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ORIGINAL ARTICLE

Angiogenesis and angiogenic factor expression in thyroid cancer

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Summary

Purpose: *Increased expression of angiogenic factors and high vascular density characterize tumors with increased invasive and metastatic capability. Anti-vascular endothelial growth factor (VEGF) therapies have shown an important potentiation of chemotherapy and radiotherapy in experimental and clinical studies. The purpose of this study was to investigate whether it could be possible to identify a subgroup of thyroid cancer patients with high angiogenic activity.*

Methods: *Formalin-fixed paraffin-embedded tissues from 25 papillary and 18 follicular thyroid carcinomas were assessed immunohistochemically for angiogenic activity, i.e. vascular density (VD) and expression of VEGF and basic fibroblast growth factor (bFGF).*

Results: *VD was significantly higher in follicular tumors (p=0.05). Tumors > 4 cm had a significantly higher VD (p=0.001). High VEGF expression was significantly related to high VD (p=0.05). There was no association of bFGF with histological characteristics.*

Conclusions: *Increased angiogenic activity is a common feature of thyroid carcinomas, particularly in follicular tumors and larger carcinomas. These results support the testing of anti-VEGF therapies in combination with radiotherapy and chemotherapy in advanced thyroid tumors.*

Key words: angiogenesis, bFGF, thyroid cancer, VEGF

Introduction

Well differentiated thyroid cancer is a common malignancy with high cure rates after thyroidectomy and, when necessary, radioiodine administration. Nonetheless, about 10-20% of patients with large tumors and extensive capsular invasion will recur in lymph nodes or the thyroid bed and a minority will also exhibit distant metastasis [1,2].

The results of radiotherapy and chemotherapy in inoperable or recurrent tumors are poor [3,4]. Any therapeutic management for this group of patients has a rather palliative role. Targeted therapies with monoclonal antibodies and specific inhibitors recognizing important biological pathways of malignancy are gradually incorporated in clinical practice. Anti-angiogenic agents, such as anti-VEGF monoclonal antibodies, have already shown promising results in various malignancies [5,6]. Although not tested yet in patients with thyroid cancer, experimental studies suggest an important role of angiogenesis in thyroid cancer progression that can be effectively suppressed with anti-angiogenic policies [7,8]. Clinicopathological studies also confirm that high VD or VEGF expression is more frequently noted in metastatic thyroid tumors [9-11].

In the present study we assessed the VD and the expression of the angiogenic factors VEGF and bFGF in a series of thyroid carcinomas treated with thyroidectomy, in an attempt to identify immunohistochemically subgroups of thyroid tumors with high angiogenic activity.

Methods

Formalin-fixed paraffin-embedded tissues from 43 patients with thyroid carcinoma (18 follicular and 25 papillary carcinomas), treated with total thyroidec-

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tomy, were retrieved from the archives of the Department of Pathology, Democritus University of Thrace, Alexandroupolis, Greece. An additional 12 normal thyroid tissue samples were retrieved and stained. The patient and disease characteristics are shown in Table 1.

Immunohistochemistry

The JC70 monoclonal antibody (Dako, A/S Denmark), recognising CD31 (platelet/endothelial cell adhesion molecule; PECAM-1) and a standard immunohistochemical technique were used for microvessel staining [12]. VEGF was detected using the VG1 monoclonal antibody [13]. For the bFGF expression we used the sc-79 polyclonal antibody (Santa Cruz Biotechnology Inc). Three-μm sections were deparaffinized and peroxidase was quenched with methanol and 3% H_2O_2 for 15 min. Thereafter, slides were placed in antigen unmasking buffer pH 6.0 (code: TAR001, ILEM, Italy) and microwaving followed (3 × 4 min). Then, JC70 antibody was applied for 90 min, while overnight incubation was allowed for the VG1 and sc-79 antibodies. Following washing with TBS, sections were incubated with a secondary mouse anti-rabbit antibody (Kwik Biotinylated Secondary, 0.69A Shandon-Upshaw, Pittsburgh, PA, USA) for 15 min and washed in TBS. Kwik Streptavidin peroxidase reagent (039A Shandon-Upshaw, Pittsburgh, PA, USA) was applied for 15 min and sections were again washed in TBS. The color was developed after 15 min incubation with DAB solution and sections were weakly counterstained with haematoxylin. Normal immunoglobulin-G was substituted for the primary antibody as the negative control at the same concentration as the primary antibody.

