

## ORIGINAL PAPER

## Evaluation of proliferation potential in thyroid normo-/hypofunctioning and hyperfunctioning nodules

MARIOARA CORNIANU<sup>1)</sup>, V. STAN<sup>2)</sup>, ELENA LAZĂR<sup>1)</sup>, ALIS DEMA<sup>1)</sup>,  
 IOANA GOLU<sup>3)</sup>, SORINA TĂBAN<sup>1)</sup>, MIHAELA VLAD<sup>3)</sup>,  
 ALEXANDRA FAUR<sup>1)</sup>, F. VĂRCUȘ<sup>4)</sup>, F. BABĂU<sup>4)</sup>

<sup>1)</sup>Department of Pathology,  
 "Victor Babeș" University of Medicine and Pharmacy, Timisoara

<sup>2)</sup>Municipal Hospital, Resita

<sup>3)</sup>Endocrinology Clinic

<sup>3)</sup>Surgery Clinic II

"Victor Babeș" University of Medicine and Pharmacy, Timisoara

### Abstract

**Introduction:** Thyroid follicular adenomas (FA) and adenomatous thyroid nodules (AN) – lesions that are frequently found in areas with iodine deficiency, can be normo-/hypofunctioning (scintigraphically cold – SCN) or hyperfunctioning (scintigraphically hot – SHN) nodules. **Aim:** Evaluation of proliferation potential in thyroid nodules on tissue samples obtained at surgery from euthyroid patients clinically diagnosed with SCN and from patients with thyroid hyperfunction and SHN. **Materials and Methods:** We investigated the proliferation activity estimated by assessing PCNA and Ki-67 proliferation markers in 20 SCN (eight FA and 12 AN) and 16 toxic nodules (six hyperfunctioning FA and 10 toxic multinodular goiters), on formalin-fixed and paraffin-embedded tissue samples, 4–5 µm thick; we used the immunohistochemical technique in LSAB system (DAB visualization) with anti-PCNA (PC10) and anti-Ki-67 (MIB-1) monoclonal antibodies. For each case, we calculated the proliferation index PI-PCNA and PI-Ki-67. The data were statistically evaluated using the *t*-unpaired test. **Results:** We observed a higher PI-PCNA in thyroid nodules than in the normal surrounding thyroid tissue, with statistically significant values for FA (14.3% vs. 3.8%;  $p < 0.029$ ) and also for AN (8.36% vs. 1.24%;  $p < 0.001$ ). The mean PI-Ki-67 in nodules vs. surrounding thyroid tissue was 1.64% vs. 1.10% in FA ( $p < 0.35$ ) and 1.07% vs. 0.51% in AN ( $p > 0.05$ ). We also noted: (1) significantly higher PI-PCNA values ( $p < 0.01$ ) in FA (14.03%) than in AN (8.36%), as compared to statistically insignificant values for Ki-67 (1.64% vs. 1.07%;  $p > 0.05$ ); (2) increased proliferation rate ( $p < 0.01$ ) in thyroid nodules with aspects of lymphocytic thyroiditis (LT) (PI-Ki-67 was 1.21%) as compared to nodules without LT (PI-Ki-67 was 0.12%); (3) a mean PI-PCNA of 8.5% and PI-Ki-67 of 4.61% in toxic thyroid nodules (TTN) vs. 3.01% and 1.5% in normal surrounding thyroid, respectively. **Conclusions:** The clinical expression of SCN is the consequence of increased thyrocyte proliferation in the nodules; the increased proliferative potential of TTN thyrocytes is a common feature of nodules, independent of their histopathological characteristics.

**Keywords:** thyroid nodules, proliferation index, Ki-67, PCNA.

### Introduction

Follicular thyroid adenomas and adenomatous thyroid nodules are lesions frequently found in geographical areas with iodine deficiency and they can be normo-/hypofunctioning (scintigraphically cold) or hyperfunctioning (hot) nodules. Their predominant clone origin suggests that they are the result of clonal expansion of a single cell, most probably following intensification of proliferation, as compared with the surrounding unaffected cells [1].

In normal thyroid tissue, cellular proliferation is reduced [2, 3]. Activation of the proliferation of thyroid follicular cells (following iodine deficiency) is known as a cause in goiter development, while focal intensification of the division of thyroid epithelial cells determines the appearance of thyroid nodules. Recent studies demonstrated an increase in proliferation, being seen also in later stages of functionally autonomous

thyroid nodules [4], activation of cyclic AMP cascade that stimulates growth and function of the thyroid being considered the most probable cause of thyroid tumors [5, 6].

Unlike hyperfunctioning thyroid nodules (also called hot nodules), in cold nodules iodine metabolism is significantly reduced, making affected epithelial cells unfunctional [7]. This mechanism that stimulates only the growth of thyroid epithelial cells and not of their function is probably responsible for the development of cold nodules.

Most of the toxic thyroid nodules (TTN) result from the clonal expansion of a single cell, caused by a somatic mutation of the thyrotropin (TSH) receptor, of  $G_{sa}$  protein or of some proteins yet unknown. The expansion of a single cell into a toxic thyroid nodule with thousands of cells suggests an intensification of cellular proliferation, as compared to unaffected surrounding cells [4].

## Aim

This paper aims to assess the proliferative potential of thyroid nodules on surgical resection pieces from euthyroid patients, clinically diagnosed with scintigraphically cold nodules, and patients with thyroid hyperfunction (scintigraphically hot nodules).

## Materials and Methods

We investigated the proliferative activity estimated through the evaluation of proliferation markers – proliferating cell nuclear antigen (PCNA) and Ki-67 antigen, in 20 benign follicular lesions and normal adjacent thyroid tissue. The study was made on surgical resection pieces from euthyroid patients clinically diagnosed with scintigraphically cold thyroid nodules and colloid nodular goiter, processing of the material involving fixation in formalin 10%, inclusion in paraffin, section (5–6 microscopic sections/block) and staining with HE.

