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Tumor angiogenesis as an independent prognostic indicator in human papillary thyroid carcinoma

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Abstract. Angiogenesis has been determined to play a very important role in either the growth of solid tumors or their recurrence in distant organs. Microvessels stained immunohistochemically for the factor VIII-related antigen were counted in one hundred patients with papillary thyroid carcinoma in order to clarify the association of tumor angiogenesis with the prognosis of the disease. The mean microvessel count (MVC) was significantly higher in tumors with distant recurrence than those without recurrence. Disease-free survival of patients with a high MVC (≥23) was significantly worse than that of patients with a low MVC (≤22), as well as the overall survival. In multivariate analysis, the MVC was most strongly correlated with disease-free survival, and was independent of other prognostic factors. In conclusion, the MVC is a potent prognostic indicator in papillary thyroid carcinoma.

Introduction

Solid tumors require neovascularization when they grow beyond a diameter of a few millimeters (1), and blood vessels of tumors are inclined to be several times more permeable to plasma proteins compared with blood vessels of normal tissues (2). These facts suggest that the distribution and the metastasis of malignant tumors correlate strongly with tumor angiogenesis. In several types of malignant tumors such as breast cancer (3,4), non-small cell lung cancer (5), head and neck squamous cell carcinoma (6) and prostatic carcinoma (7), the relationship between microvessel density of the tumor and prognosis of the patient has been reported. In thyroid cancer, tumor angiogenesis has not yet been reported. The vascular endothelial cells are regulated by certain adhesion molecules and protease in order to proliferate, migrate and build up vascular lumens (8,9). Weidner and colleagues have reported (3) vessel density evaluated by immunohistochemical staining for factor VIII-related antigen.

Microvessels of thyroid carcinomas per a 400x field in the most active area of neovascularization were counted in our study and the correlation of microvessel count (MVC) with clinicopathological features and prognosis was analyzed.

Materials and methods

Patients. One hundred patients (95 females and 5 males) with primary papillary thyroid cancer, without distant metastasis at the time of diagnosis were included in this study. They had undergone surgical procedures from June 1983 to April 1994. They had not received chemotherapy or radiation therapy prior to surgery. The age ranged from 17 to 77 (average 48.0) years. Total thyroidectomy with modified neck dissection (MND) was performed in 16 patients, subtotal thyroidectomy with MND in 72, lobectomy with MND in 9 and thyroidectomy alone in 3. Patient characteristics are shown in Table I. Pathological tumor size, pathological regional lymph node metastasis and distant metastasis was determined according to the UICC classification. Histological typing was categorized according to the WHO classification. Postoperative adjuvant chemotherapy was performed in only one patient in whom multiple lung metastases had occurred 43 months after surgery. Radiation therapy was not utilized for patients prior to the occurrence of any local or distant metastases. The median follow-up period of all the patients was 101 months.

Immunohistochemical methods. The immunohistochemical analysis was performed according to the modified method of Takei and co-workers (10). Each resected specimen was fixed in 10% formalin and embedded in paraffin. Sections from paraffin-embedded blocks were used both for histological examination with hematoxylin-eosin staining and for immunohistochemical assay. The immunoperoxidase staining by the

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Table I. Average microvessel count related to each clinicopathological finding.

	Total	Microvessel count (x400) Low-group	High-group	Mean ± SD	p-value
Total	100	61	39	22.3±13.8	
Sex and age					
Male ≥41 Female ≥51	48	28	20	21.8±11.6	
Male ≤40 Female ≤50	52	33	19	22.8±15.7	NS
Pathological tumor size					
≥5.0 cm	7	4	3	21.0±13.8	
≤4.9 cm	93	57	36	22.4±13.9	NS
Extrathyroidal invasion					
Presence	48	29	19	23.9±16.5	
Absence	52	32	20	20.8±10.7	NS
Nodal involvement					
Presence	76	44	32	23.2±15.1	
Absence	24	17	7	19.3±8.4	NS
Operation method					
Total thyroidectomy with MND	16	10	6	23.4±15.5	
Subtotal thyroidectomy with MND	72	43	29	22.1±13.7	
Lobectomy with MND	9	6	3	22.3±15.3	
Others	3	2	1	20.3±7.6	NS
Differentiation of carcinoma					
Well	94	60	34	21.0±12.0	p<0.01
Poorly	6	1	5	43.0±24.0	
Relapse site					
Distant	3	0	3	60.7±22.1	p<0.01
Local	6	1	5	27.7±7.5	
None	91	60	31	20.7±12.0	

