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Glomeruloid Microvascular Proliferation Is Superior to Intratumoral Microvessel Density as a Prognostic Marker in Non-Small Cell Lung Cancer[1]

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ABSTRACT

Glomeruloid microvascular proliferation (GMP) is a focal proliferative budding of endothelial cells (ECs) resembling a renal glomerulus. Whereas some experimental and clinical studies have suggested recently that GMPs indicate an aggressive angiogenic phenotype, the incidence and clinical significance of GMPs remains unclear. Thus, we conducted a retrospective study on GMPs in a total of 236 patients with completely resected pathological (p-) stage I-IIIA NSCLC. ECs were highlighted with immunohistochemical staining using an anti-CD34 antibody, and GMPs were defined as focal glomerulus-like aggregates of closely associated and multilayer CD34-positive ECs. Expression of vascular endothelial growth factor, angiopoietin (Ang)-1, and Ang-2 was also examined immunohistochemically. GMPs were positive in 60 (25.4%) patients, and the incidence was not correlated with age, gender, histological type, or p-stage. The mean intratumoral microvessel densities for GMP-negative tumor and GMP-positive tumor were 178.2 and 184.1, respectively, showing that the incidence of GMPs was not correlated with intratumoral microvessel density ($P = 0.676$). There was no correlation between vascular endothelial growth factor expression and the incidence of GMPs, but GMPs were more frequently seen in Ang-1-positive tumor than in Ang-1-negative tumor. The 5-year survival rate of GMP-positive patients was 54.2%, which was significantly lower than that of GMP-negative patients (72.3%; $P = 0.016$). The 5-year survival rate of higher-MVD patients (71.5%) seemed to be lower than that of the lower-MVD patients (63.7%), but the difference did not reach a statistical significance ($P = 0.137$). A multivariate analysis confirmed that the presence of GMPs was a significant prognostic factor ($P = 0.003$), whereas MVD was not. In conclusion, GMPs indicate an aggressive angiogenic phenotype associated with a poor prognosis in NSCLC.

INTRODUCTION

Angiogenesis is an essential process in development and progression of a variety of malignant tumors including NSCLC,[3] and it is generally recognized that angiogenesis can be a new marker and target in the diagnosis and therapy (1, 2). IMVD is usually used in the evaluation of angiogenesis, but some clinical studies did not show the validity of use of IMVD in identification of aggressive angiogenic phenotype (3–6).

GMP is a focal proliferative budding of ECs that resembles a renal glomerulus (7–9). GMPs are commonly seen in glioblastoma multiforme brain tumors and are one of the defining histological characteristics (10). Recent studies have revealed that GMPs are also found in other malignant tumors such as gastrointestinal carcinomas (11) and lymphomas (12), and also revealed that GMPs indicate an aggressive vascular phenotype associated with poor prognosis in melanoma, breast cancer, endometrial cancer, and prostate cancer (9). However, no studies have been reported on GMPs in NSCLC. Therefore, in the present study we have assessed the clinical significance of GMPs in NSCLC.

MATERIALS AND METHODS

Patients and Tissue Preparation. A total of 237 consecutive patients with p-stage I to IIIA NSCLC, who underwent complete resection without any preoperative therapy at Kyoto University Hospital from January 1, 1985 through December 31, 1990, were retrospectively reviewed. One patient was excluded from the study because of operation-related death, and a final total of 236 patients were evaluated (Table 1; Refs. 6, 13, 14). P-stage was re-evaluated and determined with the current tumor-node-metastasis classification (15), and histological type was also determined with the current classification by WHO (16).

For all of these patients, the inpatient medical records, chest X-ray films, whole-body computed tomography films, bone scanning data, and records of surgery were reviewed. Intraoperative therapy was not performed in any patient. As postoperative adjuvant therapy, cisplatin-based chemotherapy, radiation, and oral administration of tegafur (a fluorouracil derivative drug) were prescribed for 55, 35, and 58 patients, respectively (13). Follow-up of the postoperative clinical course was conducted by outpatient medical records and by inquiries by telephone or letter. This study was reviewed and approved by the "Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University."

Serial 4-μm formalin-fixed and paraffin-embedded sections were prepared from each tumor sample, and were served for routine H&E staining, IHS, and the terminal deoxynucleotidyltransferase-mediated nick end labeling staining. Results of IHS were evaluated by two authors independently (F. T. and S. I.) without knowledge of any clinical data.

