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*Review*

# Angiogenesis in thyroid malignant neoplasm: State of the art and advances of the modern digital pathology and nanotechnology

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**It is well known that both radiation and genetic factors are interconnected and significantly impact the morbidity of thyroid cancer and that is why Belarus has the highest incidence of that malignancy. Author describes statistical data, classification of angiogenesis and thyroid cancer, typical pathological features of malignant thyroid diseases relating to the vascular network. Nanotechnology developed for molecular imaging, differential diagnosis and drug delivery applied for thyroid cancer investigation and the free open-source image processing software applications designed for the quantitative evaluation of histological samples are discussed.**

**Key words:** Thyroid gland, angiogenesis, cancer, malignancy, image processing, tumor expansion, nanotechnology, radiation effects.

## INTRODUCTION

### Thyroid cancer statistics, the global burden of malignant disease

Thyroid cancer is multifactorial disease (Pearce and Braverman, 2009; Sipos and Mazzaferri, 2010), comprises 1 to 2% of all malignancies and occurs worldwide. 4 years after the Chernobyl disaster of April 1986, thyroid malignancy cases were noted to have increased in the surrounding regions with incidence rising 100-fold downwind in Belarus and sevenfold in the upwind regions of the nearby Ukraine (Baker and Bhatti, 2006). Although less than five cases were reported between 1986 and 1989, there were 29 cases in 1990, 55 in 1991, and 62 in 1992 (Moysich, 2002). The radioactive contamination of Ukrainian territory by I-131 was varied in 2–5 Ci/square km (Ci = activity of 1 g of radium-226). The dependence of the total number of thyroid cancer cases on time after exposure is similar in the highly contaminated areas of Belarus and Ukraine (Jacob et al., 2002). The main contribution to the inner radiation dose was caused by various isotopes of iodine (Dikiy et al., 2002). It is suggested that Chernobyl fallout had influenced cancer rates in several Asian, European and Scandinavian countries, including England (Cotterill et al.,, 2001), Denmark (Hou et al., 2003), Turkey and Greece (Moysich et al., 2002). 246,347 cancers have been diagnosed in 2007 in UK (United Kingdom) In 2006, 1,933 British people suffered thyroid cancer. Thyroid cancer is within the top 20 most common cancers for UK females (number 18), with 1,421 new cases diagnosed in 2006. This compares to 512 cases in males - giving a male: female ratio of 1:3. It has been estimated that the lifetime risk of developing thyroid cancer is 1 in 842 for men and 1 in 324 for women in the UK. These were calculated on February 2009 using incidence and mortality data for 2001-2005. Interestingly, the average annual age-standardised incidence rate (per 100,000) was 8.3 in Kuwaiti and 4.7 in non-Kuwaiti women. Similarly high relative frequency and rates of thyroid cancer among women have also been reported from other Arab countries in the Gulf region (Oman, Qatar, Saudi Arabia, United Arab Emirates) (Memon et al. 2004).

Thyroid cancer is rare in children, while in adults the incidence rates rise steadily with age. Although the rates are highest in the over 75s, there is a substantial number

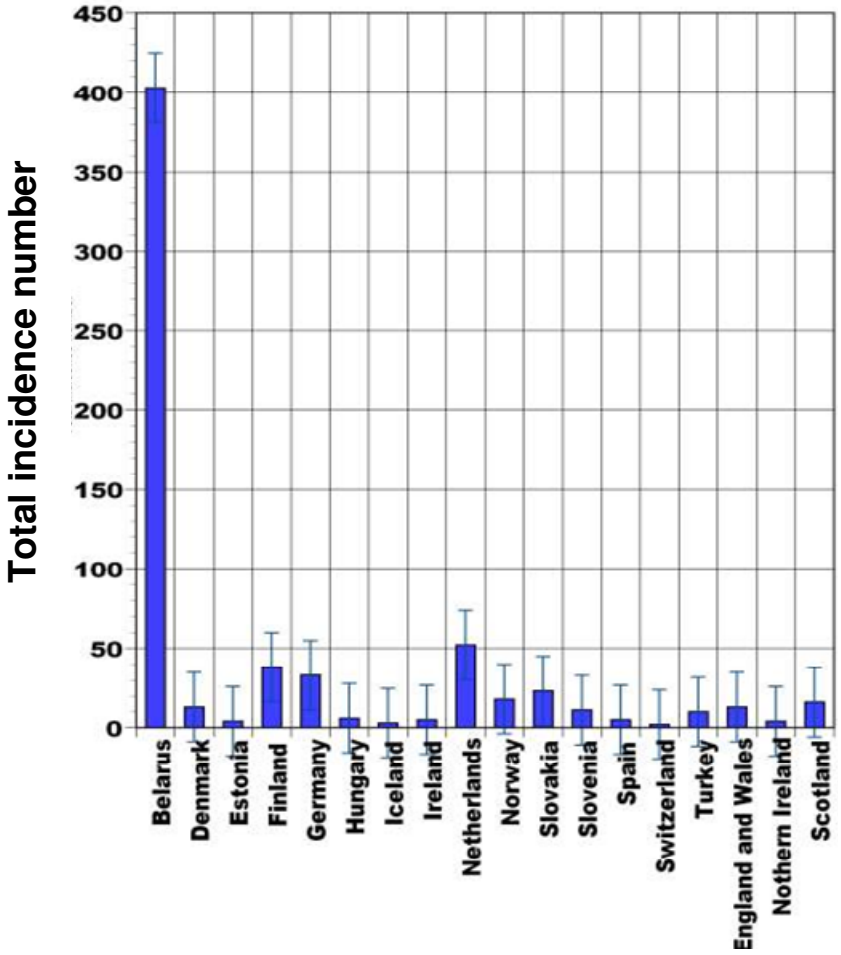


**Figure 1.** Pediatric thyroid cancer statistical data (1993-1997). Graphic is built from the European cancer registry; However, registry does not contain epidemiology on juvenile thyroid cancer in Russia and Ukraine.

of cases at younger adult age. Almost half (48%) of all cases occur in people aged less than 50 years. In the UK, the age-standardized incidence rates have increased from 1.8 to 2.9 per 100,000 population between 1993 and 2006. There has been a larger increase in female incidence rates, from 2.4 to 4.2 per 100,000 population. The highest rates for thyroid cancer in the world occur in Northern America, where the female age-standardized rate is 8.1 per 100,000 females, compared with 1.4 per 100,000 females in Western Africa. Incidence is low in all parts of Africa. Thyroid cancer incidence per million person-years at age 15–19 years in Africa during the 1993-1998, was 3.7 and 6.7 for males and females, respectively. For comparison, similar incidence for females of Hong Kong was 34.8, Shanghai 10.4, and Japan 10.6 (Stiller, 2007).

The registered case number of pediatric thyroid cancers in Belarus during 1993-1997 consisted of 403 cases, 317 of them occurred in children aged 10-14. In 2002, more than 1000 cases of thyroid cancer have been reported among approximately 2 million children younger than 15 years, who were exposed to radioactive fallout. In the Gomel region, the most contaminated area of Belarus, the incidence between 1986 and 1996 was 13 per 100000 children per year (Pacini et al., 2002). The current statistics are not published yet and the expected numbers are unknown (Figures 1 and 2). The second leading place in that epidemiologic cohort belongs to Netherlands, with 52 cases (29 persons in 15-19 age groups). During the described time period no juvenile thyroid cancer was reported in Switzerland (Anonymous, 2010c).

After exposure to external irradiation, the projected overall lifetime incidence of fatal thyroid cancer would be 7.5 cases per 0.01Gy (Gray) absorbed dose to the thyroid in a general population of one million persons. Ethnic background was found to influence the risk of radiation-induced thyroid cancer (Figure 3); for example,