avidin-biotin complex (ABC) method was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). A section (2-3 μ) from one formalin-fixed paraffin-embedded block per tumor was deparaffinized and then digested with 0.05 M Tris-saline buffer (pH 7.6) containing 0.025% trypsin and 0.025% calcium chloride at 37°C for 3 h. The sections were washed in a cold phosphate-buffered saline (PBS, pH 7.2) and were then exposed to a 0.3% solution of hydrogen peroxide in absolute methanol to inactivate the endogenous peroxidase. The sections were then incubated with normal horse serum to block non-specific binding of antibody for 1 h in a moist chamber at room temperature. Each section was reacted with 100 μl of monoclonal mouse anti-human von Willebrand factor (Dako-vWf, F8/86) at a 1:40 dilution overnight in a moist chamber at room temperature. They were then incubated with biotinylated secondary antibody for 1 h, followed by incubation with Vectastain Elite ABC Reagent for 1 h. The sections were washed in PBS for 30 min after each incubation. For visualization purposes the sections were immersed in a 0.017% diaminobenzidine tetrahydrochloride solution in 0.05 M Tris-saline buffer containing 0.01% H_2O_2 for 7 min, followed by counter-staining of nuclei with hematoxylin. Negative control was performed by substituting PBS for the primary antibody.

Vessel staining and counting. All blood vessels were highlighted by staining endothelial cells for factor VIII. Weidner and co-workers (3) established that any brown-staining endothelial cells or endothelial-cell clusters that were clearly separated from ajacent microvessels and tumor cells, and other connective tissue elements were considered as a single and countable microvessel. Vessel lumens which were thought to have been already existent before tumor-induced neovascularization were included among microvessels because of the difficulty of detecting the distinction between vessel lumens and microvessels.

Initially, areas of the highest neovascularization were found by scanning tumor sections at a low power (40x and 100x) and identifying areas of the tumor with the highest number of discrete microvessel staining for factor VIII (brown). Then individual microvessels were counted on a 400x field (i.e., 40x objective lens and 10x ocular lens; 0.19625 mm^2 per field).

MVC was performed by two investigators simultaneously using a double-headed light microscope. MVCs were determined without information of the patients outcome or any other pertinent variable.

Statistical analysis. The Fisher's exact probability test was used to analyze the correlation between the two groups of MVC and patient characteristics, and the t-test or significance tests for multiple comparison were used to analyze the difference of average MVC in each category of clinicopathological findings. Disease-free survival (DFS) and overall survival (OS) were calculated by the Kaplan-Meier method. The log-rank test was used to determine statistical differences between the survival curves. A multivariate analysis of factors related to DFS was carried out using the Cox proportional hazards regression model to assess the gravity of each prognostic factor such as age, sex, pathological tumor size, extrathyroidal invasion, neck nodal involvement, operation method and the MVC. In our model each regression coefficient (ß) was recognized as the log of odds ratio.

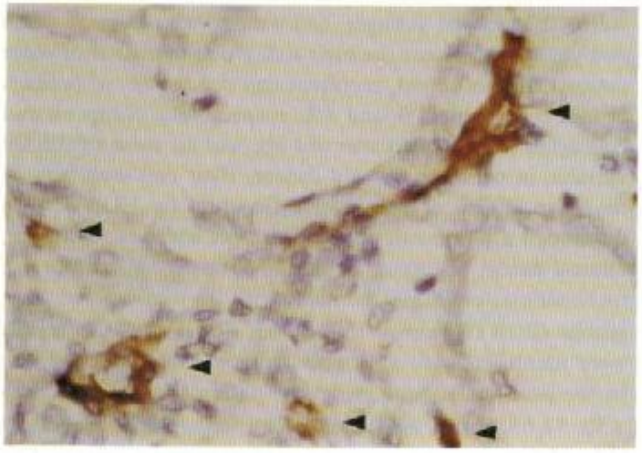

Figure 1. Microvessels were stained in the area of highest neovascularization of papillary thyroid carcinoma with high MVC (≥23). Each arrow shows the discrete immunohistochemical microvessel staining for factor VIII-related antigen with hematoxylin counterstain (original magnification, x400).

