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The Predictive Value of Vascular Endothelial Growth Factor and Nm23 for the Diagnosis of Occult Metastasis in Non-small Cell Lung Cancer

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We assessed the association of vascular endothelial growth factor (VEGF) and nm23 expression with occult micrometastasis in lung cancer. As destination sites for micrometastasis, we scrutinized lymph node (LN) and bone marrow (BM) specimens. For LN, 122 stage I patients who had received curative operations were studied. As regards BM, 203 patients in stage I–IV who underwent operations were registered. Immunohistochemical anti-cytokeratin staining was used to detect microdissemination of cancer cells. The VEGF and the nm23 expression at the primary sites were immunohistochemically studied in 285 cases in total. The percentages of the patients with microdissemination were 28.7% for LN and 42.4% for BM. The outcome for the patients with LN or BM microdissemination was significantly worse than that for patients without it. The increased VEGF and the decreased nm23 expression within primary tumors were significantly associated with LN and BM microdissemination. The results indicate possible value of using these biological markers to predict the risk of systemic micrometastasis in non-small cell lung cancer.

Key words: VEGF — nm23 — Lung cancer — Micrometastasis — Recurrence

We previously reported that vascularity within tumors might predict the risk of recurrence in lung cancer patients with curative resections in early stages.[1, 2] In a study that focused on "recurrence" after the curative resection of the primary tumor, we highlighted a novel biological indicator for the neo-formation of vessels, the vascular endothelial growth factor (VEGF). We found that this marker was strongly associated with the event of recurrence in stage I patients. This implies that angiogenesis within tumors may be of importance for metastasis. Recently, it has been discovered that VEGF influences a wide spectrum of biological functions, such as the immune systems via inhibition of the maturation of dendritic cells, the migration of tumor cells, cancer cell invasion, malignant transformation, and tumor survivability.[3–8] Besides angiogenesis, these multiple roles of VEGF may pertain to the event of recurrence/metastasis, as well as to the cancer development. The intent of this study was to assess the association of VEGF and nm23 expression with micrometastasis in non-small cell lung cancer, and also to inquire about their significance as predictors for occult metastasis.

PATIENTS AND METHODS

Patients For the assessment of nodal micrometastasis, 122 primary tumors and 2030 lymph nodes (LN) were obtained from primary lung cancer patients in stage I. The 78 men and 44 women with a mean age of 65.0±8.2 years (range, 46 to 84) underwent operations at Kanazawa University Hospital from 1988 to 1991. The pathological types were 77 adenocarcinomas, 37 squamous cell carcinomas, six adenosquamous carcinomas, one large cell carcinoma, and one mucoepidermoid carcinoma. According to TNM classification, 78 patients were T1N0M0 and 44 were T2N0M0. On the other hand, for the assessment of bone marrow micrometastasis, 203 lung cancer patients in various stages (stage I, 110; stage II, 19; stage IIIA, 44; stage IIIB, 27; stage IV, 3) were included in the study. The 139 men and 64 women with a mean age of 65.0±7.0 (range, 25 to 89) underwent operations from 1996 to 1999. Pathological types were 129 adenocarcinomas, 60 squamous cell carcinomas, seven adenosquamous carcinomas, and seven large cell carcinomas. The basic clinical features of the patients are summarized in Table I. Patients gave their informed consent for the use of clinical materials including resected specimens.

Immunohistochemistry for the detection of micrometastases To examine nodal micrometastasis, we carried out immunohistochemical staining using anti-cytokeratin antibodies, AE1/AE3 (DAKO, Carpinteria, CA) for LN and CK2 (Boehringer Mannheim Biochemica, Mannheim, Germany) for bone marrow. The paraffin-embedded lymph node samples were sectioned at 4 μm and the sections were floated onto silanized slides. Then the sections were deparaffinized and boiled for 5 min in 0.01 *M* sodium citrate solution (pH 6.0) in a microwave oven three times. Endogenous peroxidase was blocked by treatment with

