Cyclin D1 and p27 Expression as Prognostic Factor in Papillary Carcinoma of Thyroid: Association with Clinicopathological Parameters

Valdi Pešutić-Pisac[1], Ante Punda[2], Ivo Glunčić[3], Vladimir Bedeković[4], Anka Pranić-Kragić[2], Nenad Kunac[1]

[1]Clinical Department for Pathology, Split University Hospital Center, Split, Croatia
[2]Department for Nuclear Medicine, Split University Hospital Center, Split, Croatia
[3]Clinical Department for Ear Nose and Throat, Split University Hospital Center, Split, Croatia
[4]Clinical Department for Ear Nose and Throat and Surgery of Head and Neck, Sisters of Mercy Zagreb University Hospital, Zagreb, Croatia

Aim To determine the prognostic value of cell cycle regulators cyclin D1 and p27 for papillary thyroid carcinomas.

Methods Analysis included 180 patients with papillary thyroid carcinoma who underwent surgery at Split University Hospital Center between 1999 and 2001. Clinical data were obtained from clinical charts and histopathology reports. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue by antibody p27 and cyclin D1. Quantification was based on the intensity and distribution of nuclear staining.

Results Univariate analysis showed that sex ($P = 0.019$) and capsular invasion ($P = 0.010$) were significant predictors of lymph node metastases, whereas age ($P = 0.96$), histopathological variant ($P = 0.075$), size ($P = 0.556$) and multifocality ($P = 0.131$) were not. Univariate analysis also showed that overexpression of cyclin D1 ($P < 0.001$) and underexpression of p27 ($P < 0.001$) predicted lymph node metastases in papillary thyroid carcinomas. There was a significant correlation between cyclin D1 ($P = 0.024$) and p27 ($P = 0.029$) expression in two prognostic groups of low and high risk. Low risk group was cyclin D1 negative and p27 positive, while high risk group was cyclin D1 positive and p27 negative. Multivariate analysis confirmed that sex ($P = 0.041$), capsular invasion ($P = 0.027$), and p27 ($P < 0.001$) were strong independent predictors of lymph node metastases in the high-risk group.

Conclusions Immunohistochemical analysis of p27 expression may be a valuable tool for identifying risk of lymph node metastases and more aggressive behavior of papillary thyroid carcinoma.

> **Correspondence to:**
Valdi Pešutić Pisac
Clinical Department for Pathology, Clinical Hospital Split
Dubrovačka 18
21000 Split, Croatia
valdy@net.hr

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Papillary thyroid carcinoma is the most common endocrine malignancy (1). It has a low mortality rate – the overall 10 years survival rate being about 95%, but a significant number of patients present with aggressive disease. This is why it is of utmost importance to evaluate the prognostic factors and identify high-risk patients (1). A few studies have described the factors influencing prognosis and long term outcome of thyroid cancer (2,3). Some authors proposed lymph node metastasis as a prognostic factor, but many disagree with it (4). It is still unclear whether lymph node metastasis depends on the same factors that influence survival (5), such as age, sex, tumor size, tumor histopathological variant, tumor focality, and extrathyroidal extension.

Cell cycle kinases also play an important role in metastazing of thyroid carcinoma. The cell cycle is controlled by cyclin-dependent kinases (CDK), which are activated by forming complexes with cyclins. Cyclin D1 gene, located on chromosome 11q23, is a positive regulator of the cell cycle. It encodes a nuclear protein that forms complexes with CDKs 4 and 6, which phosphorylate and inactivate the retinoblastoma protein. This allows cell cycle progression from G1 to S phase (6).

Tumor suppressor gene p27 is located on the chromosome 12p13. It encodes the CDK-inhibiting nuclear protein and inhibits the formation of cyclinD1/cdk complexes during G0 and early G1 phases of the cell cycle. This inhibits inactivation of the phospho-retinoblastoma protein and prevents G1 to S phase transition (6).

Normal thyroid cells do not show nuclear immunoreactivity to cyclin D1, but do show strong immunoreactivity to p27 (7). Cyclin D1 overexpression and p27 underexpression were found in a variety of tumors and were associated with increased tumor aggressiveness, incidence of lymph node metastases, and poorer prognosis (8-10).

In the present study, we tested the hypothesis that cyclin D1 overexpression and p27 underexpression would allow us to identify the subgroup of papillary carcinomas with a potential for metastatic behavior.

