Circulating Aminopeptidase N/CD13 Is an Independent Prognostic Factor in Patients with Non–Small Cell Lung Cancer

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Abstract

Purpose: Aminopeptidase N, also known as CD13, has important roles in tumor metastasis and angiogenesis. Its expression in tumor tissue has been reported to be associated with poor prognosis. However, the clinical significance of circulating aminopeptidase N/CD13 in patients with solid tumors is unknown. We previously developed an aminopeptidase N/CD13–specific monoclonal antibody (mAb) MH8-11, which inhibits cell motility and angiogenesis *in vitro*. The aim of this study was to evaluate the clinical significance of circulating aminopeptidase N/CD13 protein detected by mAb MH8-11 in patients with non–small cell lung cancer (NSCLC).

Experimental Design: We used electrochemiluminescence immunoassay with mAb MH8-11 to determine circulating aminopeptidase N/CD13 levels in 90 healthy volunteers and 90 patients with NSCLC. Circulating aminopeptidase N/CD13 levels were measured in sera taken before treatment and evaluated for a relationship with clinical outcomes.

Results: A significant correlation was found between tumor progression and serum aminopeptidase N/CD13 concentrations ($r = 0.23$, $P = 0.029$). High serum aminopeptidase N/CD13 levels ($n = 17$) were associated with advanced stage ($P = 0.004$) or poor performance status ($P = 0.001$). The overall survival rate for patients with high serum aminopeptidase N/CD13 levels ($n = 17$) was significantly less than that of patients with low serum aminopeptidase N/CD13 levels ($n = 73$, $P < 0.0001$). In a multivariate survival analysis in patients with NSCLC, serum aminopeptidase N/CD13 levels had an independent influence on survival (relative risk, 4.1; 95% confidence interval, 1.9-8.8).

Conclusions: Our data suggest that a high level of circulating aminopeptidase N/CD13 at diagnosis is an independent prognostic factor in patients with NSCLC.

Lung cancer is a common health problem in the world, and non–small cell lung cancer (NSCLC) accounts for 75% to 80% of cases. The high rate of metastasis is an important problem in the treatment of patients with NSCLC, and the prognosis for patients with distant metastasis is extremely poor. Because systemic treatments for NSCLC with standard chemotherapy agents are still relatively ineffective, novel strategies for the development of anticancer therapies are required (1).

Aminopeptidase N is identical to the myeloid differentiation antigen cluster of differentiation 13 (CD13). It is a zinc-dependent metalloprotease cell surface protein that catalyzes the removal of NH_2-terminal amino acids from peptides with a preference for neutral residues (2, 3). Aminopeptidase N/CD13 is expressed in various cells outside the hematopoietic system, including epithelial cells of the intestine and kidney, synaptic membranes of the central nervous system, fibroblasts, endothelial cells, and tumor cells (4, 5). Aminopeptidase N/CD13 has been reported to play important roles in tumor cell invasion (6, 7) and tumor angiogenesis (8, 9). We previously developed an aminopeptidase N/CD13–specific monoclonal antibody (mAb) MH8-11. This novel murine mAb inhibited tumor cell motility, endothelial cell migration, and tube formation for angiogenesis *in vitro* (10). In patients with resected colon or pancreatic cancer, aminopeptidase N/CD13 expression determined by immunohistochemical assay with mAb MH8-11 has been reported as an independent prognostic factor (10, 11).

Aminopeptidase N/CD13 activity has been found to be elevated in the plasma of cancer patients and was associated with tumor load (12–14). The tumor environment may be an important source of circulating aminopeptidase N/CD13. However, the clinical significance of circulating aminopeptidase N/CD13 in patients with solid tumors is unknown. In this report, we investigated circulating aminopeptidase N/CD13 protein concentrations detected by functional mAb MH8-11 and evaluated its clinical significance in patients with NSCLC.