The proliferative activity of thyrocytes from the studied thyroid nodules and the surrounding thyroid tissue was assessed on 4–5 µm thick sections applied on electrostatic charged slides; for immunostaining we used the IHC technique in the LSAB (labeled streptavidin-biotin) system, the kit being delivered by the firm Dako, Carpinteria – USA. The method is based on the application of biotined secondary antibody and of streptavidin marked with peroxidase. It is a sensitive technique that offers an intense immunostaining using primary antibodies diluted in Tris-HCl buffer solution, pH 7.6.

To highlight PCNA by IHC we used the anti-PCNA mouse monoclonal antibody, clone PC<sub>10</sub> (Dako, Carpinteria, USA), using the diluted primary antibody. The final reaction product has a brown color and nuclear localization, in a few cases obtaining cytoplasmic focal immunostaining of some cells.

For each case, we calculated the proliferation index (marking index) for PCNA (PI-PCNA) and Ki-67 (PI-Ki-67).

PI-PCNA (representing the number of marked cells from the total number of cells and expressed in percentages) was calculated for 500 thyroid epithelial cells (that were evidently forming thyroid follicles, all the other cells being excluded), on identical sections, from nodular tissue and surrounding normal thyroid tissue, after examining 10 microscopic fields (selected from the most relevant areas) with an objective that magnifies 400 times.

We analyzed the IHC expression of Ki-67 antigen, using the prediluted monoclonal antibody, clone MIB-1, LSAB technique with pre-treatment by boiling (in Retrieval solution for 60 minutes at 90°C) and DAB visualization. For the positive control of the reaction, we included in the study a tonsil fragment, and for the negative control, the buffer replaced the primary antibody. The final reaction product has a brown color and nuclear localization; the reaction was considered as positive for any nuclear staining, independent of the coloring intensity.

The quantification of the reaction was made by

assessing the proliferation index of Ki-67 (PI-Ki-67), expressed as a percentage result of the number of Ki67-positive cells referred to 500 cells (Ki-67+ and Ki-67-).

Due to the reduced percentage of Ki-67-positive cells, we counted the Ki-67-reactive nuclei on 15–20 microscopic fields randomly selected.

In the studied lesions, PI-PCNA and PI-Ki-67 were assessed on the areas with the highest density of PCNA- and Ki-67-reactive nuclei. The differences in PI-PCNA and PI-Ki-67 in the cold nodules as compared to the adjacent normal thyroid tissue were statistically analyzed using the unpaired *t*-test.

To verify the hypothesis that hyperfunctional thyroid nodules have a higher proliferation potential, we studied the proliferative rate for PCNA and Ki-67 (as markers of cell proliferation) in 16 scintigraphically hot thyroid nodules and in the surrounding normal thyroid tissue.

The tissue sections (formalin-fixed and paraffin-embedded) taken from the 16 hyperthyroid patients with toxic thyroid nodules were examined IHC using the LSAB technique and processed similarly to cold nodules, the antibodies used, quantification of immunoreactions and counting of PI for PCNA and Ki-67 being similar.

## Results

The 20 cases of scintigraphically cold nodules were histopathologically evaluated and diagnosed (according to WHO criteria) as follicular adenomas in eight cases and adenomatous nodules (normo- and macrofollicular) in 12 cases.

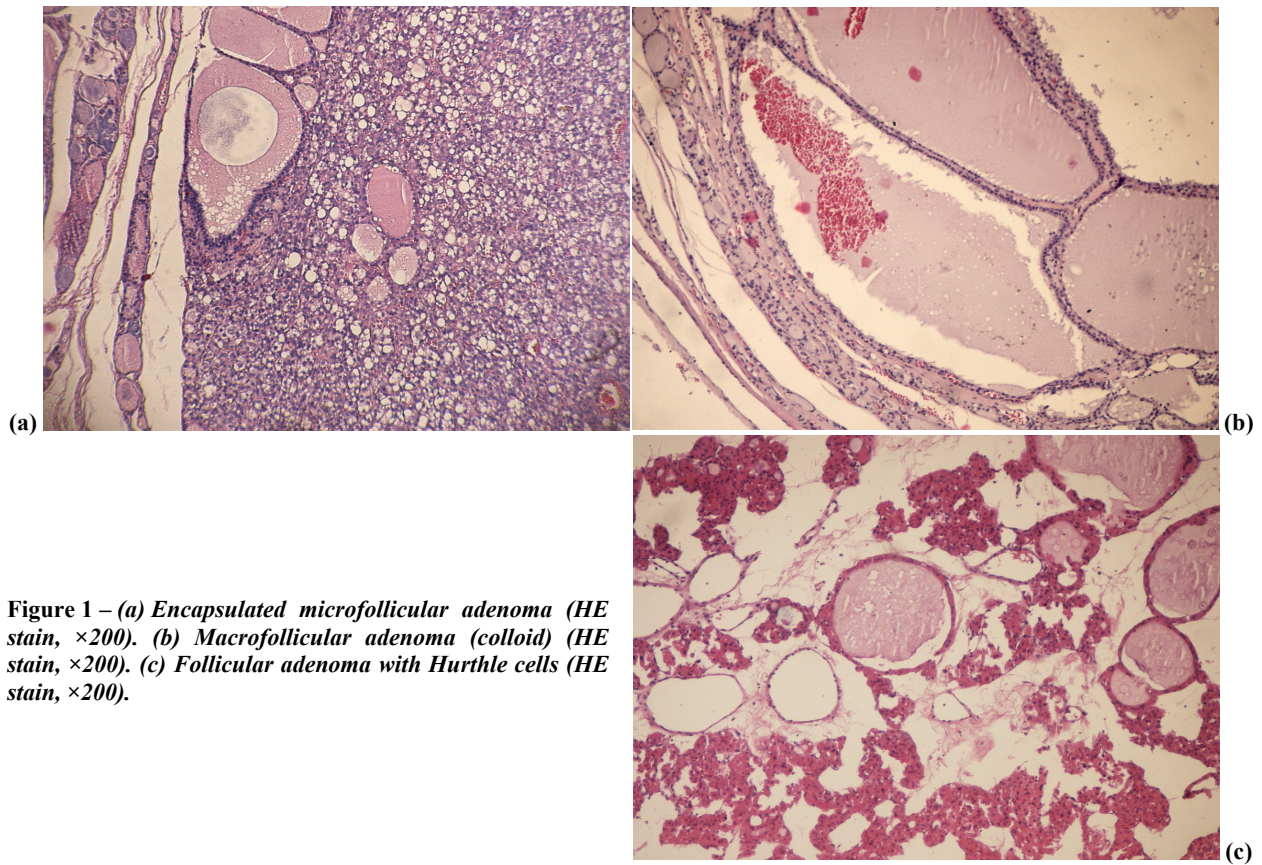
We classified as *follicular adenomas* (Figure 1, a–c) the encapsulated lesions with a uniform structure (with a micro-, normo- and macrofollicular pattern and/or with Hurthle cells), with compressive effects on surrounding structures, often associating secondary modifications like hemorrhage, edema and hyalinization of the stroma.

