Histological and immunohistochemical aspects of papillary thyroid cancer

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Abstract

Papillary thyroid carcinoma is one of the most common malignancies of the endocrine system. In recent years, its incidence has increased worldwide, including children, which concerns the entire community. Although the histopathological diagnosis seems rather easy, the existence of particular forms of thyroid cancer and the inclusion of the follicular aspect as a variant of papillary carcinoma lead to diagnostic confusion. Therefore, in the last 20 years were reviewed several immunohistochemical markers, which are useful in the positive and differential diagnosis, and which offer better data on disease prognosis. Our study included a total of 27 cases of papillary carcinomas, which we evaluated the immunohistochemical expression of Ki-67, p53, p63, E-cadherin, CD56, calretinin, and bcl-2 markers. The most intense expression was found in p53, E-cadherin, and bcl-2. Ki-67 and p63 expression were moderate and inconsistent, and CD56 and calretinin had a negative expression in all cases.

Keywords: thyroid papillary carcinoma, Ki67, CD56 antigen, immunohistochemistry.

Introduction

Thyroid cancer is the most common malignancy of the endocrine system, with approximately 19 500 new cases diagnosed each year in the United States [1, 2]. In recent decades, there is a worldwide increased incidence of thyroid cancer [3]. In the United States, the incidence of thyroid cancer has increased continuously in the recent decades, with no difference based on ethnicity. Between 1974 and 2009, in the US, the incidence of thyroid cancer has nearly tripled, rising from 4.9 to 14.3 per 100 000 inhabitants [4]. In South Korea, thyroid cancer incidence began to increase after 2000, reaching to about 52.7 per 100 000 inhabitants in 2010 [5, 6].

Some researchers believe that the increased incidence of thyroid cancer is not real because the widespread use of ultrasound and other modern techniques of medical imaging, as well as thyroid-guided puncture have lately helped in the detection of thyroid asymptomatic nodules, therefore of some forms of thyroid cancer in the early stages, which could not be achieved three or four decades ago [4]. In addition, the mortality rate in this form of cancer is stationary [4]. However, such an increase in the incidence of thyroid cancer in a relatively short time and the discovery of the disease in children suggest that there is a real increase in the incidence of thyroid cancer in many areas of the world [7, 8]. It was also noted that the incidence of thyroid cancer is increasing faster in children, adolescents, and young adults [3].

Papillary thyroid carcinoma (PTC) is the most common thyroid cancer [9], reaching to up to 80% of malignant thyroid tumors [2, 10, 11]. Sometimes, thyroid cancer is difficult to differentiate from a benign papillary hyperplasia or some forms of thyroiditis. In recent years, many immunohistochemical markers have been used for differential diagnosis between benign and malignant lesions of the thyroid gland and for choosing the best therapeutic methods [12–14].

In this study, we have decided to evaluate the immunohistochemical expression of Ki-67, p53, CD56, p63, E-cadherin, calretinin and bcl-2 markers for the papillary thyroid cancer.

Materials and Methods

The study was conducted on a total of 27 thyroid fragments obtained from the same number of patients clinically and imagistically diagnosed with thyroid cancer. Patients were hospitalized in the Surgical Clinics in Emergency County Hospital of Craiova, Romania, during the years 2012–2013, after the clinical and imaging investigations needed, underwent surgical treatment consisting of total thyroidectomy. Immediately after removal of the thyroid, biological material was sent to the Laboratory of Pathology, Emergency County Hospital of Craiova, which was fixed in 10% formalin neutral solution for 48 hours, at room temperature, and processed using the classical histological paraffin-embedding techniques.

For the histopathological study, three stains were used: Hematoxylin–Eosin (HE), trichromic Goldner–Szekely (GS) green light technique and Periodic Acid Schiff–Hematoxylin (PAS–Hematoxylin).

For immunohistochemical study, out of the paraffin-embedded biological material, 3 μm thick sections were made using a microtome (HM350 Microm) equipped with a special transfer section (Section Transfer System, STS) to water bath. The sectioned biological material was collected on special histological blades coated with a
positively charged amino acid residues, slides coated with polylysine (poly-L-Lysine) (Sigma) in order to increase the blade adhesion of the cross-sections. After a period of rapid drying on a hot plate for 5–10 minutes, the sections were transferred to an incubator at 48°C and kept overnight, during which the biological material adhered perfectly to the surface of the histological slide.

