Patterns of Cyclin E, Retinoblastoma Protein, and p21<sup>Cip1/WAF1</sup> Immunostaining in the Oncogenesis of Papillary Thyroid Carcinoma

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ABSTRACT

**Purpose:** Uncontrolled cell proliferation, a hallmark of cancer, may result from an increased expression of cell cycle up-regulators, and/or from a reduced expression of cell cycle down-regulators. In the present study, we analyzed, by immunohistochemistry, the expression of a panel of three proteins: cyclin E and two cell cycle inhibitors, p21<sup>Cip1/WAF1</sup> and retinoblastoma protein (pRb) product, in different stages of papillary thyroid carcinomas (PTC).

**Experimental Design:** We investigated immunostaining patterns of the proteins in question in 51 resected PTC in pathologic stages, ranging from pT1a to pT4, taking into consideration their relation to clinicohistopathologic factors.

**Results:** We observed a significant, progressive loss of expression of p21<sup>Cip1/WAF1</sup> with advancing tumor grade. The differences reached values of significance between pT1a [papillary thyroid microcarcinomas (PMC)] and pT2 and between PMC and pT4 stages of PTC. pRb presented a similar immunostaining pattern to that of p21<sup>Cip1/WAF1</sup> and the differences reached values of significance between pT1a and pT4, and between PMC and pT4 stages of PTC. The results of cyclin E immunostaining corresponded to our recently published result, and a negative correlation was observed between the immunostaining index of cyclin E and pRb.

**Conclusions:** The results of the present study suggest that cyclin E expression and suppression of pRb and p21<sup>Cip1/WAF1</sup> may be characteristic patterns of immunostaining for PTC with a tendency to early metastasizing. If our results are confirmed in a larger study, the diagnostic panel, constructed of the antibodies against these proteins, may become a valuable tool in predicting the metastatic potential in PTC.

INTRODUCTION

Thyroid carcinoma is the most common malignancy of the whole endocrine system and has the highest mortality rate—with the exception of ovarian cancer (1). Papillary thyroid cancer (PTC) is the most common of all thyroid carcinomas and the incidence of this malignancy has been increasing over the last decade. The prognosis for PTC is better for patients <40 years of age with PTCs, without extracapsular extensions or vascular invasions. However, despite many clinical and laboratory investigations, age seems to be the single, most important prognostic factor (2–6). Therefore, there is a clinical need for a prognostic marker for PTC, especially, considering the increasing number of diagnosed papillary thyroid microcarcinomas (PMC; ref. 7).

The biology of these small papillary carcinomas, with a maximum dimension of <1 cm, is not fully understood, although lymph node metastases from PMCs are very uncommon, a PMC may—or on rare occasions—behave aggressively and metastasize early. These tumors then result in significant morbidity and mortality (8). At present, the traditional histopathologic assessment cannot distinguish between the typical PMC, which almost always remains quiescent, and the unusual PMC, which has a potential to behave aggressively.

Deregulation 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 (9). Cyclins, the regulatory subunits of cyclin-dependent kinases (CDK), control the passage of proliferating cells through key checkpoints in the cell cycle. Among the G1 cyclins, cyclin D1 and cyclin E are the key regulators during the G1-S cell cycle transition and, perhaps, the most important checkpoint in the mammalian cell cycle (10). The overexpression of positive growth regulators (i.e., cyclins) may overwhelm the arrest mechanism of the normal cell cycle and lead to uncontrolled cell proliferation (11).

Our previous study showed a relationship between T factor of tumor-node-metastasis (TNM) scale and cyclin E expression in PTCs. Moreover, we have found that cyclin E overexpression is associated with lymph node metastases in PTC with different staging (12).

The results of the study indicate that cyclin E may have an important role in thyroid carcinogenesis and that it probably plays a key role in the progression of malignancy of PTC. Similar observations were made in several other cancers, including colorectal (13, 14), laryngeal (15), lung (16–18) and, particularly, breast cancer, in which Keyomarsi et al. (19) proposed cyclin E to be the prognostic marker. In their latest
investigation, Keyomarsi et al. (20) indicate that high levels of cyclin E, measured by Western blotting, is the most sensitive and specific prognostic indicator in patients with breast cancer than any other marker ever analyzed.

