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

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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 MAbs (MH8-11) that inhibited endothelial cell migration and tube formation (3). This epitope was a Mr 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 patients were alive at least 1 year postoperatively. The clinical data are summarized in Table 1.

Tumor Specimens. The patients were classified into two groups: solitary and resectable (group 1) and solitary and unresectable (group 2). The tumor microvessel density (IMD) was measured immunohistochemically. The mean IMD was 68.0 (range, 5.0–156.0) for group 1 and 245.0 (range, 32.0–1020.0) for group 2. The difference was statistically significant ($P = 0.0005$). The group with high IMD had a significantly shorter survival time than that with low IMD ($P = 0.0004$).

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

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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 \(-80^\circ\)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 (×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 ×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 ≥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 ≥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 ×200 magnification (4 fields of 0.785 mm² 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-CAGTACT; downstream primer: 5'-GGTGCTGATGCGCATTAACCT). As a control, specific primers for b-actin (upstream primer: 5'-GAGAGATCCCCAGTATGTT; downstream primer: 5'-ACTCCATGCCCCAGGGAAGAAG) 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 b-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 b-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 \( \chi^2 \) test.
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, T1–T2 tumors are localized in the pancreas, whereas T3–T4 tumors extend directly into surrounding organs or adjacent large vessels. All Ps 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.
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 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 months, \( P = 0.013 \); Table 2 and Fig. 3).

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

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<th>APN/CD13-positive</th>
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<td>2</td>
<td>24</td>
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<tr>
<td>APN/CD13 negative</td>
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<td>23</td>
<td>26</td>
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<tr>
<td>Total</td>
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\( ^{a} P < 0.0001 \) (\( \chi^2 \) test).

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

<table>
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<th>Median survival time (month)</th>
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<tr>
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<td>13</td>
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<td>≥60</td>
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<tr>
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<td>36</td>
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<tr>
<td>G3</td>
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<td>8.5</td>
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<tr>
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<td>9.5</td>
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\(^{a}\) NS, not significant; ND, not done; NR, not reached.
months, \( P = 0.013 \)); male patients (8.3 versus 13.2 months, \( P = 0.002 \)); \( T_2 \) tumor status (8.3 months versus not reached, \( P = 0.024 \)); \( N_0 \) nodal status (9.7 versus 27.6 months, \( P = 0.026 \)); \( M_0 \) metastatic status (11.2 versus 24.6 months, \( P = 0.015 \)); and \( G_2 \) histopathological grade (6.7 versus 13.0 months, \( P = 0.026 \)). Multivariate analysis of the 50 patients showed that \( \text{APN/CD13} \) expression was a significant independent prognostic factor (\( P = 0.016 \); 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 \( \sim 1 \) 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 MAb MH8-11 recognizing \( \text{APN/CD13} \) (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 \( \text{APN/CD13} \) expression, although not seen on normal melanocytes, was observed on malignant melanoma cells (24). \( \text{APN/CD13} \) has been shown to play an important role in the invasion of metastatic tumors in vitro (7), and an association of \( \text{APN/CD13} \) with angiogenesis was reported recently (25). Treatment of animals with \( \text{APN/CD13} \) inhibitors significant decrease of retinal neovascularization, choroidal membrane angiogenesis, and xenograft tumor growth (25). Bhagwat et al. (26) reported that \( \text{APN/CD13} \) 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 \( \text{APN/CD13} \) might regulate endothelial morphogenesis during angiogenesis (26). In this study, we found a significant relationship between \( \text{APN/CD13} \) expression and a poor prognosis in patients with pancreatic carcinoma (\( P = 0.009 \)). In addition, we showed that \( \text{APN/CD13} \) 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 \( \text{APN/CD13} \) 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 \( \text{APN/CD13} \). Bestatin has already been

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<th>Variable</th>
<th>Assigned score</th>
<th>( \beta )</th>
<th>SE</th>
<th>( \chi^2 )</th>
<th>Hazard ratio (95% CI)</th>
<th>( P )</th>
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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. Ac-
cordingly, the inhibition of APN/CD13 may be an effective new
strategy for pancreatic carcinoma therapy.

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