Papillary thyroid cancer stroma – histological and immunohistochemical study

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Abstract
Thyroid cancer is the most frequent endocrine neoplasia. The incidence of the disease has been increasing in the past few decades in many regions, especially where the population was subject to some forms of accidental exposure. Among all the histopathological forms, papillary thyroid cancer (PTC) is the most common histological subtype of malignant thyroid tumor, representing about 80–90% of all malignant thyroid tumors. Although it is generally accepted that tumor stroma plays an essential role in the development and metastasis of tumor cells, histopathological studies focused on tumor cells characteristics. In this study, we evaluated the characteristics of tumor stroma histopathologically and immunohistochemically examining a total of 18 cases of papillary thyroid carcinomas, of which 18 cases were classic papillary carcinomas, 11 cases papillary carcinomas, follicular forms, five cases were papillary carcinomas – formed with tall-cells, three cases of papillary carcinomas, solid variants and one case was interpreted as an oncocytic variant. Most papillary carcinomas have been typically characterized by the presence of neoplastic papillae, composed of a central axis of fibro-vascular, branched, and coated by one or more layers of cubic or prismatic epithelial cells. In three typical papillary carcinomas, the stroma was composed of coarse connective axes rich in collagen fibers. The predominantly fibrous stroma, consisting of connective septum was observed in four cases, while in one case of papillary carcinoma, solid variant, we have identified a hyaline stroma; in one case was revealed a myxoid stroma. Diffuse stromal calcifications have been identified in two cases only. In the tumoral stroma, there were identified inflammatory infiltrates in nine cases, formed mostly of lymphocytes, and in one case, there was observed the presence of aggregated lymphoid nodules. The immunostaining with anti-CD34 antibody showed that in papillary thyroid carcinomas there is a well-represented vascularity, mostly made of small vessels (arterioles, venules, capillaries) with diameters between 7 and 50 μm, and immunostaining with anti-vimentin and anti-α-SMA antibody showed an increased number of fibroblasts, respectively myofibroblasts in the tumoral stroma. We believe that in the same thyroid tumor there are several clones of neoplastic cells that reshape the stroma, giving it certain histopathological characteristics.

Keywords: papillary thyroid carcinoma, tumoral stroma, immunohistochemistry, thyroidectomy, trabecular pattern.

Introduction
Thyroid cancer represents, worldwidely, about 1% of all epithelial malignancies and it is considered to be the most common endocrine neoplasia [1–3]. In the last 15–20 years, the incidence of the disease increased progressively in many countries, due to the improved methods for detection of the disease, mainly through increased accuracy of thyroid ultrasound and application of fine-needle aspiration, followed by cytological examination [4–6]. After some studies, increased incidence of thyroid cancer in children less than 20 years shows that the increase in the incidence of thyroid cancer is not apparent (due to increased diagnostic accuracy), but it is real [7–11].

Of all the histopathological forms, papillary thyroid cancer (PTC) is the most common histological subtype of thyroid malignancy, representing about 80–90% of all thyroid malignancies [12–15].

In assessing the microscopic characteristics and correlations with the clinical examination, the histopathological studies focused on the characteristics of tumor cells, the nucleus aspect and genetic changes, and less on the architectural aspects and stromal changes. Until now, histopathological studies have described a number of variants of papillary thyroid carcinoma: classic, follicular, solid, encapsulated, diffuse sclerosing, tall-cell, cylindrical cell papillary, etc. [16–19]. Although it is generally accepted that tumor stroma plays an essential role in the development and metastasis of tumor cells, there are few studies that have addressed stromal changes in papillary thyroid cancer.
In our study, we analyzed some histological and immunohistocemical aspects of tumor stroma in papillary thyroid cancer.