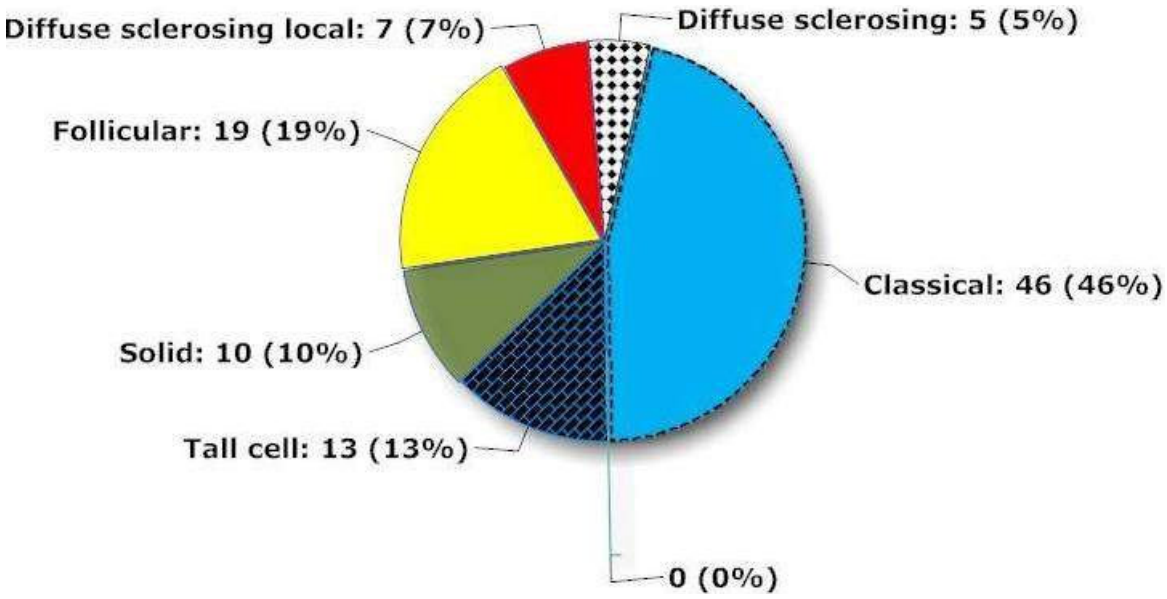


**Figure 2.** Pathology of sporadic juvenile papillary cancer in Belarus (Data from 95 patients).

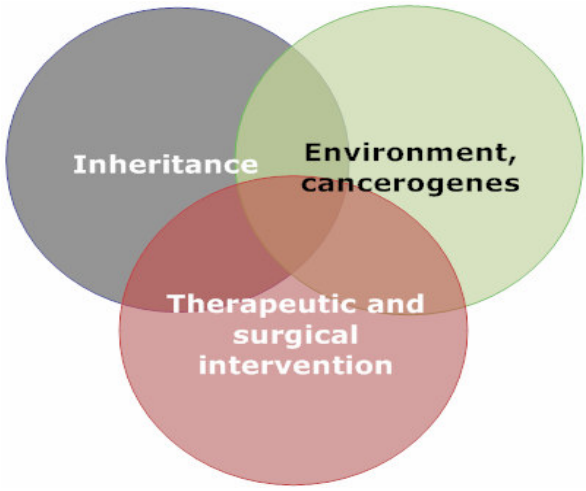


**Figure 3.** Diagram of the fundamental determinants of tumor origin, expansion and outcome.

the relative risk for Jews compared to non-Jews was about 3.5 after adjusting for gender, time since exposure, and dose (National Council on Radiation Protection and Measurements; Scientific Committee and National Council on Radiation Protection and Measurements, 2008). It had been known since 1930 that capillaries are radiosensitive structures and Ahmad et al. (2003) showed that ionizing radiation decreases capillary-like structure formation by endothelial cells *in vitro*. To the author's knowledge, no studies were performed exploring the patterns of angiogenesis in thyroid tissues exposed to ionizing radiation and toxic agents.

## PATHOLOGICAL CLASSIFICATION OF THYROID CANCER

Normal follicular cells are arranged in follicles or monolayer sheets with a honeycomb patter, well-defined borders and polarized nuclei (Gimm, 2001). It is the author's opinion, that before the comprehensive and type-specified discussion of angiogenesis in thyroid tumors, it is essential to describe the classification of the diseases on which the review is focused.

According to Baloch and LiVolsi (2008), papillary group primary thyroid cancer includes the following (Figures 4

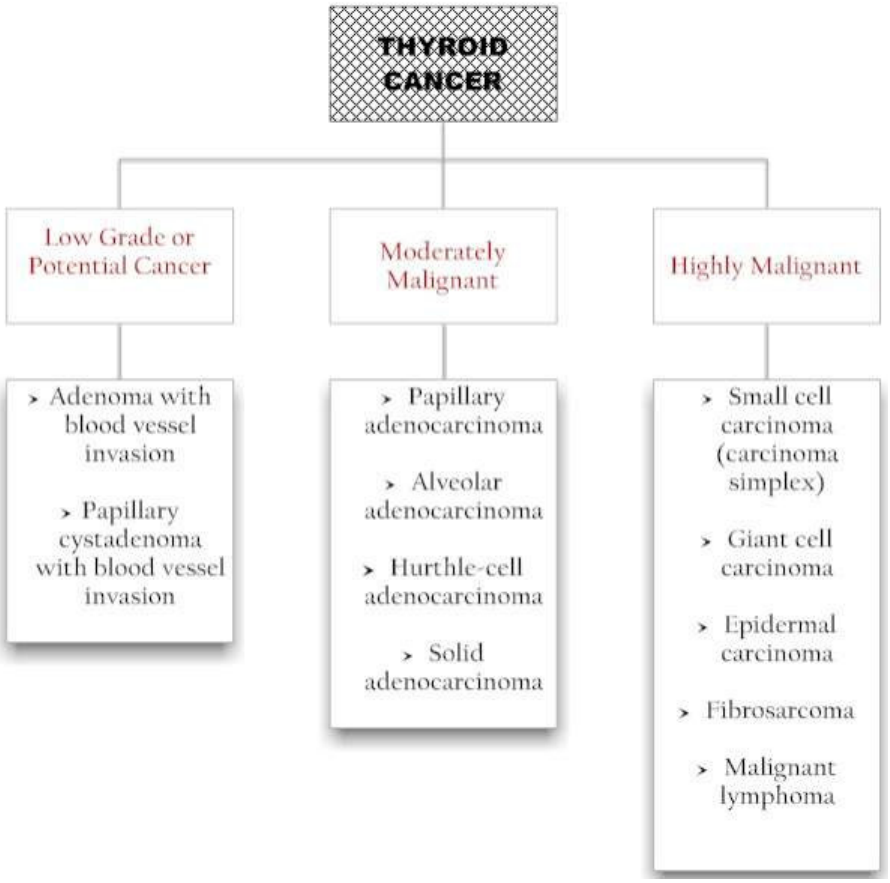


**Figure 4.** Classification of cancer described by Robert S. Pollack (Pollack and Francisco, 1951).

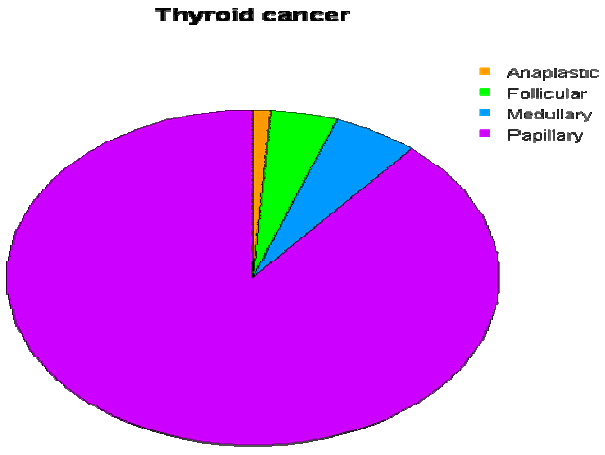


**Figure 5.** Approximate relative distribution of the occurrence of the major thyroid cancer types. Data represent the opinion of author.

and 5): (1) Papillary Hurthle thyroid cell carcinoma with lymphatic stroma, 'Warthin-like tumor' of thyroid; (2) Macrofollicular variant of papillary carcinoma; (3) Papillary carcinoma with spindle cell metaplasia; (4)

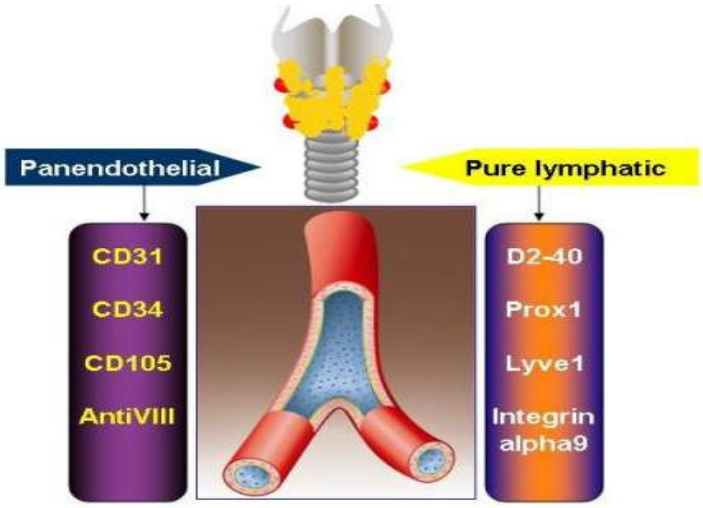


**Figure 6.** Major Immunochemical markers for angiogenesis staining in thyroid and other tissues (Baluk and McDonald, 2008; Liang et al., 2006a; Van den Eynden et al., 2007).

Papillary carcinoma with nodular fasciitis-like stroma; (5) Cribriform-morular variant of papillary carcinoma; (6) encapsulated columnar-cell carcinoma; (7) Solid variant of papillary carcinoma. As written by Sylvia L. Asa (Farid, 2004), variants of papillary carcinoma are defined as; (1) Usual papillary carcinoma; (2) Papillary microcarcinoma; (3) Cystic variant; (4) Encapsulated; (5) Hyalizing trabecular tumor (often considered of non-papillary originor defined as "paraganglioma-like" lesion; (6) Diffuse sclerosis variant; (7) Cribriform-morular variant. Features characteristical for papillary cancer include little colloid; hypercellular structure, monolayer sheets, cells with enlarged, irregular, crowded nuclei with loss of polarity, occurrence variable of psammoma bodies (Gimm, 2001). Non-papillary primary thyroid tumors derived from the follicular origin are: (1) Poorly differentiated thyroid carcinoma; (2) Spindle cell squamous carcinoma of thyroid; (3) Paucicellular variant of anaplastic carcinoma; (4) Hyalinizing trabecular neoplasm. Interestingly, the diagnosis of Hurthle-cell follicular variant papillary carcinoma remains controversial. The application of ret/PTC (RET is an abbreviation for "rearranged during transfection" analysis, PTC - papillary thyroid carcinoma). Analysis by RT-PCR (reverse transcription polymerase chain reaction) allowed the recognition of a follicular variant of Hurthle-cell papillary carcinoma as a group of lesions with no invasive behaviour at the time of diagnosis but which harbored a ret/PTC gene rearrangement. Many of these lesions exhibit irregularities of architecture, with hypereosinophilic colloid and nuclear features of papillary carcinoma, but these can be obscured by the hyperchromasia and prominent nucleoli of oncocytic change (Asa, 2004).

