

Clinical Significance of Aminopeptidase N/CD13 Expression in Human Pancreatic Carcinoma¹

Naoya Ikeda, Yoshiyuki Nakajima,
Takahiro Tokuhara, Noboru Hattori,
Masayuki Sho, Hiromichi Kanehiro, and
Masayuki Miyake²

The Fifth Department of Oncology and the Department of Thoracic Surgery, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka 530-8480 [N. I., T. T., N. H., M. M.], and The First Department of Surgery, Nara Medical University, Nara 634-8522 [N. I., Y. N., M. S., H. K.], Japan

ABSTRACT

Purpose: We previously established a novel murine monoclonal antibody (MH8-11) that recognized aminopeptidase N (APN)/cluster of differentiation antigen 13 (CD13). This monoclonal antibody inhibited human umbilical vein endothelial cells migration and capillary-like tube formation of human umbilical vein endothelial cells on Matrigel. In this study, we investigated the expression of APN/CD13 and the intratumor microvessel density (IMD) as the number of microvessel counts in 50 patients with pancreatic carcinoma.

Experimental Design: We investigated APN/CD13 gene expression using the reverse transcriptase-PCR. We also used immunohistochemistry with MH8-11 to investigate APN/CD13 protein expression. Moreover, we investigated the relationship between APN/CD13 expression and tumor angiogenesis by measuring the IMD.

Results: APN/CD13 gene expression detected by reverse transcriptase-PCR was positive in 50.0% (25 of 50) of the tumors, and APN/CD13 protein positive was detected by immunohistochemistry in 48.0% (24 of 50). APN/CD13 gene expression agreed well with the immunohistochemical findings (90.0% concordance). APN/CD13 was also significantly associated with an increase of the IMD ($r = 0.71$, $P = 0.0003$). However, APN/CD13 expression was not associated with various prognostic factors. The median survival time of patients with APN/CD13 expression was significantly shorter than that of patients without APN/CD13 expression

($P = 0.009$), and multivariate analysis showed that the APN/CD13 status was a significant independent factor ($P = 0.016$).

Conclusions: Our data suggest that APN/CD13 may be a new prognostic marker for patients with pancreatic carcinoma and may have a relationship with the angiogenesis for this cancer.

INTRODUCTION

To grow beyond the size of 1–2 mm (1), primary tumors and metastases must develop an adequate blood supply through the process known as angiogenesis (2). The induction of angiogenesis is mediated by multiple angiogenic-mitogenic molecules that are released by both tumor cells and host cells (1). Various processes are involved, including the production of proteases such as urokinase and collagenase by both tumor and endothelial cells.

The migration of endothelial cells also plays a very important role in angiogenesis. We therefore assumed that inhibiting the migration of endothelial cells would be able to suppress angiogenesis and established a novel murine MAb³ (MH8-11) that inhibited endothelial cell migration and tube formation (3). This epitope was a M_r 165,000 protein, and the sequencing analysis revealed that it was almost identical to APN/CD13. APN/CD13 is a surface glycoprotein encoded by a gene located on the long arm of chromosome 15 at bands q25–26. This 967 amino acid integral membrane protein has a large extracellular COOH-terminal domain containing a pentapeptide motif, which is characteristic of members of the zinc-binding metalloprotease superfamily (4–6). APN/CD13 has been implicated in the tumor invasion (7–9). To investigate the clinical significance of APN/CD13 expression in pancreatic carcinoma, we performed a reverse transcriptase-PCR analysis to quantify the expression of this gene in tumor tissues from 50 patients with pancreatic carcinoma. Immunohistochemical assays were also performed to confirm the results of the reverse transcriptase-PCR. In addition, we investigated the relationship between APN/CD13 expression and angiogenesis by determining the IMD.

MATERIALS AND METHODS

Patients and Surgical Specimens. We examined 50 patients with pancreatic carcinoma who underwent surgery at the First Department of Surgery of Nara Medical University (Nara, Japan) between December 1992 and April 1999. The median age of the patients was 65.0 years, with a range of 47–80 years. Eight of the patients had distant metastasis, which was both solitary and resectable. After pancreatectomy, almost all of the

Received 3/27/02; revised 11/18/02; accepted 11/20/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported, in part, from the Ministry of Education, Science, Sports and Culture of Japan Grant-in-Aids 14370421 (to M. M.) and 14770651 (to N. I.) and a grant from the Vehicle Racing Commemorative Foundation (to M. M.).

