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Patterns of cyclin A and B1 immunostaining in papillary thyroid carcinoma

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Abstract

Introduction and objectives. Cyclin A, encoded by *CCNA* (*cyclin A)* gene with *locus* in chromosome 4q27, and cyclin B1, encoded by *CCNB1* (*cyclin B1*) gene with *locus* in chromosome 5q12, are proteins that play a key role in the passage through the restriction point in G2 phase of the cell cycle. The aim of the study was to analyse immunohistochemically the expression of cyclins A and B1 in different variants of papillary thyroid carcinoma (PTC).

Material and methods. The immunostaining patterns of the proteins in question in the tissue of 40 resected PTC (20 cases of classic variant of PTC, 9 cases of PTC follicular variant and 11 cases of other non-classic variants of PTC) were investigated.

Results. On analyzing cyclin A and B1 expression, positive staining in 90% cases of PTC were observed. The study revealed a significant difference in expression of cyclins A and B1 between classic and non-classic variants of PTC. The expression of both examined cyclins was weaker in the classic variant of PTC. In the group of follicular variant of PTC, the expression of cyclins was of medium intensity and in the group of other non-classic variants of PTC, the expression was clearly higher.

Conclusions. The results of the presented study suggest that cyclins A and B1 expression may have a characteristic pattern of immunostaining for particular variants of PTC. If the obtained results are confirmed in a larger group of patients, the diagnostic panel constructed of the antibodies against these proteins may increase the diagnostic accuracy in PTC cases.

Key words

Cyclin A, cyclin B1, papillary thyroid carcinoma, immunohistochemistry

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the most common of all thyroid cancers and the incidence of this neoplasm has been increasing over the last decade. Its prognosis is associated with patient's age, tumour size, as well as histological parameters, including extracapsular invasion, extrathyroidal extension, lymph node and distant metastasis and histological variants. These histological variants, which include the classic variant of PTC, follicular variant of PTC and other variants, such as the tall-cell variant, the columnar-cell variant or the variant with diffuse sclerosis, are related to tumour aggressiveness and metastatic potential. In addition, in PTC, several molecular changes have been typically described (e,g, *RET/PTC*, *NTRK1* rearrangements and/or sporadic mutations of *BRAF* or *RAS* oncogene) [1, 2, 3].

Thyroid nodules are common ultrasound findings, detected in approximately 19–67% of the general adult population. The aim of further approach is to differentiate between benign nodule and malignancy. Ultrasound-guided fine needle aspiration biopsy (FNAB) is the most important tool for this purpose, although its accuracy is not always sufficient due to a relatively high incidence of indeterminate or suspicious cytologic features. Most of these patients undergo diagnostic thyroidectomy, which frequently reveals a benign lesion. Thus, many novel potential markers are studied to facilitate the diagnosis of malignancy and to determine tumour type and progression. Several biomarkers which might be useful adjuncts in the assessment of proliferation and apoptosis in PTC have been identified. Dysregulation of the normal cell cycle machinery, leading to unrestrained cell proliferation, is integral to the neoplastic process, and the loss of regulatory control of the cell cycle is a hallmark of cancer. Cyclins, the regulatory subunits of cyclin-dependent kinases (CDK), control the passage of proliferating cells through key checkpoints in the cell cycle. The overexpression of positive cell regulators (i.e. cyclins) may overwhelm the arrest mechanism of the normal cell cycle and lead to uncontrolled cell proliferation. An important checkpoint in the cell cycle progression is at G2/M boundary [4]. G2/M transition is regulated by the complex of CDK I and two cyclins: cyclin A, encoded by *CCNA* (*cyclin A)* gene with *locus* in chromosome 4q27 and cyclin B1, encoded by *CCNB1* (*cyclin B1*) gene with *locus* in chromosome 5q12. Cyclin A activates CDK to regulate proliferation and cell cycle progression through the S phase to the G2/M checkpoint [5]. Cyclin B1 works selectively for G2/M transition.

Cyclin A and cyclin B1 expressions have possible prognostic value in breast, hepatocellular, squamous oesophageal cancers and in non-Hodgkin's lymphoma [6, 7, 8]; however, their role in PTC has been less studied.