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Table 1. Characteristics of 90 patients with NSCLC

Characteristics	
Age (y)	
Median	67
Range	30-86
Sex (%)	
Female	27 (30.0)
Male	63 (70.0)
Performance status, *n* (%)	
0	55 (61.1)
1	22 (24.5)
2	4 (4.4)
3	6 (6.7)
4	3 (3.3)
Stage (%)	
I	17 (18.8)
II	5 (5.6)
IIIA	5 (5.6)
IIIB	23 (25.6)
IV	40 (44.4)
Histologic type (%)	
Adenocarcinoma	64 (71.1)
Squamous cell carcinoma	23 (25.6)
Adenosquamous cell carcinoma	1 (1.1)
Large cell carcinoma	2 (2.2)
Serum CEA (%)	
Normal	41 (45.6)
More than normal	49 (54.4)

Abbreviation: CEA, carcinoembryonic antigen.

Materials and Methods

Patients. Ninety patients with histologically proven and previously untreated NSCLC seen and subsequently treated in the Department of Molecular and Internal Medicine, Hiroshima University Hospital (Hiroshima, Japan), between November 2001 and June 2004, were included in this study. Histologic typing of the tumors was done according to the WHO classification. The majority of patients (n = 64, 71.1%) had adenocarcinoma. The median age was 67 years (range, 30-86 years), and 63 (70.0%) of the patients were male. The baseline characteristics of the patients are shown in Table 1.

Twenty-nine of the patients were treated either with surgery (n = 17, 18.9%), radiation therapy (n = 8, 8.9%), or chemoradiotherapy (n = 4, 4.4%). Forty-three (47.8%) patients were treated with chemotherapy, and 24 of the patients received platinum-based regimens. We assessed objective tumor response as complete response, partial response, stable disease, or progressive disease in accordance with the Response Evaluation Criteria in Solid Tumor for patients with measurable disease (15). Fifty-five patients died during the follow-up period. The median follow-up of surviving patients was 24.0 months (range, 14.2-43.4 months).

Controls. Serum aminopeptidase N/CD13 concentrations were measured in 90 healthy volunteers as controls (63 males and 27 females; median age, 62 years; range, 41-89 years). We randomly picked up sex-matched healthy individuals who showed no abnormalities in complete blood cell counts, C-reactive proteins, erythrocyte sedimentation rates, liver function tests, renal function tests, urinalyses, fecal examinations, chest X-rays, or electrocardiograms in medical check up.

Biochemical measurements. Peripheral venous blood samples were collected before initiation of any cancer therapy, centrifuged at 3,000 rpm for 10 minutes, and then stored at −80°C for later analysis. Informed consent was obtained from all patients, including the healthy volunteers. Serum aminopeptidase N/CD13 concentrations were evaluated using electrochemiluminescence immunoassay without any knowledge of patient survival or other clinical data. Serum samples (20 μL) were diluted 1:10 in matrix buffer (Dulbecco's PBS containing 1% bovine serum albumin and 10% normal rabbit serum). All samples were mixed with an aminopeptidase N/CD13–specific mAb (MH8-11)–coated magnetic beads for 2 hours at room temperature. After incubation, the samples were magnetically captured and washed thrice with matrix buffer. Ruthenium-labeled MH8-11 was diluted to 0.1 μg/mL in matrix buffer and added to all samples for 30 minutes. ORIGEN assay buffer (Igen, Gaithersburg, MD) was added to the reaction, and the electrochemiluminescence response was initiated and recorded on the ORIGEN analyzer. All analyses were carried out in triplicate. We calibrated this assay with culture supernatant derived from the human fibrosarcoma cell line HT1080, which expresses aminopeptidase N/CD13 (10). This solution was determined to have an aminopeptidase N/CD13 concentration of 128 units/mL and, subsequently, serially diluted 2-fold in matrix buffer to yield final concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 unit/mL. Aminopeptidase N/CD13 concentrations were calculated from a standard curve determined by the concurrent testing of calibrators in each analysis. The lower limit of quantitation for this assay was 0.5 unit/mL.