The group of primary "epithelial" tumors sharing the non follicular derivation consists of: (1) Thyroid paraganglioma; (2) Tumors with thymic or related branchial pouch differentiation; (3) Spindle epithelial tumor with thymus-like differentiation; (4) Carcinoma showing thymus-like differentiation; (5) Mucoepidermoid carcinoma of thyroid gland; (6) Mucoepidermoid carcinoma; (7) Sclerosing mucoepidermoid carcinoma with eosinophilia.

Primary nonepithelial tumors are: (1) Mesenchymal tumors of the thyroid gland; (2) Smooth muscle tumors of thyroid; (3) Solitary fibrious tumor; (4) Vascular tumors (hemangioma, epitheloid hemangioenothelioma, angiosarcoma); (5) Granular cell tumor; (6) MALTomas (mucosa-associated lymphoid tissue originated neoplasm) (Baloch and Livolsi, 2008).

Follicular carcinomas are divided into several categories: (1) Widely invasive follicular carcinomas; (2) Minimally invasive follicular carcinoma; (3) Vasculoinvasive follicular carcinomas (Farid, 2004).

Typical pathological findings are as follows: Little or no colloid; hyper cellular; most often microfollicles or syncytial groups; poorly defined borders; slightly enlarged round nuclei (Gimm, 2001).

Therefore, some tumors in the thyroid are not derived from the follicular thyroid epithelium. In such cases, the use of ancillary techniques including immuno-histochemistry (Figure 6) and molecular analysis can significantly improve diagnosis. However, a single marker is usually suboptimal in terms of sensitivity and specificity (Fischer and Asa, 2008).

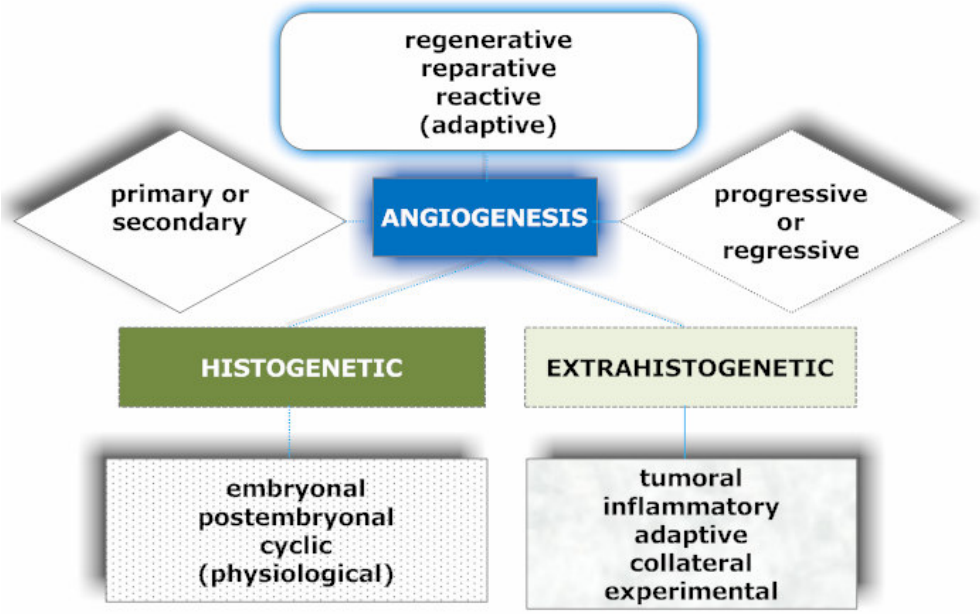


**Figure 7.** Graphical description of the classification of angiogenesis (Kupryianov et al., 1993).Text was translated into English.

To the best of the author's knowledge, there are no published data from the human studies providing the description of angiogenesis patterns of every histological entity mentioned. Moreover, there are practically no information describing morphology of angiogenesis in mouse thyroid cancer models (Kim and Zhu, 2009).

## DEFINITIONS OF ANGIOGENESIS AND ITS CLASSIFICATION

Much has been written about angiogenesis since the fundamental discoveries performed by Hunter in 18th century and Folkman, Ferrara and several others in the current era. Despite of existing thousands of articles, monographs and research reports describing practically all the facets of angiogenesis, little information is dedicated to the classification of angiogenesis and angiogenic morphologic patterns.

In the majority of research papers, angiogenesis is defined as the development of new vessels from the already existing vasculature, capillaries. Angiogenesis, essential for tumor growth and progression, does not involve a single pathway, but is a complex of many factors and signal transduction systems (Tanaka et al., 2002). Endothelial cells are the source of new blood vessels, and they have a remarkable ability to migrate, proliferate, and differentiate (Xu et al., 2001).

Several distinguishing morphological and pathological characteristics were described for the tumoral vasculature versus normal blood supply in respective tissues. Malignant vessels show increased vessel tortuosity and variable vessel diameter, poorly developed and fragile vessel walls, variable flow rates leading to micro-regional tumor hypoxia, increased interstitial pressure within the tumor and increased vessel permeability, poor connections between pericytes and endothelial cells, they demonstrate irregularly shaped endothelial cells and basement membrane as well as lack of lymphatic drainage and of vascular smooth muscle (Patterson and Rustin, 2007).

It is assumed that increased expression of vascular endothelial factor, a potent angiogenesis stimulator, is characteristic of differentiated thyroid cancers and is associated with increased growth, progression, and invasiveness of the tumor and with decreased recurrence-free survival (Sherman, 2008; Sherman et al., 2008).

Hsio et al. 2007) found that the K2578 C/A SNP (Single-Nucleotide Polymorphism) in the promoter region of the VEGF (vascular endothelial growth factor) gene may predispose the risk of development of thyroid cancer and regional lymph node metastasis. Their data also suggested a sex-specific effect and those males are under stronger genetic influence than females.

The most clear, informative and comprehensive classifications of angiogenesis and the neovascularization types I could find and interpret from the relevant literature to graphical charts are depicted on the Figures 7 and 8. In 1993, Kupriyanov et al. described these forms of angiogenesis in the monograph, published

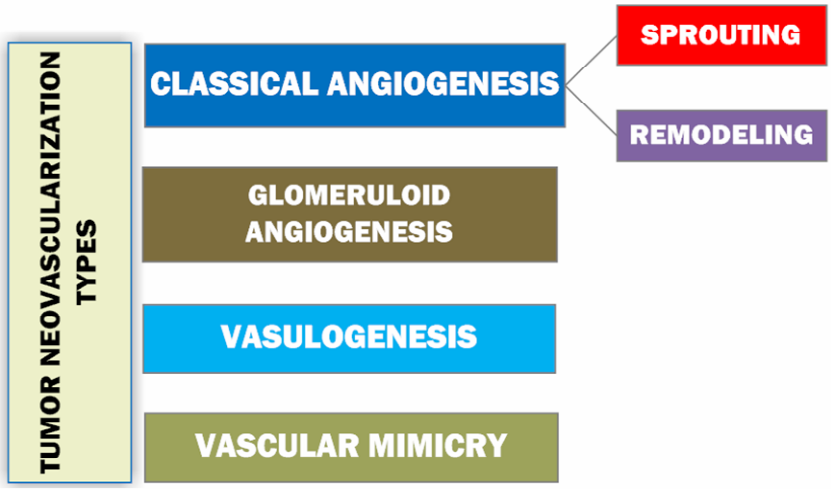


**Figure 8.** Diagram representing the classification of tumor neovascularization (Sprindzuk et al., 2009).

in Russian, and the chart represents the classification, which was assembled from several chapters of this book. Sprouting as the subtype of a classical angiogenesis is the expansive outgrowth of the vascular network. Remodeling refers to the rebuild of the vascular net and intussusceptive vascular growth. The term "vasculogenesis" is the *de novo* development of vessels from the endothelial cell progenitors. Glomeruloid angiogenesis is a formation of vascular structures that are similar to the renal glomeruli. Vascular mimicry is the tumoral ability to form capillary network composed of neoplasm cells without blood and lymphatic capillaries in the content of these tubular structures (Bamberger and Perrett, 2002; Sprindzuk et al., 2009).