² To whom requests for reprints should be addressed, at Fifth Department of Oncology and The Department of Thoracic Surgery, Kitano Hospital, Tazuke Kofukai Medical Research Institute, 2-4-20 Ohgimachi, Kita-ku, Osaka 530-8480, Japan. Phone: 81-6-6312-1221; Fax: 81-6-6312-8816; E-mail: miyakem@kitano-hp.or.jp.

³ The abbreviations used are: MAb, monoclonal antibody; IMD, intratumor microvessel density; APN, aminopeptidase N; CD13, cluster of differentiation antigen 13; TNM, tumor node metastasis.

Table 1 Correlation between APN/CD13 status and known prognostic factors

	Total (n)	APN/CD13		P
		Positive	Negative	
Age at surgery				
<60	13	4	9	NS ^{a,b}
≥60	37	21	16	
Gender				
Male	36	18	18	NS ^b
Female	14	7	7	
Tumor status				
T ₁	2	1	1	NS ^c
T ₂	10	4	6	
T ₃	21	11	10	
T ₄	17	9	8	
Nodal status				
N ₀	27	12	15	NS ^b
N ₁	23	13	10	
Metastatic status				
M ₀	42	21	21	NS ^b
M ₁	8	4	4	
Pathological stage				
Stage I	7	2	5	NS ^c
Stage II	10	5	5	
Stage III	11	6	5	
Stage IVA	14	8	6	
Stage IVB	8	4	4	
Histopathologic grade				
G1	15	6	9	NS ^c
G2	29	16	13	
G3	6	3	3	
Total number of patients	50	25	25	

^a NS, not significant.

^b χ^2 test.

^c Mann-Whitney's *U* test.

patients received intraoperative radiation therapy, with 20 Gy of radiation delivered to the retroperitoneal area, including the origins of the portal vein and the celiac artery. Postoperative systemic chemotherapy was also administered to all of the patients. Tissues were obtained from all of the resected specimens and were rapidly frozen at -80°C for storage until use. The remainder of each specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin. Tumors were classified according to the TNM staging system (10). The TNM classification and the clinical characteristics of these patients are presented in Table 1.

Immunohistochemistry. Immunohistochemical analysis was performed with a Vectastain ABC System (Vector Laboratories Inc., Burlingame, CA). After neutralization of endogenous peroxidase, sections on glass slides were preincubated in blocking serum and then were incubated overnight with hybridoma culture supernatant containing the MAb that recognized APN/CD13 and a mouse CD34 MAb (Nichirei Corporation, Tokyo, Japan) at a 1:20 dilution, respectively. After three washes in PBS, the sections were incubated for 2 h with biotinylated antimouse IgG, washed three times with PBS, incubated with avidin biotin peroxidase complex for 1 h, and again washed for 10 min with PBS. Reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride, and the slides were counterstained with hematoxylin. Sections incubated with mouse myeloma SP2 supernatant served as negative reaction controls.

Evaluation of Immunostaining and Counting of Microvessels. All of the immunostained sections were examined under low power ($\times 4$ objective) to identify regions containing low-staining invasive tumor cells. In cases of multiple areas of low intensity, five randomly selected areas were scored, and in sections where all of the staining appeared intense, one field was selected at random. The proportion of tumor cells showing high and low staining in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per $\times 400$ field. We set several cutoff points arbitrarily to select the best value preliminarily. When the percentage of APN/CD13-positive tumor cells within the tumor tissue specimen was $\geq 10\%$, significant differences were found with respect to the survival rate. As previously described (11, 12), we therefore selected 10% as the most appropriate cutoff value. Briefly, specimens with a $\geq 10\%$ APN/CD13-positive tumor cells classified as positive and as negative when it was $< 10\%$. To count microvessels, the vessels in the four most highly vascularized areas were counted at $\times 200$ magnification (4 fields of 0.785 mm^2 each), and the average count was calculated. The microvessel count of the 50 tumors ranged from 20.9 to 117.5, with a mean count of 59.1 ± 26.7 . We selected the mean value 59.1 as the cutoff point and classified the tumors into two groups as previously evaluated (11): tumors with a microvessel count > 59.1 were classified as hypervascular, and tumors with a count ≤ 59.1 were classified as hypovascular. Tumor samples were examined and classified by two pathologists who had no knowledge of the clinical status.

