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GALECTIN-3 AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) EXPRESSION IN PAPILLARY THYROID CARCINOMA

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Background and aim: **To examine the relationship between galectin-3 and cell proliferation in thyroid tumor tissue. Galectin-3, a beta-galactoside binding protein, has recently been recognized as a promising molecular marker of thyroid malignancy, due to its high expression in thyroid carcinomas and absence from normal or benign thyroid tissue. However, its exact role in thyroid tumor biology is still unknown.** ***Patients and methods:*** **We examined the relationship between galectin-3 and cell proliferation by comparative immunostaining for galectin-3 and proliferating cell nuclear antigen (PCNA) in paraffin-embedded tissues from 126 cases of papillary thyroid carcinoma.** ***Results:*** **Positive cytoplasmic immunostaining for galectin-3 was found in 115 (91.3%) cases. Nuclear staining for PCNA was detectable in 93 (74.4%) cases. A low level of PCNA staining (less than 10% positive cells) was found in 36 (28.6%) cases, moderate staining for PCNA (more than 10% but less than 30% positive cells) in 35 cases (27.8%), while highly increased PCNA expression (more than 30% positive cells) was found in 32 (25.4%) cases. Moderate or strong galectin-3 expression, found in 99 cases, was associated with highly increased PCNA staining in 28.3% of them but with no detectable PCNA expression in 24.3% of them.** ***Conclusion:*** **These results suggest that overexpression of galectin-3 is not clearly related to proliferative activity of papillary thyroid carcinoma cells as assessed by PCNA immunostaining.**

Key Words: **galectin-3, papillary thyroid carcinoma, proliferating cell nuclear antigen, immunohistochemistry.**

Galectins are a growing family of beta-galactoside binding animal lectins [1, 17]. It is assumed that, through binding to complementary glycoconjugates, galectins play important roles in various biological and pathophysiological processes, such as differentiation, cell growth and apoptosis, cell adhesion, modulation of the immune response, neoplastic transformation and invasiveness [1, 9, 16, 17, 20].

Galectin-3 (a 29-31 kDa protein) is the most extensively studied member of the galectin family. This lectin is constitutively expressed in a variety of tissues and cell types and is mainly localized in the cytoplasm, although it can also be detected in the nucleus, on the cell surface or in the extracellular environment. The demonstration of altered galectin-3 expression in several human neoplastic tissues has attracted the attention of scientific researchers and clinicians. Furthermore, galectin-3 expression was found to correlate with tumor progression in some tumor cell types [8, 27, 28].

The expression of this lectin in the thyroid has been extensively investigated during the last few years in both histological and cytological specimens. The results of such studies, published by others [2, 10, 12, 13, 15, 19, 23, 24, 29, 30] and by us [6, 7], demonstrated that thyroid carcinomas consistently express galectin-3 at a high level, whereas galectin-3 expression in normal and benign tissue is absent or weak. Thus, galectin-3 appears to be a promising molecular marker of thyroid malignancy.

However, the functional relevance of galectin-3 overexpression in the malignant thyroid cell is unknown.

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Abbreviations used: PCNA – proliferating cell nuclear antigen; PTC – papillary thyroid carcinoma.

Research aiming to elucidate the role of galectin-3 in malignant thyroid tissue using methods of molecular biology is still rare. Yoshii et al. [31] inhibited galectin-3 gene expression in papillary thyroid carcinoma (PTC) cells *in vitro*, which resulted in a marked reduction of the malignant phenotype. Recently, Takenaka et al. [26] demonstrated that overexpression of the galectin-3 gene (induced by galectin-3 cDNA transfection into normal thyroid follicular cells in culture) was associated with overexpression of the following genes: retinoblastoma, replication factor (RFC) and proliferating cell nuclear antigen (PCNA), all of which are involved in G1-S transition of the cell cycle. PCNA is a nuclear protein, a cofactor of DNA polymerase-d. Due to involvement in the replicative phase in cell cycle progression, its synthesis correlates with the proliferative state of the cell [3].

