Clinical Significance of Aminopeptidase N in Non–Small Cell Lung Cancer

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Abstract

Purpose: The aim of our study is to investigate the mechanism of metastasis, to detect novel metastasis-associated molecules, and to evaluate the molecules from the point of view of clinical application. A monoclonal antibody MH8-11, which we established, recognizes a glycoprotein that is identical to aminopeptidase N (APN/CD13). APN/CD13 degrades the extracellular matrix, while it is also involved in cell motility and improves angiogenesis.

Experimental Design: We investigated the expression of APN/CD13 in 194 cases of non–small cell lung cancer (NSCLC) by immunohistochemical analyses and reverse transcription-PCR assay to determine the significance of this prognostic factor; 95 tumors were stage I, 36 were stage II, 39 were stage IIIA, and 24 were stage IIIB. Moreover, we investigated that the relationship between the expression of APN/CD13 and angiogenesis and prognosis for patients with NSCLC.

Results: We found a correlation between the expression of APN/CD13 and angiogenesis ($r = 0.659$; $P < 0.0001$). In the 194 patients with NSCLC, we found 68 patients to be APN/CD13$^+$ and 126 patients to be APN/CD13$^-$. The 5-year survival rate in patients with APN/CD13$^+$ tumors was significantly lower than in those whose tumors had negative APN/CD13 (48.3% versus 67.1%; $P = 0.0001$).

Conclusion: Our data suggest the expression of APN/CD13 for patients with NSCLC to be associated with a poor prognosis and angiogenesis. This is the first study to show the relationship between the expression of APN/CD13 and the prognosis of patients with NSCLC. The inhibition of APN/CD13 may be an effective new molecular target therapy for patients with NSCLC.

Metastasis is one of the most important problems in the treatment of the patients with non–small cell lung cancer (NSCLC) and is composed of a complex process involving cell adhesion, motility, and the degradation of tissue and extracellular matrix by different proteases secreted by tumor cells (1–3). Angiogenesis is also essential in the progression and metastasis of cancer (4). Based on the hypothesis that the inhibition of tumor metastasis and angiogenesis leads to the effective treatment of cancer, we have established a murine monoclonal antibody, MH8-11, which inhibits cell motility and angiogenesis (5). This monoclonal antibody recognized a glycoprotein identical to aminopeptidase N (APN/CD13) and could inhibit the motility of many kinds of cancer cell lines and endothelial cell and capillary tube formation. At another laboratory, APN/CD13 has been reported to be activated by angiogenic signals and it is also essential for capillary tube formation (6). APN/CD13 is a widely expressed zinc-binding ectopeptidase that preferentially catalyzed the removal of neutral amino acids from small peptides (7). In a classic report, APN/CD13 was reported to play an important role in tumor cell invasion and extracellular matrix degradation (8–11). Pasqualini et al. reported APN/CD13 to be a receptor for the NGR peptides in the tumor vasculature, while it is also involved in angiogenesis, thus suggesting that APN/CD13 can serve as a target for delivering drugs into tumors and for inhibiting angiogenesis (12). We recently reported APN/CD13 expression in tumor tissue to be associated with a poor prognosis for patients with colon and pancreatic cancers (5, 13). Therefore, we focused our interest on the expression of APN/CD13 and angiogenesis in NSCLC.

In this study, we used a flow cytometry analysis and reverse transcription-PCR (RT-PCR) to detect the levels of APN/CD13 in lung cancer cell lines and to investigate the usefulness of APN/CD13 in predicting the clinical behavior of NSCLC and we researched for the correlation between the expression of APN/CD13 and the prognosis in 194 patients with NSCLC by an immunohistochemical assay and a quantitative RT-PCR analysis. In addition, we also investigated the relationship

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Received 2/13/06; revised 3/31/06; accepted 4/27/06.

Grant support: Ministry of Education, Science, Sports, and Culture of Japan grant-in-aid 16390400 (M. Miyake).

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Note: We thank Piroska Horvath for the excellent technical assistance.

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doi:10.1158/1078-0432.CCR-06-0338

Table 1. APN/CD13 expression on the lung cancer cell lines by flow cytometry analysis and RT-PCR

NSCLC	FACS	RT-PCR	SCLC	FACS	RT-PCR
LCMS (AD)	S	P	SBC1 (SCLC)	W	N
MAC10 (AD)	S	P	SBC2 (SCLC)	W	N
PC9 (AD)	S	P	SBC3 (SCLC)	W	N
PC14 (AD)	S	P	SBC5 (SCLC)	W	N
A549 (AD)	W	N	MOA1P23 (SCLC)	W	N
HAL8 (AD)	W	N	LU135 (SCLC)	W	N
HAL24 (AD)	W	N	LU139 (SCLC)	W	N
ABC1 (AD)	W	N	N417 (SCLC)	W	N
LC-2/ad (AD)	W	N	Lawson (SCLC)	W	N
LC-1/sq (SQ)	W	N	SCLC-Mo (SCLC)	W	N
EBC1 (SQ)	W	N	LCMA (SCLC)	M	P
LC-TK (SQ)	W	N			
LC1F (SQ)	W	N			
AOI (SQ)	W	N			
EHHSQ13 (SQ)	W	N			
LK-1 (SQ)	W	N			