Objectives. The aim of the study was to analyse immunohistochemically the expression of cyclins A and B1 in different variants of PTC. The diagnostic utility of such

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immunostaining patterns in differentiation of particular variants of PTC was also evaluated.

MATERIALS AND METHOD

The procedures, used in the study were approved by the Ethical Committee of the Medical University of Lodz, Poland.

Tissue specimens. A total of 40 PTC cases were selected from the surgical pathology files of the hospital, comprising 20 cases of classic variant of PTC (mean age of the studied patients: 45; SD 12.3 years; range: 19 – 82 years) and 20 cases of other non-classic variants of PTC (mean age of the studied patients: 43.5; SD 17.1 years; range: 24 – 69 years). The patients were diagnosed and operated on between 2002 – 2004 at the Department of Endocrine and General Surgery, Institute of Endocrinology, Medical University of Lodz. The experimental protocol included only females. All patients diagnosed with thyroid cancer before or at the time of surgery underwent total thyroidectomy and regional lymphadenectomy. The remaining patients, diagnosed with cancer during routine histopathologic examinations, were re-operated, the operation was radicalized and regional lymphadenectomy was performed. Following the approved procedures, all patients received post-operative radioactive iodine therapy, followed by L-T_4 suppression treatment. All the clinical charts and histopathology reports were reviewed for data regarding the patient's age, tumour size and the presence of lymph node metastasis. Histopathological diagnoses for carcinomas, according to the WHO Classification of Tumours [9], were obtained from pathomorphological reports (Tab.1), together with TNM classification and AJCC stage groupings [10].

Two pathologists reviewed all the cases to confirm the obtained diagnoses. At that review of H&E-stained sections, paraffin blocks were selected for immunohistochemistry to include both the representative sections of the analyzed tumours and the rims of morphologically normal thyroid tissue around them, the latter serving as comparative tissue.

Immunohistochemistry. Archival paraffin-embedded tumour tissue was analyzed by immunohistochemistry for cyclin A and B1 expressions. Formalin-fixed paraffin-embedded tissue sections (4 μm thickness) were dewaxed in xylene and rehydrated through graded alcohols to water. Antigen retrieval was performed in citrate buffer (pH 6.0; Dako, Glostrub, Denmark) inside a microwave pressure cooker.

Primary antibody characteristics, the conditions of incubation and the manufacturer's name for each of the cyclins are presented in Table 2. After incubation, the slides were washed in Tris Buffered Saline (TBS), after which the incubations were carried out using an En Vision/HRP/DAB+ kit (Dako). The slides were counterstained in haematoxylin and mounted on Faramount (Dako). For negative control, TBS replaced the primary antibody.

Evaluation of immunostaining. Immunostained tissue sections were evaluated by estimating the percentage of tumour cells staining positive with monoclonal antibody without any knowledge of pathologic diagnosis. For the evaluation of positivity, the positively stained nuclei and cytoplasm (for cyclin A and cyclin B1, respectively) were counted. The nuclear staining in the normal thyroid tissue surrounding PTC, was also evaluated at the same time as the tumour, serving as an internal control.

Staining index. All the stained sections were examined on an Olympus microscope (Olympus CHS, Olympus, Tokyo, Japan), using an eyepiece graticule (PZO, Warsaw, Poland) to facilitate cell counting, at a higher magnification (400

Table 1. Age, histopathological diagnosis, TNM classification and American Joint Committee on Cancer (AJCC) stage groupings of malignant thyroid tumours in studied patients