## THE DEVELOPMENT OF VASCULATURE IN THYROID GLAND

The rich vascular net of the thyroid was mentioned in research article by Bradley (1896) in the description of haemorrhagic cysts of thyroid gland. Cunningham (1898) investigating the pharmacology of thyroid extracts on animal models pointed out the fact that thyroid hormones might have a significant impact on vascularization in tissues other than the thyroid gland itself. The role of vascularization in thyroid cancer pathogenesis was highlighted in the old classification of that disease by Pollack and Francisco (1951) (Figure 4). Later in 1971, Folkman et al. in Harvard`s laboratories investigated the thyroid perfusion and formulated the major modern concepts of angiogenesis. In the heart and other tissues, thyroid hormone has well-documented effects on angiogenesis. Mechanistically, most of these effects are initiated at the integrin receptor for the hormone on endothelial and vascular smooth muscle cells and reflect interfaces between nongenomic and genomic mechanisms invoked by the hormone. While physiologic concentrations of T3 (triiodothyronine) T4 (thyroxine) support angiogenesis in normal tissues, it is speculated that they may be supplementally useful for the opposition of tissue ischemia (Luidens et al., 2009). Patel et al. (2003), isolated a human endothelial cell strain, confirmed by Tie-2 and factor VIII-related antigen expression and NO (nitric oxide) release in response to VEGF. These cells respond to paracrine FGF-2 (fibroblast growth factor), and VEGF, though a less potent mitogen, was able to increase FGFR1 (fibroblast growth factor receptor) expression. The cells also respond to the paracrine antiangiogenic factor TSP-1 (thrombospondin) and to angiostatin generated from the plasminogen by the action of thyroid follicular cell-conditioned medium. This, and the observation that TSH (thyroid stimulatory hormone) and thyroid hormone had no apparent effect on thyroid endothelial cells, according to the researchers, suggests that angiogenesis observed during goitrogenesis is under the control of TSH-induced paracrine factors (Patel et al., 2003). Yamada et al. (2006) demonstrated for the first time that Iodide at high concentration decreases the expression of the angiogenic factors VEGF-A, VEGF-B, and PGF (platelet growth factor), accompanied by an increase in the expression of possible antiangiogenic factors. These proangiogenic and antiangiogenic factors may at least partly account for the iodide-induced decrease in thyroid blood flow (Yamada et al., 2006). Zhang et al. (2009) showed that thyroid hormone has a substantial impact on vasculature development in the brain. Pathologically altered vascularization could, therefore, be a contributing factor to the neurologic deficits induced by thyroid

hormone deficiency. Interestingly, T4 should not be used for pro-angiogenic intent in vessels because it causes platelet agglutination (Davis et al., 2009). In the study of a hind limb ischemia model in intact rabbit, thyroid hormone administration induces new blood vessel formation. Thus, evidence from the intact animals suggests that circulating thyroid hormone supports angiogenesis (Davis et al., 2009).

## ESSENTIALS OF PATHOLOGICAL MECHANISMS OF ANGIOGENESIS IN THYROID GLAND

According to Ramsden (2000), increased vascularity in the thyroid can occur in hyperplastic goiter, Graves' disease and cancer, and may be associated with a vascular hum because of increased blood flow. In cancers of the thyroid, MVD (micro vessel density) has been shown to correlate with disease-free survival in papillary carcinoma of the thyroid and intra thyroid tumor spread in follicular carcinoma. Interestingly, in experimental induction of goiter by low Iodine and thiouracil in rats, Wollman et al. (1978) showed that the capillaries within the thyroid clearly enlarged within 3 days of treatment, and by 20 days, they surrounded the follicles with a continuous endothelial sheet. There was both fusion of capillaries and mitosis of endothelial cells. There was no change in blood vessel morphology or number in nearby extrathyroidal tissue, including the parathyroids (Ramsden, 2000). It is hypothesized that patterns for tumor behaviour and metastasic spread vary according to tumor type and whether differences in the angiogenic or lymphangiogenic phenotype influence the route for tumor metastases or determine a more aggressive behaviour has not been fully explored (de la Torre et al., 2006). It is discovered that integrin alphaVβ3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis (Bergh et al., 2005). To assess the potential role of angiogenesis factors in human thyroid tumor growth and spread, Bunone et al. (1999) analyzed their expression by semi quantitative RT-PCR and immunohistochemistry in normal thyroid tissues, benign lesions, and different thyroid carcinomas. Compared to normal tissues, in thyroid neoplasias researchers observed a consistent increase in VEGF, VEGF-C, angiopoietin-2 and in their tyrosine kinase receptors KDR (tyrosine kinase receptor), Flt-4 (Fms-related tyrosine kinase 4), and Tek (tyrosine kinase). In particular, researchers detected the overexpression of angiopoietin-2 and VEGF in thyroid tumor progression from a prevascular to a vascular phase. In fact, they found a strong association between tumor size and high levels of VEGF and angiopoietin-2. Furthermore, results of that study show an increased expression of VEGF-C in lymph node invasive thyroid tumors and, on the other hand, a decrease of thrombospondin-1, an angioinhibitory factor, in thyroid malignancies capable of hematic spread. These data suggest that, in human thyroid tumors, angiogenesis factors seem involved in neoplastic growth and aggressiveness. Moreover, the discovered findings are in keeping with a hypothesis that in the presence of VEGF, angiopoietin-2 may collaborate at the front of invading vascular sprouts, serving as an initial angiogenic signal that accompanies tumor growth (Bunone et al., 1999). Gerard, Anne-Catherine et al. (2009) proposed that an inverse relationship exists between the expansion of the thyroid microvasculature and the local availability of iodine. This microvascular trace element-dependent regulation is unique and contributes to keep steady the iodide delivery to the thyroid. Signals involved in this regulation, such as VEGF-A, originate from the thyrocytes as early TSH-independent responses to iodide scarcity. The question raised in this paper is how thyrocytes, facing an acute drop in intracellular stores of iodine; generate angiogenic signals acting on adjacent capillaries. Using *in vitro* models of rat and human thyroid cells, researchers showed for the first time that the deficit in iodine is related to the release of VEGF-A via a reactive oxygen species/hypoxia-inducible factor-1-dependent pathway (Gerard et al., 2009).

## THYROID CANCER: GENERAL EXPERIENCE IN ANGIOGENESIS INVESTIGATION

VEGF was initially discovered as a tumor-derived factor, which increased microvascular permeability. Subsequently, the protein was found to exhibit mitogenic effects exclusively on endothelial cells. In normal thyroids, VEGF was found to be present within the follicular cells and shown to be secreted in response to thyrotropin from a thyroid cancer line *in vitro*. VEGF was also elevated within goiters and, in the FRTL-5 (Fischer rat thyroid cell line), and VEGF was found to significantly reduce the ability of TSH to increase 125-I uptake (Patel et al., 2003). It is now clearly evident that VEGF-C is associated with the lymphatic tumor spreading (Liang et al., 2006b). VEGF seems to be a significant component of the regulation of angiogenesis within the thyroid and alteration in intrathyroid expression of VEGF is seen in many thyroid pathologies.

On current understanding, VEGF is a central factor in angiogenesis in the thyroid gland, as indicated by the alterations in VEGF concentrations in many pathological conditions of the thyroid. Many other factors are involved, although some of these may act through regulation of VEGF expression in follicular cells. TSH is an important regulator of VEGF, although the role of factors important in other tissues, such as hypoxia, is not known in the thyroid. The thyroid is an excellent model for the

integrated control of angiogenesis, because of the vascularity of the thyroid gland, and its capacity to increase its blood flow in disease (Ramsden, 2000). TSH has been shown to induce VEGF expression in several thyroid carcinoma cell lines (Clauss and Breier, 2005). Luboshitzky and Dharan (2004) demonstrated the utility of CD34 immunolocalization in cell block preparations as an adjunct to fine needle aspiration (FNA) diagnosis of thyroid cancers. Researchers reported linear "beaded" and "looped" pattern of angiogenesis in papillary cancer, narrow, slitlike, discontinuous, sinusoidal pattern of medullary carcinoma of the thyroid and slightly convoluted "gaping" pattern" in benign nodular goiter.

Turner et al. (2003) reviewed the published articles reporting the data where the principal objectives of investigation were the correlation of MVD with clinical and pathological parameters. Six studies were mentioned in their research paper. These experiments elucidated the fact that the higher MVD is directly proportional to the level of tumor differentiation. With regard to the survival terms and receive occurrence, conclusions of the mentioned above studies show controversial information, suggesting the idea that survival and the recurrence could be influenced by a wide spectrum of factors, including the co-morbidities and the pathological predominance of the mechanisms of tumor expansion other than angiogenesis (Turner et al., 2003). Garcia et al. (2006) studied the angiogenic and lymphangiogenic phenotypes of a relatively large but heterogenous cohort of thyroid proliferative lesions (n=191). Using immunohistochemistry for CD34, lymphatic vessel endothelial receptor-1 (LYVE-1) (specific markers for vascular and lymphatic endothelium respectively), vascular endothelial growth factor (VEGF-A), VEGF-C and fibroblast growth factor-2 (FGF-2), this study analyzed MVD, LVD (lymphatic vascular density), and expression of angiogenic and lymphangiogenic factors in normal thyroid (n=19), multinodular goiter (n=25), toxic multinodular goiter (n=8), Graves' hyperplasia (n=22), follicular adenoma (n=54), papillary carcinoma (n=27), incidental papillary microcarcinoma (n=8), follicular carcinoma (n=20) and medullary carcinoma (n=8). MVD was decreased in proliferative lesions, benign and malignant, compared with normal tissue ($P < 0.0001$). In contrast, VEGF-A expression was increased in major thyroid carcinomas when compared with papillary microcarcinomas, benign lesions and normal gland ($P < 0.0001$). LVD was higher in PC (papillary carcinoma) and PMC (papillary microcarcinoma) ($P = 0.001$), and VEGF-C expression was increased in papillary carcinomas ($P < 0.0001$). Despite higher LVD (lymphatic vessel density) and increased expression of VEGF-A and VEGF-C in thyroid cancers, these markers were not related to poor prognosis in terms of tumor size, multifocality and/or presence of lymphatic or distant metastases. In conclusion, angiogenesis is reduced in thyroid proliferative lesions compared with normal thyroid tissue. However, VEGF-A expression is upregulated in thyroid cancers. Lymphangiogenesis and VEGF-C expression are increased in thyroid tumors prone to lymphatic metastases. This may be an important mechanism underlying the differences in metastatic behaviour between papillary and follicular thyroid cancer (de la Torre et al., 2006).