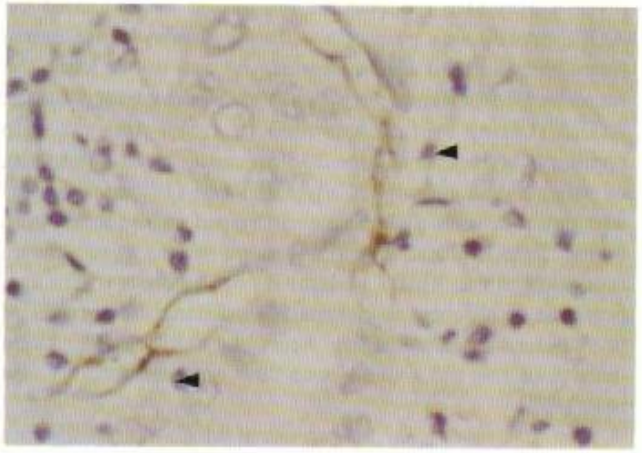

Figure 2. Microvessels stained in a papillary thyroid carcinoma with low MVC (≤22) (original magnification, x400).

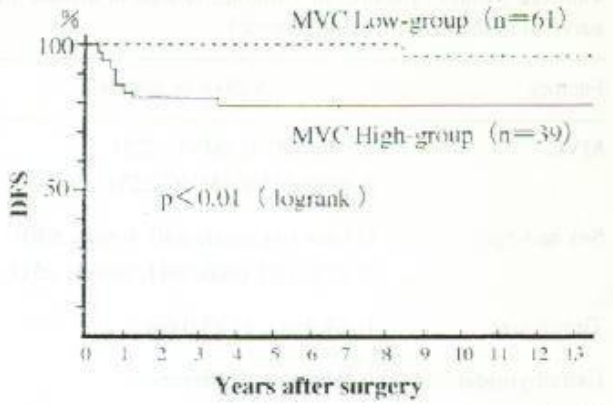

Figure 3. Disease-free survival (DFS) of patients with low MVC was better than patients with high MVC. There was a statistically significant difference between the two groups ($p<0.01$).

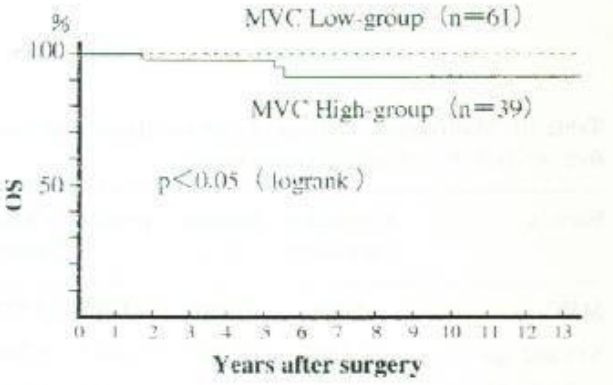

Figure 4. Overall survival (OS) of patients with low MVC was better than patients with high MVC. There was a statistically significant difference between the two groups ($p<0.05$).

Results

The areas of high neovascularization were seen at all locations in thyroid carcinoma. The MVCs in areas of the highest neovascularization ranged from 4 to 86, and the average MVC was 22.3 per 400x field. Figs. 1 and 2 show the discrete microvessel staining for factor VIII.

Patients were classified into 2 groups; high MVC (≥23) and low MVC (≤22). There were 39 patients in the high MVC group and 61 in the low MVC group (Table I). The average MVC of poorly differentiated papillary carcinoma was 43.0±24.0, which was significantly higher than of well differentiated papillary carcinoma. In addition the average MVC of patients having distantt recurrence at a later date was 60.7±22.1. This was significantly higher than patients having no recurrence, and also significantly higher than patients having local recurrence at a later date. There were no differences among the average MVC in each group in the other clinicopathological categories, such as age and sex, pathological tumor size, extrathyroidal invasion, nodal involvement or operation method.