Subjects and methods

Patients

This analysis included 180 consecutive patients with papillary thyroid carcinoma who underwent surgery at Clinical hospital center Split between 1999 and 2001. Patients with no evidence of lymph node involvement at the time of surgery underwent total thyroidectomy, while patients with gross lymph node metastases underwent total thyroidectomy with regional lymphadenectomy.

Data on patient age (a cut-off point of 45 years), sex, tumor size (a cut-off point of 2 cm), tumor focality, histopathological variants of papillary carcinoma (low risk – papillary or follicular; high risk – oncocytic, clear cell, diffuse sclerosing, tall cell, columnar cell, or solid carcinoma), presence or absence of extrathyroidal extension, and presence or absence of lymph node involvement were collected from histopathology reports.

Carcinomas were divided into low-risk and high-risk groups (1,3,11) as follows: (a) the low risk group – patient age <45 years, female sex, tumor size <2 cm, papillary or follicular variant, no extrathyroid extension, unifocal tumor, and no metastases; and (b) high-risk group – patient age ≥45 years, male sex, tumor size ≥2 cm, all variants except papillary and follicular carcinoma, extrathyroid extension, multifocal tumor, and lymph node metastases.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 3 μm-thick tissue sections were deparaffinized in toluene and rehydrated through graded alcohols to water. Endogenous peroxidase activity was

blocked in 0.5% hydrogen peroxide. Antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0) inside a microwave pressure cooker. Slides were immediately placed into a tap water bath. Sections were washed in Tris-buffered saline (TBS) for 1 × 5 minutes, placed in diluted normal serum for 10 minutes, and finally incubated with the primary antibody.

Primary antibody incubation was carried out at room temperature for 1 hour using p27 mouse monoclonal Clone 1B4 P (HIER) (W – Novocastra, Vision Bio Systems, Newcastle Upon Tyne, UK) in a 1:40 dilution and cyclin D1 mouse monoclonal Clone P3D11F11 P in a 1:40 dilution (HIER/Enzyme) (W – Novocastra).

In the next step, the slides were washed in TBS buffer for 2 × 5 minutes, incubated in biotinylated secondary antibody, again washed in TBS buffer for 2 × 5 minutes, incubated in ABC reagent, washed in TBS buffer for 2 × 5 once more, incubated in diaminobenzidine (DAB), and washed thoroughly in running tap water.

Slides were counterstained in hematoxylin, dehidrated, and mounted. Positive control for cyclin D1 and negative control for p27 was mantle cell lymphoma, according to the recommendation of Novocastra. Normal thyroid tissue was positive control for p27 and negative control for cyclin D1 (7).

Quantitation

p27 expression. The intensity of nuclear staining within tumor cells was graded 0 to 4 as follows: grade 0 – total absence of staining; grade 1 – faint nuclear staining; grade 2 – moderate nuclear staining; grade 3 – strong nuclear staining; and grade 4 – staining as strong as in normal thyroid tissue (Figure 1A). Tumors with grades 0-1 were defined as non-expressors (Figure 1B) (7).

Cyclin D1 expression. The intensity of nuclear staining was graded from 0 to 3 as fol-