Abbreviations: AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large cell carcinoma; W, weak (<20%); M, moderate (21-60%); S, strong (>61%); N, negative; P, positive; FACS, fluorescence-activated cell sorting analysis.

between the expression of APN/CD13 and CD34 as an angiogenesis factor based on the immunohistochemical assays.

Materials and Methods

Lung cancer cell lines. Twenty-seven lung cancer cell lines were investigated consisting of 11 SCLC cell lines and 16 NSCLC lines in this study. These lung cancer cell lines are all listed in Table 1. They were maintained in RPMI 1640 supplemented with 10% FCS. The expression of APN/CD13 was evaluated by RT-PCR and flow cytometry analysis.

RT-PCR analysis. Total RNA was purified from growing lung cancer cells and the frozen tumor tissues by the acid-guanidinium-thiocyanate procedure (14). Synthesis of first-strand cDNA was done with 5 μg total RNA using a cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. Based on the nucleotide sequence, 5′-GCCCAAGATGTCCACGTACT-3′ is the sense primer and 5′-GGTG-CTGATGGCATTAACCT-3′ is the antisense primer of *APN/CD13*. The primer pair for *APN/CD13* amplifies a 1,459-bp fragment. The *APN/CD13* reaction mixture was subjected to 26 PCR amplification cycles of denaturation for 40 seconds at 94°C, annealing for 40 seconds at 60°C, and extension for 90 seconds at 72°C. The *β-actin* DNA was used for 24 cycles as the internal PCR control as described previously (15). HL60 was used as a positive control of *APN/CD13* and Raji was used as a negative control of *APN/CD13*. Any tubes that contain all of the ingredients, except the templates, were included in all runs as negative reaction controls.

Flow cytometry analysis. The analysis was carried out as described previously (16). The cells were trypsinized, washed twice with 0.1 mol/L PBS containing 2% FCS, and incubated for 60 minutes on ice individually with the following APN/CD13 monoclonal antibody MH8-11. They were washed with 0.1 mol/L PBS with 2% FCS and then stained with fluorescence-conjugated goat anti-mouse IgG (MP Biomedical Inc., Montréal, Canada) for 30 minutes. After a final wash, the fluorescence intensity of the cells was determined by flow cytometry and the APN/CD13 expression was evaluated as follows: weak, <20%; moderate, 21-60%; strong, >61% (Table 1).

Clinical characteristics of the patients. All patients gave informed consent for study participation according to the regulations of the Japan health authorities. Tumor specimens were obtained from 194 of the initial 219 patients with NSCLC up to stage IIIB who underwent radical surgery at the Department of Thoracic Surgery of Kitano Hospital and the Center for Adult Diseases of Osaka from January 17, 1991 to February 6, 1996 and the retrospective study about APN/CD13 and intratumoral microvessel density (IMD) was done. Twenty-five