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0.3% hydrogen peroxide in methanol for 15 min. The sections were incubated with normal goat serum diluted 10-fold with phosphate-buffered saline (PBS) for 15 min at room temperature for blocking. After having been washed with PBS, the sections were reacted with anti-AE1/AE3 antibody (diluted 50-fold with PBS) for 1 h at room temperature. They were then reacted with biotin-labeled goat anti-mouse immunoglobulin (DAKO, CA) for 2 h, and washed with PBS. Avidin-biotin-peroxidase complex was added and color was developed with 3-3′ diaminobenzidine (Sigma, St. Louis, MO) and 0.03% hydrogen peroxide. The cytospin preparations for bone marrow (BM) aspirates were stained by the alkaline phosphatase anti-alkaline phosphatase method by using a commercial kit (DAKO, Kyoto) according to the procedures recommended by the manufacturer. The sections were reacted with 2 μg/ml of CK2 for 24 h at 4°C. Counterstaining was done with H & E. Taking into consideration morphologic features such as nuclear size, the nucleus-cytoplasmic ratio, and nucleolation, cells that reacted with anti-cytokeratin antibody were considered positive. In this study, both single and clustered tumor cells with a size of not more than 2 mm were defined as micrometastasis.[9, 10] Positivity was evaluated by two independent observers without knowledge of the clinicopathologic factors.

Table I. Clinical Background Factors

	Nodal micrometastasis (n=122)		BM micrometastasis (n=203)	
	Positive	Negative	Positive	Negative
No. of patients	35	87	86	117
Gender				
Male	22	56	67	71
Female	13	31	19	46
Mean age (years)	65.5±8.8	65.1±8.0	66.7±9.4	64.1±10.4
Histology				
Adenocarcinoma	20	57	50	77
Squamous cell carcinoma	14	23	29	34
Adenosquamous carcinoma	0	6	2	4
Others	1	1	5	2
T factor				
T1	20	58	25	63
T2	15	29	36	37
T3	—	—	11	8
T4	—	—	14	9
N factor				
N0	35	87	40	88
N1	—	—	10	8
N2	—	—	30	20
N3	—	—	6	1
M factor				
M0	35	87	84	116
M1	—	—	2	1

Immunohistochemical assessment of VEGF and nm23

We used consecutive paraffin sections of the invasive edge of the primary tumors, stained immunohistochemically by the labeled streptavidin-biotin method as described above. Primary antibodies used were the anti-VEGF polyclonal antibody (Santa Cruz Biotechnology Inc., Germany) diluted 100-fold and the anti-nm23 monoclonal antibody (DAKO, CA) diluted 50-fold. They were reacted for 2 h at room temperature. The second antibodies used were biotin-labeled goat anti-mouse immunoglobulin (DAKO, CA). The immunoreactivities for VEGF were graded as