However, the overexpression of positive growth regulators may not be the only mechanism by which cell proliferation is stimulated. Alternatively, an underexpression of negative growth regulators, such as the CDK inhibitors, can have the same effect on the cell cycle. Abnormalities in the retinoblastoma and/or p53 tumor suppressor pathways have been reported in many human malignancies, including thyroid cancer (21–28). Although some studies have shown underexpression of negative growth regulators in thyroid carcinoma, compared with benign lesions (23–34), their role in malignancy progression of thyroid cancer is still to be established. The aim of this study was to determine if the differences in cyclin E, p21Cip1/WAF1, and retinoblastoma protein (pRb) immunostaining could help in the differentiation between indolent and more aggressive PTCs. Furthermore, such immunostaining may suggest possible mechanisms for thyroid malignancy progression.

MATERIALS AND METHODS

**Tissue Specimens Studied.** A total of 51 PTC cases were selected from the surgical pathology files of the hospital, comprising 25 cases of PMC and 26 cases of PTC, with staging higher than pT1a. The patients were diagnosed and operated on between 1997 and 2003 at the Department of Endocrinological and General Surgery, Institute of Endocrinology, Medical University of Łódź. During that period, ~1,000 total and >7,000 subtotal thyroidectomies were done by the department. The experimental protocol included only females. All patients diagnosed with thyroid cancer at the time of surgery underwent total thyroidectomy and regional lymphadenectomy. The remaining patients, diagnosed with cancer during routine histopathologic examinations, were re-operated on, the operation was radicalized and regional lymphadenectomy was done. Following the approved procedures at our department, all patients received postoperative radioactive iodine therapy, followed by L-T4 suppression. All the clinical charts and histo-pathologic examinations, were re-operated on, the remaining patients, diagnosed with cancer during routine histopathologic examinations, were re-operated on, the operation was radicalized and regional lymphadenectomy was done. Following the approved procedures at our department, all patients received postoperative radioactive iodine therapy, followed by L-T4 suppression. All the clinical charts and histopathologic reports were reviewed for data regarding the patient’s age, tumor size, the presence or absence of extrathyroidal extension, and the presence or absence of lymph node involvement.

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 tumors and the rims of morphologically normal thyroid tissue around them, the latter serving as control. Normal thyroid was seen in the all 51 cases. Each of the 51 patients, selected for analysis, had typical, monofocal papillary carcinomas on histopathology. Patients whose tumors showed either tall cell or columnar cell differentiation, or foci of insular or anaplastic dedifferentiation, were not included in the study. Patients with Hürthle cell predominant papillary carcinomas were also excluded.

**Immunohistochemistry.** Archival paraffin-embedded tumor tissue was analyzed by immunohistochemistry for cyclin E, p21Cip1/WAF1, and pRb expressions. Formalin-fixed paraffin-embedded tissue sections (4 μm thick) were dewaxed in xylene and rehydrated through graded alcohols to water. Antigen retrieval was done in citrate buffer (pH 6.0; Dako, Glostrup, Denmark) inside a microwave pressure cooker.

Primary antibody characteristics, the conditions of incubation, the manufacturer’s name, and positive control tissues for each of the proteins are presented in Table 1. After incubation, the slides were washed in TBS, and secondary incubations were carried out, using an En Vision kit (Dako). After washing with TBS, the antigenic binding sites were visualized by incubation with Fast Red (Dako). The slides were counterstained in hematoxylin and mounted on Faramount (Dako). Human tissue, with known overexpression of the antigens in question, served for positive controls (Table 1). For negative control, non-immune mouse serum (Dako) replaced the primary antibody.

**Evaluation of Immunostaining.** Immunostained tissue sections were evaluated by estimating the percentage of tumor cells staining positive with monoclonal antibody without any knowledge of pathologic diagnosis. Only distinct red nuclear staining of tumor cells was considered positive. The nuclear staining in the normal thyroid tissue, surrounding PTC, was also evaluated at the same time as the tumor, serving as an internal control.

**Staining Index.** All the stained sections were examined on an Olympus microscope (Olympus CH20BIM200, Olympus, Tokyo), using an eyepiece graticule (PZO, Warsaw) to facilitate cell counting, first under low magnification (×100), in order to see the overall staining pattern and, later, at a higher magnification (×200), at which a minimum of 1,000 cells were counted in the area with positive staining. The percentage of tumor cells, stained with the antibody, was regarded as the staining index. Positive staining was divided into three grades: no expression (NE), if < 10%; expression (E) if within 11% to 50%; and overexpression (OE), if > 50% of the tumor cell nuclei were positively stained.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of the used antibodies and the incubation conditions</th>
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<tbody>
<tr>
<td>Antibody/animal</td>
<td>Mouse’s</td>
</tr>
<tr>
<td>Antibody/isotype</td>
<td>IgM</td>
</tr>
<tr>
<td>Antibody/clone</td>
<td>84-B3-1</td>
</tr>
<tr>
<td>Dilution</td>
<td>1:50</td>
</tr>
<tr>
<td>Incubation</td>
<td>Whole night (4°C)</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Novocastra</td>
</tr>
<tr>
<td>Positive control</td>
<td>Breast cancer</td>
</tr>
</tbody>
</table>
Statistical Analyses. The nonparametric Mann-Whitney U-test was used to assess whether there was a difference in the expression of the antigens in question between PTC in different stages in TNM classification. The level of statistical significance was set at $P < 0.05$. Coefficients of correlation $r$ and $P$ were calculated by using linear regression analysis and Spearman’s rank correlation analysis as appropriate.