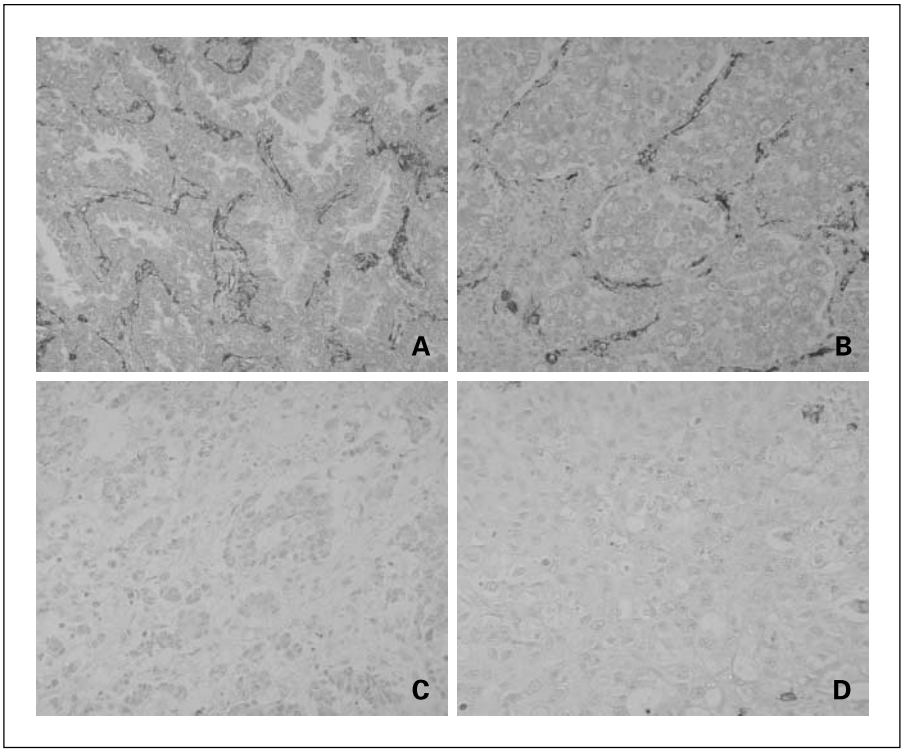

Fig. 1. Immunohistochemistry staining of APN/CD13 and CD34 expression in NSCLC was intense and was seen uniformly throughout the cell membrane and cytoplasm. *A,* adenocarcinoma positive staining; *B,* squamous cell carcinoma positive staining; *C,* adenocarcinoma negative staining; *D,* squamous cell carcinoma negative staining.

Table 2. Relationship between APN/CD13 expression and various clinical and pathologic variables

Variables	Total (*n*)	APN/CD13$^-$, *n* (%)	APN/CD13$^+$, *n* (%)	*P*
Gender				
Female	50	33 (66)	17 (34)	0.857*
Male	144	93 (65)	51 (35)	
Age (y)				
≤60	66	43 (65)	23 (35)	0.923*
>60	128	83 (65)	45 (35)	
Tumor status				
T_1	53	36 (68)	17 (32)	0.922†
T_2	89	58 (65)	31 (35)	
T_3	36	22 (61)	14 (39)	
T_4	16	10 (63)	6 (37)	
Nodal status				
N_0	119	84 (71)	35 (29)	0.217†
N_1	29	17 (59)	12 (41)	
N_2	37	20 (54)	17 (46)	
N_3	9	5 (56)	4 (44)	
Pathologic status				
I	95	66 (69)	29 (31)	0.099†
II	36	26 (72)	10 (28)	
IIIA	39	19 (49)	20 (51)	
IIIB	24	15 (63)	9 (37)	
Histology				
LA	9	6 (67)	3 (33)	0.136†
SQ	63	47 (75)	16 (25)	
AD	122	73 (60)	49 (40)	
Differentiation				
Well	34	25 (74)	9 (26)	0.513†
Moderately	103	65 (63)	38 (37)	
Poorly	57	36 (63)	21 (37)	
Smoking				
Nonsmoker	61	39 (64)	22 (36)	0.841*
Smoker	133	87 (65)	46 (35)	
IMD				
Hypovascular	104	92 (88)	12 (12)	<0.0001*
Hypervascular	90	34 (38)	56 (62)	
Total	194	126 (65)	68 (35)	

*χ^2 test.
† Mann-Whitney's method.

patients who died of causes other than NSCLC were excluded from this study. Tumor-node-metastasis staging designations were made according to the International Postsurgical Pathologic Staging System (17). All eligible patients in the specified period were included. The patients received the adjuvant treatments according to their stage. The patients with stage I and II had no more adjuvant therapy before they had the local recurrence or distant metastasis. On the other hand, postoperative adjuvant systemic chemotherapy was given based on nodal status. Among the patients with stage III, all N_2 or N_3 patients underwent mediastinal radiotherapy (50 Gy in 25 fractions for 5 weeks) after surgical resection and were then treated using two cycles of adjuvant chemotherapy, including cisplatin (80 mg/m^2 of body surface area given i.v. on day 1) and vindesine (4 mg/m^2 of body surface area given i.v. on days 1, 8, and 15). Using immunohistochemistry and RT-PCR, we studied the expressions of APN/CD13 in the patients with NSCLC. The salient clinical characteristics of the patients are presented in Table 2.

Immunohistochemical assay. APN/CD13 and CD34 were immunostained using formalin-fixed, paraffin-embedded tissue specimens as described previously (16). Deparaffinized sections at first were dehydrated through xylene and a graded alcohol series. The endogenous peroxidase activity was then quenched with 0.3% hydrogen peroxide in absolute methanol for 30 minutes and then blocked with 5% bovine serum albumin for 2 hours at room temperature. Next, the sections were individually incubated with monoclonal antibody MH8-11 directed against APN/CD13 during overnight, washed thrice in PBS, and then were incubated for 1 hour with Envision peroxidase-labeled polymer conjugated to anti-mouse IgG (DAKO Corp., Carpinteria, CA). After washing thrice in PBS, antibody binding was visualized with 3-amino-9-etylcarbazol. Similarly, the sections were incubated with mouse monoclonal antibody (Nichirei Corp., Tokyo, Japan) at a 1:20 dilution for CD34. After washing thrice in PBS, antibody binding was visualized with 3,3′-diaminobenzidine and the sections were lightly counterstained with

Mayer's hematoxylin. Sections were incubated with normal murine IgG as negative reaction controls.