Statistical methods. Comparison of non-normally distributed variables between two groups was done with the Mann-Whitney U test, and correlations were analyzed with the Spearman rank correlation. Fisher's exact test or the Mann-Whitney U test was used to compare categorical data. The overall cancer-specific survival was defined from the date of the blood sampling to the date of death due to cancer. The Kaplan-Meier method was used to estimate the probability of survival, and the differences in survival rates were assessed by log-rank test (16). Multivariate analysis of prognostic factors was done using the Cox regression model (17). Differences were considered significant when the $P < 0.05$. All statistical analyses were done using the SAS package version 8.2 (SAS Institute, Cary, NC).

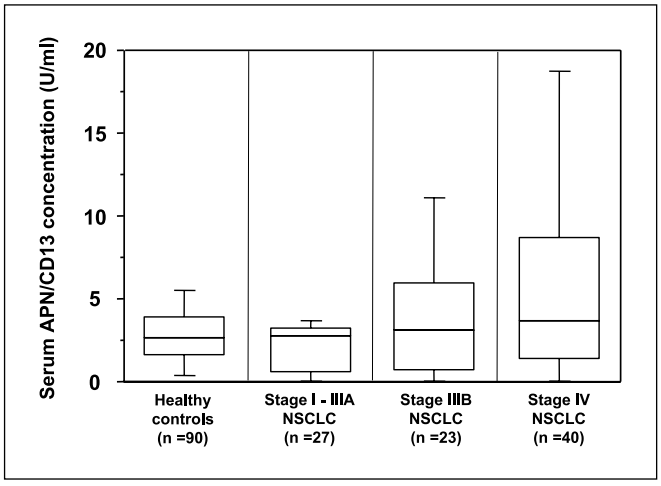

Fig. 1. Serum aminopeptidase N (*APN*)/CD13 concentrations at diagnosis in 90 patients with NSCLC and 90 healthy controls. NSCLC patients were separated into three groups according to tumor stage. A significant correlation was found between tumor progression and serum aminopeptidase N/CD13 concentration (r = 0.23, P = 0.029).

Table 2. Association of serum APN/CD13 level with clinicopathologic variables

Factor	Serum APN/CD13 (units/mL)		*P*
	High level (>6.5), *n* (%)	Low level (≤6.5), *n* (%)	
Age (y)			
≤70	14 (23.3)	46 (76.7)	0.160*
>70	3 (10.0)	27 (90.0)	
Sex			
Female	3 (11.1)	24 (88.9)	0.256*
Male	14 (22.2)	49 (77.8)	
Performance status			
0-2	11 (13.6)	70 (86.4)	0.001*
3, 4	6 (66.7)	3 (33.3)	
Stage			
I-IIIA	0 (0)	27 (100)	0.004†
IIIB	4 (13.6)	19 (86.4)	
IV	13 (36.4)	27 (63.6)	
Histologic type			
Adenocarcinoma	15 (23.4)	49 (76.6)	0.136*
Nonadenocarcinoma	2 (7.7)	24 (92.3)	
Serum CEA			
Normal	7 (17.1)	34 (82.9)	0.790*
More than normal	10 (20.4)	39 (79.6)	
Response to chemotherapy			
PR	2 (14.3)	12 (85.7)	0.007*
SD	2 (13.3)	13 (86.7)	(PR + SD vs PD)
PD	7 (58.3)	5 (41.7)	
Not evaluable	1 (50.0)	1 (50.0)	

Abbreviations: APN, aminopeptidase N; CEA, carcinoembryonic antigen; PR, partial response; SD, stable disease; PD, progressive disease.
*Fisher's exact test.
†Mann-Whitney *U* test.