*Adenomatous nodules* from multinodular goiter were diagnosed in 12 cases as hyperplastic nodules with variable degrees of encapsulation (Figure 2), some completely encapsulated nodules mimicking adenomas. In four cases, we highlighted aspects of oncocytic metaplasia (with Hurthle cells) of follicular epithelium, and in other three cases, we observed associated lesions of focal lymphocytic thyroiditis, with aggregates of lymphoid cells interstitially.

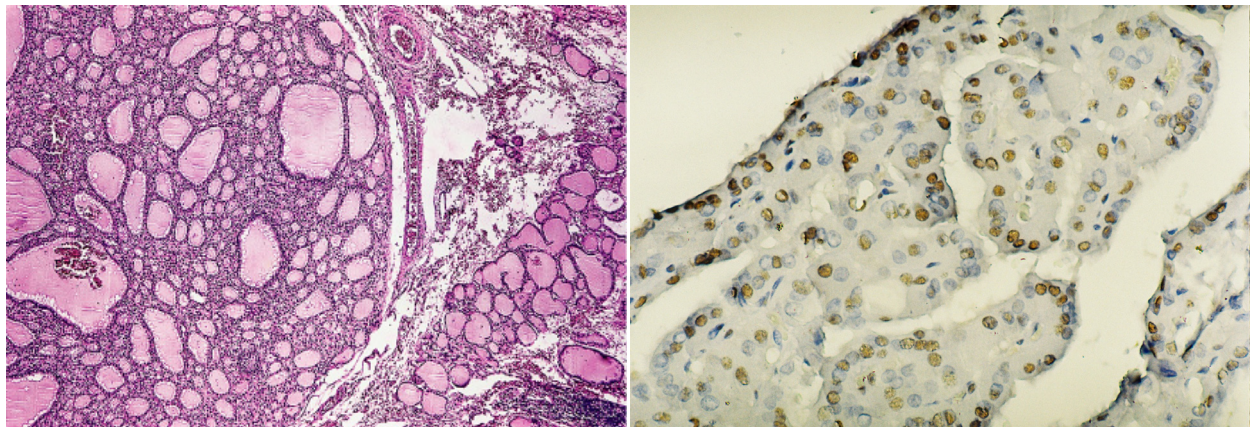
Immunoreaction for PCNA (Figure 3) and Ki-67 (Figures 4 and 5) was evaluated in all 20 scintigraphically cold nodules (including the eight follicular adenomas and 12 adenomatous nodules) and in the surrounding normal thyroid tissue.

Immunostaining of sections using the monoclonal antibody PC10 showed a heterogenous pattern of the distribution of PCNA-reactive nuclei in cold thyroid nodules as compared to the surrounding tissue, in three differentiated cases being evident even without quantifying the immunoreaction.

The proliferation indexes PI-PCNA and PI-Ki-67 were calculated for each case, both in thyroid nodules and in the surrounding normal thyroid tissue (Tables 1 and 2).

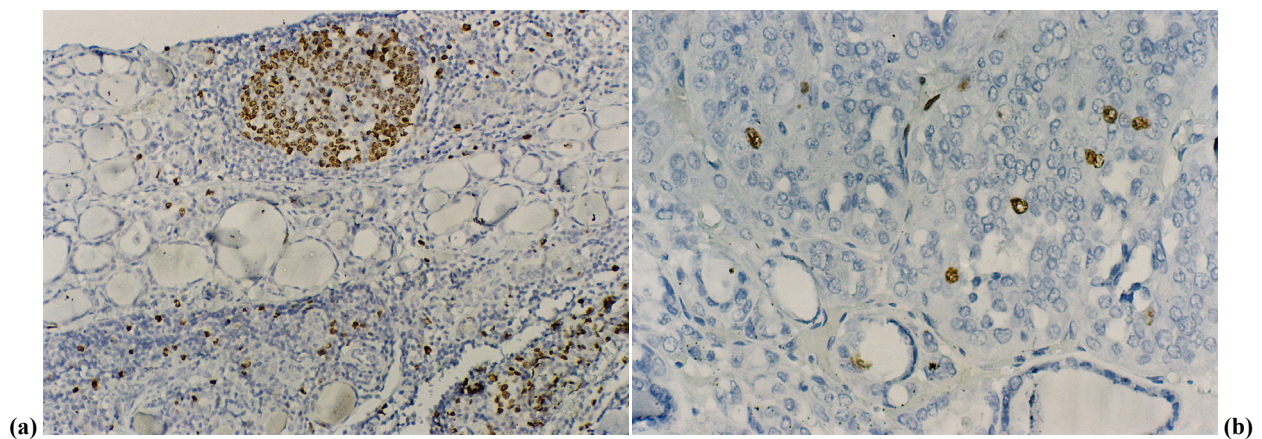


**Figure 1 – (a) Encapsulated microfollicular adenoma (HE stain,  $\times 200$ ). (b) Macrofollicular adenoma (colloid) (HE stain,  $\times 200$ ). (c) Follicular adenoma with Hurthle cells (HE stain,  $\times 200$ ).**



