The roles and role-players in thyroid cancer angiogenesis

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Abstract. Thyroid cancer is the most prevalent endocrine cancer worldwide. Angiogenesis, the formation of new blood vessels, plays a pivotal role in the development and progression of tumors. Over the past years, cancer research has focused on the ability of tumors to induce newly formed blood vessel, because tumor growth and the process of cancer metastasis mainly depends on angiogenesis. Tumor neovascularization occurs following the imbalance between pro-angiogenic and anti-angiogenic factors until the tumor switches to an angiogenic phenotype. A number of signaling factors and receptors that are implicated in the regulation of angiogenesis have been identified and characterized; most notably, the vascular endothelial growth factors (VEGFs) family and their receptors, which are the main pro-angiogenic molecules during early development and in pathological conditions such as cancer. Although thyroid is a highly vascularized organ, angiogenic switch in tumors of this organ leads to the formation of a vast network of blood vessels that favors the dissemination of tumor cells to distant organs and results in deterioration of patient conditions. Accordingly, the identification of key angiogenic biomarkers for thyroid cancer can facilitate diagnosis, prognosis and clinical decision-making and also may help to discover targeting factors for effective cancer therapy as well as monitoring response to therapy. Hence, the main purposes of this review are to summarize the types and mechanisms of angiogenesis emphasizing the prominent factors implicated in thyroid cancer angiogenesis.

Key words: Thyroid cancer, Biomarker, Angiogenesis, Metastasis

Introduction

Thyroid cancer is the most prevalent endocrine cancers, with an estimated incidence of 12.2 cases per 100,000 people per year in the United States [1]. This type of cancer occurs with various biological behaviors, ranging from indolent, well-differentiated tumors to highly aggressive, poorly differentiated or anaplastic tumors [2]. Growth of a malignant tumor critically depends on the extent of blood vessels providing a sufficient supply of

oxygen and nutrients along with the removal of waste products. Moreover, the formation of the new blood vessels plays an important role in metastatic spread to another organ [3]. According to scientists opinion, Ide, *et al.* were the first researchers that introduced the concept of angiogenesis in the tumor. They indicated that the implantation of tumor tissues in the ears of rabbits was accompanied by the formation of new capillaries suggesting the idea that angiogenesis is obligatory to support malignant tumor growth [4]. Two years later, Harry Green also showed that the growth of rabbit tumors transplanted into the anterior chamber of the eyes of guinea pigs had been accompanied by the growth of new blood vessels [5]. However, about 30 years later, Judah Folkman started a systematic study to think about cancer in a revolutionary new way. He assumed that malignant tumors need blood vessels to survive and grow, and the disruption of such vessels could result in cancer starvation and remission [6, 7]. Indeed, Folkman's pioneering study led him and others to understand the response of tumors to hypoxia, which can initiate angiogenesis and

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tumor growth [8]. It is well-known that angiogenesis occurs during the embryonic period and remains, with less extent, during adulthood [9]. Angiogenic activity has been shown to be crucial to thyroid cancer progression [10]. Accordingly, in recent years many efforts have been devoted to investigate and present the suitable biomarkers of thyroid angiogenesis [11]. Biomarkers are commonly involved in biological processes such as angiogenesis, differentiation, and growth. Thus, dysregulation of these biomarkers could result in cancer initiation, progression, and metastasis. It has been noted that these biological factors could be used as diagnostic, prognostic and therapeutic markers. Identification of important cancer biomarkers also sheds light on novel approaches in cancer therapy [12]. Hence, the main purposes of this review are to summarize the types and mechanisms of angiogenesis emphasizing the prominent factors implicated in thyroid cancer angiogenesis.

Thyroid Vascular Supply

Thyroid as an important part of endocrine system that directly releases hormones to the blood circulation has to be highly vascularized. Two major arteries supply the thyroid gland: 1) The superior thyroid artery that reaches to the superior pole of the lateral lobe of the gland where it divides into anterior and posterior glandular branches; 2) The inferior thyroid artery that supplies the inferior pole of the lateral lobe of the thyroid gland and divides into an: inferior branch, which supplies the lower part of the thyroid gland; and an ascending branch, which drains the parathyroid glands. Occasionally, a small thyroid ima artery also reaches the thyroid gland. Thyroid has three veins, namely the superior, the middle and inferior thyroid veins. Lymphatic vessels of the thyroid gland drain paratracheal and cervical nodes [13].

The Process of Angiogenesis

In the course of embryogenesis, neovascularization takes place in two different ways, including vasculogenesis, the *de novo* formation of new vessels from endothelial cell progenitors occurring in embryogenesis, and angiogenesis, as the sprouting of new vessels from existing ones. In the adults, angiogenesis can occur only transiently as part of physiologic processes such as wound healing and female reproductive cycling [14-17]. Accordingly, embryonic vascular network development is required to provide oxygen and nutrients for the fetus, while physiological angiogenesis in the adults occurs in response to the metabolic demands of tissues and is effectively triggered by hypoxia [18]. During embryogenesis, mesenchymal cells differentiate into angioblasts,