These results prompted us to investigate the possible relation between galectin-3 and cell proliferation. For this purpose, galectin-3 and PCNA expression was comparatively analyzed by immunohistochemical approach in PTC tissue for the first time.

MATERIALS AND METHODS

Tissue specimens. Formalin-fixed, paraffin-embedded tissues from 126 surgically removed human papillary thyroid carcinomas were used for this immunohistochemical analysis. Tumors were selected from the archival material of the Institute of Endocrinology, Diabetes and Metabolism, Clinical Center of Serbia, Belgrade. Histological slides from the thyroid tumor tissue stained by hematoxylin and eosin were reevaluated by two pathologists to confirm the diagnosis, according to widely accepted histological criteria [5, 18, 21, 22]. Nuclear features (ground glass nuclei, grooved nuclei and nuclear pseudoinclusions, at least two of them) regardless of

the growth pattern were taken as the gold standard for confirming the diagnosis of papillary carcinoma.

Immunohistochemistry. A rat monoclonal antibody (M3/38) against galectin-3, produced by ATCC TIB-166 (American Type Culture Collection, Rockville, MD) was kindly provided by Dr. M. E. Huflejt, La Jolla Institute for Allergy and Immunology (San Diego, CA). The same antibody was used in our previous reports on galectin-3 expression in thyroid tissue [6, 7, 25].

For immunohistochemical localization of PCNA, monoclonal mouse anti-PCNA (NCL — PCNA, Novocastra, UK) was used as the first antibody.

Immunostaining was performed on 4-6 μm thick sections using the avidin-biotin-peroxidase complex (ABC) technique with reagents supplied by Vector Laboratories (Burlingame, CA).

Following deparaffination and rehydration, endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol followed by non-immune horse serum for 20 min to block non-specific binding. The sections were then incubated with primary antibody against galectin-3 at a dilution of 1 : 200 or with primary antibody against PCNA at a dilution of 1 : 100, at 4 °C overnight. This was followed by incubation with biotinylated horse anti-mouse IgG (which also cross-reacts with the primary rat antibody) for 30 min and thereafter with the avidin-biotin-peroxidase complex (ABC reagents) for 30 min. Between each step, sections were washed three times in phosphate buffered saline (PBS). The reaction was visualised using 3, 3´-diaminobenzidine tetrahydrochloride (DAB) solution.

After counterstaining with hematoxylin, slides were dehydrated, coverslipped and examined using a Reichart-Jung microscope supplied with a Photostar automatic camera system. Controls were incubated with PBS in place of the primary antibody and no positive staining was observed. The internal positive control was represented by histiocytes.

Scoring of staining and statistical data. Galectin-3 staining was scored as follows: (-) — no staining, (+/-) — weak or focal staining, (+) — moderate staining in the majority of epithelial cells and (++) — strong staining in the majority of the epithelial thyroid cells.

PCNA staining was scored in a semiquantitative fashion as follows: (0) not detectable, (1) low level of staining, i.e. less than 10% PCNA positive cells, (2) moderate staining, i.e. more than 10% and less than 30% of PCNA detectable cells and (3) highly increased PCNA staining, i.e. more than 30% PCNA positive cells.

Statistical comparisons of data were performed by Student's *t*-test. A value $p < 0.05$ was considered to be statistically significant.

RESULTS

One hundred and twenty six cases of PTC tissues were immunohistochemically stained for galectin-3 and PCNA in parallel. The results are given in Tables 1 and 2 and some representative photographs are shown in Figure (*a-e*).

Positive immunohistochemical staining for galectin-3 was found in 115 (91.3%) cases out of the 126 analysed in this study. The intensity of staining varied from moderate or strong to weak or focal (see Table 1). Galectin-3 localization was dominantly cytoplasmic, but also membraneous or nuclear in some malignant cells. Galectin-3 could not be detected immunohistochemically in 11 cases of PTC. When present, normal or hyperplastic thyroid epithelial cells adjacent to malignant tissue of papillary carcinoma, showed no immunoreactivity for galectin-3.