Results

Serum aminopeptidase N/CD13 concentrations. Patients with advanced-stage (IIIB or IV) NSCLC had significantly higher aminopeptidase N/CD13 levels than patients with stage I to IIIA NSCLC (median, 3.3 units/mL; range, from undetectable to 87.0 units/mL versus median, 2.7 units/mL; range, from undetectable to 6.0 units/mL, respectively; $P = 0.042$, Fig. 1). There was also a significant correlation between tumor progression and serum aminopeptidase N/CD13 concentrations ($r = 0.23$, $P = 0.029$; Fig. 1). Serum aminopeptidase N/CD13 levels in patients with NSCLC were higher than those in healthy controls (median, 3.0 units/mL; range, from undetectable to 87.0 units/mL versus median, 2.6 units/mL; range, from undetectable to 9.9 units/mL, respectively), although this difference did not reach statistical significance ($P = 0.43$, Fig. 1). According to the best diagnostic accuracy to separate the healthy controls and the patients with NSCLC, we determined 6.5 units/mL as threshold value, which corresponded to a diagnostic accuracy of 0.58, a sensitivity of 0.19, and a specificity of 0.96. High serum aminopeptidase N/CD13 levels were observed in 17 of the 90 (18.9%) patients with any stage of NSCLC and 13 of the 40 (32.5%) patients with stage IV NSCLC.

Association of serum aminopeptidase N/CD13 level with clinicopathologic variables. A high serum aminopeptidase N/CD13 level was associated with advanced stage or poor performance status ($P = 0.004$ and $P = 0.001$, respectively; Table 2). Serum aminopeptidase N/CD13 levels seemed elevated in patients with adenocarcinoma, but the difference did not reach a statistical significance ($P = 0.136$; Table 2). There was also no significant association between the serum aminopeptidase N/CD13 level and age, sex, or serum carcinoembryonic antigen level at diagnosis.

Forty-one of the patients were evaluable for objective tumor response in accordance with the Response Evaluation Criteria in Solid Tumor. There were no complete responses, and the disease control rate was 70.7% (29 of 41), with 34.1% (14 of 41) of partial response and 36.6% (15 of 41) of stable disease. Six of 11 (54.5%) patients with patients with high serum aminopeptidase N/CD13 levels and 18 of 30 (60.0%) patients with low serum aminopeptidase N/CD13 levels were treated

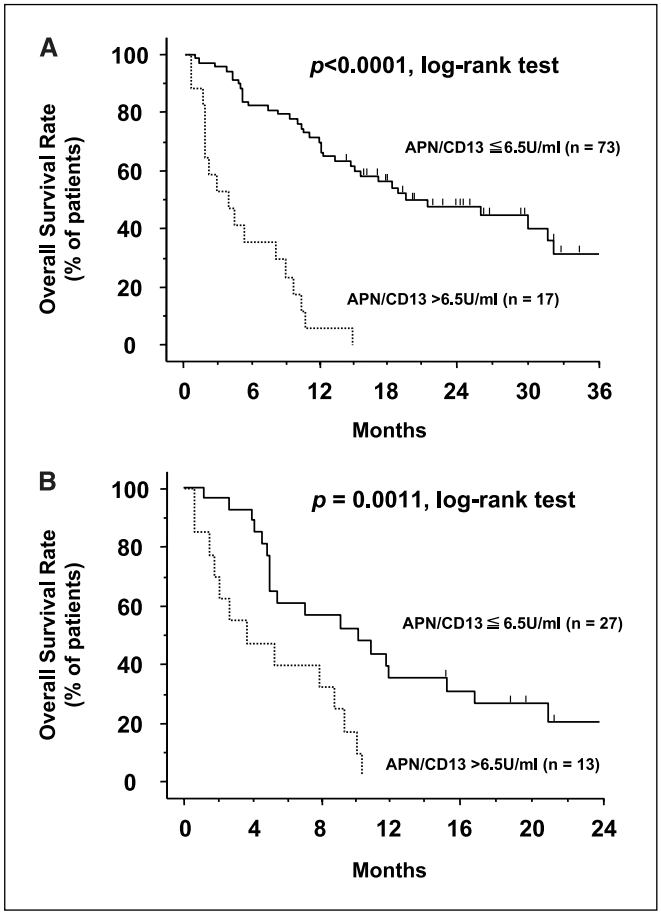

Fig. 2. Overall survival of 90 patients with NSCLC in relation to their aminopeptidase N (*APN*)/CD13 status (*A*) and overall survival of 40 patients with stage IV NSCLC in relation to their aminopeptidase N/CD13 status (*B*). The serum concentrations corresponding to the best diagnostic accuracy to separate the healthy controls and the patients with NSCLC were used as the cutoff value (>6.5 units/mL). Patients alive at the time of analysis are indicated by a vertical bar.

with platinum-based chemotherapy. The response rate for patients with high serum aminopeptidase N/CD13 levels tend to be poorer than that of patients with low serum aminopeptidase N/CD13 levels (18.2% versus 40.0 %, $P = 0.177$), and a high serum aminopeptidase N/CD13 level was significantly associated with progressive disease ($P = 0.007$; Table 2).