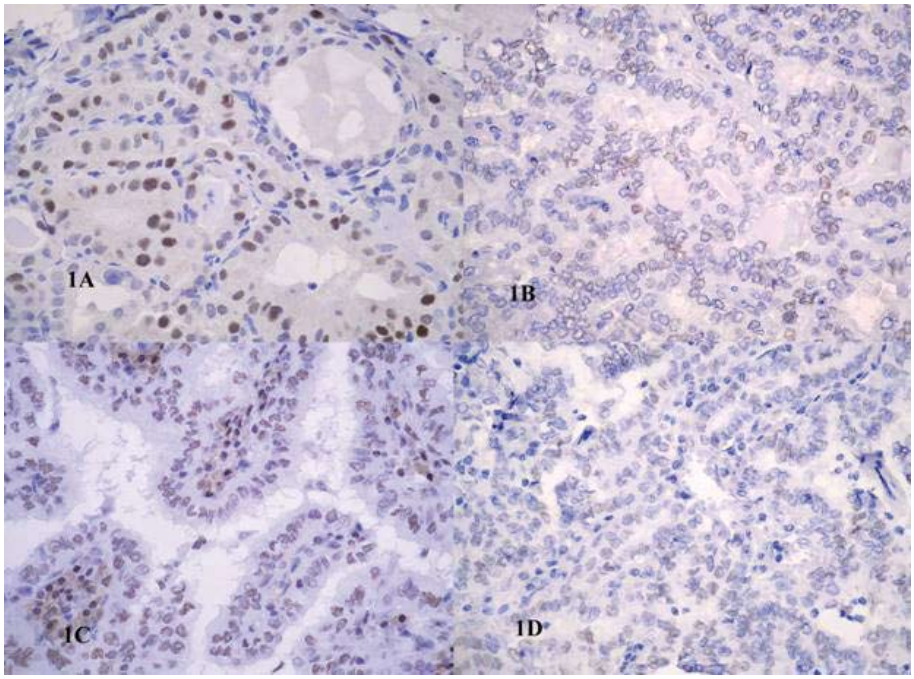

Figure 1. Expression of p27 and D1 cyclin in paplillary thyroid carcinoma. (**A**) p27 expresser. Strong nuclear staining of grade 3 (magnification ×40); (**B**) p27 non-expresser. Faint nuclear staining of grade 1 (magnification ×40); (**C**) cyclin D1 expresser. Diffuse staining of grade 2 (moderate nuclear staining) in more than 50% of tumor cells (magnification ×40); (**D**) Cyclin D1 non-expresser. Faint nuclear staining of grade 1 in fewer than 10% tumor cells (magnification ×40).

lows: grade 0 – absence of staining; grade 1 – faint nuclear staining; grade 2 – moderate nuclear staining; grade 3 – intense nuclear staining. We graded the distribution of positive cells in tumors as follows: grade 1 – focal staining in fewer than 10% of tumor cells; grade 2 – fairly widespread staining in 10%-50% of tumor cells; grade 3 – diffuse staining in more than 50% of tumor cells (Figure 1B). For the purposes of statistical analysis, tumors were divided into expressors and non-expressors. Most tumors that expressed cyclin D1 showed moderate to intense nuclear staining and widespread to diffuse distribution of positive cells within the entire tumor (Figure 1C). Non-expressors generally showed complete absence of nuclear staining within entire tumor or faint nuclear staining of grade 1 in fewer than 10% tumor cells (Figure 1D) (7).

Statistical analysis

Age, sex, tumor size, histopathological variant, tumor focality, extrathyroidal extension, and cyclin D1 and p27 expression were included in the univariate analysis to determine the predictors of lymph node metastases in papillary thyroid carcinoma. Univariate analysis using χ^2 test was also performed to determine the influence of cyclin D1 and p27 in different prognostic groups. Multivariate analysis using backward stepwise (Wald) test was performed to identify independent predictors for lymph node metastases. Level of statistical significance was set at $P < 0.05$. We used Statistical Package for the Social Sciences, version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological characteristics

Our study included 60 patients (33.3%) younger than 45 years and 120 (66.7%) older. There were 143 women and 37 men (female-to-male ratio, 3.8:1).

According to tumor size, patients were divided into two groups with a cut-off point of 2 cm (T of tumor-node-metastasis [TNM] stage 1). Tumors of ≤2 cm in maximum were found in 130 (72.2%) patients, and larger tumors were present in 50 (27.8%) patients.

Papillary thyroid carcinomas divided into low-risk and high-risk groups according to tumor histopathological variants showed expected distribution. There were 146 (81.1%) low-risk tumors 34 (18.9%) high-risk tumors.

Multifocal disease or intraglandular dissemination was found in 80 (44.4%) patients. Extrathyroidal extension was noticed in 28 (15.6%) patients. Lymph node metastases were found in 38 (21.1%) patients, and none of the patients had distant metastases.

There were 93 (51.7%) expressors for cyclin D1 and 97 (53.9%) expressors for p27.

Analysis of clinicopathological and immunohistochemical parameters

Univariate analysis of TNM stage and clinical and histopathological parameters including age, sex, tumor size, tumor variant, tumor focality, extrathyroidal extension, and cyclin D1 and p27 immunoreactivity showed that sex ($P = 0.019$), extrathyroidal extension ($P = 0.010$), overexpression of cyclin D1 ($P < 0.001$), and underexpression of p27 ($P < 0.001$) were strong predictors of lymph node metastases, whereas age ($P = 0.796$), size ($P = 0.556$), tumor variant ($P = 0.075$), and focality ($P = 0.131$) were not (Table 1).