**Figure 2 – Adenomatous nodules with variable degrees of encapsulation (HE stain,  $\times 200$ )**

**Figure 3 – Follicular adenoma with Hurthle cells and formation of papillary structures (LSAB anti-PC10, DAB,  $\times 400$ ).**



**Figure 4 – (a) Ki-67 immunoreaction in focal lymphocytic thyroiditis adjacent to adenomas (LSAB anti-MIB-1, DAB,  $\times 200$ ). (b) Ki-67 immunoreaction in follicular adenomas (LSAB anti-MIB-1, DAB,  $\times 400$ ).**

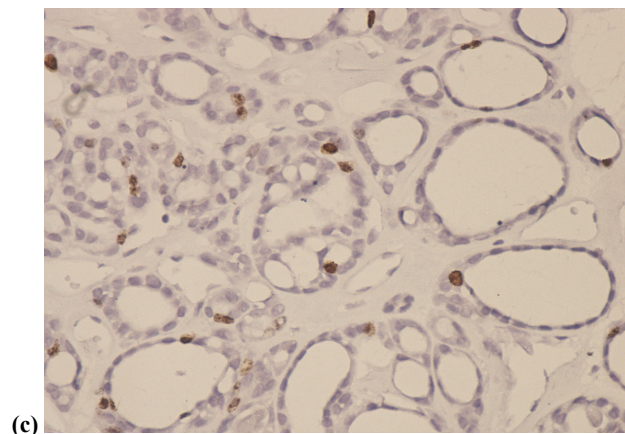


Figure 4 – (c) *Ki-67 immunoreaction in normofollicular adenomas (LSAB anti-MIB-1, DAB, ×200).*

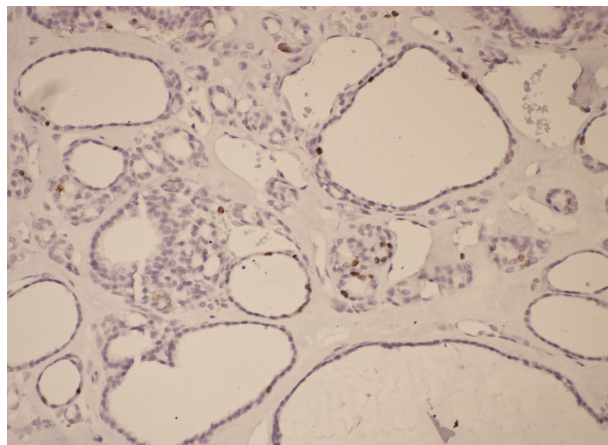


Figure 5 – *Ki-67 immunoreaction in adenomatous nodules from multinodular goiter (LSAB, anti-MIB-1, viz. DAB, ×200).*

Table 1 – *PI-PCNA and PI-Ki-67 in follicular adenomas*

No.	PI-PCNA (mean)		PI-Ki-67 (mean)	
	Nodules	Surrounding tissue	Nodules	Surrounding tissue
1.	20.2%	5.4%	1.86%	1.04%
2.	2.86%	0.8%	0.64%	0.1%
3*.	19.6%	8.1%	2.63%	2.82%
4.	22.2%	1.7%	1.48%	1.06%
5*.	5.1%	8.6%	1.02%	0.8%
6.	16.6%	2.0%	2.42%	0.9%
7.	9.48%	2.32%	1.5%	1.0%
8.	16.24%	2.9%	1.6%	1.1%
p<0.029		p<0.35		

PI – Proliferation index; PCNA – Nuclear antigen of cellular proliferation; \* – Associated lymphocytic thyroiditis.

Table 2 – *PI-PCNA and PI-Ki-67 in adenomatous nodules*

No.	PI-PCNA (mean)		PI-Ki-67 (mean)	
	Nodules	Surrounding tissue	Nodules	Surrounding tissue
1.	13.2%	1.26%	2.24%	0.28%
2.	16.1%	0.9%	1.22%	0.16%
3 <sup>DSM</sup>	3.05%	0.44%	0.2%	ID
4.	11.2%	0.34%	0.28%	0.06%
5.	8.48%	1.36%	0.12%	ND
6.	7.14%	0.9%	2.38%	0.32%
7.	8.24%	1.18%	1.92%	0.6%
8 <sup>DSM</sup>	0.5%	0.12%	ID	ID

No.	PI-PCNA (mean)		PI-Ki-67 (mean)	
	Nodules	Surrounding tissue	Nodules	Surrounding tissue
9.	4.46%	0.3%	0.2%	0.1%
10*	ID	6.24%	ID	2.6%
11.	1.48%	0.16%	1.05%	0.4%
12.	18.2%	1.70%	1.1%	0.12%
p<0.001		p>0.05		

PI – Proliferation index; PCNA – Nuclear antigen of cellular proliferation; \* – Associated lymphocytic thyroiditis; DSM – Degenerative secondary modifications; ID – Indeterminate.

We observed an increase of PI-PCNA in thyroid nodules as compared to the normal surrounding tissue, with statistically significant values for both follicular adenomas ( $p<0.029$ ) and adenomatous nodules ( $p<0.001$ ).

In four cases, we noted a proliferation index PI-PCNA significantly higher in the surrounding thyroid tissue with lymphocytic infiltrate in the interstitium as compared to the normal thyroid tissue without lesions of thyroiditis. The mean PI-PCNA in cold thyroid nodules as compared to normal thyroid tissue was 14.03% vs. 3.86% for follicular adenomas and 8.36% vs. 1.24% for adenomatous nodules, respectively (Table 3).