which are precursors of endothelial and hematopoietic cells from mesodermal tissues [19]. Angioblasts have the ability to migrate through the embryo and differentiate into endothelial cells forming vessel walls [20]. Because the thyroid gland is a highly vascularized gland, impaired angiogenesis during embryo development may lead to thyroid dysfunction [21]. Fetal angiogenic factors such as placental growth factor (PlGF) vascular endothelial growth factor (VEGF) and soluble FMS-like tyrosine kinase-1 (sFlt1) have been shown to affect thyroid function and vascular density [22]. It is noted that the vasculature of solid tumors is remarkably distinct from that of normal vasculature. For example, blood vessels that supply tumor tissue have irregular size and disorganized arrangement, where they simultaneously share features of arterioles, capillaries, and venules [23]. In normal tissue vessels, metabolic requirements of cells determine the blood flow and density of the vessels, but in tumor tissues, the blood flow appears to be sporadic, which can lead to damaged capillary network systems [24, 25]. During the aforementioned physiologic processes, there is an intricate balance between pro- and anti-angiogenic signals that are strongly maintained, leading to provide maturity and stability for newly formed vessels. However, tumor-related angiogenesis is shown a disrupted balance between these angiogenic factors, allowing unlimited development of blood vessels. This abnormal shift could result in an alteration in normal vasculature characteristics including their physical properties [26]. Tumor neovascularization has been classified into five types: 1) Angiogenesis; 2) Vasculogenesis; 3) Vascular remodeling: intussusceptive or splitting vascular growth is defined as the insertion of interstitial tissue columns into the vascular lumen and subsequent growth of these columns leading to the partitioning of the vessel lumen. In this type of neovascularization, endothelial cell division is not needed; 4) Glomeruloid angiogenesis is the formation of highly complex vascular structures that are similar to glomeruli of the kidney; 5) Vascular mimicry, referring to the ability of tumor cells to form a complete capillary network composed of neoplasm cells themselves without vascular endothelial cells that conduct blood [27]. However, some studies have described two main types of the angiogenesis: sprouting, which is the expansive growth of new capillary vessels out of pre-existing ones and intussusceptive (splitting) angiogenesis, which is defined as the rebuild of the blood vessel network [28, 29]. More interestingly, Benest, *et al.* described another form of angiogenesis termed looping angiogenesis, which is involved in the special requirements of wound healing [30]. Fig. 1 shows different types of tumor neovascularization. It is noteworthy to mention that the type of angiogenesis in a certain tissue

Fig. 1 Diagram illustrating different types of tumor angiogenesis

or organ depends upon the number of vessels already present during the initiation of the fast development of the organ or the tumor [31]. The process of angiogenesis is composed of several steps: 1) Activation of the endothelial cells, which is accompanied by overexpression of receptors such as VEGF receptors and $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins etc. Degradation of vascular basement membrane and of the extracellular matrix, by matrix metalloproteinases (MMPs) and the plasminogen pathway, also occur in this step; 2) The proliferation and migration of activated endothelial cells into interstitium leading to endothelial tube formation; 3) Differentiation of endothelial cells; 4) Synthesis of new basal membrane and the maturation of new vessels accompanied by vascular lumen formation [32–34].

Types of tumor angiogenesis

Tumor angiogenesis, as a fast growing process, is an important event in tumor biology. For about three decades, the sprouting angiogenesis originating from existing vessels was considered as an only way of tumor vascularization. However, in recent years some other mechanisms have been identified [35]. On the other hand, before the publications by Caduff, *et al.* [36] and Burri and Tarek [37] on blood vessel formation by intussusception, the sprouting model was the first known mechanism of angiogenesis. They find this new mechanism when they were studying the transformation of the capillary network in the respiratory systems of postnatal rats and humans during the investigating of postnatal lung devel-

opment [36, 38]. It has been totally accepted that angiogenesis occurs mainly through sprouting angiogenesis together with some degree of the intussusceptive mechanism [39, 40]. However, more recently, another form of tumor angiogenesis called looping angiogenesis has been revealed to occur in a non-developmental context [41].

Sprouting angiogenesis

Sprouting angiogenesis is the oldest described mechanism of angiogenesis [42]. Our knowledge of sprouting angiogenesis is originally based on results obtained from many developmental models, such as mouse and zebrafish models as well as CAM and advanced 3D *in-vitro* models [43–47]. According to some investigations, sprouting angiogenesis is started in poorly perfused tissues when terminal branches of the existing vessels, which serve as oxygen sensors, detect some degree of hypoxia that demands the formation of new blood vessels to provide the metabolic requirements of the tissue cells [48, 49]. In cancerous tissues, sprouting angiogenesis is not only implicated in primary tumors, but it also has a crucial role in the formation of metastasis and further outgrowth of metastases [14]. The process of sprouting angiogenesis is similar to the multi-step mechanism of angiogenesis mentioned above. Initiation of capillary sprouting is triggered by proangiogenic chemokines and cytokines, leading pericytes to detach from the vessel wall. Then, plasma proteins leave the vessel to form a temporary extracellular matrix, which has several roles, including cells migration guidance, storing proangiogenic molecules such as VEGF, fibroblast growth factors