Identification of GMPs and Quantification of Angiogenesis (IMVD). ECs were highlighted with IHS using an anti-CD34 mAb QBEnd10 (mouse IgG 1, κ, 50 μg/ml; DAKO, Kyoto, Japan) diluted at 1:50, and the procedure was described previously (6). IHS was performed using a sensitive streptavidin-biotinylated horseradish peroxidase complex system (TSA-Indirect kit, NEN Life Science Products, Boston, MA). According to previous reports (7–10), GMPs were defined as focal glomerulus-like aggregates of closely associated and multilayered CD34-positive ECs; lumen formation was not necessary, and tangentially sectioned vessels or nonspecific CD34-positivity in stromal components were avoided. Tumors were sampled by examining one histological slide for the presence of GMPs, and GMP was judged to be positive when one or more GMPs were present through the slide.

For evaluation of IMVD, the 10 most vascular areas within a section were selected. Vessels labeled with the anti-CD34 mAb were counted under light microscopy with a 200-fold magnification, and the average counts were recorded as the CD34-IMVD for each case (6).

Evaluation of Angiogenic Factors Expression. Expression of VEGF, Ang-1, and Ang-2 was evaluated immunohistochemically using a standard streptavidin-biotinylated horseradish peroxidase complex method (LSAB-2 kit; DAKO) as described previously (6, 14). Primary antibodies used were as follows: (*a*) anti-VEGF polyclonal antibody A-20 (rabbit IgG, 200 μg/ml; Santa Cruz Biotechnology) diluted at 1:50; (*b*) anti-Ang-1 polyclonal antibody (goat IgG, 200 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) diluted at

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; GMP, glomeruloid microvascular proliferation; Ang, angiopoietin; VEGF, vascular endothelial growth factor; IMVD, intratumoral microvessel density; EC, endothelial cell; Ad, adenocarcinoma; Sq, squamous cell carcinoma; mAb, monoclonal antibody; p, pathological; IHS, immunohistochemical staining; PI, proliferative index; AI, apoptotic index.

Table 1 *Characteristics of patients and GMP in NSCLC*

	No. of patients (%)	No. of patients (%) according to GMP status		
		GMP-negative	GMP-positive	*P*
All patients	236 (100%)	176 (74.6%)	60 (25.4%)	
Age (mean ± SD)	62.4 ± 9.7	62.7 ± 9.6	61.7 ± 10.0	0.489
Gender				
Male	170 (72.0%)	128 (75.3%)	42 (24.7%)	0.740
Female	66 (28.0%)	48 (72.7%)	18 (27.3%)	
Performance status (PS)				
0	206 (87.3%)	156 (75.7%)	50 (24.3%)	0.485
1	28 (11.9%)	19 (67.9%)	9 (32.1%)	
2	2 (0.8%)	1 (50.9%)	1 (50.0%)	
Histologic type				
Sq	85 (36.0%)	65 (76.5%)	20 (23.5%)	0.529 (Sq *vs.* Ad)
Ad	130 (55.1%)	94 (72.3%)	36 (27.7%)	
Large cell carcinoma	13 (5.5%)	12 (92.3%)	1 (7.7%)	
Others	8 (3.4%)	5 (62.5%)	3 (37.5%)	
p-stage				
I	138 (58.5%)	101 (73.2%)	37 (26.8%)	0.265 (for all p-stage)
II	26 (11.0%)	17 (65.4%)	9 (34.6%)	
IIIA	72 (30.5%)	58 (80.6%)	14 (19.4%)	

1:50; and (*c*) anti-Ang-2 polyclonal antibody (goat IgG, 200 μg/ml; Santa Cruz Biotechnology) diluted at 1:50.

VEGF expression was classified according to the following grading system as described previously (6). Briefly, a percentage score was defined as follows: score 0 if no VEGF-positive staining cell was documented, score 1 if the percentage of VEGF-positive staining cells was ≤25%, score 2 if the percentage was 26–50%, and score 3 if the percentage was >50%; a intensity score was defined as follows: score 0 if no staining was documented, score 1 if the staining intensity was weak, score 2 if the intensity was moderate, and score 3 if the intensity was high. The staining intensity of tumor cells was judged as high (score 3) when the staining intensity was comparable with that of smooth muscle cells of either bronchial wall or blood vessels. Grade of VEGF expression was represented as the sum of the percentage score and the intensity score (VEGF score), and VEGF expression was finally defined as follows: weak expression when the VEGF score is 4 or less, and strong expression when the VEGF score is 5 or 6.