Reverse Transcriptase-PCR. Total RNA was isolated using the guanidine isothiocyanate method (13) and was transcribed to cDNA using a cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. PCR amplification of APN/CD13 cDNA was carried out using specific primers cited in the GenBank database that yielded a 1459-bp product (upstream primer: 5'-GCCCAAGATGTC-CACGTACT; downstream primer: 5'-GGTGCTGATGGCATTAACT). As a control, specific primers for β -actin (upstream primer: 5'-GAGAGATGACCCAGATCATGT; downstream primer: 5'-ACTCCATGCCAGGAAGGAAGG) were used (14). Initial denaturation at 94°C for 3 min was followed by 32 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 90 s, with a final extension step at 72°C for 3 min. The same PCR conditions, but only 22 cycles, were used to amplify the β -actin DNA. PCR products were analyzed by gel electrophoresis and visualized with ethidium bromide staining and UV transillumination. A human pancreatic carcinoma cell line (PANC-1) was used as the positive control for APN/CD13 expression. The densitometric value obtained for the APN/CD13 bands from a given tumor tissue sample was divided by that for β -actin to calculate the APN/CD13 expression ratio. When the expression ratio of a given specimen was > 0.5 , it was defined as showing positive gene expression, whereas a value was ≤ 0.5 was defined as negative gene expression.

Statistical Analysis. The overall cancer-specific survival time was calculated from the date of surgery to the date of death from pancreatic carcinoma. The significance of the difference between the incidence of APN/CD13 gene expression and several clinical and pathological parameters was assessed by the χ^2