(FGFs) and promoting synergistic signaling with integrins [50, 51]. Subsequently, the release of growth factors during proteolytic degradation and remodeling of the temporary matrix leads to proliferation and migration of endothelial cells [52]. In developmental sprouting angiogenesis, branching guidance is under the control of expressed genes in the newly formed tissue, whereas guidance of sprouts in tumor context is supervised by the gradient of secreted factors in the tumor microenvironment [53-55]. It is postulated that VEGF is a key factor that plays a central role in the morphology of vessels during sprouting angiogenesis. Notably, the gradient of this factor can regulate the migration of tip cells, a subset of endothelial cells as leading cells at the tips of sprouts, and the proliferation of stalk cells, which move behind the tip cells, in a polarized manner [43, 56]. VEGFs and VEGFRs signaling represent one of the best-validated and most investigated molecular pathways in angiogenesis [57]. There are several isoforms of VEGFs and VEGFRs (Fig. 2). VEGF gene family comprises six different isoforms, including VEGFA, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) [58]. All these VEGF molecules bind to three different receptor tyrosine kinases termed VEGFR-1, VEGFR-2, and VEGFR-3 [59]. Some VEGF family members also bind to non-tyrosine kinase receptors called neuropilin (NRP) family, NRP-1 and NRP-2, which are known as co-receptors for the VEGFRs [60]. VEGFR-1, VEGFR-2, NRP-1, and NRP-2 have binding sites for VEGFA; VEGF-B binds to VEGFR-1 and NRP-1; PlGF interacts with VEGFR-1, NRP-2, and NRP-1; and VEGF-C and VEGF-D bind to VEGFR-3, VEGFR-2, NRP-1 and NRP-2 [61]. It is discovered that VEGF members have been also involved in lymphangiogenesis (generation of lymphatic vessels), hematopoiesis, recruitment of monocytes during inflammation or infection, and proliferation or survival of other cell types expressing VEGFRs or NRPs [62-65]. VEGF/VEGFR signaling pathway triggers cascades such as Mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, Phospholipase C-gamma (PLC- γ) pathway, and endothelial nitric oxide synthase (eNOS) signaling to exert their effects [66, 67]. As noted earlier, the hypoxic environment induces the angiogenesis. It is documented that hypoxia can stimulate parenchymal cell of the tissues to secrete VEGF-A (also known as VEGF) as a key proangiogenic factor that there appears to be a completely unique factor in hypoxia-induced angiogenesis [68-70]. It is also clear that the tip cell guides the developing vessel sprout through extracellular matrix toward VEGF-A gradient [49, 69]. Delta-Notch signaling pathway and its ligand, Delta-like-4 (Dll4), have an essential role in sprout formation. Interestingly, VEGF-A

has been reported to induce the production of Dll4 by tip cells leading to inhibition of disorganized sprout formation [71-73].

Intussusceptive angiogenesis

Sprouting model is a time consuming and energy intensive mechanism of angiogenesis. Thus, nature has developed a more rapid way termed intussusceptive angiogenesis to create new vessels [74]. Intussusceptive angiogenesis mechanism occurs by longitudinal splitting of existing vasculature into two functional vessels [75]. This type of angiogenesis was first described by Caduff, *et al.* in 1986 when they were studying the pulmonary capillary bed of newborn rats using electron microscopy [76]. According to results of one study, application of VEGF-A in the chick chorioallantoic membrane (CAM) could result in stimulation of intussusceptive angiogenesis in the capillary plexus and terminal feeding vessels of this membrane. These authors have also concluded that high levels of VEGF can induce endothelial cells that undergo intussusceptive angiogenesis to either sprout or fuse or maybe even both [77]. The process of intussusceptive angiogenesis includes four steps: 1) Intussusceptive endothelial cells opposite from one another within the capillary wall migrate to each other to form a transluminal pillar; 2) After initial contact, rearrangement of endothelial cells forms a central perforation in the core of the pillar forming a cylindrical bridge in the central part of the vessel; 3) Central pillars are invaded by pericytes and myofibroblasts to form an ECM in pillars; 4) Finally, the pillars increase in size and being mature to split up the initial capillary into two new one [78]. Although intussusceptive angiogenesis was discovered in developing lungs, many reports indicated that this type of angiogenesis also occurs in wide range of other tissues, including muscle, bone, kidney, retina, mammary gland, ovary and many more [79-85]. This type of angiogenesis has been reported in some tumor tissues such as breast tumors, colon tumors, gliomas, hepatocellular carcinomas [86-89]. There is some evidence indicating the escape mechanism after antiangiogenic therapy using vandetanib (a tyrosine kinase inhibitor) combined with ionizing radiation [90, 91]. Results of these studies showed that this type of combination therapy could result in an angiogenic switch from sprouting to intussusceptive angiogenesis, representing an escape mechanism and resistance of the tumor to treatment. Makanya, *et al.* studied the interplay between sprouting and intussusceptive vascular growth modes in the development of the microvascular network in the chicken embryo lung and kidney [92, 93]. In one study, they found that high expression of VEGF was related to sprouting mode of angiogenesis, whereas, strong expression of FGF was observed in the intussusceptive form of angiogenesis

Fig. 2 VEGF family and their respective receptors

[92]. In another study, they showed that the expression of VEGF-A and FGF were maintained at moderate levels during the sprouting phase of angiogenesis, while Platelet-derived growth factor-B (PDGF-B) expression was minimal. However, the expression levels of all three factors were increased during intussusceptive angiogenesis [93]. Together, it appears that the intussusceptive angiogenesis does not directly need endothelial cell proliferation and as mentioned above, migration and rearrangement of endothelial cells of the existing vessel can provide new vessel network. Thus, it can be acceptable that this type of angiogenesis is not or less dependent on proliferative pathways such as the VEGF signaling pathway and that the VEGF inhibitors do not appear to hamper the tumor growth.