The correlation between MVC and prognosis was examined in a total of 100 patients. None of the patients had distant metastasis at the time of surgery. Nine patients recurred; 6 had local recurrence only and 3 developed distant metastases. Two of the 3 patients with distant metastases and 1 of the 6 with local recurrence died as a result of the tumor. The 3 tumor deaths were included in the MVC high-group. The disease-free survival of patients with high MVC was worse ($p<0.01$) than patients with low MVC (Fig. 3), and the overall survival of patients with high MVC was worse ($p<0.05$) than patients with low MVC (Fig. 4). The Cox proportional hazards regression analysis was used to evaluate the independent prognostic effect of MVC related to disease-free survival with other prognostic factors. These factors are shown in Table II. The odds for developing metastasis for those patients with high MVC were 13.52 times higher than for those with low MVC (Table III). The MVC was proven

Table II. Values or scores of variables related to disease-free survival in the Cox regression model.

Factors	Values or scores
MVC	1: Low MVC (MVC ≤22) 2: High MVC (MVC ≥23)
Sex and age	1: Low risk (male ≤40, female ≤50) 2: High risk (male ≥41, female ≥51)
Tumor size	1: ≤4.9 cm, 2: ≥5.0 cm
Extrathyroidal invasion	1: Absence, 2: Presence
Nodal involvement	1: Absence, 2: Presence
Operation method	1: Total thyroidectomy with MND 2: Subtotal thyroidectomy with MND 3: Lobectomy with MND 4: Others

Table III. Multivariate analysis of factors related to disease-free survival in the Cox regression model.

Factors	Regression coefficient	Standard error	p-value	Odds ratio
MVC	2.6043	1.0703	0.0169	13.522
Sex and age	1.1646	0.7847	0.1411	3.2047
Tumor size	-0.3089	1.1260	0.7845	0.7343
Extrathyroidal invasion	0.5869	0.7460	0.4334	1.7985
Nodal involvement	1.1619	1.1667	0.3219	3.1960
Operation method	0.8708	0.6871	0.2082	2.3888

to be the strongest variable in disease-free survival and independent from other known prognostic factors. However, a multivariate analysis of factors related to overall survival could not be successfully undertaken because of the limited sampling of non-surviving patients.

Discussion

Prognostic factors of differentiated thyroid carcinoma have been discussed in several reports. Cady and co-workers reported the risk factor in differentiated thyroid cancer (11). Recurrent and death rates were significantly different between the high risk and low risk groups which were defined by age and sex alone, the low risk group consisted of men who were 40 years and younger, and women who were 50 years and younger, whereas the high risk group consisted of older patients. In that report, recurrent and death rates of patients at low risk were 11% and 4%, respectively. Simpson and colleagues (12) reported later that age was the most significant variable in papillary thyroid cancer. Byar and members of the Thyroid Cancer Cooperative Group of the EORTC (13) developed a prognostic index based on age, sex, cell type, clinical extent of tumor, lymph node status and number of metastatic sites in 507 patients with thyroid cancer. Five groups (EORTC scores <50, 50-65, 66-83, 84-108, ≥109) were identified with a progressively worsening prognosis. In the later study, four groups of patients (EORTC scores ≤50, 51-65, 66-83, ≥84) were identified with similar results (14). McConahey and co-workers (15) reported a retrospective study of 859 patients with papillary thyroid cancer, who had received primary treatment at the Mayo Clinic during the period between 1946 and 1970. In this report, death from thyroid cancer was highly associated with the following factors; age more than 50 years, male sex, tumor size, tumor grade, initial extent of disease and absence of Hashimoto's disease. Hay (16) reported the prognostic index named the AGES score using patient age, tumor grade, tumor extent (local invasion and distant metastasis) and tumor size. The incidence of patients with lower risk categorized by the AGES scoring system was 86% of 1,500 patients with papillary carcinomas and the 20-year cause-specific mortality of patients was only 1%. Cady and Rossi (17) reported that 310 patients with differentiated thyroid carcinomas were divided into low and high risk groups related to recurrence according to the AMES system. It consisted of patient age, presence or absence of metastasis to distant sites, extent of primary tumor and tumor size. According to the AMES system the rate of low risk patients was 89% and the mortality was only 1.8%.