Table 3 – *Mean PI-PCNA and PI-Ki-67 in follicular adenomas as compared to adenomatous nodules*

	PI-PCNA (mean)			PI-Ki-67 (mean)		
	TN	ST	p	TN	ST	p
Follicular adenomas	14.03%	3.86%	<0.029	1.64%	1.1%	<0.35
Adenomatous nodules	8.36%	1.24%	<0.001	1.07%	0.51%	>0.05

PI – Proliferation index; PCNA – Nuclear antigen of cellular proliferation; TN – Thyroid nodules; ST – Surrounding thyroid.

The intensity of immunostaining and the percentage of Ki-67-reactive cells were proven to be much more reduced as compared to PCNA immunostaining. Globally, we observed an increased proliferation in thyroid nodules as compared to normal parenchyma without associated lesions of thyroiditis. In 15 cases (seven follicular adenomas and eight adenomatous nodules), we found a higher PI-Ki-67 in thyroid nodules than in surrounding normal tissue, and in other two cases, a higher PI-Ki-67 in the normal thyroid tissue with associated lesions of lymphocytic thyroiditis.

Mean PI-Ki-67 in nodules *vs.* surrounding thyroid tissue was 1.64% *vs.* 1.10% in follicular adenomas and 1.07% *vs.* 0.51% in adenomatous nodules, respectively (Table 3), but without statistically significant values for both follicular adenomas ( $p < 0.35$ ) and adenomatous nodules ( $p > 0.05$ ).

Moreover, we compared PI-PCNA and PI-Ki67 for cold thyroid nodules and surrounding thyroid tissue, depending on size of the nodules, histological features (follicular adenomas *vs.* adenomatous nodules, micro-*vs.* macrofollicular architecture), presence or absence of lymphocytic infiltrate (LI) (Table 4), as well as other clinic characteristics of the patients (age, sex and medication).

**Table 4 – Proliferation index of PCNA and Ki-67 [%] for cold thyroid nodules based on histological characteristics**

Immunostaining		Follicular adenomas		Adenomatous nodules		<i>p</i>
		PI [%]	No. of cases	PI [%]	No. of cases	
Ki-67	TN	1.64%	8	1.07%	12	$>0.05$
PCNA	TN	14.03%	8	8.36%	12	$<0.01$
Ki-67	ST	1.10%	8	0.51%	12	$<0.01$
PCNA	ST	3.86%	8	1.24%	12	$<0.0001$
		Microfollicular pattern		Macrofollicular pattern		<i>p</i>
		PI [%]	No. of cases	PI [%]	No. of cases	
Ki-67	TN	1.41%	5	2.02%	3	$<0.18$
PCNA	TN	14.15%	5	13.76%	3	$<0.24$
Ki-67	ST	0.86%	5	1.57%	3	$<0.04$
PCNA	ST	2.44%	5	6.21%	3	$<0.001$
		Positive LI		Negative LI		<i>p</i>
		PI [%]	No. of cases	PI [%]	No. of cases	
Ki-67	TN	1.21%	2	0.12%	2	$<0.01$
PCNA	TN	12.3%	2	8.48%	2	$<0.002$
Ki-67	ST	1.81%	2	2.6%	2	$<0.015$
PCNA	ST	8.3%	2	3.8%	2	$<0.001$

PCNA – Nuclear antigen of cellular proliferation; TN – Thyroid nodules; ST – Surrounding tissue; PI – Proliferation index; LI – Lymphocytic infiltrate.

Both the heterogeneity of the reaction and the proliferation index PI-PCNA and PI-Ki-67 did not correlate with clinical parameters; we did not find any relationship between proliferation indexes and size of the nodules or clinical characteristics of patients. However, we noted: (1) significantly higher values of PI-PCNA ( $p < 0.01$ ) in follicular adenomas (14.03%)

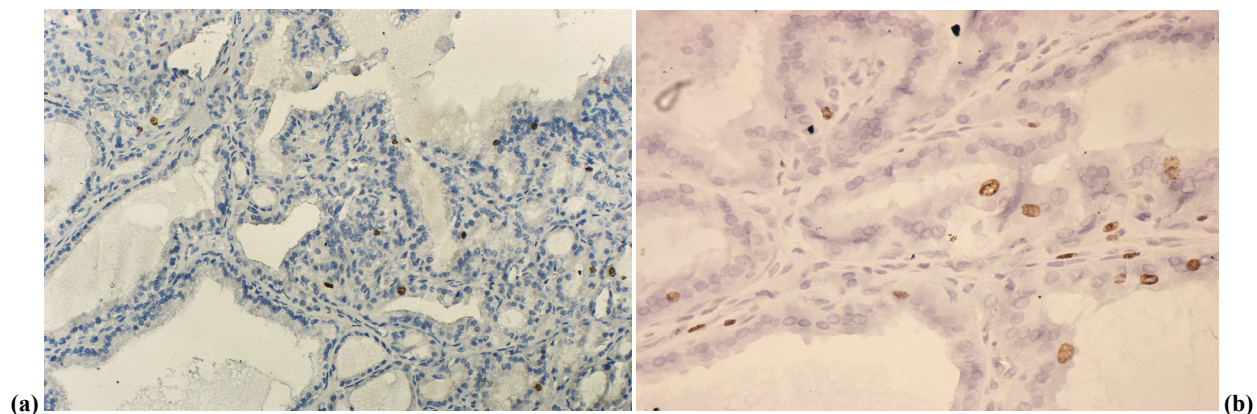
than in adenomatous nodules (8.36%), as compared to the statistically insignificant values ( $p > 0.05$ ) for Ki-67 (1.64% *vs.* 1.07%) and (2) a significantly higher proliferation rate ( $p < 0.01$ ) in thyroid nodules with aspects of focal lymphocytic thyroiditis (PI-Ki-67 of 1.21%) as compared to the nodules without lymphocytic infiltration of the surrounding tissue (PI-Ki-67 of 0.12%) (Table 4).

According to WHO criteria, we established the diagnosis of hyperfunctioning follicular adenoma in six of the 16 studied cases and the diagnosis of toxic multinodular goiter in ten cases. In three of the cases, we observed papillary aspects, oxyphylic changes (with Hurthle cells) of the follicular epithelium being evident in other three lesions.