Ang-1 or Ang-2 expression was judged to be positive when the percentage of cancer cells with positive staining exceeded 5% (14).

Evaluation of Cell Proliferation and Apoptotic Cell Death. Proliferative activity of tumor cells was evaluated IHS using a mAb against proliferative cell nuclear antigen (clone PC-10, mouse IgG2a, κ, 400 μg/ml; DAKO) as described previously (13). A total of 1000 tumor cells were counted for positive staining, and the proliferative activity was represented as the percentage of proliferative cell nuclear antigen-positive tumors cells (PI).

The terminal deoxynucleotidyltransferase-mediated nick end labeling staining was performed using *In Situ* Death Detection Kit, POD (Boehringer Mannheim, Mannheim, Germany) as described previously (13). In each case, a total of 10,000 tumor cells were evaluated, and AI was defined as the number of apoptotic cells per 1,000 tumor cells.

Statistical Methods. The χ^2 was used to compare counts. Continuous data were compared using Student's *t* test, if the distribution of samples was normal, or the Mann-Whitney *U* test, if the sample distribution was asymmetrical. The postoperative survival rate was analyzed by the Kaplan-Meier method, and the differences in survival rates were assessed by the log-rank test. Multivariate analysis of prognostic factors was performed using Cox's regression model. Differences were considered significant when $P < 0.05$. All of the statistical manipulations were performed using the SPSS for Windows software system (SPSS Inc., Chicago, IL).

RESULTS

GMPs in NSCLC. GMPs was documented in 60 patients (25.4%; Fig. 1; Table 1). No significant correlation between the presence of GMPs and characteristic of any patient was documented (Table 1).

The mean IMVDs for GMP-negative and GMP-positive tumors were 178.2 and 184.1, respectively, which showed that there was no correlation between the presence of GMPs and IMVD (Table 2). No significant correlation between the presence of GMPs and the status of VEGF or Ang-2 was documented (Table 2). However, the incidence of positive GMP was significantly higher in Ang-1-positive tumors than in Ang-1-negative tumors (55.0% *versus* 38.6%; $P = 0.034$; Table 2).

There was no significant difference in PI or AI between GMP-negative and GMP-positive tumors (Table 2).

GMPs and Postoperative Survival. The 5-year survival rate for GMP-negative patients was 72.3%, significantly higher than that for GMP-positive patients (54.2%; $P = 0.016$; Fig. 2; Table 3). In contrast, there proved to be no significant difference in the postoperative prognosis between the lower IMVD patients (IMVD <157) and the higher IMVD patients (IMVD ≥157), although the 5-year survival rate of the lower IMVD group (71.5%) seemed to be higher than that of the higher IMVD group (63.7%; $P = 0.137$).

Next, analyses of postoperative survival stratified by p-stage or histological type were performed (Table 3). For p-stage I disease, the 5-year survival rate for GMP-positive patients was significant lower than that for GMP-negative patients (62.9% *versus* 84.8%; $P = 0.018$; Fig. 3). For Ad, there was a significant difference in the postoperative survival between GMP-positive and -negative patients (Fig. 4).

A multivariate analysis confirmed that the presence of GMPs was a significant factor to predict a poor prognosis and that VEGF status or IMVD was not significant prognostic factor (Table 4).

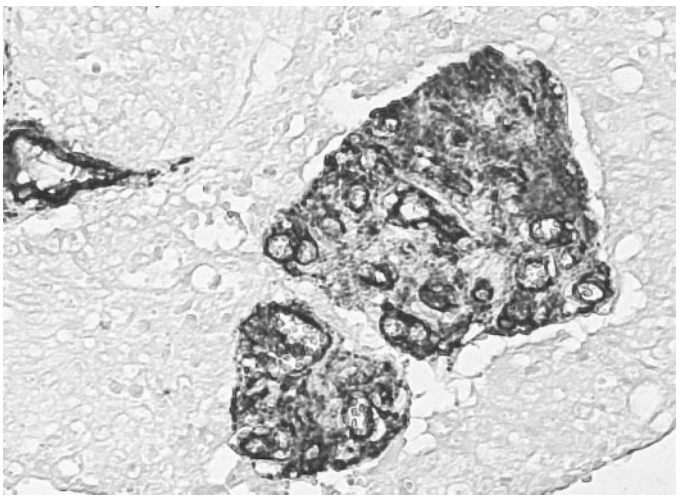

Fig. 1. GMP in NSCLC. Vessels were highlighted with IHS using an anti-CD34 antibody.