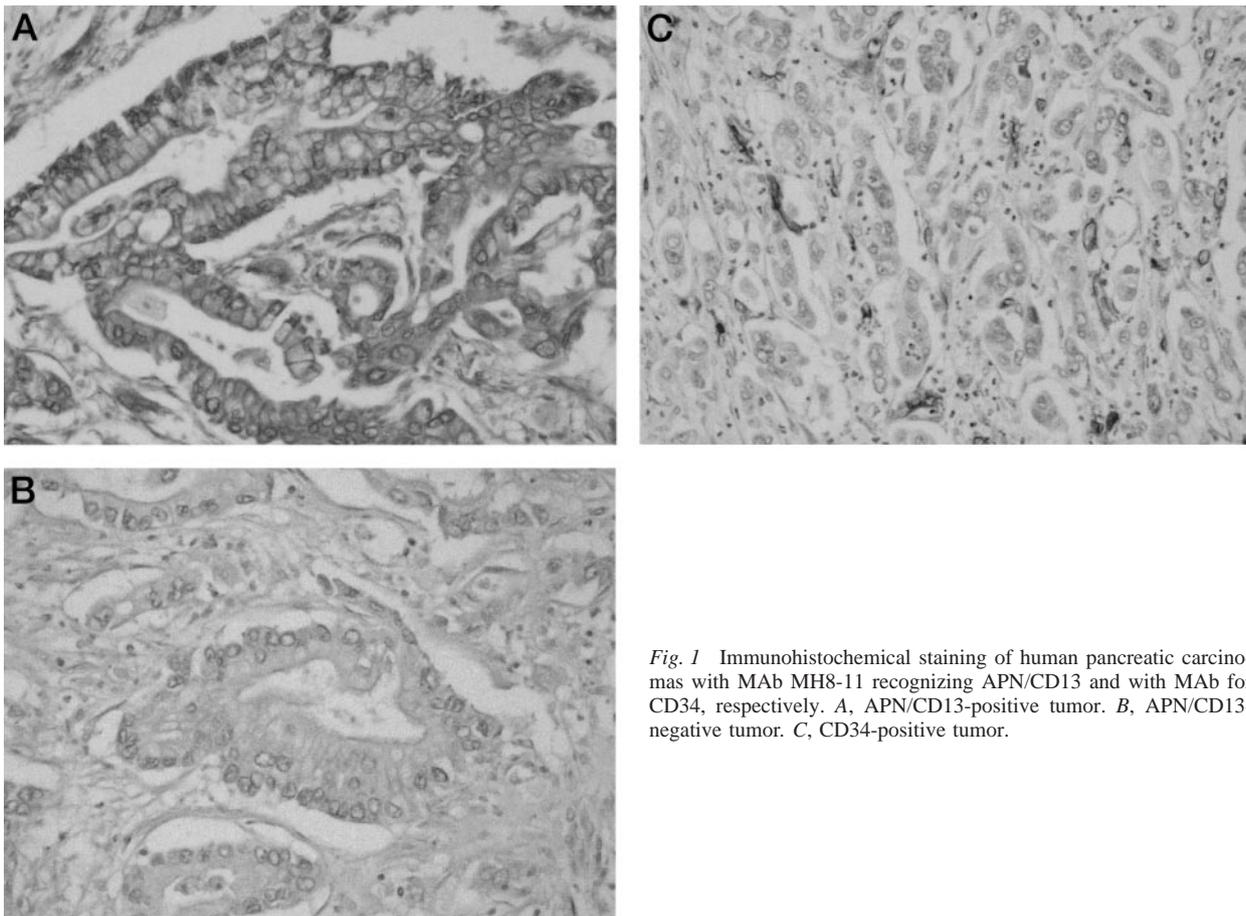


Fig. 1 Immunohistochemical staining of human pancreatic carcinomas with MAb MH8-11 recognizing APN/CD13 and with MAb for CD34, respectively. **A**, APN/CD13-positive tumor. **B**, APN/CD13-negative tumor. **C**, CD34-positive tumor.

test or by the Mann-Whitney t test. Spearman rank correlation was used to assess association between *APN/CD13* expression and the IMD. The Kaplan-Meier method was used to estimate the probability of survival, and significance was assessed with the log-rank test (15). Multivariate analysis was performed using the Cox regression model to study eight factors (*APN/CD13* status, histopathological grade, tumor status, nodal status, metastatic status, pathological stage, gender, and age at surgery; Ref. 16). We use the term of tumor status as T factor, nodal status as N factor, and metastatic status as M factor in TNM classification, respectively. In pancreatic cancer, T_1 - T_2 tumors are localized in the pancreas, whereas T_3 - T_4 tumors extend directly into surrounding organs or adjacent large vessels. All P s were based on two-tailed statistical analysis and $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Immunohistochemical Analysis of APN/CD13 Protein Expression. *APN/CD13* protein was positive by immunohistochemistry in 48.0% (24 of 50) of the tumors. *APN/CD13* protein was mainly found in the cell membrane and cytoplasm, but occasionally the nuclei of tumor cells and stromal cells (including fibroblasts and endothelial cells) were also stained (Fig. 1A). By contrast, there were 26 cases (52.0%) with negative *APN/CD13* protein expression (Fig. 1B).

Microvessel Staining. A single microvessel was defined as a row of brown immunostained endothelial cells that was separated from the adjacent microvessels, tumor cells, and other connective tissue elements (Fig. 1C). Twenty-six tumors were evaluated to be hypervascular, and 24 tumors were hypovascular.

Reverse Transcriptase-PCR Analysis of APN/CD13. Of the 50 tumors studied, 25 (50.0%) were positive for *APN/CD13* gene expression, and 25 were negative (Fig. 2). The *APN/CD13* gene expression ratio ranged from 0 to 2.53, with a median value of 0.50. *APN/CD13* gene expression evaluated by the reverse transcriptase-PCR agreed with the immunohistochemical findings in 90.0% of the patients (Table 2). When there was a discrepancy, the reverse transcriptase-PCR results were used for classification.

Correlation between APN/CD13 Expression and the IMD. There was a significant correlation between *APN/CD13* expression and the IMD. (Spearman $r = 0.71$, $P = 0.0003$; Fig. 3).

Correlation between APN/CD13 Expression and Various Prognostic Factors. The relationship between *APN/CD13* expression and various prognostic factors is shown in Table 1. There was no significant relationship between *APN/CD13* gene expression and the age at surgery, gender, tumor status, nodal status, metastatic status, pathological stage, or histopathological grade.