Table 1. Galectin-3 and PCNA immunostaining in papillary thyroid carcinoma tissue

Marker	Score of staining			
Galectin-3 staining*:	++	+	+/-	-
Number of cases (%)	67 (53.2)	32 (25.4)	16 (12.7)	11 (8.7)
PCNA staining**:	3	2	1	0
Number of cases (%)	36 (28.6)	35 (27.8)	23 (18.3)	32 (25.4)

Note: *Galectin-3 staining: (-) no staining, (+/-) weak or focal staining, (+) moderate staining in the majority of tumor cells, (++) strong staining in the majority of tumor cells; ** PCNA staining: (0) not detectable, (1) low staining, i.e. < 10% positive cells, (2) moderate staining, i.e. more than 10% but less than 30% positive cells cells, (3) highly increased PCNA staining, i.e. more than 30% positive cells.

Table 2. Relation between strong/moderate galectin-3 expression and proliferating cell nuclear antigen (PCNA) immunostaining in PTC

Marker	PCNA immunostaining**			
	3	2	1	0
Galectin-3 immunostaining* (++/+, n = 99)	28 (28.3%)[a]	30 (30.3%)	17 (17.2%)	24 (24.3%)[b]

**PCNA immunostaining: (0) not detectable, (1) low staining, i.e. < 10% positive cells, (2) moderate staining, i.e. more than 10% but less than 30% positive cells, (3) highly increased PCNA staining, i.e. more than 30% positive cells; [a, b]: Differences statistically not significant ($p > 0.05$) for a vs b.

PCNA was not detectable by immunohistochemical staining in 32 (25.4%) cases of PTC. Positive nuclear staining for PCNA was found in 94 (74.6%) cases. A low level of PCNA expression (less than 10% positive cells) was detected in 23 cases (18.3%), while a moderate level of PCNA immunostaining (more than 10% but less than 30%) was found in 35 (27.8%) cases analysed. A highly increased proliferation rate presented with a large number of malignant cells expressing PCNA immunoreactivity (i.e. more than 30% cells) was found in 36 (28.6%) out of the 126 cases of PTC.

Overexpression of galectin-3 (moderate and strong staining) was compared with the level of PCNA immunoreactivity (Table 2). Among the 99 cases of PTC showing moderate or strong galectin-3 expression, there was a similar number of cases with highly increased PCNA immunostaining (28 cases, i.e. 28.3%) and without any PCNA expression (24 cases, i.e. 24.3%). The differences between these two opposite groups were not statistically significant. These results suggest that overexpression of galectin-3 is not clearly associated with increased proliferative capacity of papillary thyroid carcinoma.

DISCUSSION

During the last few years, several studies reported high galectin-3 expression in thyroid carcinomas, while its expression in normal and benign tissue was absent or weak. Thereupon galectin-3 was proposed as a promising molecular marker of thyroid malignancy. However, understanding the role of this protein in the malignant thyroid cell is still at the beginning. Fetal thyroid cells do not express galectin-3 [25], which means that galectin-3 is not considered an oncofetal antigen,