Case No.	Age	Histopathological variant of PTC	TNM staging system	AJCC grouping system
1	48	classic	T2NxM0	II
2	25	classic	T2N0M0	I
3	31	classic	T4N1M0	I
4	70	classic	T2N0M0	II
5	30	classic	T2N0M0	I
6	60	classic	T3N1Mx	III
7	24	classic	T1aN0M0	I
8	51	classic	T1bN0M0	I
9	19	classic	T4N1Mx	I
10	49	classic	T2NxMx	II
11	22	classic	T2N1Mx	I
12	82	classic	T2NxMx	II
13	61	classic	T3N0M0	III
14	37	classic	T2N1Mx	I
15	49	classic	T1aN0Mx	I
16	64	classic	T1aN0Mx	I
17	52	classic	T1bN0M0	I
18	58	classic	T1aN1Mx	III
19	35	classic	T2NxMx	I
20	42	classic	T1aN1Mx	I
21	50	follicular	T1bN0M0	I
22	24	follicular	T2N1M0	I
23	35	follicular	T1aN0M0	I
24	42	follicular	T2N0M0	I
25	43	follicular	T1aN1M0	I
26	30	follicular	T1bN0M0	I
27	69	follicular	T3N1Mx	III
28	48	follicular	T1aN0M0	I
29	41	follicular	T2N1M0	I
30	38	tall-cell	T1bN0M0	I
31	37	tall-cell	T1aN1Mx	I
32	47	tall-cell	T1bN1Mx	III
33	66	tall-cell	T1bN0M0	I
34	47	tall-cell	T2NxMx	II
35	52	tall-cell	T1bN0M0	I
36	42	tall-cell	T2N1M0	I
37	62	columnar-cell	T3N1Mx	III
38	30	columnar-cell	T1aN0M0	I
39	24	columnar-cell	T1aNxMx	I
40	43	with diffuse sclerosis	T3N1Mx	I

Table 2. Characteristics of antibodies used and incubation conditions

	Cyclin A	Cyclin B1
Antibody/animal	Rabbit	Mouse
Antibody/isotype	IgG polyclonal	IgG_1 monoclonal
Antibody/clone	H-432; sc-751	GNS1; sc-245
Dilution	1:100	1:100
Incubation	Whole night (4° C)	Whole night (4° C)
Manufacturer	Santa Cruz Biotechnology	Santa Cruz Biotechnology

x) at which a minimum of 1,000 cells were counted in the area with positive staining. The percentage of tumour cells stained with the antibody was regarded as the staining index. Staining results were defined as negative (expression < 5%) or positive. A score (1–3) was used to classify positive cells (1: between 5–10% of positive cells; 2: between 10–50% of positive cells; 3: > 50% of positive cells).

Statistical analyses. The multivariate variance analyses (Fischer-Snedecor test) was used to assess whether there was a difference in the expression of the antigens in question between PTC of different variants. Statistical significance was determined at the level of $p< 0.05$.

Statistica for Windows 7.0 was applied for calculations.

RESULTS

Cyclin A expression. On analyzing the cyclin A expression, positive staining was observed in 90% of the cases (n=36/40). The staining signal of cyclin A was observed predominantly in the nuclei in 27% (n=11) of cases, in cytoplasm in 40% (16), and both in nuclei and cytoplasm in 23% (n=9) of the cases.

In the group of classic variant of PTC, positive staining was observed in 80% of the cases (n=16/20). The staining signal of cyclin A was observed predominantly in the nuclei in 35% (n=7) of the cases, in cytoplasm in 35% (n=7), and both in nuclei and cytoplasm in 10% (n=2) of the cases.

In the group of follicular variant of PTC, positive staining was observed in 89% of the cases (n=8/9). The staining signal of cyclin A was observed predominantly in the cytoplasm in 67% (n=6) of the cases and both in nuclei and cytoplasm in 22% (n=2).

In the group of other non-classic variants of PTC, positive staining was observed in 100 % of the cases (n=11/11). The staining signal of cyclin A was observed predominantly in the nuclei in 9.1% (n=1) of the cases, in cytoplasm in 54.5% (n=6), and both in nuclei and cytoplasm in 36.4% (n=4) (Tab. 3).

Cyclin B1 expression. On analyzing cyclin B1 expression, positive staining was observed in 90% of the cases (n=36/40). Cyclin B1 immunoreactivity was observed mainly in cytoplasm in 62.5% (n=25/40) of cases. The staining signal of cyclin B1 was observed in the nuclei in 7.5% (n=3) of the cases, and both in nuclei and cytoplasm in 20% (n=8).