Specimen classification based on APN/CD13 immunohistochemistry results. All immunostained sections were reviewed by two pathologists (Drs. Tadashi Ohbayashi and Tomoko Okuno, Department of Pathology, Kitano Hospital) who were blind to the clinical status of the patients. The proportion of high- and low-staining tumor cells in each selected field was determined by counting the individual tumor cells at a high magnification. The slides were examined under low power (4× objective) to identify any regions containing low-staining invasive tumor cells. In cases of multiple areas of low intensity, five areas selected at random were scored, and in sections where all of the staining appeared to be intense, one random field was selected. At least 200 tumor cells were scored per ×40 field. All sections were scored in a semiquantitative fashion according to this method, which considers both the intensity and the percentage of cells staining at each intensity. The intensities were classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), or +3 (very strong staining). For each slide, a value designated HSCORE was obtained by applying the following algorithm: HSCORE = ΣP_i $(i + 1)$, where P_i = 0, 1, 2, 3 (18). When ≥40 of HSCORE in a given specimen were evaluated, the sample was classified as APN/CD13$^+$; when <40 of HSCORE were evaluated, the sample was classified as reduced or APN/CD13$^-$ (19).

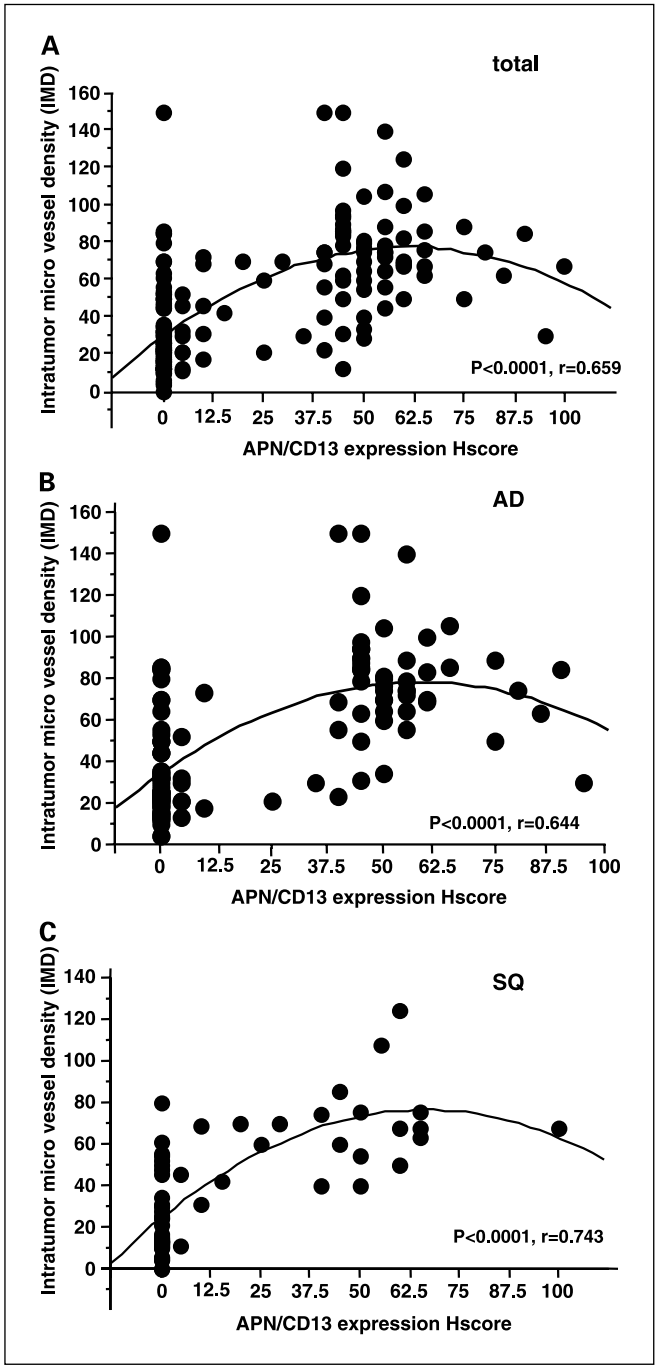

Fig. 2. Relationship between APN/CD13 expression and IMD. APN/CD13 expression was significantly associated with the IMD. *A,* total; *B,* adenocarcinoma (*AD*); *C,* squamous cell carcinoma (*SQ*).