Materials and Methods

The study was conducted on a total of 38 thyroid tumors obtained from the same number of patients, clinically and imagistically diagnosed with thyroid tumors, hospitalized in the Surgical Clinics of the Emergency County Hospital, Craiova, Romania, during the years 2010–2015. Immediately after the surgical excision, thyroid fragments were fixed in 10% neutral formalin solution and sent to the Laboratory of Pathological Anatomy, Emergency County Hospital, Craiova, where they were worked in classic histological technique of inclusion in paraffin. The sectioning of the biological material was made using a Microm HM325 microtome rotary, equipped with a section transfer system on a water bath (STS, microM) and Peltier cooling system. For the histopathological study, there were used two stainings: Hematoxylin–Eosin (HE) and light green trichrome, Goldner–Szekely (GS) technique. For the immunohistochecmical study, from the biological material embedded in paraffin, there were made sections of 3-μm thickness that were collected on histological slides coated with Polysine™ (poly-L-lysine, Sigma), in order to increase the adhesion of sections to slides. The histological cups were transferred to an incubator at 45°C and kept overnight (18 hours), during which the biological material adhered perfectly to the surface of the histological slide. The next day, the classic immunohistochemical protocol was applied, consisting of dewaxing and hydration of sections, followed by antigen unmasking by boiling the sections in a sodium citrate solution pH 6 in a microwave for 21 minutes (seven cycles of 3 minutes). Next, there was performed the endogenous peroxidase blocking by incubating the biological material in 3% hydrogen peroxide for 30 minutes at room temperature, followed by washing in distilled water for 10 minutes and a wash in a 1% phosphate-buffered saline (PBS) solution, for 5 minutes. The blocking of non-specific sites was achieved by passing sections into a 2% skim milk bath for 30 minutes. Then, the sections were incubated with primary antibodies, for 18 hours (overnight) in a refrigerator at 4°C, and the next day the biotinylated secondary antibody was applied for 30 minutes at room temperature. After washing the biological material with 1% PBS (three baths of 5 minutes), the Avidin-HRP (Horseradish peroxidase) was applied for 30 minutes, at room temperature, followed by the washing in 1% PBS 3×5 minutes. The signal was detected by using 3,3’-diaminobenzidine (DAB, Dako). The contrast washing in 1% PBS 3×5 minutes. The signal was detected by using 3,3’-diaminobenzidine (DAB, Dako). The contrast with Mayer’s Hematoxylin, dehydration in alcohol, xylene clarification and slide mounting using DPX medium (Fluka) were next. For the immunohistochecmical study, there were used the following antibodies: anti-vimentin (clone V9, 1:50, Dako), anti-α-SMA (clone 1A4, 1:100, Dako), anti-CD34 (clone QBEnd10, 1:50, Dako), anti-thyroglobulin (clone DAK-Tg6, 1:200, Dako), anti-p53 (clone DO7, 1:50, Dako), anti-E-cadherin (clone NCH-38, 1:100, Dako) and anti-Bcl-2 (clone 124, 1:100, Dako).

Results

Of the 38 fragments of thyroid tumors harvested after surgery, the histopathological study allowed us to diagnose the following variants of papillary carcinomas, 18 cases of classic papillary carcinomas, 11 cases as papillary carcinomas – follicular form, five cases were interpreted as papillary carcinomas – tall-cell forms, three cases as solid variants and one case – oncocytic variants.

The most commonly experienced tumoral cells were of prismatic or column form, with round or oval nuclei, hypochromic, with grooves and intranuclear inclusions in about 60–65% of cases.

The microscopic aspect of papillary thyroid carcinomas varied a lot from one patient to another. In addition, even in the same patient, the microscopic aspect of the tumor was variable, with conventional forms of classic papillary carcinomas, associated with areas of tall-cell or follicular papillary carcinomas (Figure 1). We believe that these microscopic aspects are due to the occurrence of different tumor cell clones under the influence of the same etiopathogenic factors.

Most papillary carcinomas were typical, well differentiated, characterized by the presence of neoplastic papillae, consisting of a central axis of fibro-vascular of various sizes, frequently branched, coated by one or more layers of cubic or prismatic epithelial cells (Figure 2). The same aspect of fine stroma was observed in the follicular variant of most papillary carcinomas (Figure 3).