Looping angiogenesis

As mentioned earlier, looping angiogenesis is a form of angiogenesis process that is involved in special requirements of wound healing [30, 94, 95]. Although looping Angiogenesis has been observed in several non-developmental settings [96], there is an emerging body of evidence showing that this type of angiogenesis occurs in tissue revascularization in post-developmental settings. Namely, the process was indicated to be the main mechanism of wound vascularization in human

surgical wounds [97]. This type of angiogenesis is not regulated by traditional way involving pericytes and endothelial cell, but rather by contractile myofibroblasts that pull the extracellular matrix and adjacent tissue into the wound area, resulting in elongation of vessels and the vascular loops formation. Thus, loop-shape expansion of the vessel by looping angiogenesis is a mechanical mode of neovascularization that there appear to be less dependent on signaling pathways such as the VEGF pathway [95]. However, Maes, *et al.* recently suggested that during endochondral development, interactions of osteoblastic precursors with the endothelium may cause the invasion of mature vessels into the cartilage, probably *via* both looping and sprouting angiogenesis mechanisms [98]. More interestingly, they also proposed that cartilage vascularization may steer by the high levels of stimulatory factors, including VEGFs. Although looping angiogenesis process has not been fully understood and has not yet been indicated to be a pivotal angiogenic process implicated in vessel growth during tumor vascular development, there are multiple pieces of evidence indicating the relation between tumor neovascularization and wound healing. Notably, tumors have formerly been described as “wounds that do not heal” [99]. In addition, identification of hypoxic tumor cell areas in squamous

cell carcinomas of the uterine cervix showed the evidence of looping angiogenesis, which was clearly visible as a finger-like protrusion that was pulled into the direction of the hypoxic center of a tumor cell aggregate [100]. The results obtained from time-lapse intravital multiphoton microscopy showed that the mechanical forces in rapidly growing tumors could extend the vessels in a process that could be considered as looping angiogenesis, which may occur with other types of angiogenesis [101].

Mechanism of Control of Angiogenesis

It is now clear that the angiogenic activity of tumor tissue is a result of the net balance between pro- and anti-angiogenic factors [102]. To date, several stimulatory and inhibitory regulators of angiogenesis have been identified (Table 1). The proportion of these opposite factors determines the angiogenic behavior of the tumor. When stimulatory factors are produced in excess of inhibitory factors, the balance is in favor of vessel expansion. On the contrary, when inhibitory factors are dominant, angiogenic activity is ceased [102-104]. It is acceptable that the up-regulation of a stimulatory growth factor is not sufficient for tumor angiogenesis, but rather the down-regulation of certain inhibitory regulators may be necessary for vessel growth [105]. However, the scenario appears to be much more complex than it at first seems. There are some other molecular mechanisms implicated in vascularization of the tumor. For example, some oncogenes, including c-Met down-regulates thrombospondin-1 (TSP-1), leading to angiogenesis and tumor progression [106]. Moreover, there are multiple oncogenes such as ras, raf, src, and erbB2, which positively regulate the expression of VEGF [107-110]. Otherwise, tumor suppressors such as von Hippel-Lindau (VHL) negatively regulate the expression of VEGF [111]. p53, as a transcription factor involved in the checkpoint of the cell cycle, can act as a tumor suppressor by activating the promoter of TSP-1 [112]. The most important factor that controls blood vessel growth is VEGF, particularly, VEGF-A, which is pivotal for many aspects of the angiogenesis being equally prominent for blood vessel development in adults and tumors [113]. VEGF also stimulates integrin $\alpha v\beta 3$ to promote both neovascularization and tumor cell invasion *via* ECM through paracrine and autocrine regulatory mechanisms [114, 115]. FGF, as a potent angiogenic inducer, has different in control of neovascularization by interaction with other growth factors and chemokines such as VEGF, hepatocyte growth factor (HGF), PDGF, and Monocyte chemoattractant protein-1 (MCP-1). However, it appears that the unique role of this factor is the organi-

Table 1 Different stimulatory and inhibitory regulators of angiogenesis

Stimulators	Inhibitors
VEGFs	Angiostatin
FGF	Anti-angiogenic anti-thrombin III
MMPs	TIMPs
PDGF	Vasostatin (calreticulin fragment)
Angiopoietin-1	Canstatin
HGF	Endostatin (collagen XIII fragment)
Leptin	Fibronectin fragment
MCP-1	Heparinases
β -Estradiol	IFN- α , β , γ
NOS	IL4, IL12, IL18
IL-8, IL-1	Plasminogen activator inhibitor
TNF- α	PEDF
Angiogenin	Prolactin 16 kDa fragment
TGF- α/β	TSP-1
Ephrins	Retinoids
integrins $\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha 5\beta 1$	Tumstatin
COX-2	Platelet factor-4 (PF4)
Histamine	Kringle 5 (plasminogen fragment)
Plasminogen activator	Fragment of SPARC
PIGF	Angioarrestin
Bfrf	Restin
IGF-i	Vasoinhibin

zation of different angiogenic pathways and coordination of cell-cell interactions in this process [116]. ECM has multiple components around the vessels, of which many are proteins and have stimulatory or inhibitory effects on angiogenesis [117]. The proteolytic release of matrix-bound VEGF isoforms has been proved to be a substantial regulator of VEGF bioavailability and pattern of the vasculature [118]. ECM proteases such as MMP9, on the one hand, can release matrix-bound VEGF and promote angiogenesis, for example as an important phase in tumorigenesis; on the other hand, these enzymes can also suppress pro-angiogenic activities of VEGF [119]. In addition, it has been thought that physical contact between endothelial cells and pericytes may result in the induction of quiescent phenotype without sprouting of the vessels [120, 121].