Jebreel et al. (2007) evaluated the relation between VEGF, its receptors (VEGFR-1 and VEGFR-2) and MVD in thyroid diseases. In that study, immunostaining for VEGF and VEGF receptors was performed in 66 specimens of thyroid tissue, comprising 17 multi nodular goiter (MNG), 14 Graves' disease, 10 follicular adenoma, 8 Hashimoto's thyroiditis, 7 papillary carcinoma and 10 normal thyroid specimens. Thyrocyte positivity for VEGF and VEGF receptors was scored 0 to 3. Immunohistochemistry for CD31, and CD34 on the same sections was performed to evaluate MVD. Immunohistochemical staining of VEGF in thyrocytes was positive in 92% of all the thyroid tissues studied. Using an immunostaining intensity cut off of 2, increased thyrocyte staining was seen in follicular adenoma specimens, MNG and normal thyroids compared with Hashimoto's thyroiditis and Graves' disease ($P < 0.05$). Similarly, VEGF thyrocyte expression in Graves' disease was less than other pathologies ($P < 0.05$). VEGFR-1 expression and the average MVD score did not differ between the different thyroid pathologies. VEGF distribution and expression was lower in the autoimmune pathologies of Graves and Hashimoto's than those associated with autonomous growth processes. Conversely, both VEGFR-1 and VEGFR-2 were widely expressed in the thyrocytes of both benign and neoplastic thyroid disease, suggesting that up-regulation of VEGF and not its receptors occurs as tissue becomes autonomous. There was no clear relationship between MVD measurement and thyroid pathology. According to scientists, no attempt was made to make any correlation between MVD and clinicopathological parameters (Jebreel et al., 2007).

## ANGIOGENESIS IN PAPILLARY THYROID CANCER

Papillary carcinomas have a MVD that is, on average, threefold higher than that present in the peritumoral normal thyroid tissue. Moreover, intratumoral blood vessels have distinctive morphological and immuno-histochemical features. In fact, aggregated vascular complexes (glomeruloid structures) have been identified in the stroma of tumor papillae, but not in the normal thyroid or in follicular adenomas or carcinomas. In addition, the oncofetal fibronectin EDB (extradomain B) has been demonstrated in the tumor stroma and in the vascular basement membranes of tumor papillae (Scarpino et al., 2003).

The first report demonstrating that the balance between angiogenic and antiangiogenic factors correlates with distinct invasion to other organs and neovascularization of papillary thyroid carcinoma belongs to Tanaka et al. (2002). VEGF expression strongly correlated with other angiogenic factors. The cytoplasm of cancer cells stained positive for all factors. Tie-2 and TSP-1 receptor also appeared in endothelia of microvessels. TSP-1 inversely correlated with the degree of invasion of the primary tumor to other adjacent organs and with MVC (mean micro vessel count). A higher MVC correlated with poorer survival. To clarify the balance between angiogenic and antiangiogenic factors in the same tumor, investigators calculated the ratio of each angiogenic factor against TSP-1 as the antiangiogenic factor. The ratios VEGF/TSP-1, VEGF-C/TSP-1, and Ang-2/TSP-1 significantly correlated with a higher MVC. Furthermore, the ratios VEGF/TSP-1 and Ang-2/TSP-1 significantly correlated with the degree of infiltration. However, in their study, there was no correlation between the clinical data, such as age, gender, and tumor size, and the expression levels of angiogenesis-related factors (Tanaka et al., 2002).

Akslen and Livolsi (2000) examined a series of 128 papillary carcinomas with respect to MVD and patient survival. Follow-up was obtained for all cases (median, 145 months). Scientists found a mean MVD of 216 per $mm^2$ (range, 35-751), and there was an average of 3.14 times more vessels in the tumors, when compared with surrounding non-neoplastic thyroid tissue. MVD was inversely related to age, tumor diameter, histological grade, and primary tumor extent. Furthermore, increasing MVD tended to be associated with improved survival ($P = 0.056$). Scientists concluded that their data indicate that angiogenesis is important for the development and maintenance of papillary thyroid carcinomas, although it was not identified as a prognostic factor.

Scarpino et al. (2003) described two types of vessels which are involved in providing vascular support to the papillary thyroid carcinomas: A delicate network of capillary network of capillary vessels located in the stroma of tumor papillae that were weakly stained for EC-NOS (endothelial cell nitroxide-synthase), and large venous spaces, sometimes with a muscular coat, located in the peritumoral fibrous tissue. They were intensely stained for EC-NOS, a marker of endothelial activation; some endothelial cells were positive for Ki-67, a marker of proliferation. The venous spaces were often in close contact with infiltrating tumor cell nests and provided images of `vascular invasion. In that study, scientists found that papillary carcinoma cells contain RNAs (ribonucleic acid) for VEGF, VEGF-C, and angiopoietin, and produce large amounts of VEGF. Moreover, they suggested an idea that, besides tumor cell migration towards blood vessels, VEGF and/or other angiogenic factors released by tumor nests are capable of attracting endothelial cells. According to Scarpino et al., (2003), the frequent occurrence of vascular invasion in thyroid tumors might be derive from a combination of mutual events involving both migration and attraction of tumor cells and endothelial cells.

Another investigation of angiogenesis in thyroid nodular lesions and in papillary cancer in particular was performed by Rzeszutko et al., 2004. Using immunohistochemistry, the authors of that study evaluated number of vessels in various nodular lesions of the thyroid (54 cases). Expression of CD34 antigen and MVD were evaluated in sections of archival paraffin blocks originating from the local institutions. MVD was assessed in ten different fields per section in "hot spots". Expression of CD34 was quantified using computerized image analysis and, then, MVC and MVA (microvessel area) were calculated. In thyroid tissue with benign lesions, the MVC (31.7) was higher than in neoplastic lesions (22.3), although no differences in MVA were observed. This observation, according to the opinion of the researchers, points to differences in the size of newly formed vessels in individual nodular lesions of the thyroid (Rzeszutko et al. 2004).

Stabenow et al. (2005) based on their experimental results suggested that angiogenesis is more intense among the classic and tall cell variants of metastatic tumors, showing that microvessel count can be an indicator of metastatic potential in these histological subtypes of papillary thyroid carcinoma. In their research, patients that developed recurrent disease had a tendency to exhibit higher angiogenesis; however, there was no association between MVD and prognostic index groups. Finally, authors of that experimental work demonstrated the importance of the study of angiogenesis in papillary thyroid carcinoma.

## ANGIOGENESIS IN FOLLICULAR THYROID TUMORS

It is assumed that thyroid follicular neoplasms (adenoma and carcinoma) may pose difficulties to the differential diagnosis. Because such a distinction is not possible at FNA, surgery is often performed. Clinical information such as age, sex, and node size is important in case of suspected carcinoma. Follicular carcinoma is characterized by capsular invasion, vascular invasion, and metastatic dissemination mainly by the hematogenic pathway. This invasion depends on collagen degradation in capsule and in subendothelial basement membrane. Collagen degradation has been widely researched in the angiogenesis process and in the hematogenic dissemination mechanism (Frigugliett et al. 2000).

As early as in 1996, Karl Segal et al. investigated 30 parrafin-embedded samples of follicular thyroid tumors obtained from the local archives. Endothelial cells were immunostained with anti von Willebrand factor,

antiperoxidase, diaminobenzidine and counterstained with Carazzi`s hematoxylin with consequent standard washing procedures. A marginal difference was noted in the degree of vascularity between the follicular thyroid adenomas and the follicular thyroid carcinomas; both types were relatively vascular. Eight of the 15 adenomas examined were more vascular than the other seven. The same was true for the follicular carcinomas. However, a major difference in vascularity was noted among different areas within the follicular carcinomas. Areas of follicular carcinomas adjacent to and, especially, infiltrating the capsule showed significantly increased vascularity, with a ratio of one blood vessel to two tumor cells. Tumor areas distant from the capsule had a ratio of only one vessel to 10 tumor cells on average. Areas of adenomas adjacent to the capsule did not have prominent vascularity, in contrast to the carcinomas. Their ratio of blood vessels to tumor cells was uniformly around 1:10. Solid areas with marked pleomorphism within the follicular carcinomas, suggesting a higher degree of malignancy, again showed a higher ratio of vascularity than areas with no marked pleomorphism or solid formation. A 1:2 blood vessel tumor cell ratio was noticed in the pleomorphic sites and solid areas, whereas a much lower ratio 1:10 was observed in other zones. In comparing the degree of vascularity between follicular adenomas and adenocarcinomas, no significant differences were found. However, a definite difference in vascularization was noted in different areas within the follicular carcinomas. Researchers showed that the more malignant appearing areas, as indicated by pleomorphism and solidity, had a higher rate of vascularization. Areas of tumor adjacent to or penetrating the capsule were also characterized by high vascularity. Thus, although vascularity did not seem to be a distinguishing feature of follicular carcinoma, the higher vascularity in the more malignant areas of the tumor suggests that vascularity may indeed play a role in tumor aggression. The presence of high vascularity in the pericapsular area also suggests that vascularity may be important in tumor potential for extracapsular extension and expansion.