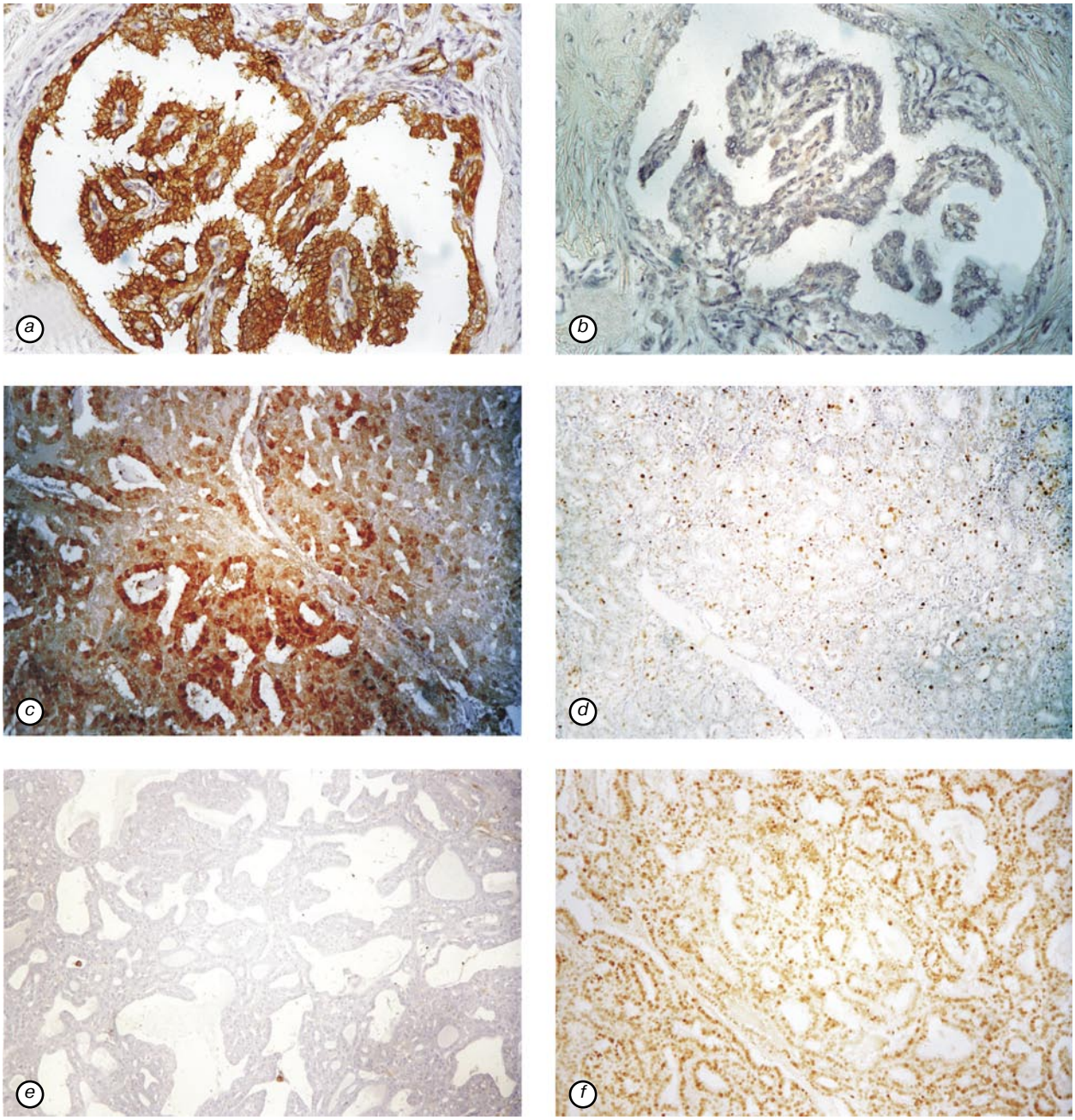

Figure. Comparative immunohistochemical staining for galectin-3 (*a, c, e*) and PCNA (*b, d, e*) in papillary thyroid carcinoma tissue, shown for three cases (indirect immunoperoxidase, hematoxylin — diaminobenzidine). *a, b*: Strong galectin-3 expression without PCNA immunostaining; *c, d*: strong galectin-3 expression and moderate PCNA staining; *e, f*: Strong galectin-3 expression associated with highly increased PCNA staining

but is newly expressed during neoplastic transformation. Galectin-3 is assumed to contribute to different events associated with cancer biology, including neoplastic transformation, cell cycle regulation, apoptosis, cell adhesion, migration and inflammation.

In this study we tried for the first time to examine the relationship between galectin-3 and cell proliferation in thyroid malignant tissue by comparative immunohistochemical staining for galectin-3 and PCNA, a typical marker of proliferation, in a series of 126 cases of PTC. The results showed positive galectin-3 immunoexpression in 115 (91.3%) cases of PTC, which is in agreement with the previously reported sensitivity of galectin-3 immunostaining in confirming the diagnosis of this type of thyroid carcinoma.