Intratumoral microvessel density. For the IMD, the three most highly vascularized areas were counted in a ×200 field (×20 objective lens and ×10 ocular lens, 0.739 mm^2/field), and the average counts of the three fields were recorded (20). The median IMD among the 194 tumors was chosen as the cutoff point to divide the patients into two groups.

Specimen classification based on RT-PCR results. The amplified DNA samples were subjected to electrophoresis on a 1% agarose gel, and the bands were visualized with ethidium bromide and photographed with a Polaroid camera (Funakoshi, Tokyo, Japan). A densitometric analysis of the photographs was then used for the quantification of the bands. The densitometric value obtained for the *APN/CD13* bands of a tumor sample was divided by that of the corresponding *β-actin* band, and it was called the *APN/CD13* expression ratio (15). The expression ratio of the tumor was then divided by that of HL60 to obtain the *APN/CD13* relative ratio. When the relative rate value of a given specimen was ≥0.8, it was considered to indicate a conserved positive *APN/CD13* gene expression, and if the value was <0.8, then it was considered to denote a nonconserved negative expression. The cutoff point of RT-PCR was closely consistent with the cutoff point for immunohistochemistry.

Statistical analysis. The statistical significance of the difference between the expressions of APN/CD13 and several clinicopathologic variables was assessed by the χ^2 test and Mann-Whitney test. The overall cancer-specific survival was defined from the date of the operation to the date of death due to cancer. An analysis of the overall survival was done using the Kaplan-Meier method (21). Differences between curves for censored survival data were assessed with the log-rank test (22). All *P*s were based on a two-tailed statistical analysis and $P < 0.05$ was considered to indicate statistical significance. The Spearman rank correlation was used to assess associations between APN/CD13 expression and IMD. The joint effect of covariables was analyzed. A multivariate analysis for prognosis value in overall survival was done using the Cox regression model. We analyzed by statistical program StatView version 5.

Results

Expression of APN/CD13 in lung cancer cell lines by RT-PCR and a flow cytometry analysis. Among the 27 lung cancer cell lines, 1 (3.7%) SCLC cell line expressed APN/CD13 moderately, whereas 4 (14.8%) cell lines of NSCLC strongly expressed APN/CD13. The remaining 22 (81.5%) cell lines did not express APN/CD13 (Table 1). In the 16 NSCLC cell lines, 4 (25.0%) cell lines were APN/CD13$^+$. The mRNA expression completely agreed with the expression of the protein level.

Clinical characteristics of the patients. The median age at operation was 63 years (range, 22-80 years) and the median follow-up period of the surviving patients was 116.9 months (range, 61.2-173.9 months). This report includes the follow-up data on June 13, 2005. One hundred one patients died during the follow-up period.

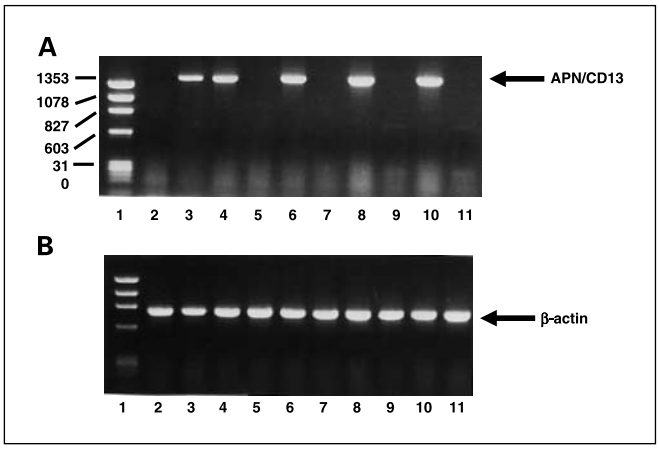

Fig. 3. RT-PCR. *A, APN/CD13*. *Lane 1,* size marker; *lane 2,* Raji (negative control); *lane 3,* HL60 (positive control); *lanes 4* and *6,* adenocarcinoma of *APN/CD13*$^+$; *lanes 5* and *7,* adenocarcinoma of *APN/CD13*$^-$; *lanes 8* and *10,* squamous cell carcinoma of *APN/CD13*$^+$; *lanes 9* and *11,* squamous cell carcinoma of *APN/CD13*$^-$. *B, β-actin* (internal control).

APN/CD13 protein expression analyzed by immunohistochemistry. In these tumors, the APN/CD13 expression resembled that by normal renal proximal tubular epithelial cells (positive control), with intense immunostaining being seen uniformly throughout the cell membrane and cytoplasm (Fig. 1). Of the 194 primary lung cancers studied, a total of 68 (35.1%) tumors were classified as positive for APN/CD13, whereas 126 (64.9%) tumors were classified as negative for APN/CD13.