Frigugliett et al. (2000) performed clinical and histopathologic assessment of 74 follicular neoplasms, as well as immunohistochemical reactions for CD-34 protein to estimate angiogenesis and for metalloproteinase-9, an enzyme that degrades type IV collagen. The research was carried out retrospectively in 74 patients who had surgery and were followed up at the local institutions. Clinical, histologic, and immunohistochemical variables were compared among the groups of follicular neoplasms and a control group of 36 patients with colloid goiter. No significant statistical difference was found between patients with follicular adenoma and thyroid follicular carcinoma concerning sex ($P = 0.092$), age ($P = 0.098$), thyroid node size ($P = 0.426$), vascularization ($P = 0.388$), and immunostaining intensity for metalloproteinase-9 ($P = 0.055$). The proportion of immunoreactive cells for metalloproteinase-9 in follicular carcinoma cases was higher than that observed in follicular adenoma cases ($P < 0.001$). Patients in more advanced stages of carcinoma were more than 45 years old ($P = 0.006$), presented extensive invasion ($P < 0.001$), had less vascularization ($P = 0.046$), and had a higher proportion of immunoreactive cells for metalloproteinase-9 ($P < 0.001$). Finally, researchers calculated that the proportion of immunoreactive cells for metalloproteinase-9 in follicular carcinoma was higher than that observed in follicular adenoma, with a significant statistical difference ($P < 0.001$). Researchers proposed an idea that the described method must be developed to apply in material obtained by FNA to differentiate follicular adenoma from carcinoma.

Caroline Kim et al. (2007) based on their experiments, suggested that deletion of the pituitary tumor-transforming gene in TRbPV/PV (thyroid receptor beta) mouse thyroids decreases cell proliferation, up-regulates p21, reduces angiogenic factors such as FGF2 and leads to an overall improvement in survival. Their study highlights the fact that pituitary tumor-transforming gene promotes angiogenesis in a mouse model of follicular cancer similar to the tumors localizing elsewhere.

## ANGIOGENESIS IN MEDULLARY THYROID NEOPLASM

Medullary thyroid cancers are characterized by the presence of abundant cells; loose groups; poorly defined borders; often multi- or binucleation; eccentrical (plasmacytoid) nuclei; detection of amyloid (Gimm, 2001). As I could find, no data are available on the description of typical morphological patterns of angiogenesis in medullary carcinomas of the thyroid and only several reports regarding the angiogenic activities in that disease are published. Zhang et al. (2005) in laboratory investigation showed that ASODN (antisense oligonucleotide) can suppress endothelial cell growth and inhibit tumor angiogenesis possibly by specifically blocking VEGF expression in medullary thyroid carcinoma. Bugalho et al. (2008), measured VEGF levels in medullary cancer patients. Researchers concluded that serum VEGF levels in medullary thyroid cancer patients are not significantly different from those found in healthy patients and did not correlate with the extension of disease. Thus, the serum VEGF levels in medullary thyroid cancer patients do not appear useful to select potential candidates for therapies with tyrosine kinase inhibitors. Failure to demonstrate high levels of serum VEGF in medullary thyroid cancer patients with distant metastases, in contrast to what happens in patients with differentiated thyroid cancer suggests the involvement of other proangiogenic factors. Petrangolini et

al. (2006) tested the antitumor activity of RPI-1 (the indolinone RET tyrosinase kinase inhibitor) against large established subcutaneously thyroid tumor xenograft, a human medullary thyroid carcinoma harboring oncogenic MEN-2A-type RET mutation. Oral treatment with RPI-1 caused growth arrest or regression in 81% treated tumors. An additional finding in that study was the significant reduction of MVD in thyroid tumors from mice receiving RPI-1 treatment. Although a direct inhibitory effect on endothelial cells cannot be ruled out, an indirect antiangiogenic effect could be related to inhibition of the oncogene-dependent angiogenic phenotype in light of the marked inhibitory effect of RPI-1 in VEGF expression/secretion by the thyroid tumor cells. Such property is of particular relevance considering that VEGF secretion has been found constitutively activated in some thyroid cancers including medullary thyroid cancers. Even the loss of cellularity observed in treated tumors might be at least in part the consequence of tumor hypoxia.

## ANGIOGENESIS IN ANAPLASTIC THYROID CANCER

The expression of VEGF *in vitro* has been shown to correlate with *in vivo* aggressiveness of the tumors, with anaplastic tumors having the greatest level of expression of VEGF (Ramsden, 2000). Xu et al. (2001) investigated the anticancer effects of combined manumycin (a farnesyltransferase inhibitor) and paclitaxel (a microtubule inhibitor) against anaplastic thyroid carcinoma. Scientists presented data showing that the combination of paclitaxel (found in the bark of the Pacific yew tree, is an inhibitor of microtubule function) and manumycin (a natural product of Streptomyces parvulus, inhibits farnesyltransferase by competing with the farnesyl pyrophosphate substrate) provides improved antineoplastic activity *in vivo* without increased toxicity. They observed that the tumor xenografts that were treated with manumycin were paler than those not exposed to manumycin. A hypothesis that can explain this observation is that manumycin inhibits angiogenesis. Researchers concluded that manumycin plus paclitaxel is an effective combination against anaplastic thyroid carcinoma, and inhibition of angiogenesis plays a role in the antineoplastic effect of this combination (Xu et al. 2001). A large percentage of anaplastic thyroid carcinomas have been shown to harbor the V600E B-Raf point mutation, leading to the constitutive activation of the mitogen-activated protein kinase pathway. Anaplastic thyroid carcinoma`s invasion, metastasis, and angiogenesis are in part dependent on the gelatinase class of MPMP (matrix metalloproteinases). The explicit targeting of these two tumor markers may provide a novel therapeutic strategy for the treatment of anaplastic thyroid carcinomas. The MMP-activated anthrax LeTx (lethal toxin), a novel recombinant protein toxin combination, shows potent mitogen-activated protein kinase pathway inhibition in gelatinase-expressing V600E B-Raf tumor cells *in vitro.* Based on the literature data and their own experimental work, Alfano et al. (2009) suggested that the MMP-activated LeTx could be used not only in the clinical management of V600E B-Raf ATC (anaplastic thyroid cancer) but potentially in any solid tumor Alfano et al. (2009 ). Kim et al. (2007) showed that Sorafenib, a multikinase inhibitor of the B-raf, VEGF receptor-2, and platelet-derived growth factor receptor beta kinase exerts significant antitumor activity in an orthotopic xenograft model of anaplastic thyroid neoplasias via a potent antiangiogenic effect (Kim et al. 2007b). Recently, Zhu et al. (2009) in the experimental research showed that triptolide, a small molecule from a Chinese herb, may function as inhibitor of tumor angiogenesis and invasion and may provide novel mechanistic insights into the potential therapy for human anaplastic thyroid cancers.

Therefore, literature data from the mentioned sources show that the majority of studies investigating angiogenesis in anaplastic thyroid cancer are pharmacology-oriented.

## THE ROLE OF SURVIVIN AND ITS RELATIONSHIP WITH TUMOR ANGIOGENESIS IN DIFFERENTIATED THYROID CARCINOMAS

Survivin is a member of the inhibitor of apoptosis protein family and has been implicated in both apoptosis inhibition and cell cycle control. It is aberrantly expressed in various kinds of cancer cells but is undetectable in normal differentiated adult tissues, except testis, thymus, and placenta. Several studies have reported that the expression rate of survivin in tumor tissues is associated with tumor progression and unfavorable clinopathologic variables: Poor prognosis, shorter survival rates and chemoresistance. Survivin is expressed in human carcinomas, but its expression levels in tissues are different, that is associated with the poor outcome in patients (Liguang et al., 2007). Sugawara et al. (2002) showed that measuring survivin mRNA levels or immunohistochemistry of the protein expression can be useful to aid the diagnosis of thyroid lymphoma when histologic diagnosis is difficult.

According to Ito et al. (2003) survivin was only occasionally expressed in normal follicular cells, whereas in follicular and papillary carcinomas, about 20% of cases were positive for survivin. The incidence was significantly higher in advanced stage papillary carcinoma ($P = 0.0080$) and papillary and follicular carcinomas with poorly differentiated lesions ($P = 0.0150$). In anaplastic carcinoma, survivin positivity was observed in 84% of the cases, which was in significantly higher incidence than in papillary or follicular carcinoma ($P < 0.0001$). These

results suggest that survivin is strongly related to the dedifferentiation of thyroid carcinoma.