Analysis of PCNA immunostaining in the same cases of PTC demonstrated great variability in PCNA expression, i.e. from an undetectable level to a large number of malignant cells expressing positive nuclear PCNA immunoreactivity. Comparison of galectin-3 and PCNA expression, which was the main focus of this study, revealed that among the PTC cases with moderate/strong galectin-3 expression, there were similar numbers with undetectable PCNA expression (24.3%) and with highly enhanced proliferative rates (28.3%). Thus, our results suggest that up-regulation of the galectin-3 gene is not clearly correlated with proliferative activity in PTC.

The role of galectin-3 in cell proliferation is controversial. Galectin-3 has not been reported to regulate epithelial cell proliferation directly. Moreover, it was

shown that galectin-3 has no effect on the growth of either colon or breast carcinoma cells [4, 14]. On the other hand, intracellular synthesis of galectin-3 is reported to be relevant to the proliferating state in fibroblasts [11]. It seems likely that galectin-3 stimulates mesenchymal cells but not epithelial cells to proliferate and that galectin-3 plays some other role in the malignant thyroid epithelial cell.

As a multifunctional protein, galectin-3 may also act as an anti-apoptotic molecule. Galectin-3 protects cells from apoptosis by inducing G1 arrest in response to loss of cell anchorage [14]. Resistance to apoptosis is essential for cancer cell survival and plays an important role in tumor biology. Thus, the molecular mechanisms underlying the role of galectin-3 in the thyroid malignant cell, particularly its possible roles in apoptosis and the cell cycle, deserve further investigations.

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ЭКСПРЕССИЯ ГАЛЕКТИНА-3 И ЯДЕРНОГО АНТИГЕНА ПРОЛИФЕРИРУЮЩИХ КЛЕТОК (PCNA) В ПАПИЛЛЯРНЫХ КАРЦИНОМАХ ЩИТОВИДНОЙ ЖЕЛЕЗЫ ЧЕЛОВЕКА

Обоснование и цель: **исследовать взаимосвязь между экспрессией галектина-3 и пролиферацией клеток опухолей щитовидной железы (ЩЖ) человека. Галектин-3, бета-галактозид связывающий белок, был недавно признан потенциальным молекулярным маркером злокачественных новообразований щитовидной железы ввиду своей высокой экспрессии в клетках карцином ЩЖ и отсутствия таковой в нормальной ткани и доброкачественных новообразованиях ЩЖ. Однако роль этого белка в биологии опухолей ЩЖ остается неизученной.** ***Методы:*** **проведен сравнительный иммуногистохимический анализ экспрессии галектина-3 и ядерного антигена пролиферирующих клеток (PCNA) и взаимосвязи между экспрессией галектина-3 и пролиферацией клеток в парафиновых срезах тканей 126 образцов папиллярной карциномы ЩЖ человека.** ***Результаты:*** **позитивное цитоплазматическое иммуногистохимическое окрашивание галектина-3 было выявлено в 115 (91.3%) случаях, ядерное окрашивание PCNA в 93 (74.4%) случаях. Низкий уровень окрашивания PCNA (более 10% положительно реагирующих клеток) был отмечен в 36 (28.6%) случаях, умеренный (более 10%, но менее 30% положительно реагирующих клеток) — в 35 (27.8%), высокий (более 30% положительно реагирующих клеток) в 32 (25.4%) случаях. Умеренная/высокая экспрессия галектина-3, выявленная у 99 больных, была ассоциирована с интенсивным окрашиванием PCNA в 28.3% случаев и с отсутствием экспрессии этого маркера — в 24.3%.** ***Выводы:*** **результаты исследования свидетельствуют, что методом иммуногистохимии не удалось установить прямой связи между гиперэкспрессией галектина-3 и пролиферативной активностью клеток папиллярной карциномы ЩЖ человека.**

Ключевые слова: **галектин-3, папиллярная карцинома щитовидной железы, ядерный антиген пролиферирующих клеток, иммуногистохимия.**