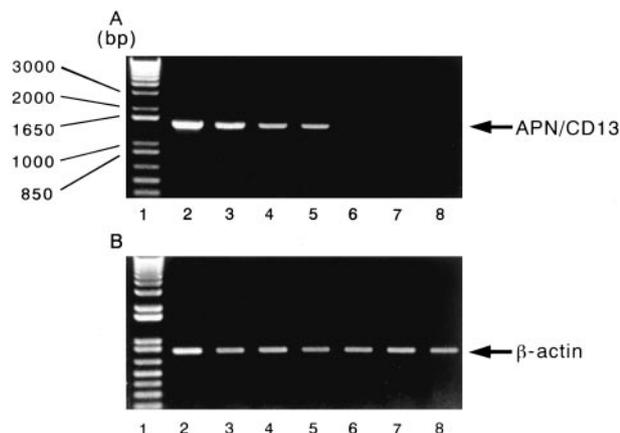


Fig. 2 Agarose gel electrophoresis of the reverse transcriptase-PCR-amplified 1459-bp fragment of *APN/CD13* (A) and the amplified internal control (β -actin; B). Lane 1, size marker; Lane 2, a human pancreatic cancer cell line (PANC-1) used as the positive control for *APN/CD13* expression; Lanes 3–5, pancreatic adenocarcinomas showing *APN/CD13* positivity; Lanes 6–8, tumors evaluated as negative for *APN/CD13*.

Table 2 Relationship between immunohistochemical and reverse transcriptase-PCR results of *APN/CD13*^a

Immunohistochemistry	Reverse transcriptase-PCR		Total
	<i>APN/CD13</i> -positive	<i>APN/CD13</i> -negative	
<i>APN/CD13</i> positive	22	2	24
<i>APN/CD13</i> negative	3	23	26
Total	25	25	50

^a $P < 0.0001$ (χ^2 test).

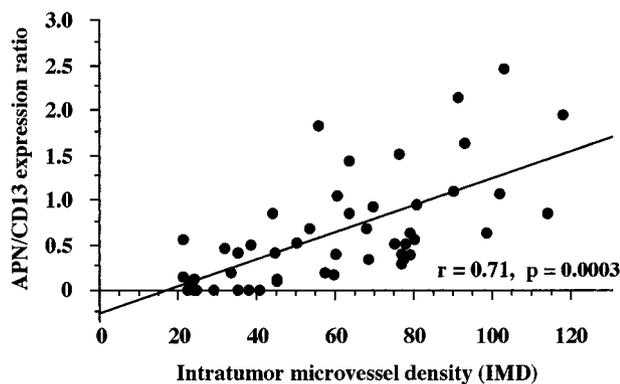


Fig. 3 Correlation between *APN/CD13* expression and the IMD. The *APN/CD13* expression ratio was significantly associated with the IMD.

Correlation between *APN/CD13* Expression and Overall Survival. The prognosis of the patients with pancreatic carcinoma was very poor, and the overall survival rate was generally 0%. Among the 50 patients, the median survival time of those with tumors showing positive *APN/CD13* expression was shorter than that of the patients with negative tumors (9.5

Table 3 Correlation between *APN/CD13* expression and overall survival of patients with pancreatic carcinoma

	Total (n)	Median survival time (month)		P
		<i>APN/CD13</i> Positive	<i>APN/CD13</i> Negative	
Age at surgery				
<60	13	4.9	24.6	0.013
≥ 60	37	9.7	11.0	NS ^a
Gender				
Male	36	8.3	13.2	0.002
Female	14	14.3	10.6	NS
Tumor status				
T ₁	2	NR	NR	ND
T ₂	10	8.3	NR	0.024
T ₃	21	12.7	13.0	NS
T ₄	17	6.7	10.6	NS
Nodal status				
N ₀	27	9.7	27.6	0.026
N ₁	23	8.5	8.7	NS
Metastatic status				
M ₀	42	11.2	24.6	0.015
M ₁	8	5.0	8.7	NS
Pathological stage				
Stage I	7	24.3	NR ^a	NS
Stage II	10	14.0	27.6	NS
Stage III	11	11.2	13.0	NS
Stage IVA	14	6.3	10.6	NS
Stage IVB	8	5.0	8.7	NS
Histopathological grading				
G1	15	11.2	27.6	NS
G2	29	6.7	13.0	0.026
G3	6	8.5	4.2	NS
Total number of patients	50	9.5	13.2	0.009

^a NS, not significant; ND, not done; NR, not reached.