Immunoreactivity to cyclin D1 ($P = 0.024$) and p27 ($P = 0.029$) was significantly associated with a carcinoma risk group (Table 2). High-risk papillary carcinomas were cyclin D1 overexpressors and p27 underexpressors, while low-risk papillary carcinomas were cyclin D1 underexpressors and p27 overexpressors.

Sex ($P = 0.041$), capsular invasion ($P = 0.027$), and p27 ($P < 0.001$) also showed a

Table 1. Clinical, histopathological, and immunohistochemical parameters in 180 patients with thyroid papillary carcinomas

Characteristics	No. (%) of tumors with nodal status		*P**
	negative	positive	
Age (years):			
<45	48 (33.8)	12 (31.6)	0.796
≥45	94 (66.2)	26 (68.4)	
Sex:			0.019
male	24 (16.9)	13 (34.2)	
female	118 (83.1)	25 (65.8)	
Tumor size (cm):			0.556
<2	104 (73.2)	26 (68.4)	
≥2	38 (26.8)	12 (31.6)	
Histopathological variant:†			0.075
low risk	119 (83.8)	27 (71.1)	
high risk	23 (16.2)	11 (28.9)	
Tumor focality:			0.131
unifocal	83 (58.5)	17 (44.7)	
multifocal	59 (41.5)	21 (55.3)	
Extrathyroidal extension:			0.010
negative	125 (88.0)	27 (71.1)	
positive	17 (12.0)	11 (28.9)	
Cyclin D1 expression:‡			<0.001
negative	79 (55.6)	8 (21.1)	
positive	63 (44.4)	30 (78.9)	
p27 expression:§			<0.001
negative	54 (38.0)	29 (76.3)	
positive	88 (62.0)	9 (23.7)	

*χ2 test.
†Histopathological variants of papillary carcinoma: low risk – papillary and follicular; high risk – oncocytic, clear cell, diffuse sclerosing, tall cell, columnar cell, and solid carcinoma (1).
‡Negative – non-expresser, complete absence of nuclear staining within entire tumor; Positive – expresser, showing moderate to intense nuclear staining and widespread to diffuse distribution of positive cells within the entire tumor (7).
§Negative – non-expresser: grade 0 – total absence of staining; grade 1 – faint nuclear staining. Positive – expresser: grade 2 – moderate nuclear staining; grade 3 – strong nuclear staining; grade 4 – staining as strong as in normal thyroid tissue (7).

strong independent predictive value for lymph node metastases in multivariate analysis (Table 3).

Discussion

Although the field of thyroid oncology has much profited from a large body of information obtained by immunohistochemistry, cytogenenetics, and molecular biology, the issue of identification of the most aggressive papillary carcinoma has not been solved (1,12-22). Pathologists still debate about clinical and histopathological parameters and their predictive value (1,3,5,11), gradually turning toward new possible markers or combinations of the old and new markers.

In this study, we confirmed that cyclin D1 overexpression and p27 underexpression

Table 2. Immunoreactivity to cyclin D1 and p27 in low- and high-risk group of patients with thyroid papillary carcinomas

Expression	No (%) of patients*		*P*†
	low-risk group	high-risk group	
Cyclin D1 expression:‡			0.024
negative	15 (17.2)	72 (82.8)	
positive	6 (6.5)	87 (93.5)	
p27 expression:§			0.029
negative	5 (6.0)	78 (94.0)	
positive	16 (16.5)	81 (83.5)	

*Low risk –<45 y old; female sex; tumor size <2 cm; papillary or follicular variant; no extrathyroid extension; unifocal tumor; no metastases. High risk –≥45 y old; male sex; tumor size ≥2 cm; oncocytic, clear cell, diffuse sclerosing, tall cell, columnar cell, or solid variant; extrathyroid extension; multifocal tumor; lymph node metastases.
†χ2 test.
‡Negative – non-expresser, complete absence of nuclear staining within entire tumor. Positive – expresser, showing moderate to intense nuclear staining and widespread to diffuse distribution of positive cells within the entire tumor (7).
§Negative – non-expresser: grade 0 – total absence of staining; grade 1 – faint nuclear staining. Positive – expresser: grade 2 – moderate nuclear staining; grade 3 – strong nuclear staining; grade 4 – staining as strong as in normal thyroid tissue (7).