On the second day, we began applying the classic immunohistochemical protocol, consisting of unwaxing and moisturizing of the sections. Antigen unmasking was performed by boiling sections in a sodium citrate solution, pH 6, for 21 minutes (seven cycles of 3 minutes) in a microwave oven. After cooling the prepared substances, staining protocol was continued by washing the slides in distilled water for 15 minutes (three baths of 5 minutes). To block the endogenous peroxidase, we incubated the slides in 3% hydrogen peroxide for 30 minutes at room temperature followed by washing in distilled water for 10 minutes and a wash in a solution of 1% phosphate-buffered saline (PBS), 5 minutes. Blocking non-specific sites was achieved by passing sections in a bath with 2% skimmed milk for 30 minutes. Thus prepared, the sections were incubated with primary antibodies for 18 hours (overnight) in a refrigerator, at 4°C. The next day we applied the secondary biotinylated antibody for 30 minutes at room temperature and then we performed a washing in 1% PBS (three baths of 5 minutes), and then applied to the Streptavidin–HRP for 30 minutes, at room temperature, followed by washing the slides in 1% PBS 3×5 minutes. The signal was detected using 3,3’-diaminobenzidine (DAB) (Dako) and the reaction was stopped in 1% PBS.

Contrasting with Mayer’s Hematoxylin followed, then dehydration in alcohol, xylene clarifying and mounting slides using DPX (Fluka). In our study, we used the following immunomarkers (Table 1).

### Table 1 – Antibodies used for immunohistochemical study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Code</th>
<th>Clone</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ki-67</td>
<td>M 7240</td>
<td>MIB-1</td>
<td>EDTA, pH 9</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-p53</td>
<td>M 7001</td>
<td>DO-7</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-CD56</td>
<td>M 7304</td>
<td>123C3</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:250</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-p63</td>
<td>M 7247</td>
<td>4A4</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-E-cadherin</td>
<td>M 7361</td>
<td>NCH-38</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:100</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-calretinin</td>
<td>M7245</td>
<td>DAK-Calretinin</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:50</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-bcl-2</td>
<td>F 7053</td>
<td>124</td>
<td>Sodium citrate buffer, pH 6</td>
<td>1:100</td>
<td>Dako</td>
</tr>
</tbody>
</table>

**Results**

In our study, the microscopic appearance of the neoplastic lesions was quite varied; most of the tumors were papillary projections of cubical-cylindrical cells placed on very fine conjunctive-vascular structures (Figure 1). The ratio between the epithelial and stromal component was clearly in favor of the epithelial cells. The shape and dimensions of the tumor cells were varied in the same patient; most cells were of cubical-cylindrical shape, but we also highlighted round cell, oval ones, or flattened ones, arranged in cords, islands or as microfollicles (Figure 2). In poorly differentiated papillary cancers cells had round, small shapes, and the nuclei were hyperchromic. Generally, tumor cell nuclei were round or oval shaped, larger than the normal thyroid cells and looked hypochromic in well-differentiated cancers. Chromatin looked fine, powdery and the nuclei were mostly eccentric. Often they reveal forms of “Orphan Annie eyes” nuclei, pinholes and nuclear grooves, giving nuclear nuclei the appearance of “coffee bean nuclei” (Figure 3). The combination of oval appearance of the nucleus, with hypochromasias, incisions and nuclear grooves, are important diagnostic features of thyroid cancer. In all the cases we studied, we identified more or less numerous microfollicular structures. However, there was no evidence that these follicular structures were the most tumor volume, which lead us to believe that all the cases were papillary thyroid cancers.

Some microfollicular structures contained eosinophilic (which stained intensely with eosin) and PAS-positive material, while other microfollicles did not stain with Eosin and were PAS-negative (Figures 2 and 4). The histopathology findings show that the thyroid tumor cells undergo genetic changes variations, some preserving their secretory capacity, while others have completely lost this ability. Although papillary carcinoma cells derive from thyroid follicular cells, most of them lose their secretory capacity.