RESULTS

The rims of normal thyroid tissue in all of the 51 cases showed either expression or overexpression of both pRb and p21$^{Cip1/WAF1}$. However, only three cases of PMC showed overexpression of p21$^{Cip1/WAF1}$ and one case was negative for the expression of the protein in question (Fig. 1C). From the group of PTC with TNM higher than pT$1a$, only six showed the expression of the p21$^{Cip1/WAF1}$, yet none of them overexpressed that protein (Fig. 1C). The analyzed cases of pT$4$ PTCs were negative for p21$^{Cip1/WAF1}$ expression (Fig. 1C). The p21$^{Cip1/WAF1}$ staining index was significantly different between the PMC (pT$1a$ PTCs) and the rest of the investigated cancers (23.2 ± 14.6% versus 7.67 ± 3.86%, respectively; $P < 0.00005$; Table 2). Analyzing the p21$^{Cip1/WAF1}$ staining index according to the TNM scale, we observed a significant decrease in the levels of p21$^{Cip1/WAF1}$ immunostaining with an increasing pathologic stage of the cancer (T factor). The differences reached values of significance between pT$1a$ and pT$2$ and PMC and pT$4$ stages of PTC (23.2 ± 14.6% versus 7.98 ± 4.12%, respectively; $P < 0.0001$, and 23.2 ± 14.6% versus 6.7 ± 2.9%, respectively; $P < 0.0005$; Fig. 2B).

Analyzing pRb expression, we observed positive staining in 90% of the cases (47/51). Out of 25 microcarcinomas, 14 showed expression and 9 overexpressed pRb (Fig. 1B). Similarly, 23 out of 26 cancers, with TNM higher than pT$1a$, showed expression of pRb but there were no cases of overexpression and three cases were negative for pRb expression (Fig. 1B). Out of the pT$4$ cancers, only three expressed pRb (Fig. 1B). The pRb staining index was significantly different between the PMC (pT$1a$ PTCs) and the other investigated cancers (38.9 ± 21.5% versus 22.4 ± 11.3%, respectively; $P < 0.005$; Table 2). Analyzing the pRb staining index, following the TNM scale, we observed a decrease of its value along with increasing cancer staging. The differences reached values of significance between pT$1a$ and between pT$2$ and PMC and pT$4$ stages of PTC (38.9 ± 21.5% versus 24.5 ± 11.5%, respectively; $P < 0.02$, and 38.9 ± 21.5% versus 15.46 ± 8.2%, respectively; $P < 0.02$; Fig. 2B).

Normal thyroid tissue was immunonegative in all the cases, but 78% (40/51) of the analyzed thyroid papillary carcinomas were immunopositive for cyclin E expression. We observed an increase in the number of cases positive for cyclin E expression along with PTC staging (Fig. 2A). Cyclin E expression was observed in 68% of microcarcinomas (17/25). In that group, there were 15 cases of expression and 2 cases of overexpression of cyclin E (Fig. 1A). In the group of cancers with TNM, higher than pT$1a$, we observed positive staining in 23 cases (88%); there were also 10 cases overexpressing cyclin E (Fig. 1A). Analyzing the six cases of pT$4$ thyroid carcinomas (included in the group of PTC with staging higher than pT$1a$), we observed cyclin E immunopositiveness in all the cases, although five out of six pT$4$ PTCs (83%) presented overexpression of the protein in question (Fig. 1A). The remaining 20 cases of PTC were pT$2$ cancers of which, 13 presented expression and 5 presented overexpression of cyclin E. Two of the pT$2$ cancers did not express cyclin E (Fig. 1A). The staining index was significantly different between

![Fig. 1](cyclinE.png)
the PMC and the other investigated cancers (16.4 ± 13.7% versus 31.6 ± 23.6%; P < 0.02; Table 2). Analyzing the cyclin E staining index, following the TNM scale, we observed an increase of its value along with increasing cancer staging (Fig. 2A). The differences reached the values of significance between PMC and pT2 stages of PTC (16.4 ± 13.7% versus 48.7 ± 18.8%; P < 0.005).