Table 3. Multivariate analysis of predictors of lymph node metastases in patients with thyroid papillary carcinomas

Predictors	*P*	Odds ratio (95% confidence interval)
Age (<45, ≥45)	0.427	0.692 (0.279-1.718)
Sex (male, female)	0.041	2.478 (1.039-5.909)
Tumor size (<2 cm, ≥2 cm)	0.301	0.621 (0.251-1.534)
Histopathological variant (low-risk, high-risk)*	0.581	1.329 (0.483-3.658)
Focality (unifocal, multifocal)	0.380	1.441 (0.637-3.260)
Extrathyroid extension (–,+)	0.027	2.876 (1.130-7.318)
Cyclin D1(–,+)†	0.200	1.938 (0.075-5.332)
p 27(–,+)‡	0.000	5.850 (2.460-13.913)

*Histopathological variants of papillary carcinoma: low risk – papillary or follicular; high risk – oncocytic, clear cell, diffuse sclerosing, tall cell, columnar cell, or solid carcinoma (1).
†Negative – non-expresser, complete absence of nuclear staining within entire tumor. Positive – expresser, showing moderate to intense nuclear staining and widespread to diffuse distribution of positive cells within the entire tumor (7).
‡Negative – non-expresser: grade 0 – total absence of staining; grade 1 – faint nuclear staining. Positive – expresser: grade 2 – moderate nuclear staining; grade 3 – strong nuclear staining; grade 4 – staining as strong as in normal thyroid tissue (7).

could be useful prognostic markers for identification of papillary carcinomas with a potential for metastatic behavior. This allows easier identification of patients who are at higher risk of metastatic carcinoma and should be controlled more frequently. For example, if the patient is a young woman, with a low-risk histopathological variant of the tumor of 1 cm in size, unifocal, without extrathyroidal extension, and without metastases, she has excellent prognosis, but if that tumor is cyclin D1 positive and p27 negative, it has a propensity to metastasize to lymph nodes. Our results are in agreement with the findings on other carcinomas, such as gastric, colorectal, or breast carcinoma (8-10)

Other studies that analyzed the importance of cell cycle kinases in thyroid tumors analyzed either a single cell cycle regulator, a single microcarcinoma, or only differentiated and undifferentiated papillary carcinomas (15-22), whereas our study group was composed of a large number of patients (n = 180) with well differentiated papillary carcinomas for both cyclin D1 and p27, combined with all relevant clinicopathological parameters.

We included six clinical and histopathological parameters that had been shown as survival predictors (1,5). Univariate analysis showed that sex, extrathyroidal extension, overexpression of cyclin D1, and underexpression of p27 were strong predictors of lymph node metastases, unlike age, size, tumor variant, and focality. In our study, tumor size was not found to be predictive because there were no cancers larger than 5 cm, which was the cut-off point proposed by Age, Metastases, Extent and Size (AMES) risk classification (1). We used the cut-off point of 2 cm because it is a new T1 tumor classification in the latest World Health Organization classification (1). Tumor variant did not appear to be a predictor, because only a few unfavorable high-risk histopathological variants were included in the study, due to their low frequency (1). Multifocality was not found to be a predictor because it is a result of multicentric transformation of follicular epithelium rather than the process of intrathyroid lymph vessel spread (1).

Multivariate analyses showed that sex, capsular invasion, and p27 were strong independent predictors of lymph node metastases. Although overexpression of cyclin D1 did not retain independent predictive value in multivariate analysis, its identification as predictor of lymph node metastases by univariate analysis is still an important finding. Although our study included a large number of patients, multivariate analysis did not confirm the predictive value of expression of cyclin D1. A possible explanation could be either that expression of cyclin D1 is associated with other factors or that the immunohistological method of analysis is not sensitive enough. Therefore, other additional methods, such as polymerase chain reaction, should be used.

We also combined prognostic factors and formed two groups of patients under high or low risk of metastases. High risk papillary carcinomas were cyclin D1 overexpressors and p27 underexpressors, while papillary low risk carcinomas were cyclin D1 underexpressors and p27 overexpressors. This result may serve as additional information in prognosis assessment and risk classifications such as AMES, MACIS (distant metastasis, patient age, completeness of resection, local invasion, and tumor size) or AGES (patient age, presence of distant metastases, extent and size of the primary tumor) (1). However, when dealing with single factors, we advise that only p27 immunoreactivity be used and interpreted as a predictor of lymph node metastases.

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