Regarding the degree of tumor differentiation, we sometimes observe that the same histological slides there were well-differentiated papillary carcinoma areas and areas with poor or moderately differentiated carcinoma appearance. Poorly differentiated thyroid cancers looked as if they were composed of small cells arranged in islands, with smaller, rounded, and hyperchromic nuclei (Figure 5). Sometimes, at the periphery of the tumor tissue, we identified a “conjunctive capsule” made up of neatly arranged collagen fibers (Figure 5).

In order to evaluate the molecular aspects of tumor cells of papillary thyroid cancer, we conducted a number of six immunohistochemical tests.

It is known that cell proliferative activity is one of the important factors for evaluating the biological behavior of cancer cells. Currently, the most widely used marker for assessing the proliferative capacity of tumor cells is the Ki-67 antigen. In our study, immunohistochemical expression of Ki-67 antigen was very low, less than 5% of the nuclei of tumor cells were positive for this antigen, which explains the relatively low capacity for growth of this type of cancer.

One of the most affected genes in the process of carcinogenesis is the TP53 gene, which is expressed by change in the p53 protein. In our study, of the 27 cases of papillary carcinoma, a positive expression of p53 (Figure 6) was found in 12 (44.44%) cases, while for the other 15 (55.56%) cases, p53 protein expression was negative (Figure 7). The variability of response intensity shows a varying degree of alteration of the cellular genome. The presence of increased amounts of p53 protein...
in the nucleus and cytoplasm of tumor cells show an excessive production of p53 protein due to mutations occurring in the TP53 gene. In addition, overexpression of p53 shows a loss of the normal function of “guardian of the genome” for p53 through epigenetic mechanisms.

Another protein that intervenes both in the normal processes of cell proliferation and differentiation, and in neoplastic processes is the p63 protein. In our study, p63 was intensely positive only in three (11.11%) cases (Figure 8), moderately positive in four (14.81%) cases and negative in 20 (74.08%) cases. We believe that the importance of p63 protein in diagnostics of papillary thyroid cancer is much smaller than the p53 protein.

Given the fact that the loss of intercellular adhesion is overall a characteristic of tumor cells and it correlates with the loss of differentiation and a more aggressive tumor behavior, in this study, we investigated the presence and responsiveness of two molecules involved in this process, namely E-cadherin and CD56. From our images (Figure 9), E-cadherin was intensely expressed in the well-differentiated papillary thyroid carcinoma. Conversely, for the weak forms or moderately differentiated carcinoma, E-cadherin reaction had a much lower intensity, and in some areas, it was absent.

CD56 is a neural cell adhesion molecule found in the normal thyroid follicular epithelial cells where it seems to regulate cell motility. In our study, the immunohistochemical reaction of the CD56 marker for papillary thyroid cancer was negative in all cases (Figure 10). These data make us believe that tumor cells are unable to synthesize this protein due to genetic changes they underwent.

The calretinin expression has been less investigated in thyroid lesions, the immunohistochemical marker is found primarily in the retina, particularly in the nervous tissue. We investigated the presence of this antigen because we were looking for the presence of papillary carcinomas of neuroendocrine cells. The immunohistochemical expression was negative in all cases.

Bcl-2 protein is regarded as an important anti-apoptotic protein; its expression is encoded by an oncogene. The deterioration of the bcl-2 gene, outlined by genetic studies, has been identified in a number of cancers and the immunohistochemical expression of the bcl-2 protein has always been found in genetic modifications. Investigation of this protein in papillary thyroid carcinoma is still precarious. In our study, of the 27 cases of papillary carcinoma, five had an intense expression of bcl-2 (Figure 11), four cases showed a moderate expression, and three cases a weak expression of the bcl-2 protein. Negative expression of the bcl-2 protein (Figure 12) was identified in 15 (55.55%) cases.

Figure 1 – Well-differentiated papillary thyroid carcinoma, with cubical-cylinder cells, arranged along fine connective septa. Trichromic GS staining, ×200.

Figure 2 – Papillary carcinoma with cells arranged in cords, islands, or follicles. HE staining, ×200.

Figure 3 – Thyroid papillary carcinoma image, in which we can notice the hypochromasia presence, the pinholes, and nuclear grooves, and “Orphan Annie eyes” nuclei. HE staining, ×600.