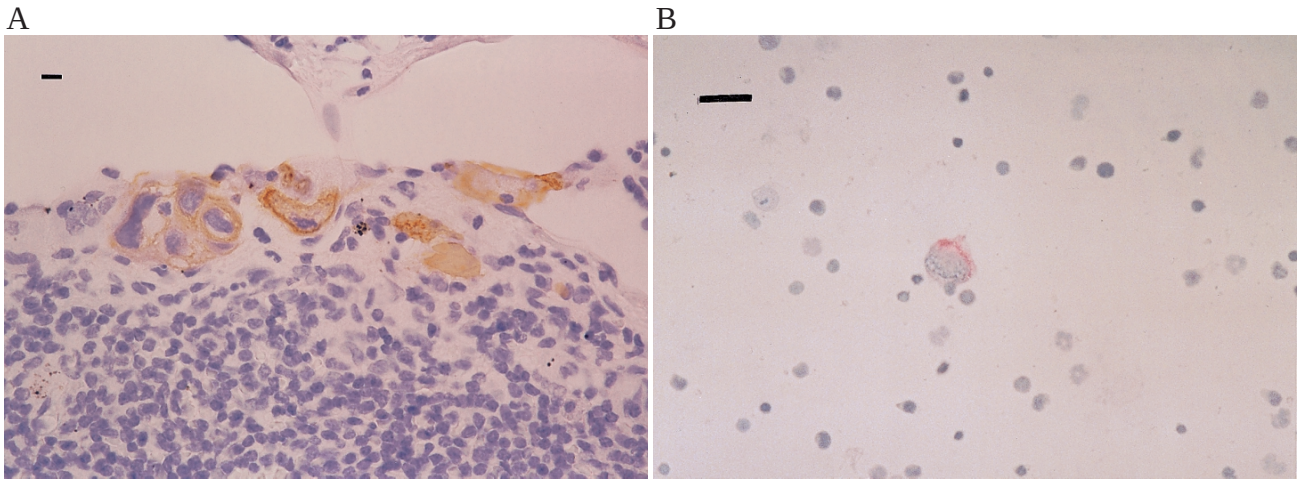

Fig. 1. Photomicrographs of lymph node with microspread of cytokeratin-positive tumor cells (A) and BM specimen with microspread of cytokeratin-positive tumor cells (B). Scale bar indicates 30 μm.

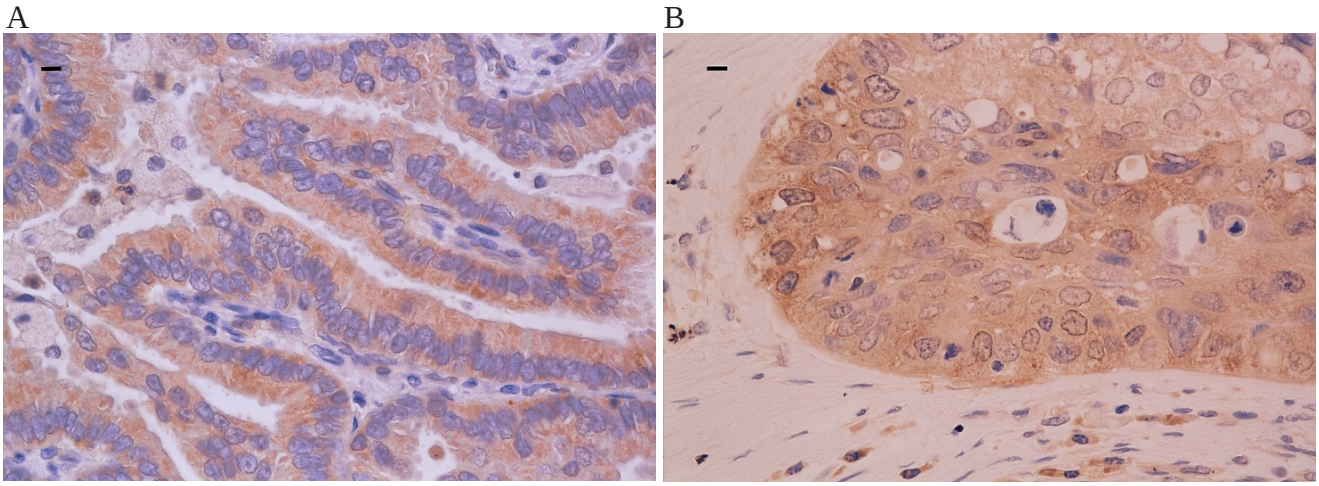

Fig. 2. Immunohistochemical staining for VEGF (A) and nm23 (B). VEGF antigens were mainly identified in the cytoplasm of tumor cells in addition to vascular endothelial cells. Nm23 antigens were mainly found in the epithelial component and were mainly cytoplasmic in tumor cells. Scale bar indicates 30 μm.

Table II. VEGF and Nm23 Expression in Tumors and Their Association with Nodal and BM Micrometastasis

Factors	Nodal micrometastasis			BM micrometastasis		
	Positive (%) (n=35)	Negative (%) (n=87)	P value	Positive (%) (n=69)	Negative (%) (n=94)	P value
VEGF+[a)]	88.6	77.0	0.15	97.1	85.1	0.0110
VEGF++[b)]	68.6	31.0	0.0001	68.1	25.5	0.0001
Nm23−[c)]	80.0	54.0	0.008	69.6	45.7	0.0023

a) More than 10% positive staining area in tumor cells.
b) More than 50% positive staining area in tumor cells.
c) Defined as negative if any epithelial cells in tumors showed negative staining.
The association between different variables was analyzed by the χ^2 test.