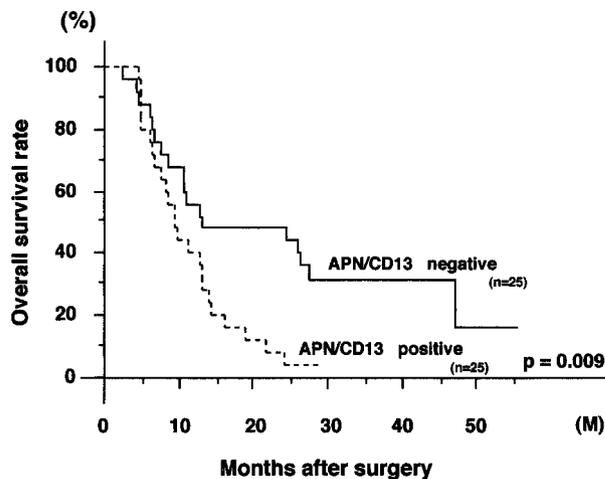


Fig. 4 Overall survival of 50 patients with pancreatic carcinoma in relation to their *APN/CD13* status.

versus 13.2 months, $P = 0.009$; Table 3 and Fig. 4). In addition, significant differences were noted between patients with tumors positive and negative for *APN/CD13* expression in the case of the following variables: younger patients (4.9 versus 24.6

Table 4 Multivariate cox analysis of overall survival of 50 patients with pancreatic carcinoma

Variable	Assigned score	β	SE	χ^2	Hazard ratio (95% CI)	<i>P</i>
APN/CD13 status		-0.913	0.379	5.805	0.401 (0.191–0.844)	0.016
Negative	0					
Positive	1					
Histopathological grading		0.272	0.365	0.554	1.312 (0.642–2.683)	0.457
G1	1					
G2	2					
G3	3					
Tumor status		0.269	0.350	0.588	1.308 (0.659–2.598)	0.443
T ₁	1					
T ₂	2					
T ₃	3					
T ₄	4					
Nodal status		-0.146	0.398	0.134	0.864 (0.396–1.887)	0.714
N ₀	0					
N ₁	1					
Metastatic status		-0.273	0.913	0.089	0.761 (0.127–4.555)	0.765
M ₀	0					
M ₁	1					
Pathological stage		0.478	0.306	2.434	1.612 (0.885–2.939)	0.119
I	1					
II	2					
III	3					
IVA	4					
IVB	5					
Gender		-0.097	0.398	0.059	0.908 (0.416–1.981)	0.808
Male	0					
Female	1					
Age		-0.078	0.399	0.038	0.925 (0.423–2.024)	0.846
<60	0					
≥60	1					

months, $P = 0.013$); male patients (8.3 versus 13.2 months, $P = 0.002$); T₂ tumor status (8.3 months versus not reached, $P = 0.024$); N₀ nodal status (9.7 versus 27.6 months, $P = 0.026$); M₀ metastatic status (11.2 versus 24.6 months, $P = 0.015$); and G₂ histopathological grade (6.7 versus 13.0 months, $P = 0.026$). Multivariate analysis of the 50 patients showed that APN/CD13 expression was a significant independent prognostic factors ($P = 0.016$; Table 4).

DISCUSSION

In Europe and North America, the incidence of pancreatic carcinoma has remained unchanged over the past 20 years, and it currently ranks as the fifth most common cause of cancer-related death in Western countries (17). Because of the high rate of recurrence, surgery alone seems to be inadequate for the treatment of pancreatic carcinoma. To improve the poor prognosis of this tumor, new clinical approaches are required. Recently, various antiangiogenesis therapies have been reported (18–20).