Figure 4 – Papillary carcinoma with microfollicles containing PAS-positive and PAS-negative. PAS–Hematoxylin staining, ×200.
Figure 5 – Poorly differentiated papillary carcinoma area with cells arranged in islands or nests, separated by fine connective septa. At the periphery of the tumor structure there can be seen a conjunction with peritumoral capsule appearance. Trichromic GS staining, ×200.

Figure 6 – Papillary thyroid carcinoma image with intense reaction to p53 antibody. Immunostaining with anti-p53, ×100.

Figure 7 – Papillary thyroid carcinoma with p53 negative reaction. Immunostaining with anti-p53 antibody, ×200.

Figure 8 – Papillary thyroid carcinoma with intense reaction to p63. Immunostaining with anti-p63 antibody, ×200.

Figure 9 – Papillary thyroid carcinoma with E-cadherin intense reaction. Immunostaining with anti-E-cadherin antibody, ×200.

Figure 10 – Papillary thyroid carcinoma well differentiated negative reaction to CD56. Immunostaining with anti-CD56 antibody, ×200.
Discussion

The papillary thyroid carcinoma is a tumor with insidious evolution, most often asymptomatic, incidentally discovered most often by ultrasound examination of the anterior region of the neck. The tumor occurs more frequently in women, and the peak incidence is between 35 and 55 years. In recent years, the incidence of thyroid cancer has increased, ranking the sixth form of cancer in women in the US, in 2009 [15–17].

Although the histopathological diagnosis of the papillary thyroid carcinoma seems relatively easy due to changes in the thyroid tissue architecture, introducing the follicular shape as a variant of papillary thyroid carcinoma created some confusion among pathologists. In addition, the existence of inflammatory lesions such as Hashimoto’s thyroiditis or thyroid adenoma may create confusing diagnoses [9]. Follicular or encapsulated variants of papillary thyroid carcinoma are often a diagnostic challenge, in which the differential diagnosis includes other follicular lesions, such as follicular adenoma and follicular carcinoma, because the microscopic criteria of differential diagnosis are sometimes difficult to apply, and they are subject to a high degree of subjectivity on behalf of the pathologists. [18–20].

In our study, we selected only those papillary thyroid carcinomas in which the positive and differential diagnosis was clear. We also excluded from the study the follicular variant of papillary thyroid carcinoma in order to reduce diagnostic confusion.

At present, it is known that all papillary carcinomas and follicular variants arise from the follicular cell, but the mechanism of carcinogenesis is not yet specified. Also, the etiopathogenic conditions involved in the malignant transformation of the follicle cells are not known. It is admitted that environmental factors and genetic predisposition are involved in thyroid carcinogenesis. Among environmental factors, the most involved are the ionizing radiation, diet, and carcinogenic chemicals. Among the internal factors chronic inflammatory processes (Hashimoto’s thyroiditis), obesity, adipokines, insulin resistance and sex hormones have been involved lately [21–24].

In the past 20 years, numerous studies of molecular biology, genetics and immunohistochemistry sought to better characterize thyroid carcinoma, to increase positive and differential diagnostic accuracy, for the correct therapeutic approach in each case of thyroid tumor.

Ki-67 is one of the most commonly used immunohistochemical markers in the differential diagnosis between benign and malignant tumor lesions in human pathology. The thyroid neoplasm, the Ki-67 expression was assessed by several studies, but their results are conflicting. Thus, some studies show that lesions of thyroid tumors are characterized by a high Ki-67 expression [25], while other studies show that there is no significant difference in the expression of Ki-67 between thyroid carcinoma and adenoma [26]. Song et al. (2011) [15] showed that there is no statistically significant difference between the Ki-67 marker expression in benign thyroid lesions (nodular goiter or follicular adenoma) and papillary thyroid carcinoma. In our study, Ki-67 expression was very low, which suggests a slow evolution of this type of tumor and a favorable prognosis after the appropriate treatment.

Regarding p53 immunohistochemical reaction, it should be noted that it is an altered TP53 gene. After some studies [27, 28] in about 50% of all human cancers, mutations of TP53 tumor suppressor gene are found, and it is the most frequent genetic alteration present in malignant cells.