Haghpanah et al. (2006) showed potential value of survivin in discrimination between follicular thyroid adenoma and follicular thyroid carcinoma. In their investigation, survivin expression was significantly ($P < 0.005$) higher in the cases that received a diagnosis of carcinoma in comparison with follicular adenomas cases. Odds ratio of follicular carcinoma for survivin expression was 21.375.

In research work of Tirro et al. (2006), increased expression of c-IAP1 and survivin contributed to the acquisition of permanent resistance to cytotoxic compounds. Du et al. (2006) reported that ASODN (antisurvivin oligonucleotides) inhibit growth and induce apoptosis in human medullary thyroid carcinoma cells and these suggest that survivin plays an important role in MTC (medullary thyroid cancer) independent of the conventional clinicopathologic factors, and ASODNs is a promising survivin-targeted gene therapy for MTC.

Dong et al. (2006) detected the expression of Survivin, VEGF and MVD by immuno-histochemical staining in 47 differentiated thyroid carcinomas, 10 thyroid adenoma and 10 normal thyroid tissues and their content were measured by pathological image analysis system. The expression of Survivin and VEGF in differentiated thyroid carcinomas was 46.8 and 61.7%, respectively. No expression of Survivin was detected in thyroid adenoma and normal thyroid tissues. The expression of Survivin was significantly correlated with the expression of VEGF and MVD in differentiated thyroid carcinomas. The expression of Survivin was significantly associated with clinical stages, tumor complete capsulae and lymph node metastasis. Researchers suggested that the up-regulation of Survivin expression and its close relationship with angiogenesis may play an important role in carcinogenesis and development of differentiated thyroid carcinomas.

Xiang et al. (2007) investigated expression of survivin gene in thyroid carcinoma using Western blotting and RT-PCR in order to detect mRNA and protein expression of survivin in the carcinoma tissue and the tissue around the carcinoma. Survivin gene was expressed in most of the thyroid carcinoma. The positive expression rate of survivin is significantly linked to cell proliferating activity, lymph node metastasis, and hematogenous metastasis. According to the researchers, survivin gene will be a new target of gene therapy in thyroid carcinoma.

Antonaci et al. (2008) discovered that survivin and cyclin D1 are jointly expressed in thyroid papillary carcinoma and microcarcinoma. Survivin showed only cytoplasmic expression. As researchers concluded, cyclin D1 and survivin overexpression are probably early events in tumorigenesis of thyroid papillary carcinoma but their full role in the process of tumor progression and their clinical value should be investigated further. Zhang et al. (2009) observed higher mRNA expression and positive immunostaining for survivin and VEGF in thyroid cancer compared with thyroid adenoma and normal thyroid, with a significant positive correlation among them and significant correlations with histological typing, clinical staging and lymph node metastasis in thyroid cancer, but researchers could not find any significance of caspase-3 in thyroid cancer and its significant relationship with survivin expression. Results of that study indicated that survivin and VEGF are unfavorable molecules for thyroid cancer evolution and prognosis, and possess positive correlation in thyroid cancers.

## THE EXPERIENCE OF THE IMAGE PROCESSING SOFTWARE PACKAGES FOR THE THYROID TISSUES PATHOLOGY INVESTIGATION

Karl et al. (1996), wrote: "There is to date no accurate, reliable method by which benign thyroid nodules can be differentiated from malignant tumors. As a result, many patients with thyroid nodules undergo unnecessary surgery. Depending on the series quoted, 60-80% of thyroid operations performed because of suspected cancer could be prevented if a reliable marker for thyroid malignancy were available. The large number of unjustified operations places a considerable burden on health care systems.

In order to search for new diagnostic and prognostic criteria, Kavantzas et al. (2002) investigated the nuclear and angiogenetic profile in different types of thyroid neoplasms and performed statistical comparison between the different nuclear morphometric and angiogenetic parameters. Sixty-two cases of thyroid neoplasms were classified as follows: 31 papillary carcinomas, 10 follicular neoplasms (5 adenomas and 5 carcinomas), 5 undifferentiated carcinomas, 6 Hurthle-cell carcinomas and 10 medullary carcinomas. Using an image analysis system, six nuclear morphometric and eight angiogenic variables were measured for each case. Concerning nuclear morphometric variables, statistical differences were found mainly between undifferentiated and overall subtypes of differentiated carcinomas, as well as between follicular adenomas and carcinomas. Concerning angiogenesis variables, statistical differences were found only in the vessel's minor axis length between undifferentiated and overall subtypes of differentiated carcinomas, between MVD of follicular adenomas and carcinomas respectively, as well as between MVD of medullary carcinoma and follicular cell carcinomas generally. In conclusion, scientists assumed that the nuclear morphometry and quantitation of angiogenesis could offer two additional parameters in the distinction between follicular adenomas and carcinomas. However, according to the opinion of scientists, they cannot serve as absolute diagnostic criteria since they are only based on statistical differences. From a prognostic point of view,

nuclear morphometry may have some relevance as far as follicular cell neoplasms are concerned since the more aggressive anaplastic carcinomas have a distinct morphometric profile. This study clearly revealed differences in the angio genetic profile between medullary and follicular cell carcinomas (Kavantzas et al. 2002).

Tseleni-Balafouta et al. (2006) performed the comparative study of angiogenesis in thyroid glands with GD (Graves disease) and Hashimoto's thyroiditis. In that research, multiple morphologic characteristics of microvessels were measured in and compared between 18 cases of GD, 29 cases of Hashimoto's thyroiditis, and 15 control cases. All histologic sections were immunostained for CD31. Quantification of MVD, major axis length, minor axis length, area, perimeter and shape factor was performed by image analysis. MVD was increased significantly in both forms of autoimmune thyroid disease. Significantly higher values were found in GD in comparison to Hashimoto's thyroiditis. In contrast, major axis length, minor axis length, and area had significantly higher values in Hashimoto's thyroiditis than in GD. The statistical analysis revealed MVD as the unique significant morphometric factor discriminating the two autoimmune entities (Tseleni-Balafouta et al., 2006).

## SOFTWARE PACKAGES FOR THE IMAGE PROCESSING OF BIOMEDICAL DATA AND THE CONCEPTS OF THE DIGITAL PATHOLOGY

Computer-assisted analysis of digital images was used for the first time in 1980 to quantify immunostaining. Digital images can be translated into numerical values, and these are able to describe staining intensity quantitatively, which is considered more precise that the visual qualitative observation. De Matos et al. (2006) compared semi-quantitative method with immunochemistry quantification by a digital computer-assisted method for the evaluation of well-differentiated thyroid carcinoma samples stained with galectin-3. According to scientists, both methods correlated significantly. Several studies have been performed applying the so-called computerized morphometry for the cell nuclear image analysis (Ambros et al., 1989; Gupta et al., 2001). One study, carried out by Kent et al. (2005), investigated the vascularization of the thyroid neoplasms in dogs. Researchers prepared and stained the specimens with antibodies against human CD31 or factor VIII-related antigen (factor VIII-rag). The areas of highest vascularity were identified in CD31- stained sections, and corresponding areas were then identified in factor VIII-rag-stained sections. In that study, image analysis was used to calculate the total vascular density in each section, and neovascularization, expressed as a percentage, was determined as the absolute value of the total vascular density derived from factor VIII-rag-stained sections minus the vascular density derived from CD31-stained sections.

Mean vascular density of thyroid gland carcinomas derived from CD31-stained sections was significantly greater than density derived from factor VIII-rag-stained sections. This incremental difference was presumed to represent degree of neovascularization. However, significant differences were not detected between vascular densities derived from CD31 and factor VIII-rag-stained sections for either normal thyroid gland tissue or thyroid gland adenomas. No significant correlations were found between vascular density in thyroid gland carcinomas and survival time following surgery (Kent et al., 2005). Image processing in the spheres of biomedical sciences is developing in a fast pace according to the demands of the health care professionals, who need computer technologies and should be taught how to use computers and software packages in order to improve the service of medical practice and to make easier the research job. Digital pathology and immunochemistry as the integration of several basic sciences now have the tools to perform the experiments applying the modern computer-assisted approach to the diagnosis of diseases, which require the interpretation of the image reflecting the picture of the disease and the normal tissues. In contrast to the statistical packages, which spectrum includes such valuable free sources as R language tools, the realm of digital pathology lacks the robust, user-friendly high-quality software for the needs related to the practical diagnostic activities and complex laboratory research quantitative tasks, for example the calculation of the specific immunostained structures, the estimation of their morphologic characteristics (area, perimeter, axis length, color intensity, fractal ant syntactic parameters). The following packages are suitable and available for the free download and use in the spheres of basic and clinical health sciences:

(1) Angioquant;
(2) Image J;
(3) ImageLab;
(4) GIMP (GNU image manipulation program);
(5) Cell Profiler;
(6) Cell analyst;
(7) AQuaL (Boettcher et al., 2009).

Among these software packages, to the author’s opinion, “Image J” and “Cell Profiler” are the most multifunctional and modifiable in terms of the availability of several additional modules. It is necessary to note, that all the mentioned software rather difficult in use for a biomedical scientist if there are no consultations and help of IT (Informational technology) professionals are available.