The Role of Angiogenesis in Tumor Development and Metastasis

Several lines of experimental and clinical investiga-

tions verify the concept that angiogenesis is a key element for invasion, progression, and metastasis of the tumors [122, 123]. Angiogenesis dependency of the tumor was confirmed by multiple pieces of evidence that indicated that tumor growth and metastasis are inhibited by anti-angiogenic factors [124, 125]. Most tumors remain in the stage, which is called carcinoma *in situ*, without angiogenesis for a long time. This stage is considered as a pre-cancerous neoplasm that may be terminated by vascularization of the tumor during the phase termed angiogenic switch when the tumor requires to keep growing [126]. Folkman believed that angiogenesis itself should be considered as “organizing principle”, which means that there are basic connections between molecular mechanisms apparently unrelated phenomena, not as a sign of malignancy [127]. This point of view has prominent consequences for developing therapeutic approaches in cancer and other unrelated diseases, leading to a discovery in one field to be utilized to describe events in another field [128]. Although pathological dysregulation of cell cycle control was found that is the first step in the development of a tumor, it is now accepted that most of the dysregulated pathways that lead to cancer are the consequence of deregulated angiogenesis and functions of tumor vasculature [129]. Notably, the tumor vasculature may involve in the deregulated tumor metabolism, genetic instability, inflammation, and metastatic tumor growth [130, 131]. Tumors are often hypoxic and this hypoxic state results in advanced but dysfunctional vascularization and can alter different aspects of tumor metabolism [132]. Tumor-hypoxia was reported as the main cause of highly reactive oxygen species (ROS) production within the tumor cells [133] and also pathological metabolism and acidosis in the tumor tissue [134, 135]. Pathological angiogenesis of tumor, microvascular leakage and hypoxia-induced apoptosis in the central part of the tumor together lead to tremendous recruitment and activation of inflammatory cells, including macrophages, lymphocytes, neutrophils, and mast cells. These immune cells contribute to produce massive growth and pro-angiogenic factors and cytokines *via* communication with a complex network of signaling pathways [136-138]. The inflammatory cytokines interleukin-1 α and interleukin-1 β , as well as other angiogenic factors such as VEGF and MMPs, may implicate in angiogenesis, development of a tumor, and cancer metastasis [139, 140]. The deregulated tumor vasculature in relation with hypoxia can induce angiogenesis in cancer-associated fibroblasts (CAFs), which involved in the tumor progression and resistance to treatment [141]. On the other hand, these cells are rich sources of tumor angiogenic growth factors and cytokines, playing an important role in sustaining tumor angiogenesis and rendering resistance to

anti-angiogenic agents [142]. Angiogenesis has a key role in tumor metastasis to the distant organ (s). In some tumor types such as neurological cancers, which mostly metastasize locally, dissemination of tumor cells often occurs through the vasculature as tumor cells intravasate into blood vessels and invade the neighboring tissue by crawling along the endothelium [143]. In addition, most malignant tumors also depend on blood vessels to spread to the distant secondary organ(s). Of note, several factors such as the origin of cells, intrinsic features of the tumor, organ tropism and circulation patterns determine not only the sites of tumor dissemination but also the temporal course and severity of metastasis to other organs [144].

Factors Involved in Thyroid Cancer Angiogenesis

It has been shown that the maintenance of thyroid vasculature is mainly regulated by several ligands and its receptors, including VEGFs and their receptors (VEGFRs), and angiopoietins (Angs) and the Tie2 receptor [145-148]. A large body of evidence indicates that alterations of such crucial factors and some of other factors and receptors could implicate in a variety of thyroid abnormalities [149-152].

VEGF and VEGFR

As mentioned earlier, there are several VEGFs and VEGFRs that each VEGF ligands bind to their specific receptor(s) [61]. It is supposed that the over-expression of VEGF is a feature of differentiated thyroid carcinomas and is related to increased growth, progression, and invasiveness of the tumor [153-156]. Hsiao, *et al.* reported that the A allele of -2578C/A SNP (single nucleotide polymorphism) in the promoter of VEGF gene may increase the risk for thyroid cancer development and regional lymph node metastasis, suggesting the role of VEGF in tumor growth and spreading, the two processes which depend on angiogenesis [157]. To evaluate the potential role of angiogenic factors in human thyroid tumor growth and spread, Bunone, *et al.* analyzed their expression by semiquantitative RT-PCR and immunohistochemistry in normal thyroid tissues, benign lesions, and different thyroid carcinomas [158]. They found a consistent increase in VEGF, VEGF-C, and angiopoietin-2 and in their tyrosine kinase receptors VEGFR2, VEGFR3, and Tek (TEK receptor tyrosine kinase). They also reported a strong correlation between tumor size and over-expression of VEGF and angiopoietin-2. The results of that study also showed an augmented expression of VEGF-C in lymph node invasive thyroid tumors and a reduced TSP-1 expression in

