Neuroendocrine cell differentiation of poorly differentiated colorectal adenocarcinoma correlates with liver metastasis

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Abstract. Poorly differentiated (PD) adenocarcinoma often retains the capacity for neuroendocrine (NE) cell differentiation; however, it is difficult to distinguish the NE cell differentiation by routine hematoxylin and eosin staining. It is important to detect the presence of NE cell differentiation in advanced colorectal carcinomas because these carcinomas have been shown to produce distant metastasis at the time of diagnosis and to have a particularly poor prognosis. In this study, the characteristics of PD adenocarcinoma with NE cell differentiation and its biological metastatic mechanisms were investigated. Forty-eight of 2204 colorectal cancer patients, diagnosed as having PD adenocarcinoma (2.2%) were enrolled in this study. Immunohistochemical analysis was performed with anti-chromogranin A anti-synaptophysin, anti-CD34, anti-D2-40, and anti-VEGF antibodies. The clinicopathological factors for PD adenocarcinoma with NE cell differentiation were compared with those for PD adenocarcinoma without NE cell differentiation. Microvessel density (MVD) was assessed using immunostained slides with anti-CD34 antibody and vascular endothelial growth factor (VEGF) expression in PD adenocarcinoma with NE cell differentiation was confirmed by *in situ* hybridization. By immunohistochemical staining for chromogranin A and synaptophysin, NE cell differentiation was detected in eight of 48 patients (16.7%) with PD adenocarcinoma. The frequency of liver metastasis at the time of diagnosis was significantly higher in patients having PD adenocarcinoma with NE cell differentiation (p=0.03). Moreover, MVD and VEGF expression level tended to be higher in patients having PD adenocarcinoma with NE cell differentiation (p=0.13 and 0.068, respectively). NE cell differentiation in PD adenocarcinoma may produce liver metastasis through microvessel formation in the tumor induced by VEGF. In PD colorectal adenocarcinoma, immunohistochemical analysis of NE markers is important for establishing the presence of NE cell differentiation and further study is necessary to evaluate the effectiveness of anti-angiogenic drugs to PD adenocarcinoma with NE cell differentiation.

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Introduction

Clinically, poorly differentiated (PD) colorectal adenocarcinoma can rapidly metastasize to distant organs and has a poor prognosis compared with well- or moderately differentiated adenocarcinoma. PD colorectal adenocarcinoma is comparatively rare. Particularly in Japan, the frequency of PD adenocarcinoma is less than 5.0% (1), whereas a frequency of 10 to 25% has been reported in western countries (2-4).

Some PD colorectal adenocarcinomas show neuroendocrine (NE) cell differentiation in some parts of the tumor; however, it is not easy to diagnose the NE cell differentiation by routine hematoxylin and eosin (H&E) staining. Histopathologically, adenocarcinomas with NE cell differentiation, carcinoid tumors and NE carcinomas contain NE granules in their cytoplasm. Carcinoid tumors are characterized by being composed of small cells containing regular well-rounded nuclei and tumors with increased atypical nucleus, high number of mitotic figures, or areas of necrosis have in the past been broadly termed 'atypical' or 'anaplastic' carcinoids. These tumors have more recently been classified as either well-differentiated or poorly differentiated NE carcinomas (5). NE carcinomas in the colon and rectum are a heterogeneous group of tumors that display aggressive clinical behavior (6). Compared with common adenocarcinomas, NE carcinomas of the colon and rectum have a significantly poor prognosis (7-9).

A previous study demonstrated that NE cell differentiation can be used as an independent prognostic factor in colorectal cancer of stages III and IV, and NE cell differentiation was found more frequently among PD tumors than in well- or moderately differentiated tumors (10). Tumor cells with NE cell differentiation exhibit characteristic neurosecretory granules in the cytoplasm as shown by electron microscopy. By immunohistochemistry, NE cells are shown to be stained by NE markers, such as synaptophysin, chromogranin, or neuron-specific enolase (NSE). The definitive pathological diagnosis of these tumors is important because treatment options by alternative chemotherapy have been administered with promising responses, although transiently, in particular to cisplatin-based chemotherapy (11).

Tumor angiogenesis plays a critical role in cancer progression and metastasis. One of the major factors that have been demonstrated to be involved in cancer angiogenesis is vascular endothelial growth factor (VEGF). VEGF mRNA expression is induced by exposure to low oxygen tension, and several major growth factors (TGF-α, TGF-ß, keratinocyte growth factor/FGF-7, insulin-like growth factor-1, fibroblast growth factor 4/FGF-4 and platelet-derived growth factor) and inflammatory cytokines (IL-1α and IL-6) are expressed under various pathophysiological conditions (12-14). Some of these factors are highly expressed in colorectal cancer tissues (15,16). Previous studies of human colon cancer have shown that VEGF is associated with microvessel density (MVD) and metastasis (17,18), and is considered as a prognostic factor (19-21).