Relationship between APN/CD13 protein expression and IMD. The mean IMD in the area of highest neovascularization was 31.8 and the SE was 2.3 (range, 0-150). The IMD of APN/CD13$^+$ tumors was 74.7 $\pm$ 2.5 and that of APN/CD13$^-$ tumors was 22.7 $\pm$ 1.3. There was also a significant correlation between the expression of APN/CD13 and the IMD (Table 2; Fig. 2). We found a significant correlation between the APN/CD13 expression and IMD in the patients with squamous cell carcinoma (Spearman $r = 0.743$; $P < 0.0001$; Fig. 2C). We also found a significant correlation in adenocarcinoma (Spearman $r = 0.644$; $P < 0.0001$; Fig. 2B).

APN/CD13 ***gene expression in clinical lung tumor tissues analyzed by quantitative RT-PCR.*** Using RT-PCR, we found that the relative ratio of *APN/CD13/β-actin* expression ranges from 0 to 2.0 in the tumor specimens, and the distribution is shown in Figs. 3 and 4. The mean *APN/CD13* relative ratio was 0.8.

Relationship between APN/CD13 expression and clinicopathologic variables in patients with NSCLC. No significant relationships were observed between the APN/CD13 expression and the patient's gender, age at surgery, tumor status, lymph node status, pathologic stage, histologic subtype, grade of differentiation, or smoking history (Table 2). There was significant relationship between the APN/CD13 expression and the IMD.

Univariate analysis. The variables used in a univariate regression analysis are shown in Table 3. Four variables (APN/CD13 status, T status, N status, and pathologic stage) were found to be significant prognostic factors for survival ($P = 0.001$, $P = 0.0041$, $P < 0.0001$, and $P < 0.0001$, respectively).

Multivariate analysis. Three variables (APN/CD13 status, T status, and N status) used in Cox multivariate regression analysis are shown in Table 3. APN/CD13 was found to be independent prognostic factors for overall survival (hazard ratio, 1.765; 95% confidence interval, 1.191-2.615). Other variables (T status and N status) were also found to be significant for prognostic factors of overall survival (hazard ratios, 1.257 and 1.824; 95% confidence intervals, 1.012-1.561 and 1.490-2.233, respectively).

Discussion

APN/CD13 is type II integral membrane protein and composed of 967 amino acids and exists as a homodimer. APN/CD13 molecular weight is 100 kDa. The human *APN/CD13* gene is localized to chromosome 15q25-26 and composed of 20 exons. APN/CD13 is expressed on granulocyte, myeloid progenitor, bronchial epithelial cells, fibroblasts, renal proximal tubular epithelial cells, and small intestinal epithelial cells (7, 23). APN/CD13 promoter presents two different tissues. The epithelial promoter is closest to the coding part of the gene and the myeloid promoter is placed upstream of the epithelial promoter (23–26). APN/CD13 is considered as an auxiliary adhesion molecule localized at site of cell-cell contact in melanoma cell colonies and tightly associated with extracellular matrix component (27). Transforming growth factor-β increases the APN/CD13 expression in monocytic cells (28). Hypoxia and angiogenic growth factors, such as vascular endothelial growth factor and basic fibroblast growth factor, up-regulate endogenous APN/CD13 levels in cell lines (6). The zinc controls the invasion and adhesion of tumor cells through the regulation of APN/CD13 (29). The mechanism of regulating APN/CD13 remains an important unsolved problem.

Interestingly, there were no reports about the relationship between the APN/CD13 protein expression and gene expression in cell lines especially in NSCLC cell lines. APN/CD13 protein expression has been shown to correlate with *APN/CD13* gene expression. Astonishingly, the number of APN/CD13$^+$ NSCLC cell lines was smaller than expected. Only 4 (25%) NSCLC cell lines were positive for APN/CD13 expression. This rate was apparently smaller than that using resected

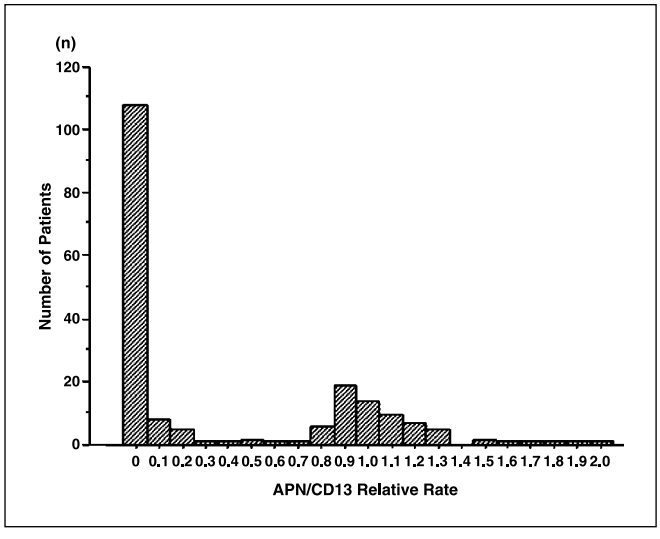

Fig. 4. Distribution of *APN/CD13* relative ratio. Values were obtained from the expression ratio of a given sample divided by that of a promyelocytic leukemia cell line, HL60, used as a positive control.