Immunostaining with PCNA (Figure 6) and Ki-67 (Figure 7) was studied in all 16 toxic nodules (and in the surrounding thyroid tissue) from patients with clinical signs of hyperthyroidism, the proliferation index being established.

Because the pattern of distribution of PCNA-positive cells was heterogeneous, we did not observe notable differences after the comparative visual examination of hyperfunctioning thyroid nodules and surrounding tissue. However, in ten of the 16 toxic nodules that were analyzed, we observed a PI-PCNA 2–3 times higher in hyperfunctioning nodules as compared to the proliferative index of the surrounding thyroid tissue (with a mean PI-PCNA of 8.5% *vs.* 3.01%). On the sections colored with MIB-1, the number of Ki67-positive (marked) cells was lower, as compared to PCNA immunostaining, presenting also a heterogeneous pattern of distribution. In three cases, PI-Ki-67 was 2–3 times higher in hyperfunctioning (toxic) nodules than in the surrounding thyroid tissue (with PI-Ki-67 mean values of 4.61% *vs.* 1.5%), in the rest of cases the differences being insignificant (Figures 8–10).

The increase of marking indexes for both proliferation markers was similar for thyroid adenoma (defined as a histological entity) and adenomatous nodules, this aspect suggesting that proliferation enhancement of thyroid epithelial cells is a common feature for most hyperfunctioning thyroid nodules, independent of their histological features.



**Figure 6 – (a, b) Positive PCNA immunoreaction in hyperfunctional thyroid nodules (LSAB anti-PC10, viz. DAB, a –  $\times 200$ , b –  $\times 400$ ).**

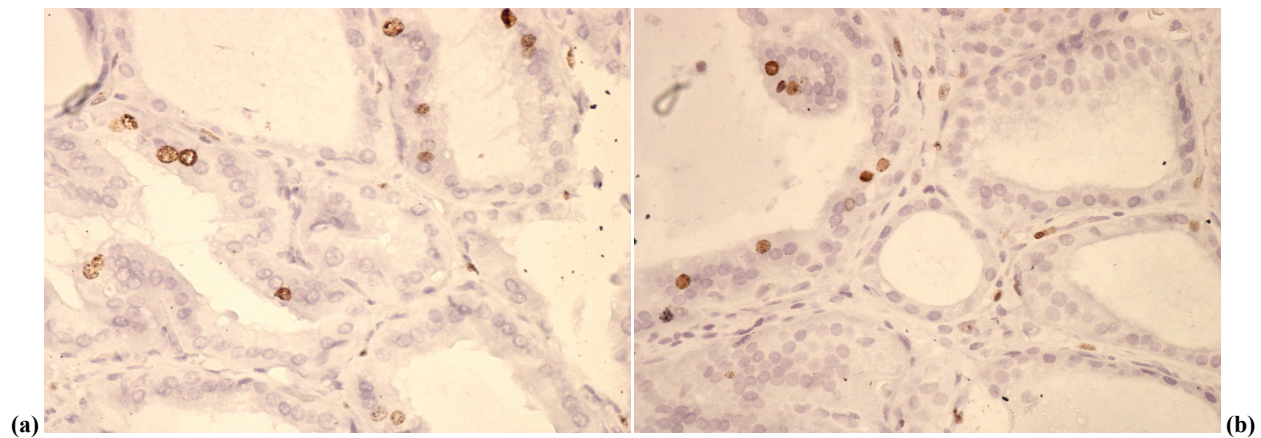


Figure 7 – (a, b) Ki-67 immunoreaction in hyperfunctional thyroid nodules (LSAB anti-MIB-1, DAB,  $\times 400$ ).

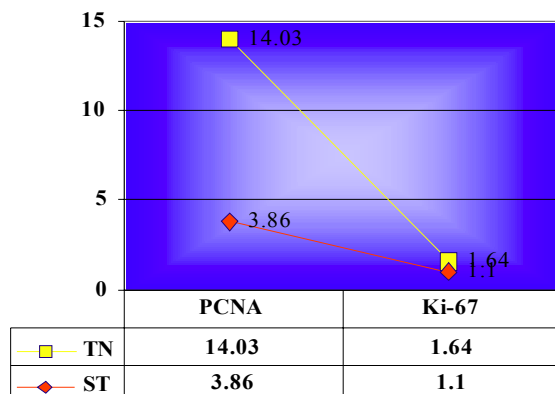


Figure 8 – Mean PI-PCNA and PI-Ki-67 in follicular adenomas (TN – thyroid nodules, ST – surrounding tissue).

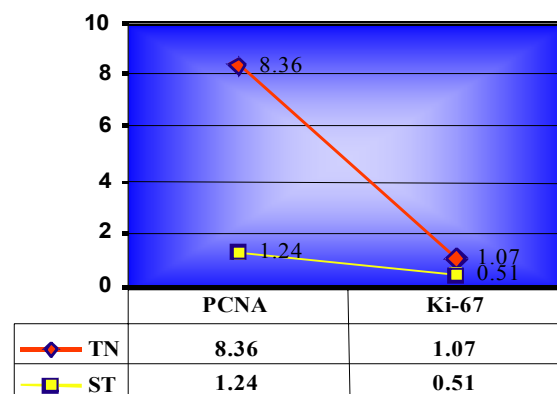


Figure 9 – Mean PI-PCNA and PI-Ki-67 in adenomatous nodules (TN – thyroid nodules, ST – surrounding tissue).

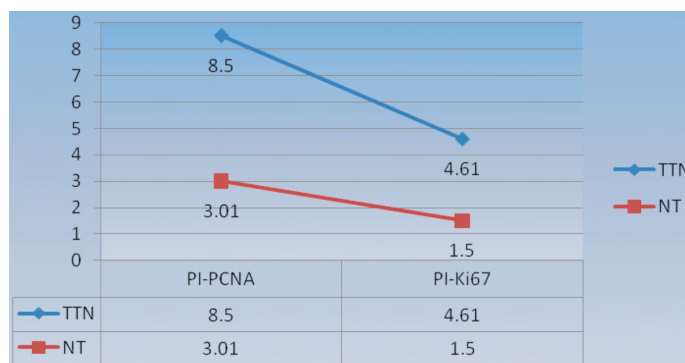


Figure 10 – The proliferation activity in hyperfunctioning thyroid nodules and adjacent thyroid (TTN – toxic thyroid nodules, NT – normal thyroid).