**Table 2** Comparison of the immunostaining indexes of the analyzed proteins between PMc and PTC with staging higher than pT1a

<table>
<thead>
<tr>
<th>Analyzed protein</th>
<th>Grading, TNM</th>
<th>Number of cases</th>
<th>Immunostaining (%), mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NE</td>
<td>E</td>
<td>OE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&gt;1</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pT1a</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&gt;1</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p21Cip1/WAF1</td>
<td>pT1a</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p&gt;1</td>
<td>21</td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviations: NE, no expression; E, expression; OE, overexpression.

All the lymph node metastases coexisted with cyclin E expression and most of them, but not all, coexisted with cyclin E overexpression. All the metastasizing PTC, except for one PMC, were negative for p21Cip1/WAF1 expression and had low levels of pRb immunostaining (12 cases of expression and 2 cases pRb-immunonegative).

Interestingly, there was a negative correlation between the pRb and cyclin E staining indexes ($r = -0.47$, $P < 0.0005$; Fig. 3). However, there were no relationships among other protein staining indexes. There was a positive relationship between the cyclin E staining index and T factor of the TNM scale and, more impressively, there was a progressive loss of p21Cip1/WAF1 and pRb expression with an increasing tumor grade. All the groups of the patients were age-matched (45.8 ± 12.2 years for PMC patients, 42.8 ± 15.4 years for pT2 patients, 44.5 ± 22.5 years for pT4 patients, and 43.2 ± 16.7 years for patients with TNM higher than pT1a).

**DISCUSSION**

Malignant transformation, as an effect of uncontrolled cell proliferation, may result from an increased expression of cell cycle up-regulators, such as cyclins, and/or a reduced expression of the cell cycle down-regulators, such as CDK inhibitors (9–11).

The role of tumor suppressor gene loss in carcinogenesis has been documented in multiple tumors. Protein p53 being the most notable example, the expression of this protein has been described in thyroid tumor genesis (24–36); however, several other oncogenes, or tumor suppressor genes, have been shown to contribute to malignancy progression and dedifferentiation of thyroid cancer (12, 23, 27–34).

Our previous study suggested that an increased expression of cyclin E could play an important role in PTC carcinogenesis (12). Moreover, other interesting studies have proved the important role of cyclin D1 and p27 in the tumor genesis of PTCs (29–34). However, the role of other cell cycle regulatory proteins in PTC progression remains largely undefined.

In the present study, we attempted to analyze cell cycle regulators in different stages of PTC by immunohistochemical expression of a panel of three proteins. We selected cyclin E and two cell cycle inhibitors, p21Cip1/WAF1 and pRb, because the proteins in question exert a very important influence on cell cycle progression, although reciprocally affecting their own activity.

Protein p21Cip1/WAF1 is a well-characterized mediator of p53-induced growth arrest and has been the first identified CDK inhibitor (36).
The amino-terminal domain of p21<sup>Cip1/WAF1</sup>, similar to the corresponding domains of p27 and p57, is both necessary and sufficient to bind and inhibit cyclin CDK complexes. The unique carboxyl terminal domain of p21 Cip1/WAF1 associates with the proliferating cell nuclear antigen, a subunit of DNA polymerase, and can inhibit DNA replication directly (36). Moreover, p21Cip1/WAF1 inhibits the retinoblastoma pathway and participates in several other specific protein-protein interactions that relate to cell cycle control, apoptosis, and differentiation (37).

Retinoblastoma was the first discovered tumor suppressor gene (38–40) and pRb product is expressed in all the cells, where it exists in an active, hypophosphorylated, and an inactive hyperphosphorylated state. When the cells are stimulated by growth factors, pRb is inactivated by phosphorylation, allowing the cells transversing the G1-S checkpoint. The hypophosphorylated pRb achieves cell cycle arrest by forming a complex with the E2F family of transcription factors. These complexes bind to DNA and actively inhibit the transcription of S-phase genes, thereby preventing cell division (41–45). The germ line loss or mutation of the retinoblastoma gene predisposes the development of retinoblastoma (38–40). The presence of retinoblastoma mutation or p21Cip1/WAF1 gene deletion in thyroid neoplasms has been documented in a small number of cases (25, 26, 46, 47). Investigations on pRb and p21 Cip1/WAF1 immunohistochemistry in malignant thyroid tumors have brought about controversial results in the few published studies, so no definitive conclusions can be drawn, this being, in particular, due to the heterogeneity and the small size of studied populations (23, 25, 27, 28, 48–53). Moreover, the analyzed tumors were not homogenous with respect to tumor size and histology. In selecting patients for this study, we sought for homogeneity of the patient population with respect to patient sex, tumor histology, and size. Tumors with certain histologic features can show markedly different behavior patterns, when compared with more typical lesions and, therefore, we excluded patients whose tumors presented with either tall cell or columnar cell differentiation, or foci of poorly differentiated, insular, or anaplastic dedifferentiation. Hürthle cell PTCs were also excluded from that analysis. The exclusion factors also included multifocality of the tumor and male patients. Considering tumor size, we divided the tumors into two groups: a group of microcarcinomas (pT<sub>1a</sub> PTCs) and a group of PTC with TNM above pT<sub>1a</sub>.

