid carcinoma, four (23.5%) patients with high proEx C LI at the first diagnosis developed anaplastic thyroid carcinoma (p-value < 0.001), two (11.8%) developed follicular carcinoma and two (11.8%) developed poorly differentiated carcinoma.

Patients with high proEx C LI at the first diagnosis had significantly shorter time before the second diagnosis than patients with low proEx C LI at the first diagnosis where the mean time before the second diagnosis was  $7.52 \pm 1.17$  years vs.  $16 \pm 1.61$  years respectively (p-value < 0.001).

Patients with high proEx C LI at the first diagnosis had significantly higher proEx C LI at the second diagnosis than patients with low proEx C LI at the first diagnosis where the mean proEx C LI at the second diagnosis was 43.55 vs. 24.25 respectively (p-value < 0.001; Table III).

#### Relationship between TERT mutation/ proEx C expression at first diagnosis and parameters at second diagnosis (including neoplastic transformation) among TUMP patients (n = 28; Fig. 5)

There was a significant association between TERT/proEx C immunohistochemical staining at the first diagnosis and second diagnosis where 100% of patients with positive TERT/High proEx C LI at the first diagnosis developed thyroid carcinoma (p-value < 0.05).

There was a significant association between TERT/proEx C immunohistochemical staining at the first diagnosis and the type of thyroid carcinoma at the second carcinoma where 40% of patients with high positive TERT/High proEx C LI at the first diagnosis developed anaplastic thyroid carcinoma (p-value < 0.001).

Patients with positive TERT/High proEx C LI at the first diagnosis were significantly older at the second diagnosis than patients without positive TERT/High proEx C LI at the first diagnosis, where the mean age at the second diagnosis was 66.80 years vs. 55.38 years respectively (p-value < 0.05).

Patients with positive TERT/High proEx C LI at the first diagnosis had significantly shorter time before the second diagnosis than patients without positive TERT/High proEx C LI at the first diagnosis, where the mean time before the second diagnosis was  $8.2 \pm 2.61$  years vs.  $12.33 \pm 4.57$  years respectively (p-value = 0.026; Table IV).

**Table IV.** Relation between +ve TERT promoter mutation/High proExc LI at first diagnosis and second diagnosis clinicopathological parameters among patients with TUMP (n = 28)

PARAMETERS	+ve TERT/HIGH PROEXC LI AT 1 <sup>ST</sup> DIAGNOSIS				P-VALUE
	ABSENT		PRESENT		
	No.	%	No.	%	
2 <sup>nd</sup> diagnosis	(n = 18)		(n = 10)		
Thyroid carcinoma	8	44.4	10	100	< 0.05 ‡
Multinodular goiter	10	55.6	0	0	
Papillary thyroid carcinoma	8	44.4	1	10	< 0.001 ‡
Follicular carcinoma	0	0	2	20	
Poorly differentiated carcinoma	0	0	3	30	
Anaplastic carcinoma	0	0	4	40	
Multinodular goiter	10	55.6	0	0	
Age 2 <sup>nd</sup> diagnosis (years)					
Mean ±SD	55.38 ±11.34		66.80 ±11.29		< 0.05 •
Median (range)	51 (40-79)		63.50 (52-83)		
Time between diagnoses (years)					
Mean ±SD	12.33 ±4.57		8.20 ±2.61		0.026 •
Median (range)	14 (6-19)		7.50 (6-15)		
proExc LI at 2 <sup>nd</sup> diagnosis					
Mean ±SD	32.15 ±12.66		42.84 ±9.98		0.023 •
Median (range)	30.20 (14.60-59.70)		39.40 (30.40-58.90)		
proExc IHC at 2 <sup>nd</sup> diagnosis					
Low LI	2	11.1	0	0	0.524 ‡
High LI	16	88.9	10	100	
+ve TERT/High proExc LI 2 <sup>nd</sup> diagnosis					
Absent	18	100	0	0	< 0.001 ‡
Present	0	0	10	100	

Categorical variables were expressed as number (percentage); • Mann-Whitney U test; ‡ Chi-square test; p < 0.05 is significant.