Metastasis is a multistep process in which tumor cells gain access to the vasculature in the primary tumor, survive in the circulation, arrest in the microvasculature of the target organ, disperse from this microvasculature, and proliferate in the target tissues. Tumor cells rarely come into the circulation before vascularization of the primary tumor, and they cannot grow to a detectable size until such vascularization is established. Thus, angiogenesis is necessary at the beginning as well as at the end of the metastatic cascade (18).

Immunohistochemical staining for factor VIII-related antigen with the peroxidase-anti-peroxidase technic as a marker for some types of endothelial cells and for the isolated elements in the better-differentiated areas of malignant tumors (19). Our data confirm the finding that papillary thyroid carcinoma presents a substantial heterogeneity in vascularity, having a particular intensity ('hot spots') (5,18,20-22). Staining endothelial cells for factor VIII does not discriminate between blood-vessel endothelial cells in normal tissues and the tumor-induced neovascularization.

Weidner and co-workers (3) counted microvessels within the initial invasive breast carcinomas of 49 patients and reported that the incidence of metastatic disease increased as vessel counts increased, reaching 100% among patients with counts above 100 per 200x field. Weidner and colleagues (18) also documented that both relapse-free survival and

overall survival rate were clearly decreased by stages of the factor VIII-positive cell count of 1-33, 34-67, 68-100 and over 100, and the prognostic value of vessel density was higher than that of nodal metastasis in a 5-year follow-up study. Bosari and colleagues (20) and Toi and co-workers (4) also reported that vessel density was an important prognostic indicator in breast carcinoma. On the other hand, van Hoef and colleagues (23) and Hall and co-workers (24) disagreed with these data. Van Hoef and co-workers (23) reported that there was no difference in vascular counts or density for relapsed patients with breast cancer compared to non-relapsed patients or for patients with local relapses compared to distant relapses. They also reported that survival analysis by the log-rank test showed no significant correlation between disease-free or overall survival and vessel counts at 100x magnification, at 200x magnification or vascular density groups. In other carcinoma such as prostatic carcinoma (7,21), head and neck squamous cell carcinoma (6), non-small cell lung carcinoma (5), cutaneous melanoma (25) and uterine cervix carcinoma (26,27), tumor angiogenesis may be used to predict the risk of tumor metastasis.

Carcangiu and co-workers (28) described that papillary thyroid carcinoma with solid areas, resulting in a diagnosis of poorly differentiated carcinoma, had a slightly higher incidence of nodal and blood-borne metastases, but the differences did not reach a statistically significant level. They also described that the prognosis of the disease was not influenced by the pattern of tumor growth, initial presence or subsequent development of cervical lymph node metastases, type of initial thyroid operation, performance of neck dissection or prophylactic administration of radioactive iodine. On the other hand Sakamoto and colleagues (29) reported that the prognosis of poorly differentiated carcinoma was statistically worse than that of well differentiated carcinoma.

Patients with differentiated thyroid cancer are thought to have a good prognosis, but a few will suffer from recurrent thyroid tumors or later distant metastases. In this study angiogenesis of thyroid cancer was confirmed as a prognostic factor. Only one patient with low MVC recurred and none of the patient died of a thyroid tumor, while eight patients with high MVC (20.5%) recurred and three died (7.7%). MVC was significantly correlated with the differentiation and metastasis of papillary thyroid carcinoma, and was also significantly correlated with disease-free and overall survival rates of patients with post-operative papillary thyroid carcinoma. A low MVC may indicate a low risk group, and a high MVC may indicate a high risk group of thyroid carcinoma. The combination of age with sex in the multivariate analysis for disease-free survival was the second most critical variable and the odds ratio was 3.205, which indicate that the high risk patients with respect to the defined combination of age with sex were inclined to have metastatic disease.

This is the first report on the evaluation of tumor angiogenesis of thyroid cancer relative to the prognosis of patients with malignant thyroid neoplasm. We conclude that MVC of human papillary thyroid carcinoma is a reliable prognostic indicator.

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