Association of serum aminopeptidase N/CD13 level with overall survival. The median survival time of patients with high serum aminopeptidase N/CD13 level was less than that of patients with low serum aminopeptidase N/CD13 levels (3.8 versus 21.4 months, $P < 0.0001$; Fig. 2A). In addition, significant differences were noted between patients with high versus those with low serum aminopeptidase N/CD13 levels in the 40 patients with stage IV NSCLC (3.8 versus 10.4 months, $P = 0.0011$; Fig. 2B).

Multivariate survival analysis of the 90 patients with NSCLC showed that a high serum aminopeptidase N/CD13 level at diagnosis (relative risk, 4.1; 95% confidence interval, 1.9-8.8), the stage at diagnosis (relative risk, 8.6; 95% confidence interval, 3.0-25.4) and the performance status (relative risk, 3.4; 95% confidence interval, 1.5-8.0) independently influenced survival, whereas age, sex, histologic type, and serum carcinoembryonic antigen level at diagnosis did not (Table 3). A high serum aminopeptidase N/CD13 level at the time of diagnosis was found to be a significant prognostic factor ($P < 0.001$).

Discussion

In this study, we determined serum aminopeptidase N/CD13 concentrations in patients with NSCLC using an aminopeptidase N/CD13–specific mAb (MH8-11), which inhibits cell motility and angiogenesis *in vitro* (10). Serum aminopeptidase N/CD13 concentrations were significantly elevated in patients with advanced-stage NSCLC. When the serum aminopeptidase N/CD13 values corresponding to the best diagnostic accuracy to separate the healthy controls and the patients with NSCLC were used as the cutoff value, the sensitivity of aminopeptidase N/CD13 was poorer than that of serum carcinoembryonic antigen; this suggests that serum aminopeptidase N/CD13 is not valuable as a diagnostic marker. However, a high serum aminopeptidase N/CD13 was significantly associated with established adverse prognostic factors in NSCLC, such as advanced stage, poor performance status, and poor response to chemotherapy. Serum aminopeptidase N/CD13 levels were also associated with overall survival in univariate analyses and had an independent influence on survival in a multivariate analysis of patients with NSCLC. Although we must consider that our findings are based on a retrospective study with a relatively small number of patients, these findings suggest that circulating aminopeptidase N/CD13 may be associated with proliferative and survival pathways in NSCLC. Circulating aminopeptidase N/CD13 level can be quickly measured with electrochemiluminescence immunoassay and may provide a useful marker of survival in patients with NSCLC.

The origin of circulating aminopeptidase N/CD13 is unclear at present. In our series, serum aminopeptidase N/CD13 levels were significantly elevated in patients presenting at an advanced stage with a large tumor mass, lymph node metastasis, or distant metastasis. In addition, we showed a significant correlation between tumor progression and serum aminopeptidase N/CD13 concentrations. These findings are

Table 3. Multivariate Cox analysis of overall survival of 90 patients with NSCLC

Factor	β	SE	χ^2	*P*	Hazard ratio (95% confidence interval)
APN/CD13					
≤6.5	1.418	0.388	13.361	<0.001	4.1 (1.9-8.8)
>6.5					
Age (y)					
≤70	−0.122	0.346	0.125	0.724	0.9 (0.4-1.7)
>70					
Sex					
Female	−0.055	0.341	0.026	0.871	0.9 (0.5-1.8)
Male					
Performance status					
0-2	1.227	0.434	7.994	0.005	3.4 (1.5-8.0)
3, 4					
Stage					
I-IIIA	2.154	0.551	15.277	<0.001	8.6 (3.0-25.4)
IIIB, IV					
Histology					
Nonadenocarcinoma	−0.514	0.386	1.767	0.183	0.6 (0.3-1.3)
Adenocarcinoma					
Serum CEA					
Normal	0.054	0.331	0.026	0.872	1.1 (0.6-2.0)
More than normal					