## Discussion

We compared the proliferation activity of thyrocytes in scintigraphically cold thyroid nodules and in the surrounding normal thyroid tissue, using PCNA and Ki-67 as immunohistochemical (IHC) markers. We showed that the expression of these proliferation markers is significantly elevated in the majority of cold nodules, as compared to the normal thyroid tissue, therefore confirming the hypothesis stating that the clinical manifestation of cold thyroid nodules is the result of cellular proliferation intensification, confined to the thyrocytes inside the lesion. The increase of proliferation potential in the thyroid tissue surrounding the nodules with aspects of lymphocyte thyroiditis (as compared to normal tissue without lymphocyte infiltration) suggests that the lymphocyte infiltrate secretes factors that stimulate thyrocyte proliferation [1].

The differences in the proliferation rate between autonomously functioning thyroid nodules and cold nodules suggest the existence of differences in growth stimulation.

It is considered that the molecular alterations involved in the appearance and manifestation of lesions are aberrant signals resulted from genomic mutations [8]. The nature of these genetic abnormalities and their consecutive accumulation determine a certain tumor phenotype that can conserve the normal function of cells, or the affected cells become non-functional.

Both cold thyroid nodules and autonomously functioning nodules are based on this principle. In the latter nodules, growth stimulation takes place in parallel with elevated hormonal synthesis, causing the phenotype of a hyperfunctioning nodule. The most probable molecular event responsible for this phenotype

is the activation of cyclic AMP signal through somatic mutations of TSH receptor [9], or more rarely, mutations of  $G_{sa}$  protein [10] that are powerful stimuli for the functions of thyroid epithelial cells and for proliferation. The molecular event that causes the tumor, responsible for cold thyroid nodules, most probable stimulates cell division, but nor their function as well.

Krohn K *et al.* (2003) suggest that a cascade of signals with mutational potential stronger than cyclic AMP is responsible for molecular defects in cold thyroid nodules; such a stronger mutational potential could explain the elevated frequency of non-functional nodules that evolve to malignant transformation [1].

Numerous studies showed differences in immunoreactivity for proliferation markers between benign thyroid tumors and thyroid cancer [11].

In the 20 benign follicular lesions analyzed we observed a heterogeneity of proliferation markers expression with a significant increase of proliferation rate in cold thyroid nodules as compared to the surrounding thyroid tissue and a mean PI-PCNA in nodules vs. surrounding thyroid tissue of 14.03% vs. 3.86% in follicular adenomas and 8.36% vs. 1.24% in adenomatous nodules, respectively. Although the intensity of immunostaining and the percentage of Ki-67-reactive nuclei were much more reduced as compared with PCNA, however we observed a mean PI-Ki-67 slightly higher in thyroid nodules as compared to the surrounding normal tissue (PI-Ki-67 of 1.64% vs. 1.10% and  $p < 0.35$  for follicular adenomas, and PI-Ki-67 of 1.07% vs. 0.51% and  $p > 0.05$  in adenomatous nodules, respectively), but the values are statistically insignificant.

We did not observe significant differences in the proliferation rate of cold thyroid nodules depending on the micro- vs. macro-follicular growth pattern, nor a relationship between PI and the size of nodules or other clinical variables, like age and sex of the patient, or medication.

We observed otherwise a higher proliferation rate in cold thyroid nodules (with statistically significant values for PCNA,  $p < 0.01$ ) based on histological characteristics (adenoma vs. adenomatous nodule), with a mean PI-PCNA of 14.03% in follicular adenomas vs. 8.36% in adenomatous nodules, as compared to the insignificant values ( $p > 0.05$ ) for Ki-67 (PI-Ki-67 1.64% vs. 1.07%). Moreover, we noted an increase of proliferation potential ( $p < 0.01$ ) in thyroid nodules with aspects of lymphocyte infiltration of surrounding tissue (PI-Ki-67 1.21%), as compared to nodules without aspects of associated lymphocyte thyroiditis (PI-Ki-67 0.12%).

Similar results were reported also in autonomously functioning thyroid nodules [4], the intensity and heterogeneity of staining for proliferation markers being otherwise diminished in hyper-functioning nodules.

Beside unknown intrinsic causes of growth stimulation, immunoreactivity variability for the studied proliferation markers can be the result of a phase growth response, or of a focal intense immunostaining in cold thyroid nodules, these aspects being dependent on the place from where the tissue was taken.

Until the present, only the tyrosine-kinase aberrant signaling through *ras* oncogene was studied, as a molecular cause of thyroid adenoma [12, 13]. In the studied cold nodules, Krohn K *et al.* (2003) identified only one *n-ras*-type mutation (on codon 61), immunoreactivity for PCNA and Ki-67 being insignificant [1]. Although most cold thyroid nodules are monoclonal [14], it is not known which of the molecular defects determines this aberrant mitogenic response.

In what concerns the immunoreactivity of PCNA and Ki-67 evaluated on histological sections from autonomously hyperfunctioning thyroid nodules, quantitative differences were found, similar to cold thyroid nodules. The two markers do not react with cells presenting active replication (like the incorporation of bromodeoxyuridine in DNA), but they can recognize epitopes of proteins expressed during certain phases of the replication. In comparison with the incorporation of bromodeoxyuridine, the marking index for PCNA tends to be higher because this protein has a longer half-period, and that is why it is often considered that it overestimates the number of proliferated cells [15]. On the other hand, the epitope marked with MIB-1 antibody is attainable only after an additional preparation (pretreatment with microwaves) of tissue sections [16], determining the underestimation of proliferation index. However, both marking methods are procedures meant to assess the proliferation rate on histological sections [17, 18]. In our study, we used these methods to assess the marking in cold thyroid nodules and in the surrounding normal thyroid tissue, on identical tissue sections, eliminating the differences between the two markers and the other variables like tissue sampling, fixing, inclusion and preservation.