thyroid malignancies capable of hematic metastasis. Together, these findings suggest that, in human thyroid tumors, angiogenesis factors appear implicated in neoplastic growth and aggressiveness. Finally, their results are in keeping with a recent 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 [158]. De la Torre, *et al.* analyzed microvascular density (MVD), lymphatic vascular density (LVD), and expression of angiogenic and lymphangiogenic factors in normal thyroid, multinodular goiter (MNG), toxic multinodular goiter (TNG), Graves' hyperplasia, follicular adenoma (FA), papillary thyroid carcinoma (PTC), incidental papillary microcarcinoma (PMC), follicular carcinoma (FC) and medullary carcinoma (MC) [159]. MVD was decreased in all samples, however, LVD was increased in PTC and PMC. Moreover, the expression levels of VEGF-A and VEGF-C in thyroid cancers have been increased. However, these markers have not been correlated with the presence of multifocal disease, distant metastases at diagnosis or increased tumor size [159]. Results of another nearly similar study showed an increase in both the distribution and intensity of VEGF immunopositivity in MNG, FA and PTC as compared with the autoimmune diseases of Graves' disease and Hashimoto's disease. The expression of VEGFR1 and VEGFR2 showed minimal variations between all samples except for Graves' disease, suggesting the importance of the up-regulation of VEGF expression rather than its receptors in these diseases. Furthermore, an increased MVD in PTC was found in comparison with normal controls. However, the authors of that study have reported no clear relationship between MVD measurement and thyroid pathology [160]. The other similar study showed a strong expression of VEGF and high MVD in differentiated malignant thyroid cancers compared to poorly differentiated thyroid tumors and other thyroid tissue samples, indicating the role of VEGF as a hypoxia-inducible angiogenic factor, which is overexpressed, concomitant with hypervascularity, in malignant thyroid tumors, which need more oxygen to proliferate, than in benign follicular tumors [161]. High levels of VEGF expression and related hypervascularity in follicular tumors have also been reported in different studies [162, 163]. Tanaka, *et al.* studied the expression patterns and levels of some angiogenic and anti-angiogenic factors in PTC samples [164]. According to the results of that study, VEGF expression strongly correlated with other angiogenic factors. The cytoplasm of cancer cells stained positive for all factors. Tumor invasiveness and microvessel count (MVC) had an inverse correlation with the levels of TSP-1. The authors calculated the ratio

of each angiogenic factor against TSP-1 as the antiangiogenic factor to assess the balance between angiogenic and antiangiogenic factors in the same tumor. The ratios VEGF/TSP-1, VEGF-C/TSP-1, and Ang-2/TSP-1 remarkably correlated with a higher MVC. In addition, the ratios VEGF/TSP-1 and Ang-2/TSP-1 significantly correlated with the degree of infiltration. There are also several lines of evidence indicating the role of VEGF in the induction of angiogenesis as well as the correlation between invasiveness and metastatic properties of PTCs [165-167]. Gwiedzinska, *et al.* conducted a study to compare the serum VEGF concentration between patients with metastatic and non-metastatic thyroid cancer, multinodular goiter, and healthy subjects [168]. The study group consisted of 71 patients, with differentiated thyroid cancer (50 papillary, 17 follicular, and 4 oxyphilic), treated in their department during the years 2003–2006. They found that the levels of serum VEGF were remarkably higher in patients with distant metastases than those in remission or healthy patients, suggesting that it may be used as a marker of thyroid cancer with distant metastases. Bugallho, *et al.*, however, found that serum concentration of VEGF in MTC patients were not significantly different from those found in healthy subjects and did not correlate with the extension of disease [169]. Narita, *et al.* also reported a decreased VEGF-D serum levels in patients with metastases of differentiated thyroid cancer, regardless of the degree of metastatic spread [170]. They suggested that it is possible that some other molecule produced by the tumor could affect the VEGF-D, which is physiologically produced from different tissues, leading to a decrease in the serum levels of VEGF-D of these patients. Capp, *et al.* found an overexpressed VEGF-A, VEGFR-1, and VEGFR-2 proteins in MTC lesions [171]. Accordingly, they concluded that the increased expression of these molecules might be involved in tumor progression, but this might not provide prognostic information regarding the spread or outcome of MTC [171]. Results of another study, however, showed that overexpression and activation of VEGFR2 and EGFR in MTCs is related to metastatic characteristics of the tumor [172]. It has been demonstrated that the expression of VEGF *in-vitro* has a correlation with *in-vivo* aggressiveness of the tumors, with anaplastic tumors having augmented expression of VEGF [10].

Thyroid stimulating hormone

Thyroid stimulating hormone (TSH) is a glycoprotein produced by thyrotropic cells of the anterior pituitary gland, which binds to specific cell surface receptors in the thyroid gland where it stimulates the synthesis of thyroid hormone [173]. More importantly, TSH has been