The diagnosis of thyroid disease is under the significant subjectivity of a human researcher. The consequences of the established and written in the patient’s files diagnosis

are first the decisions of doctors providing the treatment to the patient. Therefore, histological diagnosis based on FNA provides a surgeon the reason for the estimation of the volume of operation and affects the choice of drugs. IT specialists, developing the software for the performance of tasks related to the differential diagnosis require the collection of images with a perfectly precise established diagnosis which can serve as the definition of the diagnostic pattern. If that requirement is not satisfied correctly, the produced software will calculate and report wrong data (example of the “garbage in, garbage out” rule).

## NANOTECHNOLOGY AND THYROID CANCER RESEARCH

Biomolecular nanotechnology defined as “the three-dimensional positional control of molecular structure to create materials and devices to molecular precision" recently has grown to the multifaceted industry providing new approaches to the old problems (Freitas, 1999).

Nanodevices can receive the natural chemical messages transmitted from and between cells simply by eavesdropping on the natural molecular message traffic. Of course, mechanical (e.g., cytoskeletal) and electrical (e.g., ionic) cellular emanations may also be detectable by nanodevices (Freitas, 1999). In study of Maksimenko et al. (2003) the nanospheres and nanocapsules were used for delivering structured antisense oligonucleotides (AON). With AON in nanospheres, researchers showed that the mRNA of EWS-Fli-1 was specifically down regulated, confirming the antisense activity of the targeted AON. The application of nanoparticle-label-based all-in-one dry-reagent immunoassay for thyroid-stimulating hormone was reported by Huntinen et al. (2004). The magnetic properties of magnetic nano particles (MNPs) allow them to be imaged via magnetic resonance imaging (MRI) and targeted to a particular region by an externally applied MF (magnetic field). Once loaded with drug, MNPs can be targeted to a region of interest through an externally applied MF, and the drug released over a period of weeks. This helps to achieve optimal dosing by reducing the systemic toxicity of the drug, and decreases the likelihood of drug resistance that would result from insufficient drug present. Through MRI imaging, the biodistribution of MNPs and indirectly the drug concentration may be determined (Schulz, et al., 2009).

Nakagawa et al. (2007) determined the number of repeat in the microsatellite region (AATG, 5-14) of the human thyroid peroxidase gene (TOPX) using an automated DNA analysis system with nano-scale engineered biomagnetite. Furthermore, rapid determination of repeat numbers was possible by measuring fluorescence intensities obtained by probe dissociation at 56, 66, 40, 60, and 80°C for signal normalization. He et al. (2008) performed E1A gene transfection of human undifferentiated thyroid cancer cell line HTC/3 by nanoparticles. It is interesting that a typical DNA ladder pattern of apoptosis in HTC/3-E1A cells was observed by electrophoresis, but not found in HTC/3-Vect and HTC/3 cells. Moreover, apoptosis could be induced by x-ray in the E1A gene-transfected cells. Liao et al. (2009) proposed the method for ultra-sensitive detection of mutated papillary thyroid carcinoma DNA using square wave stripping voltammetry method and amplified gold nanoparticle biomarkers. According to authors, that approach was successful in differentiating between the mutant and wild type BRAF sequences that are present in genuine 224-nucleotides DNA.

Mousa et al. (2009) described methods of treating subjects having conditions related to angiogenesis including administering an effective amount of a polymeric nanoparticle form of thyroid hormone agonist, partial agonist or an antagonist to promote or inhibit angiogenesis in the organism. Huang et al. (2010) demonstrated an implementation of a time-resolved luminescence (TRL) assay for the sensitive and selective detection of thyroglobulin (Tg), a thyroid cancer marker, in homogeneous solution using water-soluble alpha-D-mannose-conjugated Au nanodots (Man-Au NDs). Researchers concluded that the concanavalin A/Man-Au NDs system in a label-free manner allows the detection of Tg at concentrations as low as 90 pM in the presence of BSA (bovine serum albumin) at 50 µM by TRL. With its advantages of rapid and specificity, that approach, as authors reported, showed a great potential for diagnosis of cancers.

Yalcin et al. (2010) recently showed that tetraiodothyroacetic acid (tetrac) and tetraiodothyroacetic acid nanoparticle effectively inhibit the growth of human follicular thyroid cell carcinoma. Tetrac is a deaminated analogue of L-thyroxine that blocks the actions of L-thyroxine and 3,5,3'-triiodo-L-thyronine at the cell surface receptor for thyroid hormone on integrin alpha v beta 3. Tetrac blocks the proliferative effects of thyroid hormone on tumor cells and the proangiogenesis actions of the hormone. In the absence of thyroid hormone, tetrac also blocks angiogenesis induced by various growth factors. Covalently linked to poly (lactide-co-glycolide), tetrac nanoparticles (tetrac NP) do not gain access to the cell interior and act exclusively at the integrin receptor. Moreover, Yalcin et al. (2009) reported that acting via a cell surface receptor; tetrac and tetrac NP inhibited growth of h-MTC cells and associated angiogenesis in chick chorioallantoic membrane and xenograft models. Tetrac blocks angiogenic and tumor cell proliferation actions of thyroid hormone initiated at the cell surface hormone receptor on integrin alphavbeta3. Tetrac also inhibits angiogenesis initiated by vascular endothelial growth factor and basic fibroblast growth factor. Similar

effects of tetrac were observed in breast cancer treatment (Glinskii et al., 2009). Nikolic et al. (2009) suggested radiolabeling procedure might have possible applications in cancer radiotherapy. In their study, the higher accumulation of radiolabeled nanoC (60) was observed in liver and spleen, while accumulation in thyroid, stomach, lungs and intestines was significantly lower in comparison to Na (125)I (Sodium-Iodine-125 radioactive tracer).

Development of efficient *in vivo* delivery nanodevices remains a major challenge to achieve clinical application of siRNA (small interfering RNA) (de la Torre et al., 2008, 2010). De Martimprey et al. (2010) showed that poly (alkylcyanoacrylate) nanoparticles coated with chitosan are suitable carriers to achieve *in vivo* delivery of active siRNA to tumor including after systemic administration. In that study, the loading of the nanoparticles with siRNA was achieved by adsorption.

The biological activity of the siRNA-loaded nanoparticles was assessed on mice bearing a papillary thyroid carcinoma after intratumoral and intravenous administration.

Huang et al. (2010) employed mannose-modified gold nanodots (Man-Au NDs) as a luminescence sensor for the detection of the thyroid-cancer marker thyroglobulin (Tg) in homogeneous solutions. Because luminescence quenching of the Man-Au NDs by Con A is inhibited by Tg selectivity, scientists obtained a highly sensitive and selective assay for Tg.

The aforementioned literature data shows that nanotechnology provides the real tools for the live tumor imaging, differential diagnosis and experimental treatment. Many drugs can be delivered as NP and the opportunities for applying single and combined experimental medical therapy are almost inexhaustible.

## CONCLUSIONS AND FUTURE WORK

In a review article, the author described the facts regarding the angiogenesis in thyroid cancer. The current data from the conducted studies are insufficient to draw valid conclusions regarding the power of correlation between the MVD and pathological and clinical parameters. The major limitations of the conducted studies are the minor sample numbers, differences among methods applied (e.g. Chalkley and computer-assisted MVD calculation) and the lack of research data concerning correlative analysis of a comprehensive set of values including the comorbidities, inheritance patterns, exposure to toxic substances, etc. Future studies should be performed in order to clarify the differences between angiogenic patterns in various histological types of thyroid neoplasm stained with a wide armamentarium of immunochemical substances, not only with conventional stains (CD34, CD31, F8 etc.). Though VEGF is the major proangiogenic factor in tumors and in benign disease and normal tissues, new angiogenesis markers should be investigated for a better recognition of differences of the pathological entities. The other idea is to investigate the effect of ionizing radiation and cancerogens on vascular structures observed in thyroid tissues. That research might elucidate the pathological changes similar to those which occur in nuclear atomic disaster survivors and provide knowledge for the implementation of drugs for radiation damage prevention and cancer treatment. And in conclusion, novel research opportunities are provided by the recent advances in digital pathology and image processing. Various parameters derived via the image analysis should be correlated with the available clinical and laboratory information in order to discover new mechanisms, correlative chains, pathways and predictors. Regarding the nanotechnology applied to the sphere of endocrine cancers and thyroid disease in particular, it is difficult to predict all the effects of NP to the alive human organism and that is why the major sources of experimental evidence are animal studies where tumors are produced unnaturally and NP are delivered during various stages of pathological process. Based on the data reporting benefits of application of the nanotechnology for the experimental treatment of MTC and follicular neoplasms assuming that anaplastic cancer is the most lethal, it is logical to apply the described nano approaches to that disease. Moreover, for the tumoral angiogenesis suppression, it can be hypothesized that NP can serve as a highly selective vascular disrupting agents. The idea of creating nanorobots for the goals of intervention into the process of the disease expansion can be criticized as being unrealistic and ridiculous. However, what is already done on the problem can show some encouraging promise. Enormous volumes of literature data describing nanotechnology and relevant issues are now available due to the growth of free internet-based libraries and new research tools for medical and technical data mining, processing and application. These allow an effective collaboration of scientists worldwide. It seems that one of the major problems regarding the implementation of nanotechnology methods for the prevention and treatment of endocrine malignant tumors is what chain of the disease pathology to impact and how to perform it practically in conditions of the laboratory.

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