In their study, Krohn K *et al.* does not observe significant differences in what concerns PI between TTN with or without mutations of the TSH receptor or between toxic thyroid nodules with polyclonal origin vs. monoclonal origin, the linear regression for PCNA and Ki-67 immunostaining showing a strong correlation between the two markers ( $r = 0.61$ ;  $p \leq 0.005$ ) [4].

Although the increased proliferation of many toxic thyroid nodules appears frequently because of TSH receptor mutations, the cause of increased proliferation in toxic thyroid nodules without mutations remains unclear.

It is supposed that the advantage of growth is essential for the manifestation of the somatic mutation. As a result, the somatic mutations of thyrotropin (TSH) receptor trigger an intense proliferation process, determining the appearance of a toxic thyroid nodule and clinical manifestations of hyperthyroidism.

Unlike in other cells, the AMPc signal (cyclic adenosine monophosphate) in thyrocytes mediates the development of mitotic activity [6]. The induction of AMPc cascade demonstrated the increase of proliferation activity of thyroid epithelial cells in mice *in vivo* [19] and *in vitro* [20].

To see if the proliferative activity is similar for toxic thyroid nodules with or without mutations of TSH receptor and for the nodules with monoclonal vs. polyclonal origin, the toxic thyroid nodules were studied

for the mutations that appear in the gene of TSH receptor and the *gsp* gene [21], as well as for their clonal origin [22], assessing in the same time the differences between adenoma and adenomatous nodules [23]. PCNA immunostaining was also studied in non-functional thyroid adenomas [24] and in thyroid neoplasms classified using WHO histological criteria [25].

Hamacher C *et al.* (1995) noted significant differences in PCNA immunostaining between non-functional adenomas and normal thyroid tissue; in toxic thyroid nodules PI-PCNA and PI-Ki-67 were calculated, making a quantitative comparison between these and the surrounding normal thyroid tissue [24].

In this study, we tried to assess the proliferative activity of thyrocytes from toxic thyroid nodules, as compared to the surrounding normal thyroid tissue, using the IHC staining method for the expression of PCNA and Ki-67 as proliferation markers. In general, the expression of these proliferation markers is significantly elevated in most toxic thyroid nodules, as compared to the surrounding normal thyroid tissue. This affirmation supports the hypothesis that the appearance of toxic thyroid nodules is realized by the increase of cell proliferation, confined to the thyrocytes inside the lesion.

PI-PCNA and PI-Ki-67 were calculated for the nodular tissue and the surrounding normal thyroid tissue. In ten of the 16-hyperfunctional thyroid nodules, we noted a PI-PCNA of 8.5% – more than two times higher as compared to the proliferation index of the surrounding thyroid tissue (PI-PCNA of 3.01%); in the other lesions, we did not observe significant differences in the PI of toxic thyroid nodules as compared to the surrounding tissue.

Ki-67 immunostaining and PI-Ki-67, respectively, were a lot more reduced, only six of the toxic thyroid nodules having a significant PI (PI-Ki-67 of 4.61%) as compared to the surrounding thyroid tissue (PI-Ki-67 of 1.5%), with a similar increase of PI (for both markers), in hyperfunctioning adenoma as well as in adenomatous nodules.

Toxic thyroid nodules include a heterogeneous entity from the histopathological and molecular criteria point of view. Functionally, they are autonomous nodules scintigraphically characterized by the increased absorption of technetium, suppression of neighboring normal tissue being a uniform characteristic that defines these lesions. According to the observations of Krohn K *et al.* (1999), elevated proliferation represents a common feature for most of the toxic thyroid nodules, independent of their histological characteristics [4].

The elevated level of AMPc in thyrocytes leads to their growth as well as of their functionality [5], explaining the increased proliferation in toxic thyroid nodules with mutations of the TSH receptor or  $G_{sa}$ , stimulating the cascade of AMPc. On the other hand, we can only speculate about the stimulus that causes the increased proliferation of toxic thyroid nodules without a somatic mutation of these genes and about the mediation through the AMPc cascade. The clone origin of toxic thyroid nodules without mutations of TSH

receptor or  $G_{sa}$  protein suggests the presence of a mutation of a protein yet unknown. It is very probable that toxic thyroid nodules with polyclonal origin do not have a somatic mutation, the increased proliferation being the result of an extrinsic stimulation (ex., the altered or aberrant expression of a growth factor).

The IHC expression of PCNA and Ki-67 is considered a method of evaluating the proliferative activity on histological sections [17, 18]. The PCNA antibody stains a nuclear protein with molecular weight of 37 kD that is necessary for the replication and progression of cell cycle. This staining is evident in phases G1, S and G2 of the cell cycle. On the other hand, the MIB-1 antibody stains a nuclear protein with high molecular weight that is present in all the phases of cell cycle with the exception of G0 [15].

## Conclusions

The enhancement of proliferative potential and the increase of marking indexes in scintigraphically cold thyroid nodules as compared to the adjacent normal thyroid tissue, support the statement that the clinical expression of cold thyroid nodules is the consequence of elevated proliferation of thyrocytes inside the nodules.

The increase of proliferation rate in thyroid nodules with aspects of lymphocyte thyroiditis of the surrounding tissue suggests a stimulation of thyrocyte proliferation through the growth factors secreted by the lymphocyte infiltrate.

The elevated proliferation of follicular thyroid cells detected in the majority of hyperfunctioning thyroid nodules confirms the hypothesis that the intensification of proliferation and function are common features of toxic thyroid nodules, independent of their histopathological characteristics.

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#### Corresponding author

Marioara Cornianu, MD, Department of Pathology, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40724–527 296, e-mail: marioaracornianu@yahoo.com

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