## Discussion

Borderline thyroid tumors, as defined by the World Health Organization, 2017 consist of a hyalinizing trabecular tumor (HTT), a well-differentiated tumor of uncertain malignant potential (WDT-UMP), a follicular tumor of uncertain malignant potential (FT-UMP) and a non-invasive follicular tumor with a nuclear papillary function (NIFTP). They have different pathological characteristics from each other. However, they are difficult to diagnose preoperatively with imaging, fine needle aspiration (FNA) or core biopsy. The diagnosis is usually made after a patient's lobectomy. Owing to the fairly indolent nature of these tumors, the main surgical complications associated with borderline tumors do not require complete thyroidectomy. Sadly, any of these tumors may be classified preoperatively as a malignant tumor. The other surgical issue is whether or not thyroidectomy is per-

formed following the diagnosis of lobectomy. Decision making is difficult given the fact that lobectomy alone is commonly considered appropriate [20].

To identify vulnerable borderline/precursor lesions that may recur or metastasize and cause cancer mortality in a large proportion of patients when left untreated, it is important to establish more precise histological parameters. As they can be treated with simple excision, it is also essential to exclude benign follicular adenoma and indolent borderline/precursor tumors from deadly cancers. Unfortunately, tumors of the borderline/precursor have often been treated equally and dramatically in Western clinical practice as lethal malignant thyroid tumors. While borderline/precursor tumors were ignored in Asian practice, they were treated as if they were entirely benign tumors. We need a clear border between the two activities to resolve the gaps [21].

### Are classic thyroid driver oncogenes (RET/PTC, BRAF, RAS) beneficial in detecting malignant behavior in thyroid tumors of uncertain malignant potential?

A driver mutation is an oncogenesis implication in cancer stem cells and is positively selected in the microenvironment of the tissue where cancer starts and is not required for the preservation of final cancer. BRAF, RAS mutations, and RET/PTC rearrangements are most commonly associated as a regulator of thyroid oncogene. Given all these findings, there is still no clear supporting evidence showing the classical prognostic function of BRAF and RAS mutations and RET/PTC re-arrangements. It is true, however, that RET/PTC rearrangements are associated with radiation exposure and are more frequent in patients with radio-induced PTC [22, 23, 24].

Regarding expression of the BRAF and RET mutations, it was completely absent in the entire cohort study by Duan *et al.* [22] including 49 thyroid tumors of uncertain malignant potential (TUMP), 48 samples from follicular thyroid adenoma, and 55 samples from follicular thyroid carcinoma. Moreover, they were not detected in 30 cases of TUMP and 35 benign nodules in a study by Min [23]. Similar results were obtained by Hofman *et al.* [24], who reported their complete absence in 31 cases of TUMP; in the same context were Liu *et al.* [5], who also reported the absence of BRAF in all studied 30 TUMP cases; these results were in agreement with Guerra *et al.* [25], who stated that the BRAF V600E mutation in PTCs occurs as a late clonal event during tumor development.

Regarding RAS mutations, RAS has been shown to have inferior sensitivities and specificities for thyroid cancer and their major oncogenic role stems from their conjunction with other mutations. The picture is now clearer with the light shed by wonderful recent research from Medici *et al.* [26, 27]; they investigated the diagnostic value of RAS mutations and the clinical behavior of RAS-positive thyroid tumors and confirmed the low diagnostic sensitivity and specificity of RAS mutations when used alone. Moreover, a significant novel result indicated that RAS mutations were positive, but the thyroid nodules displayed excellent stability without any radiographic development or negative clinical effects following long-term clinical follow-up (mean 8.3 years) and act as true stable nodules and can be controlled conservatively over the long term. Such results by Medici *et al.* [26] are consistent with the Durante *et al.* report [28]. Likewise it demonstrated relatively long-term survival and stable prognosis of stable thyroid nodules with RAS mutation. These clinical results further reinforce that RAS mutation alone plays a limited role in the development of benign thyroid nodules. The significant observation in the earlier [26, 27]

research is that, even though histologically reported to be malignant, tumors that are only RAS mutation positive have minimal aggressiveness, as opposed to those associated with TERT-promoting mutations that have aggressiveness and poor clinical results for thyroid cancer [29]. RAS mutations and TERT promoter mutations are strongly linked to one another in thyroid cancer, and the co-existence of the two mutations is associated with a slightly higher recurrence of the tumor compared to RAS mutations alone in FTC [30]. Generally, cytologically stable thyroid nodules, particularly though harboring RAS mutations, have an outstanding good prognosis and can be treated comfortably and conservatively at fairly long time intervals; differentiated thyroid cancer harboring RAS mutations alone without any coexisting genetic changes usually lacks aggressive behavior [29]. Furthermore, this idea is confirmed by a mouse model in which K-RAS mutation alone induced only benign thyroid neoplasm and its co-existence with PTEN deletion in the PI3 K pathway induced the transformation of the tumor into FTC with metastases [31].