For VD assessment, sections were first scanned at low power (×40 and ×100). Three areas of high VD were chosen at low power (×100), but vessel counting was performed on ×250 fields. If more than 3 areas of high vascularization were found at low power the vessel scoring was performed in all these areas to avoid erroneous omission of optical fields that would give the highest score. The final vessel score was the mean of the vessel counts obtained from 3 fields that gave the highest score. Blood vessels with a clearly defined lumen or a well defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting. The median VD (lower vs. higher/equal) was used to group cases into categories of low and high VD.

VEGF and bFGF have a cytoplasmic localization. The percentage of cancer cells with cytoplasmic reactivity was calculated in all ×250 optical fields, after scanning the whole tumor area. The mean value of these readings was used to provide a final percentage for each case. The median value of this score was the cut off point to divide cases in two groups of low vs. high reactivity.

Statistical analysis

Statistical analysis and graphic presentation were performed using the GraphPad Prism® 4.0 package (GraphPad, San Diego CA, www.graphpad.com). The Fisher's exact test or the Yates' continuity corrected chi-square test were used for testing relationships between categorical variables, as appropriate. A p-value ≤0.05 was considered significant.

Table 1. Clinical and pathological characteristics (n=43)

Characteristics	*No. of patients*	*%*
Sex		
Male	9	20.9
Female	34	79.1
Age (years)		
Range	18-84	
Median	47	
Histopathology		
Follicular carcinoma	18	41.9
Papillary carcinoma	25	58.1
Focality		
Unifocal	33	76.7
Multifocal	10	23.3
Size (cm)		
Range	0.2-8	
Median	2	
Vascular invasion		
No	38	88.4
Yes	5	11.6

Results

Vascular density

Normal thyroid gland exhibited a median VD of 34 vessels (range 26-40), showing that normal thyroid gland has a very rich vasculature. The VD in carcinomas ranged from 7 to 52 vessels per ×200 optical field with a median of 13. Using this median value, 21 cases were of low and 22 of high VD. Table 2 shows the relation of VD with patient characteristics and histopathological features. There was no statistical association with any of the parameters examined. There was, however, a trend for follicular carcinomas to fall into the high VD group. Further analysis of the VD as a continuous variable in the histopathology groups showed a significantly higher VD in follicular carcinomas compared to papillary tumors (mean VD 17.8±11 vs. 13±5;

Table 2. Vascular density and VEGF status in relation to clinical and pathological characteristics

Parameter	*Vascular density*			*VEGF status*		
	Low	*High*	*p-value*	*Low*	*High*	*p-value*
Sex						
Female	18	16	NS (0.45)	19	15	NS (0.71)
Male	3	6		6	3	
Age						
Years (±SD)	43±13	48±19	NS (0.40)	47±16	44±18	NS (0.58)
Range	18-66	20-84		18-79	20-84	
Histopathology						
Follicular carcinoma	7	11	NS (0.35)	10	8	NS (0.99)
Papillary carcinoma	14	11		15	10	
Size						
cm (±SD)	2.0±1.3	2.6±2.0	NS (0.30)	2.2±1.6	2.5±1.8	NS (0.60)
range	0.2-5	0.7-8		0.2-7	0.7-8	
Focality						
Unifocal	18	15	NS (0.28)	20	13	NS (0.71)
Multifocal	3	7		5	5	

VEGF: vascular endothelial growth factor, NS: non significant, SD: standard deviation

p=0.05; Figure 1). Figure 2A shows a typical immunohistochemical imaging of anti-CD31 immunostaining in a follicular thyroid carcinoma.

We further analysed the VD in tumors according to their dimensions (Figure 3). Although the number of cases did not permit reliable statistical analysis there was a clear trend for tumors exceeding 2 cm to exhibit a higher VD. Carcinomas larger than 4 cm had a significantly higher VD compared to smaller tumors (24.4±14 vs. 13.3±5; p=0.001).