NSCLC specimens, which remains unknown. Initially, according to these results, we investigated both APN/CD13 protein expression and gene expression in resected NSCLC specimens. We found a significant inverse relationship between the APN/CD13 expression and the prognosis for the patients with NSCLC. In the 194 patients with NSCLC, 68 (35.1%) patients had APN/CD13$^+$ tumors (Table 2). The 5-year survival rate of APN/CD13$^+$ NSCLC patients was shorter than that of individuals whose tumors had an APN/CD13$^-$ expression (48.3% versus 67.1%; $P = 0.001$; Table 3; Fig. 5). However, the 5-year survival rate of APN/CD13$^-$ patients with stage I disease was 79.0% and that of APN/CD13$^+$ was 69.0%. In addition, the median survival time of APN/CD13$^-$ was 138.5 months and that of APN/CD13$^+$ was 128.2 months. We could not find the statistical significance.

In addition, we investigated the relationship between APN/CD13 expression and angiogenesis. Bhagwat et al. reported that APN/CD13 controls endothelial cell morphogenesis (6). However, there were no reports regarding the relationship between APN/CD13 expression and angiogenesis in the patients with NSCLC. It was reported that microvessels stained using anti-CD34 antibodies might provide useful and reliable

Table 3. Univariate and multivariate analysis of 194 patients with NSCLC in relation to clinical and pathologic variables

Variables	5-y Survival rate (%)	Univariate analysis		Multivariate analysis	
		P	Hazard ratio (95% confidence interval)	*P*	Hazard ratio (95% confidence interval)
Gender					
Female	63.2	0.631	1.113 (0.719-1.721)		
Male	59.6				
Age (y)					
≤60	58.8	0.593	1.117 (0.745-1.076)		
>60	61.1				
Tumor status					
T_1	72.5	0.0041*	1.369 (1.105-1.698)	0.0386*	1.257 (1.012-1.561)
T_2	58.9				
T_3	54.0				
T_4	38.9				
Nodal status					
N_0	74.6	<0.0001*	1.910 (1.574-2.319)	<0.0001*	1.824 (1.490-2.233)
N_1	56.8				
N_2	27.0				
N_3	11.1				
Pathologic status					
I	75.9	<0.0001*	1.646 (1.384-1.957)		
II	61.0				
IIIA	38.9				
IIIB	29.4				
Histology					
LA	74.5	0.117	1.335 (0.926-1.923)		
SQ	66.7				
AD	53.2				
Differentiation					
Well	75.0	0.141	1.315 (0.984-1.756)		
Moderately	58.0				
Poorly	56.2				
Smoking					
Nonsmoker	63.1	0.555	1.133 (0.747-1.718)		
Smoker	59.1				
APN/CD13					
Negative	67.1	0.001*	1.910 (1.292-2.825)	0.0046*	1.765 (1.191-2.615)
Positive	48.3				
IMD					
Hypovascular	60.3	0.952	0.975 (0.660-1.442)		
Hypervascular	60.1				

*Significance.