(−), (+), and (++) according to the staining intensity of the tumor cells: (−) represents zero or less than 10% positive staining area, (+) represents 10−50% positive staining area, and (++) represents more than 50% positive staining area. In this study, we defined a tumor with the strongest stain (++) as VEGF-overexpressing.[2, 11)] For the assessment of nm23 protein expression, tumors were considered positive if all the epithelial cells in the lesion showed cytoplasmic staining. If any of the epithelial cells were unstained, they were considered negative.[12)]

Statistics The association between variables was analyzed by the χ^2 test. Survival curves were obtained by the Kaplan-Meier method, and compared by the log-rank test. The criterion for statistical significance was $P<0.05$ and the mean values are shown with ±SD.

RESULTS

The detection of cytokeratin-positive tumor cells Positive staining for AE1/AE3 and CK2 was found in all normal epithelial cells and tumor cells in primary sites irrespective of pathologic types. Among 122 patients in stage I, 35 patients (28.7%) had microdissemination of tumor cells within the LN. There were no significant differences in the percentage of the patients with or without nodal microdissemination of cancer cells according to age, gender, pathological type, and T factor. In our series, most of the nodal microdissemination showed clustered tumor cells (Fig. 1A). On the other hand, the incidence of BM microdissemination was 42.4% (86/203 patients) (Fig. 1B). There were no significant differences in the percentage of BM microdissemination according to age, M factor, and pathological types. The positivity of BM microdis-

Table III. Sensitivity and Specificity for the Detection of Nodal Micrometastasis by Using VEGF and Nm23

	VEGF++[a)]	Nm23−[b)]	VEGF++/nm23−
Nodal micrometastasis			
Sensitivity[c)]	68.6% (24/35)	80.0% (28/35)	62.9% (22/35)
Specificity[d)]	69.0% (60/87)	46.0% (40/87)	81.6% (71/87)
BM micrometastasis			
Sensitivity[e)]	68.1% (47/69)	69.6% (48/69)	30.4% (21/69)
Specificity[f)]	74.5% (70/94)	54.3% (51/94)	91.5% (86/94)

a) A tumor was included in VEGF high expressing group (++) if the positive staining area in tumor cells was more than 50%.
b) Defined as negative if any epithelial cells in tumors showed negative staining.
c) Percentage of patients with each factor expression among the patients with nodal micrometastasis.
d) Percentage of patients without each factor expression among the patients without nodal micrometastasis.
e) Percentage of patients with each factor expression among the patients with BM micrometastasis.
f) Percentage of patients without each factor expression among the patients without BM micrometastasis.

Table IV. VEGF and Nm23 Expression and Its Association with Recurrence and Nodal Micrometastasis

	VEGF++[a)]	Nm23−[b)]	VEGF++/nm23− (%)
Nodal micrometastasis positive group			
Recurrent group (n=18)	15 (83.3)	17 (94.4)	15 (83.3)*
Non-recurrent group (n=17)	9 (52.9)	11 (64.7)	7 (41.2)*
Total (n=35)	24 (68.6)	28 (80.0)	22 (62.9)***
Nodal micrometastasis negative group			
Recurrent group (n=23)	9 (39.1)	17 (73.9)	6 (26.1)**
Non-recurrent group (n=64)	18 (28.1)	30 (46.9)	10 (15.6)**
Total (n=87)	27 (31.0)	47 (54.0)	16 (18.4)***

a) A tumor was included in VEGF high expressing group (++) if the positive staining area in tumor cells was more than 50%.
b) Defined as negative if any epithelial cells in tumors showed negative staining.
* P=0.0099, ** P=0.2667, *** P<0.0001.

semination was correlated to T factor (T1, 2 vs. T3, 4, P<0.005), N factor (N0 vs. N1–3, P<0.0005), and pathological stages (stage I, II vs. stage III, IV, P<0.001). Basic clinical characteristics are shown in Table I.