In the present study, the expression of p21<sup>Cip1/WAF1</sup> presented with striking differences between various stages of PTCs. We observed a significant loss of expression of p21<sup>Cip1/WAF1</sup> in PTC with staging higher than PT<sub>1a</sub> in the TNM scale, when compared with PMC. Moreover, the expression of this protein in normal and malignant thyroid tissue was negatively correlated with T factor of the TNM scale, demonstrating a progressive loss of expression of the protein with an increasing histologic stage of PTC. That negative correlation implies that, with advancing tumor grade, the loss of the key cell cycle regulator—p21<sup>Cip1/WAF1</sup>—may have a novel prognostic value in PTC, the more so as all the metastasizing PMCs, except for one, were p21<sup>Cip1/WAF1</sup>-immunonegative.

Protein pRb presented an immunostaining pattern similar to that of p21<sup>Cip1/WAF1</sup>, although the differences were statistically less significant and no correlation was observed between the expression rates of those proteins. The results, considering cyclin E immunostaining, corresponded with the results, which we have recently published (12). Briefly, the cyclin E staining index was significantly different between PMC and the other investigated cancers and a positive relationship was observed between the staining index and factor T of PTC staging. All the lymph node metastases coexisted with cyclin E expression and most of them, but not all, coexisted with cyclin E overexpression. Furthermore, in the present study, we observed a negative correlation between the immunostaining index of cyclin E and pRb. Detailed analyses of the correlation presented in Fig. 3 reveals that two populations of PTC with low levels of pRb immunostaining exist. One, with low levels of cyclin E immunostaining and, the other one that overexpress cyclin E. Therefore, the observed inverse correlation is not simple or absolute, which may suggest that pRb decrease plus second event is required for cyclin E elevation. Furthermore, in the group of PTC expressing low levels of pRb and overexpressing cyclin E there are all but one (PMC) metastasizing PTCs. The relationship between cyclin E and pRb is, at present, controversial and there are currently two models explaining.

![Fig. 3 Correlation between cyclin E immunostaining and pRb: r = −0.47, P < 0.0005. □, metastasizing PTC; ●, nonmetastasizing PTC.](https://clincancerres.aacrjournals.org)
this connection (54, 55). In one, an initial phosphorylation of pRb by CDK 4/6 partially inactivates its negative regulatory functions. This inactivating event, however, is sufficient to facilitate cyclin E expression. This, in turn, allows for subsequent phosphorylation of pRb by cyclin E-CDK2 complexes, leading to its full inactivation (54). In the other model, cyclin E exerted its influence directly via CDK2’s substrates other than pRb. Both mechanisms reveal attractive features and, ultimately, both probably participate (54). This is, to our knowledge, the first study showing a correlation between cyclin E and pRb expression rates and this observation may extend the understanding of the key role of cyclin E deregulation in PTC tumor genesis.

In conclusion, either the expression or overexpression of cyclin E, together with suppression of p21\(^{Cip1/WAF1}\) and pRb, may potentially construct an excellent condition for the development of aggressive, early metastasizing PTCs.

The antibodies against the three proteins in question construct an interesting diagnostic panel, which may allow not only our distinguishing between indolent and very aggressive types of PTC, but it may also suggest which cancer will have a tendency for early metastasis. However, there is a need for a more extensive prospective study to confirm that elevated cyclin E levels and the suppression of p21\(^{Cip1/WAF1}\) and pRb are the immunostaining patterns for aggressive early-metastasizing PTC. The recognition of a potentially aggressive and metastasizing PTC would be particularly useful in selecting PMC patients that will really benefit from early introduction of intensive surgical and adjuvant therapy.

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Clinical Cancer Research

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