It is now widely accepted that angiogenesis plays an important role in tumor development because a tumor nodule is unable to grow larger than $\sim 1 \text{ mm}^3$ without creating a new blood supply (2). Vessels migrate into the tumor, and then the tumor grows toward the vessels, after which the endothelial basement membrane undergoes degradation, and new vascular loops are created (21). We have targeted endothelial cell migration and have established a novel murine MAAb MH8-11 recognizing APN/CD13 (3). In 1989, human myeloid plasma membrane glycoprotein CD13 was shown to be identical to APN (22). It is expressed by many tissues,

particularly the brush border membrane of the small intestine, the proximal renal tubules, synaptic membranes in the central nervous system, and of monocytes and granulocytes (4, 23). On the other hand, it was reported that APN/CD13 expression, although not seen on normal melanocytes, was observed on malignant melanoma cells (24). APN/CD13 has been shown to play an important role in the invasion of metastatic tumors *in vitro* (7), and an association of APN/CD13 with angiogenesis was reported recently (25). Treatment of animals with APN/CD13 inhibitors significant decrease of retinal neovascularization, chorioallantoic membrane angiogenesis, and xenograft tumor growth (25). Bhagwat *et al.* (26) reported that APN/CD13 mRNA and protein expression were transcriptionally up-regulated in endothelial cells, and cell lines under conditions that are characteristic of the tumor microenvironment such as hypoxia and increased angiogenic growth factor concentrations, as well as by signals regulating capillary formation and xenograft tumor growth. They also suggested that APN/CD13 might regulate endothelial morphogenesis during angiogenesis (26). In this study, we found a significant relationship between APN/CD13 expression and a poor prognosis in patients with pancreatic carcinoma ($P = 0.009$). In addition, we showed that APN/CD13 was an independent prognostic factor that was unrelated to various clinicopathological variables in 50 patients with pancreatic carcinoma ($P = 0.016$). Moreover, there was a significant correlation between APN/CD13 expression and the IMD ($r = 0.71$, $P = 0.0003$; Fig. 3).

Bestatin is a dipeptide that was discovered in the culture filtrate of *Streptomyces olivoreticuli* (27) and is known to be a competitive inhibitor of APN/CD13. Bestatin has already been

used clinically to treat acute myeloid leukemia in Japan. Anti-angiogenesis therapy is currently being investigated around the world, including the use of gene therapy, recombinant anti-angiogenic proteins, monoclonal antibodies, and various drugs (28, 29). Although some of these agents are undergoing clinical trials, no treatment is widely available at present. Our findings suggested that bestatin may be useful as an antiangiogenic agent for patients with pancreatic carcinoma.

In summary, we showed that APN/CD13 is a possible new prognostic marker for pancreatic carcinoma and that it may be one of the angiogenic factors in patients with this cancer. Accordingly, the inhibition of APN/CD13 may be an effective new strategy for pancreatic carcinoma therapy.

ACKNOWLEDGMENTS

We thank Drs. Tadashi Ohbayashi and Tomoko Okuno for the histological examination of the tumor samples, Sinji Hirai for his technical assistance, and Mitsuko Shirata for her assistance in preparation of the manuscript.