The expression of VEGF and nm23 in primary sites, and their association with microdissemination VEGF antigens were mainly identified in the cytoplasm of cancer cells and the endothelial cells of vessels alike (Fig. 2A). Nm23 antigens were found in the epithelial component and the cytoplasm of cancer cells (Fig. 2B). Although the VEGF antigen was detected in large amounts in both tumors with nodal microdissemination (88.6%) and those without nodal microdissemination (77.0%, P=0.15), the percentages of patients with the strongest VEGF staining were 68.6% (24/35) in tumors with nodal microdissemination and only 31.0% (27/87) in those without it (P=0.0001). Concerning patients with BM microdissemination, the percentages of the patients with positive and the strongest VEGF staining were 97.1% and 68.1%, respectively, significantly greater as compared to those without BM microdissemination (85.1% and 25.5%, respectively) (P<0.01 and P<0.0001). On the other hand, the nm23 expression was inversely correlated with nodal microdissemination (P=0.008) and with BM microdissemination (P=0.0023). Table II gives an overview of the results. With respect to the diagnosis of nodal microdissemination, the sensitivity and the specificity were 68.6% and 69.0% for VEGF++, 80.0% and 46.0% for nm23−, and 62.9% and 81.6% for the combination, VEGF++/nm23−, respectively (Table III). Among the 35 patients with nodal microdissemination, 18 developed recurrence including 15 (83.3%) with VEGF++/nm23−. On the other hand, among 64 recurrence-free patients without nodal microdissemination, only 10 (15.6%) had the VEGF++/nm23− pattern. Among the patients with nodal microdissemination, the percentage with the VEGF++/nm23− pattern was significantly greater as compared to those without microdissemination (P<0.0001) and the

VEGF++/nm23− pattern was also associated with recurrence in the nodal microdissemination-positive group (P=0.0099) (Table IV). As to BM microdissemination, the sensitivity and the specificity were 68.1% and 74.5% for VEGF++, 69.6% and 54.3% for nm23−, and 30.4% and 91.5% for VEGF++/nm23−, respectively (Table III).

Nodal or BM microdissemination and outcome The 5-year survival rate for the patients with nodal microdissemination was 54.3%, being significantly worse as compared to that (n=87, 75.9%) for the patients without it (P=0.006). Although the follow-up is still too short to allow a definitive conclusion on survival of patients with/without BM microdissemination, the outcome for the patients with BM microdissemination was significantly worse than that for the patients without it in the assessment of the 157 cases who underwent complete resection of the primary tumor (P<0.0001).

DISCUSSION

Microdissemination of cancer cells in distant organs, such as LN, BM, and peripheral blood circulation, generally results in recurrence even after a curative operation.[13–17] Therefore adjuvant therapies may be required to inhibit metastasis. In this study, we carried out immunostaining for cytokeratin fragments to examine the microdissemination of cancer cells in LN and BM specimens. To better delineate disseminated tumor cells with/without metastatic capacity, assessment of their biological characteristics including invasive behavior is needed. In a cohort of lung cancer patients, we also studied the expression of the angiogenesis-associated factor, VEGF, and the metastasis-associated factor, nm23, within primary tumors. As a result, we found that the increased expression of VEGF was significantly associated with both nodal and BM microdissemination, together with reduced expression of nm23. These results suggest that neovessels mediated by VEGF are closely associated with the establishment of nodal and BM micrometastasis.

With respect to the association of VEGF to nodal metastasis, we have already confirmed that the *VEGF* gene expression at primary sites was greater in lung cancer patients with nodal metastasis than in those without it.[18] This finding in non-small cell lung cancer has been supported by other researchers.[19, 20] Non-angiogenic functions of VEGF may also play a role in the development of metastasis. On the other hand, although there are conflicting results, nm23 has been reported to be a putative anti-metastatic gene with an inverse correlation to nodal metastasis.[21–23] In accordance with these previous reports on nodal metastasis diagnosed by conventional methods, negative nm23 expression was also significantly associated with nodal microdissemination and with BM microdissemination in our series. The concurrent analysis of VEGF and nm23 demonstrated that the percentage of patients with VEGF++/nm23− pattern was significantly high among patients with nodal microdissemination as compared to patients without it, accounting for 83.3% of the patients with recurrence in the nodal microdissemination-positive group. Although the specificity of nm23 was not high as a marker of nodal or BM microdissemination, VEGF retained high sensitivity and specificity. The VEGF++/nm23− pattern appears to be of value especially for the diagnosis of nodal micrometastasis. The evaluation of these markers within tumors may aid the diagnosis of systemic micrometastasis in non-small cell lung cancer.

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