In the group of classic variant of PTC we observed positive staining in 85% of the cases (n=17/20). The staining signal of cyclin B1 was observed predominantly in the nuclei in 10% (n=2) of the cases, in cytoplasm in 65% (n=13) of the cases and both in nuclei and cytoplasm in 10% (n=2) of the cases.

In the group of follicular variant of PTC, positive staining was observed in 89% of the cases (n=8/9). The staining signal of cyclin B1 was observed predominantly in the cytoplasm in 67% (n=6) of the cases and both in nuclei and cytoplasm in 22% (n=2).

In the group of other non-classic variants of PTC, positive staining was observed in 100 % of the cases (n=11/11). The staining signal of cyclin B1was observed predominantly in the nuclei in 9.1% (1) of the cases, in cytoplasm in 54.5% (n=6), and both in nuclei and cytoplasm in 36.4% (n=4) (Tab. 4).

Table 3. Cyclin A staining index values for particular variants of PTC

	Cyclin A					
	Nuclear expression			Cytoplasmic expression		
	Classic variant of PTC (20)	Non-classic variants of PTC		Classic variant of PTC (20)	Non-classic variants of PTC	
		Follicular variant of PTC (9)	Other non-classic variants of PTC (11)		Follicular variant of PTC (9)	Other non-classic variants of PTC (11)
Expression Staining Index ≥5%	9 (45%)	4 (44.44%)	7 (63.64%)	9 (45%)	7 (77.78%)	9 (81.82%)
Lack of expression Staining Index <5%	11 (55%)	5 (55.56%)	4 (36.36%)	11 (55%)	2 (22.22%)	2 (18.18%)

Table 4. Cyclin B1 staining index values for particular variants of PTC

	Cyclin B1					
	Nuclear expression			Cytoplasmic expression		
	Classic variant of PTC (20)	Non-classic variants of PTC		Classic variant of PTC (20)	Non-classic variants of PTC	
		Follicular variant of PTC (9)	Other non-classic variants of PTC (11)		Follicular variant of PTC (9)	Other non-classic variants of PTC (11)
Expression Staining Index ≥5%	4 (20%)	2 (22.22%)	5 (45.45%)	15 (75%)	8 (88.89%)	10 (90.92%)
Lack of expression Staining Index <5%	16 (80%)	7(77.78%)	6 (54.55%)	5 (25%)	1 (11.11%)	1 (9.09%)

A statistically significant difference in total (cytoplasmic and nuclear) expression of cyclins among particular variants of PTC (p=0.0034) was documented. In the group of other non-classic variants of PTC, the total expression of cyclins was clearly higher, and in the group of follicular variant of PTC it was of medium intensity, while in the group of classic variant of PTC the total expression was the weakest (Fig. 1).

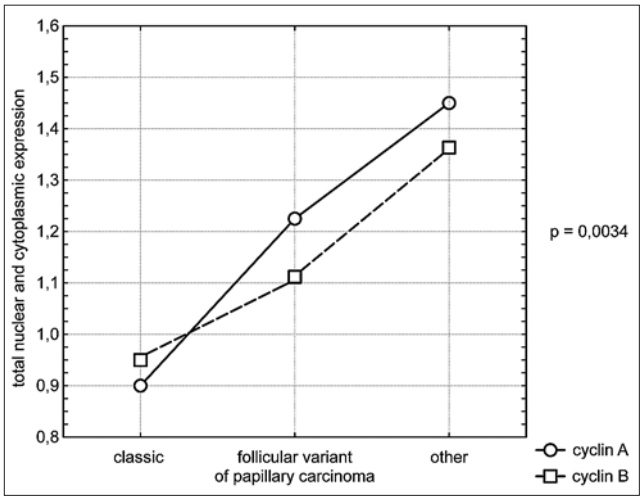

Figure 1. Total nuclear and cytoplasmic expression for particular variants of PTC

A statistically significant difference was observed in the cytoplasmic expression of cyclins among particular variants of PTC (p=0.0386). In the group of other non-classic variants of PTC, the cytoplasmic expression of cyclins was clearly higher, and in the group of follicular variant of PTC it was of medium intensity, while in the group of classic variant of PTC the cytoplasmic expression was the weakest (Fig. 2).