In three typical papillary carcinomas, the stroma was composed of coarse connective axes, rich in collagen fibers and with low vascularity (Figure 4), and in one case a myxoid stroma was revealed (Figure 5).

Most cases of papillary cancers were presented as nodular formations bounded by a peripheral fibrous capsule, made of collagen fibers, that limits the tumoral lesion from the rest of the thyroid gland. The thickness of this capsular structure was varied: in some cases, it was very thick, while in others only sketched. A histopathological feature observed in all papillary cancers was the fact that this capsular structure was coated by 1–2 rows of tumor cells (Figure 6). Of 38 cases of papillary carcinomas evaluated, in 16 (48%) cases we identified tumoral cells penetrating or infiltrating the capsular structure in the form of islands of tumor cells, or as tumoral chords (Figures 7 and 8).

The predominantly fibrous stroma, consisting of wider or narrower connective septa that divided the tumor structure into compartments was observed only in four cases (Figure 9), while in one case of papillary carcinoma, solid variant, we identified a hyaline stroma (Figure 10). Diffuse stromal calcifications were identified only in two cases (Figure 11).

In the tumoral stroma, there were identified inflammatory infiltrates in nine (24%) cases, formed mostly of lymphocytes, mostly arranged at the periphery of the tumor, subcapsularly, of mild and moderate intensity (Figure 12). In one case, there was observed the presence of aggregated lymphoid nodules (Figure 13).

The evaluation of tumoral stroma microvascularity
was performed by using the anti-CD34 antibody that marks the endothelial cells. Contrary to the histopathological aspects obtained in classic staining, the immunohistochemical study showed that in papillary thyroid carcinomas there is a well represented vascularity, mostly formed of small vessels (arterioles, venules, capillaries) with diameters between 7 and 50 μm, some in intimate contact with the basal membrane of the papillary growths (Figures 14 and 15).

Also, in the stroma, we identified many CD34 positive, isolated cells, unrelated to stromal vascular structures.

The highlighting of fibroblastic cells was performed by using the anti-vimentin antibody. A large amount of connective cells were identified in the coarse connective septa and in the tumoral capsule (Figure 16), and around the follicular structures in the follicular variant of papillary carcinomas (Figure 17). A variant of stromal fibroblasts, involved in the synthesis of connective matrix is represented by myofibroblasts. The highlighting of myofibroblastic cells was performed by using the anti-α-SMA (anti-alpha-smooth muscle actin) antibody. In all papillary carcinomas, there were highlighted large amounts of myofibroblasts in the connective stroma (Figures 18 and 19). A positive reaction to α-SMA was also recorded in the blood vessels due to the presence of α-actin in the structure of pericytes.

The use of thyroglobulin, anti-p53, anti-Bcl-2 and anti-E-cadherin antibodies allowed us to identify isolated and metastatic tumor cells, disseminated in connective septa, and present intratumorally in the tumoral capsule or at distance, in the perithyroidal tissues (Figures 20–23).
Figure 5 – Typical papillary carcinoma with myxoid stroma. GS trichrome staining, ×400.

Figure 6 – Papillary carcinoma confined to the periphery of a fibrous capsule, well developed, rich in collagen fibers, where many nests of tumor cells are identified. On the front tumor capsule there is observed a row of tumor cells lining the capsule. GS trichrome staining, ×100.

Figure 7 – Islands of tumor cells penetrating into the capsule. GS trichrome staining, ×400.

Figure 8 – Cords of papillary carcinoma infiltrating the capsule. GS trichrome staining, ×100.

Figure 9 – Papillary carcinoma with fibrous stroma. HE staining, ×100.

Figure 10 – Hyaline stroma in a papillary carcinoma: solid variant. HE staining, ×200.
Figure 11 – Tumor stroma with diffuse calcifications. HE staining, ×200.

Figure 12 – Moderate inflammatory infiltrate present in the tumoral stroma. HE staining, ×200.