The modifying of p53 protein expression in thyroid carcinoma seems correlated with the degree of tumor differentiation. Thus, in poorly differentiated carcinomas, the overexpression of p53 protein can be found between 40% and 62%, while in well-differentiated carcinomas, it does not exceed 25% [29]. In our study (which examined the protein p53 expression depending on the degree of tumor differentiation), the overexpression of p53 protein was found in 44.44% of patients, while in 55.56% of cases, p53 protein expression was negative. Our data are similar to those reported by other authors [11, 30]. After some studies, TP53 gene mutations occur quite late in the process of thyroid carcinogenesis [31]. However, an intensely positive reaction of p53 protein is an aggravating prognostic factor in thyroid tumor lesions.

Studies on the involvement of p63 protein in thyroid tumor processes are few and they have contradictory results. P63 protein is a member of p53 family of trans-
cription factors having a major role in regulating the epithelial proliferation and differentiation processes [32, 33]. It attaches itself to specific regions of DNA and controls the gene activity nearby. Immunohistochemical expression of p63 is evident in some types of carcinomas [34, 35]. Studies on p63 protein reaction in thyroid cancers show that this positivity can go from 6.9% [36] to 74% [37]. In our study, p63 appeared positive only in seven cases, accounting for only 25.92% of papillary carcinoma studied. Some studies have found a higher positivity of p63 in undifferentiated forms of thyroid cancer or sclerosis-diffuse forms of papillary thyroid carcinoma. Other authors [38] consider that p63 could promote proliferation, invasion, and metastasis of malignant cells of papillary thyroid cancer.

E-cadherin is one of the most investigated intercellular adhesion molecules, as it is involved in the process of morphogenesis of epithelial tissues. In normal thyroid epithelium, it is highly and uniformly expressed on the surface of follicular cells [39]. In our study, we observed an intense reaction of E-cadherin in well-differentiated papillary carcinomas and very weak in poorly differentiated carcinomas, which shows that E-cadherin may be used as a marker of cell differentiation in thyroid cancers. According to some authors [39–41], reducing the E-cadherin expression is associated with the ability of remote metastases, and with local recurrences thyroid carcinoma.

CD56 molecule is an adhesive glycoprotein found on the surface of neurons, glial cells, skeletal muscle cells, and on the surface of epithelial cells. It acts physiologically in the intercellular adhesion processes or cell–matrix adhesion. In our study, for all papillary thyroid carcinoma cases, the immunohistochemical expression of CD56 was negative. Several studies have shown that in the thyroid, CD56 is present on normal follicular cells and in benign lesions, but it is absent in papillary carcinoma [9, 41–44]. Our study confirms these data.

Calretinin is a diagnostic marker for certain human diseases, nerve tissue diseases, and some forms of cancer. Rating the expression of the calretinin in the thyroid tumors was less investigated. Nasr et al. (2006) [2] showed that calretinin was positive in three out of 10 papillary thyroid carcinomas, but had no positive expression in the benign thyroid tumors. In our study, all 27 thyroid papillary carcinoma cases had negative expression in calretinin.

Regarding the bcl-2 protein expression in thyroid tumors, medical database provide little studies. A recent study [45] showed that in 70 adult papillary thyroid carcinomas occurred in Ukraine after the Chernobyl irradiation, 53 cases had bcl-2 immunohistochemical positive reaction. Also, the same authors found that there was a positive correlation between the expression of bcl-2 and BRAF mutations found at the genetic level.

**Conclusions**

Papillary thyroid carcinoma is a tumor that seems easy to diagnose histologically if the main morphological features are outlined: the presence of papillary projections, with the development of mostly epithelial tumor compared to stroma, the presence nuclear hypochromia in notch ditches, and in nuclear inclusions, and also of “Orphan Annie eyes”. In our study, even for classic papillary carcinomas formations, we identified quite frequently microfollicular colloid formations, containing PAS-positive eosinophilic colloid, or, on the contrary, uncolored colloid, giving the appearance of “empty follicle”. These histopathological changes are the expression of multiple genetic changes, which occur during the process of thyroid carcinogenesis. Among immunohistochemical markers investigated for papillary thyroid carcinomas, the most intense and constant expressions were those of protein p53, E-cadherin, and bcl-2. A moderate but less frequent expression was shown by p63 and Ki-67, while CD56 and calretinin were negative in all samples.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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