REFERENCES

- Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, *1*: 27–31, 1995.
- Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst. (Bethesda)*, *82*: 4–6, 1990.
- Hashida, H., Takabayashi, A., Kanai, M., Adachi, M., Kondo, K., Kohno, N., Yamaoka, Y., and Miyake, M. Aminopeptidase N is involved in cell motility and angiogenesis: its clinical significance in human colon cancer. *Gastroenterology*, *122*: 376–386, 2002.
- Dixon, J., Kaklamani, L., Turley, H., Hickson, I. D., Leek, R. D., Harris, A. L., and Gatter, K. C. Expression of aminopeptidase-N (CD13) in normal tissues and malignant neoplasms of epithelial and lymphoid origin. *J. Clin. Pathol. (Lond.)*, *47*: 43–47, 1994.
- Look, A. T., Peiper, S. C., Rebentisch, M. B., Ashmun, R. A., Roussel, M. F., Rettenmier, C. W., and Sherr, C. J. Transfer and expression of the gene encoding a human myeloid membrane antigen (gp150). *J. Clin. Investig.*, *75*: 569–579, 1985.
- Sakai, K., Hattori, T., Sagawa, K., Yokoyama, M., and Takatsuki, K. Biochemical and functional characterization of MCS-2 antigen (CD13) on myeloid leukemic cells and polymorphonuclear leukocytes. *Cancer Res.*, *47*: 5572–5576, 1987.
- Saiki, I., Fujii, H., Yoneda, J., Abe, F., Nakajima, M., Tsuruo, T., and Azuma, I. Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. *Int. J. Cancer*, *54*: 137–143, 1993.
- Ishii, K., Usui, S., Sugimura, Y., Yoshida, S., Hioki, T., Tatematsu, M., Yamamoto, H., and Hirano, K. Aminopeptidase N regulated by zinc in human prostate participates in tumor cell invasion. *Int. J. Cancer*, *92*: 49–54, 2001.
- Fujii, H., Nakajima, M., Saiki, I., Yoneda, J., Azuma, I., and Tsuruo, T. Human melanoma invasion and metastasis enhancement by high expression of aminopeptidase N/CD13. *Clin. Exp. Metastasis*, *13*: 337–344, 1995.
- Sobin, L. H., and Wittekind, C. H. (eds). *TNM classification of malignant tumors*, Ed. 5, pp. 87–90. New York: John Wiley & Sons, Inc, 1997.
- Ikeda, N., Adachi, M., Taki, T., Huang, C., Hashida, H., Takabayashi, A., Sho, M., Nakajima, Y., Kanehiro, H., Hisanaga, M., Nakano, H., and Miyake, M. Prognostic significance of angiogenesis in human pancreatic cancer. *Br. J. Cancer*, *79*: 1553–1563, 1999.
- Ikeda, N., Nakajima, Y., Sho, M., Adachi, M., Huang, C., Iki, K., Kanehiro, H., Hisanaga, M., Nakano, H., and Miyake, M. The association of K-ras gene mutation and vascular endothelial growth factor gene expression in pancreatic carcinoma. *Cancer (Phila.)*, *92*: 488–499, 2001.
- Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, *162*: 156–159, 1987.
- Nakajima-Iijima, S., Hamada, H., Reddy, P., and Kakunaga, T. Molecular structure of the human cytoplasmic β -actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA*, *82*: 6133–6137, 1985.
- Mantel, N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother. Rep.*, *50*: 163–170, 1966.
- Cox, D. R. Regression models and life tables. *J. R. Stat. Soc. B*, *34*: 187–220, 1972.
- Rosewicz, S., and Wiedenmann, B. Pancreatic carcinoma. *Lancet*, *349*: 485–489, 1997.
- O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, *88*: 277–285, 1997.
- Griscelli, F., Li, H., Bennaceur-Griscelli, A., Soria, J., Opolon, P., Soria, C., Perricaudet, M., Yeh, P., and Lu, H. Angiostatin gene transfer: inhibition of tumor growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. *Proc. Natl. Acad. Sci. USA*, *95*: 6367–6372, 1998.
- Fathallah-Shaykh, H. M., Zhao, L. J., Kafrouni, A. I., Smith, G. M., and Forman, J. Gene transfer of IFN- γ into established brain tumors represses growth by antiangiogenesis. *J. Immunol.*, *164*: 217–222, 2000.
- Harris, A. L. Antiangiogenesis for cancer therapy. *Lancet*, *349*: 13–15, 1997.
- Look, A. T., Ashmun, R. A., Shapiro, L. H., and Peiper, S. C. Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N. *J. Clin. Investig.*, *83*: 1299–1307, 1989.
- Ashmun, R. A., Holmes, K. V., Shapiro, L. H., Favaloro, E. J., Razak, K., de Crom, R. P. G., Howard, C. J., and Look, A. T. CD13 (aminopeptidase N) cluster workshop report, leukocyte typing V. White cell differentiation antigens, pp. 771–775. Oxford: Oxford University Press, 1995.
- Menrad, A., Speicher, D., Wacker, J., and Herlyn, M. Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. *Cancer Res.*, *53*: 1450–1455, 1993.
- Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R. A., Shapiro, L. H., Arap, W., and Ruoslahti, E. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res.*, *60*: 722–727, 2000.
- Bhagwat, S. V., Lahdenranta, J., Giordano, R., Arap, W., Pasqualini, R., and Shapiro, L. H. CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. *Blood*, *97*: 652–659, 2001.
- Umezawa, H., Aoyagi, T., Suda, H., Hamada, M., and Takeuchi, T. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiot. (Tokyo)*, *29*: 97–99, 1976.
- Ferrara, N., and Alitalo, K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat. Med.*, *5*: 1359–1364, 1999.
- Lau, K., and Bicknell, R. Antiangiogenic gene therapy. *Gene Ther.*, *6*: 1793–1795, 1999.