Figure 13 – Aggregated lymphoid nodules present in the tumoral stroma. HE staining, ×40.

Figure 14 – Typical papillary carcinoma with a well-developed vasculature. Anti-CD34 antibody immunostaining, ×100.

Figure 15 – Tall-cell papillary carcinoma, with many blood capillaries in the stromal connective axis. Anti-CD34 antibody immunostaining, ×200.

Figure 16 – Papillary carcinoma with coarse connective septa, with many connective cells intensely reactive to vimentin. Anti-vimentin antibody immunostaining, ×100.
Figure 17 – Papillary carcinoma, follicular variant, with intense reaction of stromal cells to vimentin. Anti-vimentin antibody immunostaining, ×100.

Figure 18 – Papillary carcinoma with connective stroma rich in myofibroblasts. Anti-α-SMA antibody immunostaining, ×200.

Figure 19 – Myofibroblastic cells of large sizes, intensely reactive to α-SMA, unevenly disseminated in the papillary connective stroma. Anti-α-SMA antibody immunostaining, ×400.

Figure 20 – Papillary thyroid carcinoma with intense reaction to thyroglobulin. Anti-thyroglobulin antibody immunostaining, ×100.

Figure 21 – Disseminated tumor cells in the extrathyroidal connective stroma positive to E-cadherin. Anti-E-cadherin antibody immunostaining, ×200.

Figure 22 – Thyroid stroma infiltrated with many tumor cells positive to Bcl-2. Anti-Bcl-2 antibody immunostaining, ×200.
Discussion

Papillary thyroid cancer, like any other solid tumor, is composed of two separate, still interrelated, components: carcinoma cells and tumoral stroma. The tumoral stroma is reshaped by tumoral cells in order to ensure the growth, proliferation, invasion of the neighboring tissue structures and distant metastasis. In the last 10–15 years, more and more studies have pointed out that the tumoral stroma plays an important role in tumor phenotyping and that the stromal compartment is essential for tumor development. This knowledge opened new areas of research that could improve the diagnosis, treatment and prognosis of cancer [20–22].

The tumoral stroma we described above in papillary thyroid cancer is totally different from the normal thyroid gland stroma, which is mostly made up of loose connective tissue. Most of the papillary thyroid carcinomas we studied showed growths made up of fine connective axes, coated by tumor cells. However, in the papillary cancer variants identified by us, we found a stroma consisting of a whole extracellular matrix with multiple stromal cells, particularly fibroblasts and myofibroblasts, inflammatory cells and blood vessels. We believe that the formation of tumoral stroma is a complex process involving both tumor cells and stromal cells, between the two types of cells existing bidirectional relationships. The genetically altered epithelial cells (cancer cells) have the ability to change the behavior of the host stromal cells, so as to create a supportive environment and at the same time lead to proliferation, migration and metastasis of tumor cells, which makes cancer a pathological entity that invades the body.

According to some studies, fibroblasts and myofibroblasts are main connective cells that cooperate with tumor cells, being capable of producing all elements of the extracellular connective matrix, synthesizing and secreting growth factors and cytokines and promoting the formation of new blood vessels [23–25].

The basic fibroblast growth factor is one of the cytokines secreted by various normal, inflammatory or tumor cells, which act as a mitogenic factor for smooth muscle cells, fibroblasts and myofibroblasts, involved in the intercellular signaling processes, in angiogenesis, neoplastic invasion and metastasis [26–29].

We believe that the occurrence of a fibrous stroma is due to the stimulation of fibroblasts and myofibroblasts for producing fibrillar collagen in higher quantities. Some studies showed that the interaction between tumor cells and myofibroblasts is achieved through direct cell-cell contacts, but also through paracrine signals [30]. Myofibroblasts, commonly called “activated fibrocytes” were been identified in many types of cancers. According to some studies, in certain types of cancer, the presence of a large number of fibroblasts in the tumoral stroma is associated with an increased aggressiveness of the tumor, with a poor prognosis and with more frequent local recurrences [31].