VEGF expression

VEGF expression was purely cytoplasmic. The percentage of cells with VEGF reactivity ranged from 0 to 100% (median 40). Figure 2B shows immunohis-

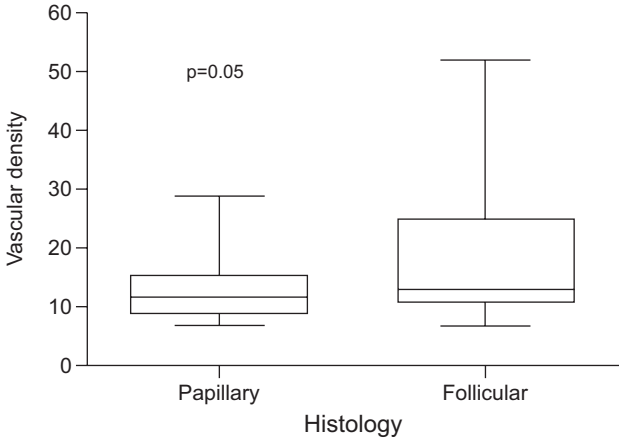

Figure 1. Vascular density according to the histological type of thyroid carcinomas.

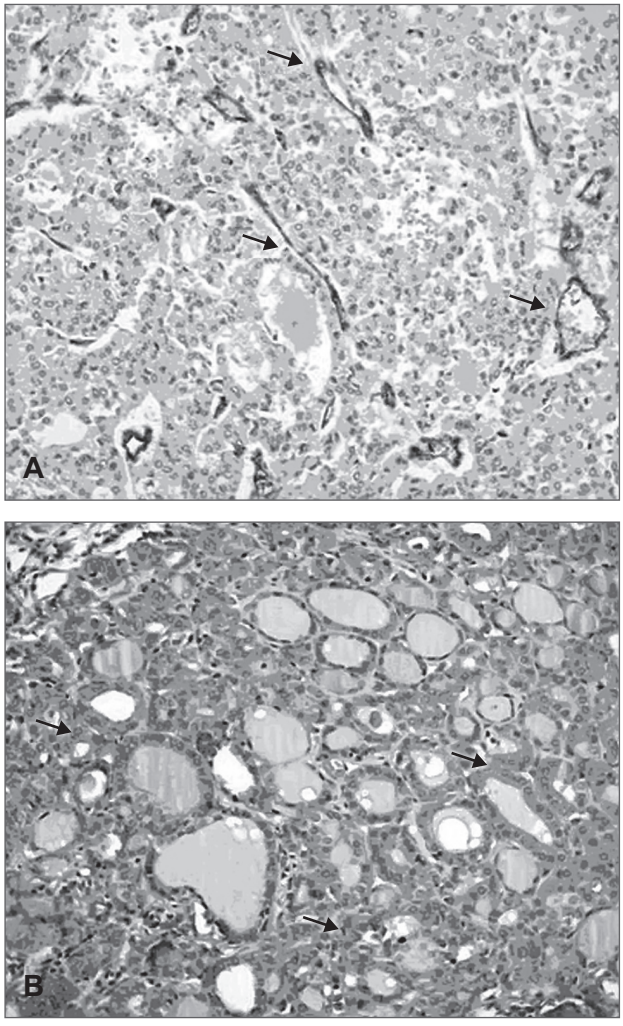

Figure 2. A: Highlighting of vessels (arrows) using anti-CD31 immunostaining in a follicular thyroid carcinoma. **B:** VEGF cytoplasmic expression in cancer cells (arrows) in a follicular thyroid carcinoma.

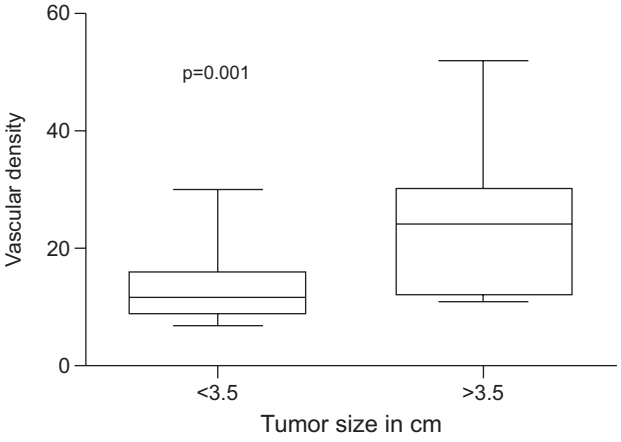

Figure 3. Vascular density according to the tumor size.