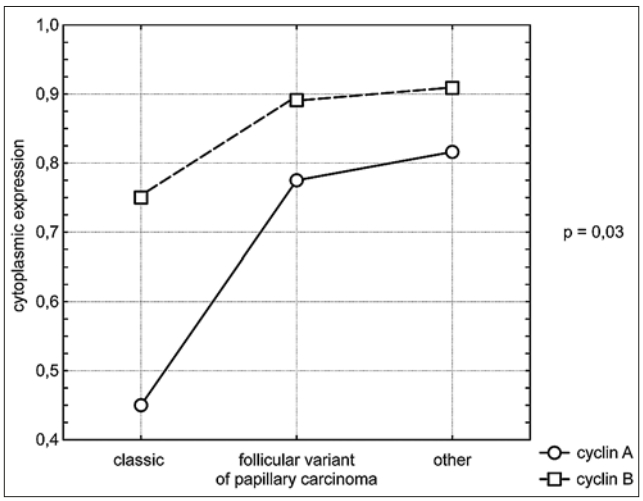

Figure 2. Cytoplasmic expression of cyclin A and B1 for particular variants of PTC

A statistically significant difference was found in the expression of cyclins A and B1 between classic and non-classic variant of PTC (p=0.0099). The expression in the classic variant of PTC was weaker. The difference reached the values of significance between the cytoplasmic and nuclear expression in the group of classic and non-classic variant of PTC (p= 0.0000). The nuclear expression was weaker. The difference depended on the kind of cyclin (p= 0.0036). For cyclin A the difference was smaller (Fig. 3).

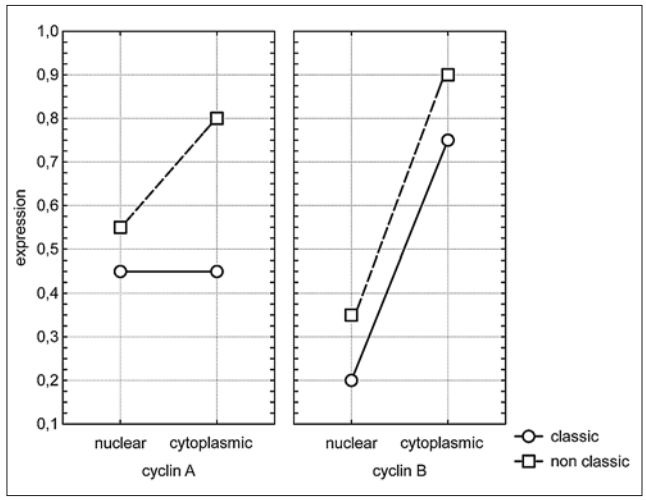

Figure 3. Nuclear and cytoplasmic expression of cyclin A and B1 for classic and non-classic variants of PTC

DISCUSSION

The pathogenesis of malignant transformation in the thyroid gland is still poorly understood. Carcinogenesis, as a result of uncontrolled cell proliferation, may result from an increased expression of cell cycle up-regulators, such as cyclins [11].

Previous studies by the authors of the presented study suggested that an increased expression of cyclin D, Pin 1 (peptidyl-prolyl *cis/trans* isomerase, which also plays a critical role in cell restriction points – particular in G1/S phase) and cyclin E, could play an important role in PTC carcinogenesis [11, 12, 13, 14]. However, the role of other cell cycle regulatory proteins in PTC remains largely undefined.

In the present study, an attempt was made to analyze cell cycle regulators in different variants of PTC by immunohistochemical expression of a panel of two cyclins. Cyclin A and cyclin B1 were selected because these proteins exert a very important influence on cell cycle progression, and also because their role in PTC development has been less studied.