considered as a key factor that stimulates the number, size, and activity of thyrocyte cells from which differentiated thyroid cancers arises [174]. Moreover, it has been shown that TSH stimulates thyroid cancer growth, invasion, and angiogenesis. Thus, an inevitable treatment for patients with thyroid cancer includes supraphysiologic doses of thyroid hormone analogs to suppress the TSH secretion [175]. A recent study conducted to investigate the role of TSH in PTC tumor growth, focusing on tumor microenvironment [176]. In that study, Cho, *et al.* implanted the tumorigenic clone of PTC cells (BHP10-3SCp) in nude mice, following recombinant human TSH (rhTSH) or saline for 3 weeks ($n = 10/\text{each}$). Larger tumor, higher MVD and higher density of macrophages were reported in the rhTSH group compared with the saline group from day 15 to day 20. The authors also evaluated the mechanism of TSH actions on tumor angiogenesis. To meet this purpose, they treated the BHP10-3SCp cells with TSH and subsequently, measured the expression of VEGF. They showed that VEGF expressions were up-regulated by 3-fold and 9-fold at 48 and 72 hours, respectively. The authors concluded that TSH stimulates PTC tumor growth by enhancing tumor angiogenesis and macrophage recruitment into tumor microenvironment and its action on PTC tumor angiogenesis was partly mediated by VEGF, which may be a potential therapeutic target in TSH-dependent PTC progressions [176]. Soh, *et al.* quantified the concentration of VEGF in conditioned medium of cultured thyroid cancer cell lines derived from PTC, FTC, MTC, and Hürthle cell carcinoma (XTC-1) as well as normal thyroid cells by means of enzyme-linked immunosorbent assay. The results showed that basal VEGF secretion was similar in normal and thyroid cancer cells, except XTC-1, which had high basal secretion. Upon TSH stimulation, all thyroid cancer cells secreted considerably higher VEGF concentrations than normal thyroid cells. According to these results, authors suggested that constitutive secretion of VEGF in some thyroid cancers is a result of TSH stimulation; thus TSH may promote the thyroid tumor growth by stimulating VEGF secretion and angiogenesis [177]. Hoffmann, *et al.* conducted a study to 1) assess the effect of TSH on VEGF levels as well as 2) to evaluate the TSH signal transduction of this effect, and 3) to screen other growth factors for the ability to modulate VEGF in thyroid cancer cell lines [178]. They used HTC, a follicular cancer cell line lacking endogenous TSH receptor (TSHr), its receptor positive variant (HTC TSHr), and an XTC cell line in order to achieve these goals. VEGF gene and protein expressions were analyzed after stimulation with TSH and some other growth factors. According to results of the study, TSH could induce VEGF mRNA and protein in a dose-dependent

manner in HTC TSHr and XTC cells by up to 40% involving protein kinase C pathway. However, it appears that EGF and transforming growth factor beta (TGF- β) could increase the capacity of thyroid cancer cells to produce VEGF more effectively than TSH. It seems that TSH stimulation has no above-mentioned effects on serum levels of VEGF in thyroid cancer patients. Tuttle, *et al.* proposed that serum VEGF in patients with no evidence of disease and 48 patients with local or distant metastases would be further increased by TSH stimulation [179]. Conversely, they found that no remarkable increase in serum VEGF concentrations can be detected 72 h after short-term TSH stimulation with recombinant human TSH. More interestingly, some investigations show that the endogenous and exogenous TSH stimulation even can significantly decrease the serum levels of VEGF, suggesting that TSH may exert its regulatory effects *via* receptors located outside the thyrocytes and may exert its effects on VEGF production from tissues other than the thyroid gland, respectively [168, 180].

Iodine

Iodine is the key material to produce thyroid hormones (T4 and T3). Iodine is absorbed and carried in the bloodstream as iodide. Iodide is incorporated into thyrocytes by the sodium/iodide symporter (NIS). Intracellular iodide is then transported in the lumen of thyroid follicles and is oxidized by thyroid peroxidase (TPO). Finally, iodine is integrated into tyrosyl residues of thyroglobulin (Tg), the protein upon which thyroid hormones are produced [181]. Iodine deficiency in normal thyroid has detrimental effects on thyrocytes due to the importance of this ion in thyroid hormones production. This deficiency could lead to hypothyroidism and goiter [182]. Using *in-vitro* models of rat and human thyroid cells, Gerard, *et al.* showed that iodine deficiency is associated with the release of VEGF-A *via* a reactive oxygen species/hypoxia-inducible factor-1 (ROS/HIF-1) related pathway [183]. Besides, deficiency of this ion also has interesting effects on thyroid cancer cells. Recent data show that iodine deficiency induces a long-lasting angiogenic phenotype in thyroid cancer cell lines that occurs *via* VEGF induction through a pathway partially mediated by hypoxia-inducible factor-1 (HIF-1). The results of that study indicate that induction of angiogenesis in cancer cells *via* alternative and likely less controlled pathways could result in uncontrolled growth [184]. More recently, Craps, *et al.* studied the involvement of mammalian target of rapamycin (mTOR) as a positive regulator and AMP-activated protein kinase (AMPK) as a negative feedback regulator of ROS/HIF-1/VEGF pathway to describe alterations of microvasculature in iodine-deficient models, including human thyrocytes and

two murine models of goitrogenesis: normal NMRI and RET-PTC mice (a PTC model) [185]. Iodine deficiency (ID) significantly increased the phosphorylation of ribosomal S6 kinase (p70S6K), a downstream target of mTOR, whereas rapamycin completely inhibited the ID-induced increase in p70S6K phosphorylation, thyroid blood flow, and VEGF-A expression in the RET-PTC and *in-vitro* models. However, these effects have not shown in NMRI model. The authors concluded that mTOR is needed for early ID-induced thyroid microvascular activation, however, AMPK negatively regulates this pathway [185]. On the other hand, excess iodine can have suppressing effects on thyroid dependent angiogenesis. Suzuki, *et al.*, based on *in-vivo* studies showing that iodine in excess suppresses thyroid function and blood flow *in-vivo*, described the molecular mechanisms of these effects [186]. They reported that excess iodide coordinately suppresses the expression of the sodium/iodide symporter (NIS) and VEGF genes in FRTL-5 thyroid cells. The results of this study suggest that excess iodide likely affects thyroid vascular function through VEGF molecule [186]. A few years later, Nascimento, *et al.* showed that inhibitory effect of excess iodine on NIS expression involves activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [187].