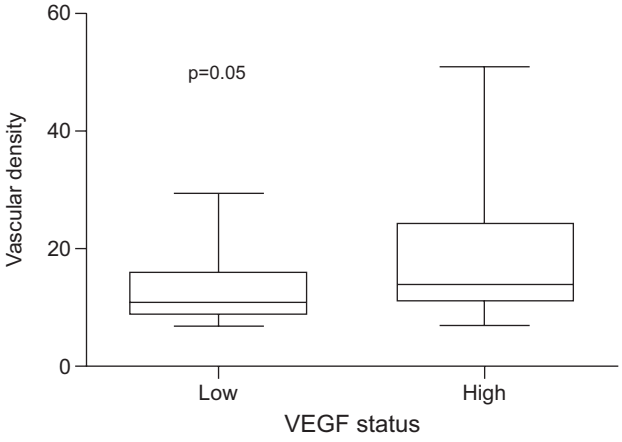

Figure 4. Vascular density according to the VEGF expression levels.

tochemical patterns of VEGF expression in a follicular thyroid carcinoma. Using the median score, 25 cases were of low and 18 of high VEGF expression. Table 2 shows the association of VEGF with patient and disease characteristics. There was no association with any of the variables examined. Normal thyroid gland showed a weak homogeneous cytoplasmic VEGF staining.

Analysis of the VD expression according to the VEGF expression status revealed a significant statistical relation of high VEGF expression with high VD. The median VD was 12±5 and 18±11 in the low and high VEGF expression cases, respectively (p=0.05; Figure 4).

bFGF expression

bFGF expression was purely cytoplasmic. The percentage of cells with bFGF expression ranged from 0-100% (median 15). 15/43 (34.8%) cases showed expression of the bFGF in more than 50% of cancer cells. Using the median value or the 50% as cut off points, we found no association of bFGF with any of the histopathological or clinical characteristics ($p > 0.05$). bFGF expression was not associated with VD or VEGF expression ($p > 0.05$). Normal thyroid gland showed a weak homogeneous bFGF expression.

Discussion

This study shows clearly that thyroid tumors are commonly associated with intensive angiogenic activity/increased VD and overexpression of angiogenic factors. As matter of fact, increased VD was noted particularly in follicular carcinomas, which traditionally are believed to bear a poorer prognosis. This finding is in accordance with a study by Fontanini et al. indicating that follicular carcinomas have a higher VD compared to papillary tumors [14].

Another interesting finding was the increased VD in tumors larger than 2 cm, showing that angiogenic activity is intensified after a certain point of the malignant thyroid growth. An alternative explanation would be a close relation of high angiogenic activity with the growth rate of thyroid cancer. This suggestion is supported by a previous study by Klein et al. where high expression of the angiogenic factor VEGF was linked with increased mitogenic activity in differentiated thyroid carcinomas [15].

VEGF was highly expressed in thyroid cancer cells with half of the tumors in the series showing expression of this angiogenic factor in 40-100% of malignant cells. This finding confirms previous studies where cancer cells contain high levels of VEGF mRNA and VEGF protein [15-17]. In a recent study by Jebreel et al. VEGF strong expression was noted in follicular adenomas compared to thyroiditis and Graves' disease suggesting that VEGF upregulation occurs as tissue becomes autonomous [18]. In our study, high VEGF expression was significantly associated with increased VD in thyroid cancer, a finding consistent with the angiogenic function of VEGF [19].

Regarding bFGF, we noted an extensive expression of this factor in thyroid cancer cells in about one third of the tumors examined. This is in accordance with a study by Daa et al [20]. We found, however, no association of bFGF expression with tumor size or angiogenesis, showing that bFGF is a rather weak angiogenic factor in thyroid cancer.

In conclusion, increased angiogenic activity characterizes a large percentage of thyroid carcinomas, particularly those with a follicular architecture. High angiogenic activity is linked with larger tumors, presumably indicating a faster tumor growth. VEGF is an important

angiogenic factor in thyroid carcinomas, being strongly expressed in more than half of the tumors. There is, therefore, a substantial biological background that supports the testing of anti-VEGF therapies in combination with radiotherapy and chemotherapy in inoperable or recurrent thyroid tumors.

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