Table 2 *Correlation between GMP status and other biomarkers*

Biomarkers	GMP-negative	GMP-positive	*P*
PI (%)[a]	45.3 ± 2.1	49.4 ± 3.6	0.322
AI (per 1000 tumor cells)[a]	19.3 ± 1.5	17.3 ± 3.1	0.574
IMVD[a]	178.2 ± 7.6	184.1 ± 11.8	0.676
Angiogenic factors			
Vascular endothelial growth factor (VEGF) expression			
VEGF-score[a]	3.5 ± 1.8	3.8 ± 1.7	0.310
Angiopoietin (Ang)-1 expression[b]			
Negative	108 (61.4%)	68 (38.6%)	0.034
Positive	27 (45.0%)	33 (55.0%)	
Angiopoietin (Ang)-2 expression[b]			
Negative	148 (84.1%)	28 (15.9%)	0.550
Positive	48 (80.0%)	12 (20.0%)	

[a] Each value is shown as the mean ± SE.
[b] Each figure represents the number of patients.

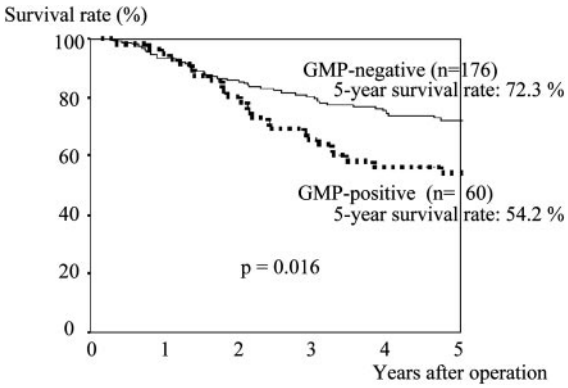

Fig. 2. Postoperative survival of patients with completely resected p-stage I-IIIA NSCLC. Comparison of postoperative survival of GMP-negative patients and GMP-positive patients.

Table 3 *GMP and postoperative survival in non small cell lung cancer NSCLC*

	5-year survival rate (%)		
	GMP-negative	GMP-positive	*P*
All patients	72.3	54.2	0.016
p-stage			
I	84.8	62.9	0.018
II	74.8	50.0	0.073
IIIA	46.9	28.0	0.234
Histologic type			
Sq	71.9	57.4	0.317
Ad	71.6	54.2	0.043

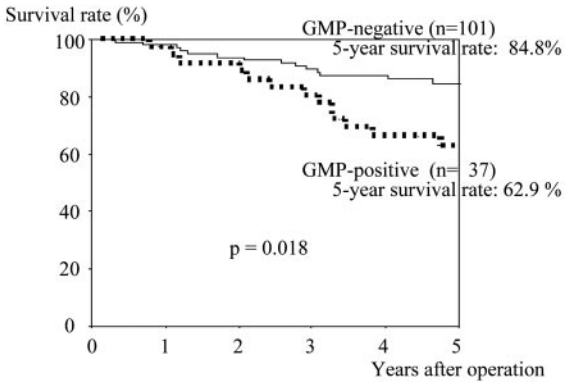

Fig. 3. Postoperative survival of patients with completely resected p-stage I NSCLC. Comparison of postoperative survival of GMP-negative patients and GMP-positive patients.