Expression of cyclin A and B1 was assessed in a number of neoplasms in humans, among others in cancers of the breast, colon, stomach and lung. In some of them, the expression of these proteins is considered to be a prognostic factor with proven clinical utility. Breast cancer seems to be the best example, as the intense expression of cyclin A in primary tumour cells is proven to be associated with an increased risk of relapse, and poor response to treatment with tamoxifen [15]. Baldini et al. [16] suggest using the expression of cyclin A as an additional factor to proceed in some patients into a group with high risk of relapse. Numerous studies have also demonstrated the usefulness of the evaluation of cyclin B1 expression as a prognostic factor for breast cancer. The expression level correlated with tumour size, presence of distant metastases and disease free survival [17].

Several studies have revealed overexpression of cyclin A and B1 in thyroid cancers, including follicular thyroid carcinoma (FTC), also its poorly differentiated form and thyroid lymphoma [18, 19, 20]. Mainly, cyclin A has been overexpressed in anaplastic thyroid carcinoma [21]. Ito et al. [21] have found that cyclin A immunoexpression was

frequently observed in undifferentiated thyroid carcinoma, indicating that this cyclin and not cyclin B1 plays a crucial role in its development. In the immunohistochemical assessment of PTC of different sizes, Dinets et al. [22] have showed that cyclin A expression has been significantly higher in PTC of size over 2 cm, which may indicate a role of cyclin A in thyroid carcinoma de-differentation. In thyroid lymphoma, both cyclins A and B1 play an important role in the cell cycle progression [20].

However, recent reports suggest that both cyclins A and B1 may be associated with progression of thyroid carcinoma. Nar et al. [23] have shown cyclin A and B1 overexpression in differentiated (follicular and papillary) carcinoma, but not in Hürthle cell carcinoma. The expression of both cyclins have not been shown in medullary carcinoma. Kebebew et al. [24] used multigene assays as diagnostic markers to identify differentially expressed genes in benign lesions *versus* malignant thyroid neoplasms. The cyclin B1 gene was up-regulated in malignant thyroid neoplasms (PTC, follicular variant of PTC, follicular thyroid carcinoma).

The results obtained in the presented study also indicate that both cyclin A and B1 are expressed in PTC, and has demonstrated statistically significant difference in cyclin A expression between different histological variants of PTC. The strongest expression was observed in tumours known to have the worst prognosis (tall-cell variant, columnar-cell variant), weaker in the follicular variant of PTC, and poor expression in the most promising classic variant of PTC. The overexpression of cyclins observed in other than the classic variant of PTC may indicate their role in tumour aggressiveness and dedifferentiation potential. These results are in agreement with recent reports by other investigators [21, 22, 23]. Ito et al. [21] demonstrated cyclin A overexpression in poorly-differentiated and undifferentiated thyroid cancers. Consistently, overexpression of both cyclin A and B1 has been observed in malignant but not in benign thyroid tumours [23].

Although expression of the studied cyclins was weak in the classic variant of PTC, it is still possible to distinguish cyclin-positive and cyclin-negative cancers within this group. As cyclin overexpression has been demonstrated to be a marker of the worst prognosis, such differentiation might serve as a useful tool in predicting the course of the diseases. Further studies confirming this hypothesis in a large group of PTC cases of the classic variant are needed.

Patients with PTC are not a homogeneous group and the disease course and prognosis depend significantly on the variant of PTC. Therefore, from a clinical point of view, it seems important to find a marker that will not only help to distinguish between well- and poorly- differentiated thyroid cancer, but also enable the differentiation of PTC variants. To the best of the authors' knowledge, this is the first report in the literature which analyzes by immunohistochemistry, the expression of cyclins A and B1 in different variants of PTC.

In the presented study, cyclin B1 was characterized by greater difference between nuclear and cytoplasmic expression than cyclin A. This fact can be explained by an active transport of cyclin B1/CDK1 complex and its accumulation in the cytoplasm during the G2 phase. Similar observations concerning the predominance of cytoplasmic over nuclear expression of cyclin B1 was made by Suzuki et al. [25] in cases of cancer of the breast, pancreas, colon and lung.

CONCLUSIONS

The results of the presented study suggest that cyclin A and B1 expression may have a characteristic pattern of immunostaining for particular variants of PTC. If the results obtained are confirmed in a larger group of patients, the diagnostic panel, constructed of the antibodies against these proteins, may increase the diagnostic accuracy in PTC cases.

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