Abbreviations: APN, aminopeptidase N; CEA, carcinoembryonic antigen.

in accordance with a previous report of biological soluble CD13 activity in plasma (14) and support the hypothesis that circulating aminopeptidase N/CD13 is derived from the tumor milieu (e.g., from tumor cells or tumor-associated endothelial cells). Although the relationship of soluble aminopeptidase N/CD13 with tumor load suggested a possible role for aminopeptidase N/CD13 as a tumor marker, our results indicate that soluble aminopeptidase N/CD13 is not sensitive enough to serve as a tumor marker, at least not in NSCLC.

The expression of aminopeptidase N/CD13 in tumor tissue had been found to be associated with poor outcomes in solid tumors, including NSCLC. Analysis of 132 patients with NSCLC undergoing radical surgery revealed that patients with aminopeptidase N/CD13 gene expression–positive tumors (n = 56) had a significantly lower 5-year survival rate than patients with aminopeptidase N/CD13 gene expression–negative tumors (n = 76; 54.1% versus 67.2%; P = 0.047; ref. 18). In pancreatic cancer, aminopeptidase N/CD13 gene expression agreed well with protein expression detected by immunohistochemistry (11). Aminopeptidase N/CD13 gene expression was also significantly associated with increased intratumor microvessel density and poor prognosis in patients with pancreatic cancer (11). The association between aminopeptidase N/CD13 expression and poor prognosis has also been reported in patients with colon cancer or acute lymphoblastic leukemia (10, 19). In our series, high levels of serum aminopeptidase N/CD13 were associated with poor prognosis in patients with NSCLC. High levels of circulating aminopeptidase N/CD13 may, at least in part, reflect high levels of aminopeptidase N/CD13 expression in tumor tissue, which generally seem to be associated with poor prognosis.

On the other hand, recently, the epoch-making progress might have been made for the treatment of NSCLC. Platinum-based regimens have been the main treatment for NSCLC for many years. However, all recent randomized studies of platinum-based combinations with newer agents have yielded similar results, with findings indicating median survival of 7.8 to 8.6 months and 1-year survival of 32% to 38% (20, 21). Advances in understanding 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 molecular-targeted therapy. Biological agents that are being investigated for use in NSCLC include agents targeting cell growth factor receptors, angiogenesis inhibitors, and signal transduction inhibitors. Aminopeptidase N/CD13 expression is exclusively up-regulated in tumor cells, as well as in endothelial cells within tumors, and has been shown to be a molecular target for anticancer therapy (8). Recently, a number of agents targeting aminopeptidase N/CD13 have undergone evaluation. The use of gene therapy for the delivery of a tumor-specific gene-targeting aminopeptidase N/CD13 has been shown to be promising in preclinical studies (22). The oral administration of bestatin, a strong aminopeptidase inhibitor isolated from a *Streptomyces olivoreticuli* culture filtrate, prolonged the survival of patients with resected stage I squamous cell lung cancer (23, 24). In addition, we developed novel human aminopeptidase N/CD13-specific mAbs.[3] Like various kinds of mAb treatments (e.g., Herceptin and Rituxan), such human mAbs would be useful for the molecular target therapy of the patients with aminopeptidase N/CD13–positive tumors. Patients with high levels of serum aminopeptidase N/CD13 may also be good candidates for treatment with agents targeting aminopeptidase N/CD13.

In conclusion, our data show that serum aminopeptidase N/CD13 levels are significantly elevated in NSCLC patients with advanced-stage disease or poor performance status, and that a high serum aminopeptidase N/CD13 level at diagnosis is an independent prognostic factor in such patients. The circulating aminopeptidase N/CD13 level may be a useful prognostic indicator in NSCLC and potentially play a role in the development of customized therapy. Further prospective study should be conducted to confirm our conclusion.

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