Fibroblasts and myofibroblasts are the main cells that reshape the tumoral stroma. The change in the activity and phenotype of fibroblasts and their transformation from normal fibroblasts into “tumor fibroblasts” (often called “carcinoma-associated fibroblasts”) apparently occurs in the early stages of carcinogenesis [32]. The two types of connective cells, along with inflammatory cells and vascular endothelial cells create a “reactive stroma” that initially is not specific to tumor, but is similar to that which occurs in healing any wounds and plays a role in restoring local tissue homeostasis [33–35]. Fibroblasts and myofibroblasts synthesize and secrete a multitude of molecules (collagen I, collagen III, fibronectin, tenascin, etc.) that contribute to the formation of the tumoral stroma [32, 36], and matrix-metalloproteinases (MMPs) that reshape the extracellular matrix and create the environment for the development and metastasis of tumor cells [37–40].

In our study, using the anti-vimentin and anti-α-SMA antibodies, we identified many fibroblasts, respectively myofibroblasts, in the tumoral stroma, thus confirming that in papillary thyroid cancer these cells interfere in the formation of tumoral stroma. The origin of these carcinoma-associated fibroblasts is an issue that triggered many controversies. Most studies consider that fibroblasts have a mesenchymal origin, being in fact local fibroblasts changed by the relationship with neoplastic cells [41]; other studies suggested that they originate in stem cells from the red bone marrow [42, 43] or even in endothelial cells [44].

In our study, we identified two cases of papillary thyroid cancer with diffuse calcifications in the stroma. Some studies showed that calcification is a common histological feature of papillary thyroid carcinoma, but not a defining characteristic of papillary cancer, because stromal calcifications were also reported in benign thyroid lesions [45, 46]. They are caused by calcium phosphate salts deposits in the fibrous stroma. The clinical significance of stromal calcifications in papillary thyroid carcinomas remains unclear. Relatively recent studies showed that certain clones of cells of papillary thyroid carcinoma are able to synthesize bone morphogenetic proteins, capable of inducing stromal calcifications and even bone tissue formation in the thyroid tumoral stroma [47].

Another particular aspect of the tumoral stroma in papillary thyroid cancers observed by us was the presence of...
of chronic inflammatory infiltrates, composed mainly of lymphocytes and plasma cells. In one case, we found aggregated lymphoid nodules. We believe that lymphoid infiltration is an immune response to certain tumor antigens. Although it is a defense reaction of the host against neoplastic cells, many studies showed that stromal lymphoid cells can produce growth factors that stimulate the growth of tumor cells and facilitate the development of a stromal microenvironment favorable to tumor growth and metastasis [48].

The use of anti-thyroglobulin, anti-p53, anti-Bcl-2 and anti-E-cadherin antibodies allowed us to identify isolated tumor cells or islands of disseminated tumor cells in the stroma or in extrathyroidal soft tissues and make the difference with other types of stromal cells.

S Conclusions

In this study, we evaluated the peculiarities of tumor stroma by histopathologically and immunohistochemically examining a total of 38 cases of papillary thyroid carcinomas. Most papillary carcinomas were typical, characterized by the presence of neoplastic papillae, consisting of a central axis of fibro-vascular, branched, and coated by one or more layers of cubic or prismatic epithelial cells. A predominantly fibrous stroma, consisting of connective septa was observed in four cases, while in one case of papillary carcinoma, we have identified a hyaline stroma; in one case was revealed a myxoid stroma. Diffuse stromal calcifications were identified only in two cases. In the tumoral stroma, there were identified inflammatory infiltrates in nine cases, formed mostly of lymphocytes, and in one case, there was observed the presence of aggregated lymphoid nodules. The immunostaining with anti-vimentin and anti-α-SMA antibody showed an increased number of fibroblasts, respectively myofibroblasts in the tumoral stroma. We believe that in the same thyroid tumor there are several clones of neoplastic cells that reshape the stroma, giving it certain histopathological characteristics.

Conflict of interests

The authors declare that they have no conflict of interests.

References

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