FGF and FGFR

Fibroblast growth factors (FGFs) are a family of heparin-binding proteins that exert their effects *via* specific binding to their cell surface receptors (FGFRs) that are equipped with tyrosine kinase activity, leading to the growth, differentiation, and function in many cell types [188]. They also have potent angiogenic characteristics and, as such, may be pivotal in the growth of solid tumors, where a rich blood supply is necessary [189]. There are several studies showing an augmented expression of FGFs and FGFRs in thyroid tumors, linking these ligands and respective receptors to etiology and development of these type of tumors [188, 190, 191]. Basic fibroblast growth factor (bFGF) or FGF2 was shown to have a key role in the development of PTC with induction of angiogenesis as well as the proliferation of the parenchymal cells [192]. Although there is an interdependency between FGF and VEGF in many tissues, it seems reasonable that FGF alone may act as an angiogenic factor in the thyroid, with direct effects on both endothelial and follicular cell growth [10]. Pituitary tumor-transforming gene (PTTG), as an oncogene, is a multifunctional protein that plays several potential roles in tumorigenic mechanisms, including angiogenesis [193]. Boelaert, *et al.* described a relationship between early recurrence of thyroid cancer and PTTG expression

in the original tumor suggesting the role of PTTG in the promotion of tumor growth, either directly or *via* stimulation of FGF-2. Consistent with this point of view is the observed association between FGF-2 expression and tumor staging at presentation, which supports a role for FGF-2 in mitogenesis and angiogenesis in thyroid tumors [194]. Interestingly, Redler, *et al.* observed the reduced expression of FGFR-2 in follicular adenoma and PTC samples [195]. They speculated that FGFR-2 down-regulation might be an early event in thyroid carcinogenesis, supporting the potential use of this receptor as an early marker for the diagnosis of thyroid cancers. However, there are several lines of studies showing the potential use of FGFRs as therapeutic targets to cease thyroid tumor growth and spreading [196-198].

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of endopeptidases which contain a zinc ion at their active site, for proteolytic activity. MMPs are produced by tumor cells and can affect tumor development in several ways, including the promotion of tumor growth, invasion and migration, angiogenesis and apoptosis inhibition. MMPs support angiogenesis by releasing pro-angiogenic factors and degrading ECM [199]. Among these proteolytic enzymes, MMP-2 and MMP-9 have been shown to have promoting effects on tumor invasion and angiogenesis by activation of TGF- β [200]. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous proteins, which are known to inhibit MMPs [201]. There are multiple investigations indicating the expression and overexpression of some MMPs and TIMPs related to the diagnosis and prognosis of thyroid cancers. For example, Cavaleiro, *et al.* reported that the ratio of MMP-2 to TIMP-2 expression is an important and novel prognostic factor to anticipate consequences of surgically treated MTC [202]. In another study, They also showed a significant correlation between the expression of MMP-2 and clinical characteristics of MTC patients, suggesting the use of this factor as an indicator of prognosis of this cancer [203]. Marko, *et al.* also reported a role for both MMP-2 and TIMP-2 in development of thyroid tumors and suggested the potential use of immunohistochemical positivity of these factors for diagnosis of PTC and prediction of the adverse behavior of the tumor [204]. Some studies revealed that overexpression of some MMPs is accompanied by an increased expression of pro-angiogenic factors such as VEGF and FGF, which are related to tumor growth and invasiveness [205, 206]. Table 2 shows the main factors involved in thyroid cancer angiogenesis.

Table 2 Factors involved in thyroid cancer angiogenesis

Factor	Type	Role in thyroid cancer angiogenesis	Effect on MVD ^a
VEGF	Growth factor	Pro-angiogenic	Increase
VEGFR	Growth factor receptor	Pro-angiogenic	Increase
TSH	Hormone	Pro-angiogenic	Increase
Iodine	Ion	Pro-angiogenic	NR ^b
FGF	Growth factor	Pro-angiogenic	NR ^b
FGFR	Growth factor receptor	Pro-angiogenic	NR ^b
MMP	Proteolytic enzyme	Pro-angiogenic	NR ^b

^a MVD, microvascular density^b NR, not reported

Conclusion

As mentioned earlier, there are several factors with some roles in the promotion of thyroid cancer angiogenesis. However, the effects of some factors on the angiogenic activity of this type of cancer are more remarkable than the others. Accordingly, VEGFs and its receptors appear to be the main role players in the angiogenesis process of thyroid tumors. Thus, other players seem to have an upstream regulatory effect on this central factor(s). However, all factors that mentioned in this review have potential to consider as suitable diagnostic, therapeutic and prognostic markers, but it is not possible to have a more comprehensive discussion on the role of these factors in thyroid cancer development and progres-

sion due to lack of sufficient data on their determining role in these processes. Hence, there appears a need for more appropriate and comprehensive studies to uncover different aspects of thyroid cancer angiogenesis with the emphasis on different angiogenic and antiangiogenic factors.

Conflict of Interest

The authors have no conflict of interest.

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