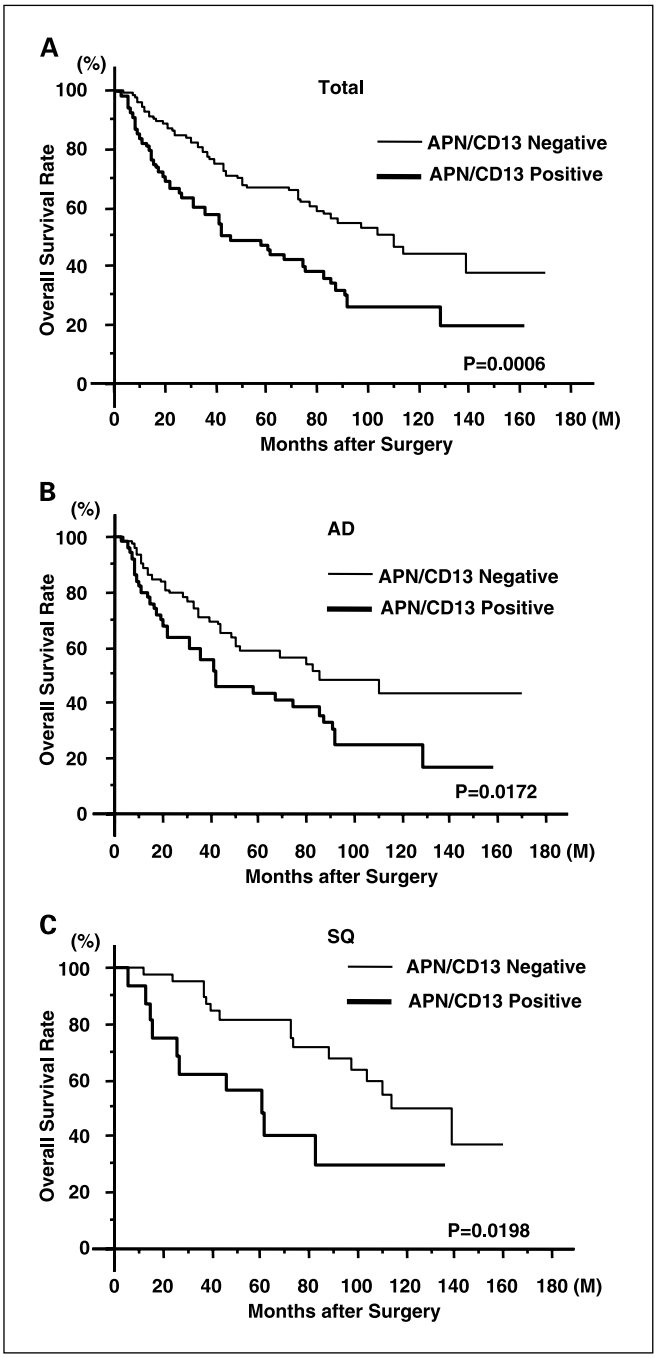

Fig. 5. Overall survival of patients with NSCLC in relation to tumor APN/CD13 status. *P* was determined by the log-rank test. *A,* total (194 patients); *B,* adenocarcinoma (122 patients); *C,* squamous cell carcinoma (63 patients).

information. There have been several reports using CD34 in the patients with various kinds of cancers, which revealed the high microvessels counts to be important as a predictor of a poor prognosis for these patients (20). We thus investigated the relationship between the APN/CD13 expression and CD34 expression as an angiogenesis factor by immunohistochemical double staining. We found a significant correlation between APN/CD13 expression and IMD in the patients with squamous cell carcinoma ($r = 0.743$; $P < 0.0001$; Fig. 4C). Similarly, we could find a correlation in adenocarcinoma ($r = 0.644$; $P < 0.0001$; Fig. 4B). This is a very interesting result; an APN/CD13 inhibitor, ubenimex, was reported to be a useful drug for the patients with squamous cell carcinoma (30, 31).

Platinum-based regimens have been the main treatment for advanced NSCLC for many years. Patients assigned to cisplatin-based adjuvant chemotherapy following a resection have shown significantly higher survival rate than those assigned to observation [5-year survival rate, 44.5% versus 40.4% in stage I-III (32) and 69% versus 54% in stage IB-II (33)]. In addition, another randomized clinical trial of adjuvant chemotherapy with carboplatin and paclitaxel following a resection in stage IB had a significantly higher survival rate [4-year survival rate, 71% versus 59% (34)]. However, all recent randomized studies of platinum-based combinations with these newer agents have yielded similar results for advanced stage IIIB-IV patients, with findings indicating a median survival from 10.2 to 12.5 months (35).

Advances in understanding the molecular and biological aspects of carcinogenesis have led to the development of new agents that act on specific biological pathways in the disease in an approach that has been termed "targeted therapy." Especially in the near future, some prognostic molecular factors might play important roles as targeted therapy in NSCLC (36). Biological agents that are being investigated for use in NSCLC include agents targeting cell growth factor receptors, angiogenesis inhibitors, and signal transduction inhibitors. Such biological agents were initially described as "cytostatic," in contrast to the cytotoxic agents used in conventional chemotherapy, because it was believed that they would not produce tumor responses when used alone; however, it has been shown that some are indeed capable of producing objective response in single-agent use (37).

The currently envisioned goal for targeted therapy is that agents showing activity on biological pathways for specific tumor types can be integrated with surgery, conventional chemotherapy, or radiation therapy at all stages of disease, including maintenance therapy and chemoprevention. Ubenimex is a potent aminopeptidase inhibitor that shows an immunostimulant and antitumor activity. A prospective randomized trial reported that the postoperative adjuvant treatment with ubenimex could prolong the survival of patients with completely resected stage I squamous cell lung carcinoma (30). We evaluated APN/CD13 from the point of view of clinical application; thus, we are going to make a plan to treat patients with NSCLC. Because APN/CD13$^+$ patients showed a poor prognosis based on the results in this study, APN/CD13$^+$ patients were thus selected and should be divided into groups who require adjuvant therapy. The inhibition of APN/CD13 using ubenimex may therefore be an effective new therapeutic strategy of therapy for APN/CD13$^+$ patients with NSCLC.

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