DISCUSSION

In the present study, we first demonstrated that GMPs were present in one quarter of NSCLC, and that the presence of GMPs was a significant factor to predict a poor prognosis. These findings are entirely consistent with results documented in other malignant tumors such as breast cancer (9), which strongly suggest that GMPs are useful markers of aggressive angiogenesis in a variety of malignant tumors. Tumor angiogenesis is generally evaluated with IMVD, and many clinical studies have revealed that higher IMVDs are significant factors to predict poor prognoses in malignant tumors including NSCLC (17). However, some clinical studies failed to document the prognostic significance of IMVD (3, 4, 5, 6), and the clinical significance of IMDV has not been established. In the present study, we failed to document a prognostic significance of IMVD, whereas we documented a significantly poor prognosis for positive GMPs tumors. These results suggest that GMPs are superior to IMVD in the evaluation of tumor angiogenesis.

In the present study, the presence of GMPs was not correlated with IMVD; the mean IMVDs for GMP-negative tumor and GMP-positive tumor were 178.2 and 184.1. One of the most probable reasons why IMVD was not a significant prognostic factor or was not correlated with the presence of GMPs may be the variability in the reactivity of anti-EC antibodies used to highlight intratumoral microvessels (18, 19). Antibodies against pan-EC markers including CD34 are not entirely specific for ECs (20, 21), and these pan-EC antibodies may not be an ideal reagent to visualize tumor-associated blood vessels, because pan-EC antibodies react not only with newly forming vessels but also stable vessels just trapped in tumors. ECs in GMPs that are highlighted with an anti-CD34 antibody may be layered "activated" vessels.

We demonstrated that the presence of GMPs was significantly correlated not with enhanced VEGF expression but with enhanced Ang-1 expression. Ang-1 and Ang-2 have been identified as ligands for Tie2, which is a receptor tyrosine kinase specifically expressed on ECs, and Angs play critical roles in angiogenesis in concert with VEGF (2). Ang-1 binds to Tie2, and maintains and stabilizes mature vessels by promoting interaction between ECs and surrounding extracellular matrix. Ang-2 competitively binds to Tie2, and antagonizes the stabilizing action of Ang-1, which result in destabilization of vessels. These destabilized vessels may undergo regression in the absence of angiogenic factors such as VEGF; however, in the presence of VEGF these destabilized vessels may undergo angiogenic changes. Thus, angiogenesis is controlled by dynamic balance between vessel regression and growth mediated by VEGF, Ang-1, and Ang-2 (2). Taken with these experimental results, enhanced expression of Ang-1 that stabilizes ECs is essential for the presence of GMPs. In contrast, enhanced expression of VEGF was not correlated with the presence of GMPs in the present study, which suggested that baseline or minimal VEGF might be adequate for the formation of

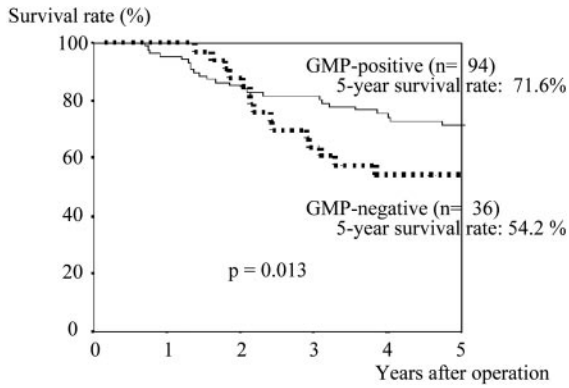

Fig. 4. Postoperative survival of patients with completely resected p-stage I-IIIA Ad. Comparison of postoperative survival of GMP-negative patients and GMP-positive patients.

Table 4 *Multivariate analysis of prognostic factors (Cox's proportional hazard model)*

Factors	β	*P*	Hazard ratio (95% confidence interval)
Gender (male/female)	−0.327	0.271	0.721 (0.402–1.292)
Age	0.026	0.073	1.028 (0.997–1.059)
Performance status (0/1/2)	0.252	0.387	1.287 (0.727–2.279)
Histology (Nonadenocarcinoma/adenocarcinoma)	−0.041	0.210	0.960 (0.901–1.023)
Pathologic stage (I/II/IIIa)	0.673	<0.001	1.961 (1.517–2.534)
VEGF-expression (weak/strong)	0.299	0.222	1.348 (0.835–2.177)
IMVD (lower/higher)	0.457	0.134	1.450 (0.893–2.455)
GMP (Negative/positive)	0.743	0.003	2.103 (1.282–3.449)

GMPs. In conclusion, GMPs indicate an aggressive angiogenic phenotype and a novel prognostic factor in NSCLC.

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