These results are consistent with the previous findings of Giordano *et al.* and Agrawal *et al.* [32, 33], who indicated that RAS mutations were associated with improved differentiation of thyroid cancer as seen in the normal or near-normal expression of thyroid iodide-handling genes. In another study, by Bae *et al.* [34], follicular nodules with NRAS mutations demonstrated no major variations in clinicopathological features beyond tumor size relative to NRAS-negative tumors. The occurrence of NRAS mutations was inversely associated with the size of the tumor, independent of the existence of malignancy. Additionally, Guerra *et al.* [25] reported that poor specificity and sensitivity hinder the ability of the RAS test to differentiate between FTC and benign nodules. Some studies have shown a correlation between weak tumor activity and RAS mutations in FTC and poorly differentiated thyroid carcinoma; it is likely to indicate the co-existence of RAS mutations with additional oncogenic modifications, such as TERT promoter mutations [29].

To the best of our knowledge, this is the first study to investigate TERT promoter mutation and proEx C in malignant transformation in tumors of uncertain malignant potential. In our study, we had 28 patients suffering from thyroid nodules diagnosed as TUMP; 10 of them had TERT promoter mutation and 17 had high proEx C LI, underwent partial thyroidectomy and after a mean period of  $7.68 \pm 1.98$  years, they developed a recurrent thyroid nodule, and underwent a complementary thyroidectomy. We found that, out of 28 TUMP cases, 20 patients developed thyroid carcinoma, whereas only one case diagnosed first as thyroid adenoma turned into thyroid carcinoma

at the second diagnosis, and no cases turned into cancer, among colloid nodule patients.

The enzyme telomerase consists of a protein portion with reverse transcriptase activity, encoded by the reverse transcriptase telomerase (TERT) gene. Telomerase activation is linked to the malignant transformation of many tumors, supplying longevity by the preservation of telomeric duration across multiple cell divisions [35]. We proved that TERT promoter mutation was significantly correlated with malignant transformation of borderline tumors of uncertain malignant potential, as we found that all patients with a positive TERT promoter mutation (10 patients) at the first diagnosis developed thyroid carcinoma at the second diagnosis with a shorter time (mean  $8.2 \pm 2.61$  years) before the second diagnosis compared with other cases with negative TERT mutation with a mean time of  $12.33 \pm 4.57$  years. This view was supported by Hysek *et al.* [35], who stated that TERT promoter constitutes a useful tool for proper detection of malignant transformation in histologically equivocal cases, and is in line with Paulsson *et al.* [36], who found that TERT expression was observed in 6/32 TUMP and 28/65 thyroid cancer cases and suggests that identical TERT aberrant profiles in the FTC and TUMP groups indicates that a subset of tumors in the latter group can experience potential malignant recurrences. Therefore, TERT promoter mutation may be a positive future marker for the assessment of malignant potential in thyroid follicular tumors. ProEx C is a mixture of two antibodies, the repair protein 2 minichromosome (MCM2) and topoisomerase 2A (TOP2A). TOP2A is an enzyme responsible for unlinking DNA strands during replication. MCM2 is a protein that is involved in the G1 phase of the cell cycle and maintains DNA synthesis via loading the pre-replication complex onto DNA; it has helicase activity resulting in the unwinding of DNA [37].

Moreover, as far as we know, this is the first study to investigate proEx C in TUMP and thyroid carcinoma. Previous studies dealt with its component topoisomerase II-alpha (Top2A) and minichromosome maintenance protein-2 (MCM2) individually in thyroid tumors.

In our study, we proved that proEx C expression was significantly correlated with malignant transformation of tumors of uncertain malignant potential. We found that all TUMP patients with high proEx C LI (17 patients) at the first diagnosis developed thyroid carcinoma at the second diagnosis with a shorter time between the two diagnoses (mean  $7.52 \pm 1.17$  years), compared with the long period of other cases of low and negative proEx C that had a mean period of  $12.31 \pm 3.57$  years. Our results were partially in line with Karayan-Tapon *et al.* [11], who found that all (30) cases of thyroid carcinoma showed high LI

of TOP2A, whereas in our study 17/28 cases of thyroid carcinoma showed high LI of proEx C.

In conclusion, TERT promoter mutation and high proEx C LI expression are significantly associated with malignant transformation of tumors of uncertain malignant potential.

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