The underlying mechanism of the aggressiveness of NE carcinomas has not yet been clarified. The aim of this study is to detect NE cell differentiation in PD adenocarcinoma by immunohistochemistry and to clarify its clinicopathological significance. We report that PD adenocarcinoma with NE cell differentiation correlates with the frequency of liver metastasis and tends to correlate with MVD and VEGF expression.

Materials and methods

Patients. From January 1990 to December 2003, 2204 colorectal cancer patients underwent surgery at Nippon Medical School Hospital (Bunkyo-ku, Tokyo, Japan) or Nippon Medical School Chiba-Hokusou Hospital (Inba, Chiba, Japan). Forty-eight patients (2.2%) diagnosed as having PD adenocarcinoma were enrolled in this study. Patients having carcinoid tumor, atypical carcinoid, adenocarcinoma composite carcinoid features (so-called adenocarcinoid tumors), and NE carcinoma were excluded. The obtained tissue samples were fixed in 20% formalin for 18-20 h and then embedded in paraffin. This study was carried out in accordance with the principles of the Declaration of Helsinki 1975.

Immunohistochemistry. Paraffin-embedded tissue sections (3.5 μm) were immunostained using a Histofine Simple Stain PO (R) or (M) Max kit (Nichirei, Tokyo, Japan). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. The tissue sections were then incubated with an appropriate antibody [1:1000 dilution for the rabbit polyclonal anti-chromogranin A antibody (DakoPatts, Glostrup, Denmark); 1:500 dilution for the rabbit polyclonal anti-synaptophysin antibody (DakoPatts), 1:150 dilution for the mouse monoclonal anti-CD34 antibody (Nichirei, Tokyo, Japan), 1:200 dilution for the mouse monoclonal anti-D2-40 antibody (Dako Cytomation, Glostrup), 1:200 dilution for the rabbit polyclonal anti-VEGF antibody (A-20, Santa Cruz Biotech., CA, USA)] in PBS containing 1% bovine serum albumin (BSA) for 16 h at 4˚C. Bound antibodies were detected with Simple Stain PO (R) or (M) Max reagents using diaminobenzidine-tetrahydrochloride (DAB) as the substrate, and the sections were counterstained with Mayer's hematoxylin. Negative controls were prepared by following the same procedure without the primary antibody staining.

PD adenocarcinoma with NE cell differentiation. PD adenocarcinoma with NE cell differentiation was determined by immunohistochemical staining with NE markers (chromogranin A and synaptophysin). When the immunoreactivity of either of the markers was >2% in the tumor cells, a diagnosis of PD adenocarcinoma with NE cell differentiation was given according to a previous report (10,22). The NE cells of the non-neoplastic mucosa served as positive control for the immunoreaction.

Microvessel density. MVD was determined according to the international consensus report (23). Immunostained sections with anti-CD34 antibody were scanned at x100 to identify the areas with the highest number of microvessels. Each image was captured using the Olympus DP12 digital camera system (Olympus Optical, Tokyo, Japan) attached to an Olympus AX80 microscope at x200. Each x200 magnification area measured 0.64 mm^2. Microvessel counting was performed on five separate areas. All stained endothelial cells or cell clusters were counted as one microvessel. If two or more positive foci seemed to belong to a single continuous vessel, they were counted as one microvessel. Furthermore, we observed that CD34-positive endothelial cells were not positive for D2-40, which has been demonstrated to react with lymphatic endothelial cells but not with vascular endothelial cells (24,25). The presence of microvessel lumen was not required for the counting. The mean of the counts was used for analysis.

Estimation of VEGF expression by immunostaining. VEGF was considered to be present when unequivocal cytoplasmic staining was observed in the tumor cells, regardless of the number of cells stained. VEGF expression was analyzed in the invasive front of the tumor away from the tumor center where necrosis and hypoxia may induce VEGF expression. The intensity of staining for VEGF was graded as follows: -, no detectable staining; +, moderate staining; and 2+, strong staining in one field at x400 according to a previous report (26). Vascular smooth muscle cells in the tissue samples were used as positive internal control for the VEGF staining. Two investigators (S.S. and T.I.) separately assessed the degree of staining without knowledge of the clinical data.

In situ hybridization. *In situ* hybridization was performed as previously described (27,28). Tissue sections were deparaffinized and incubated at RT for 20 min with 0.2 N HCl and then

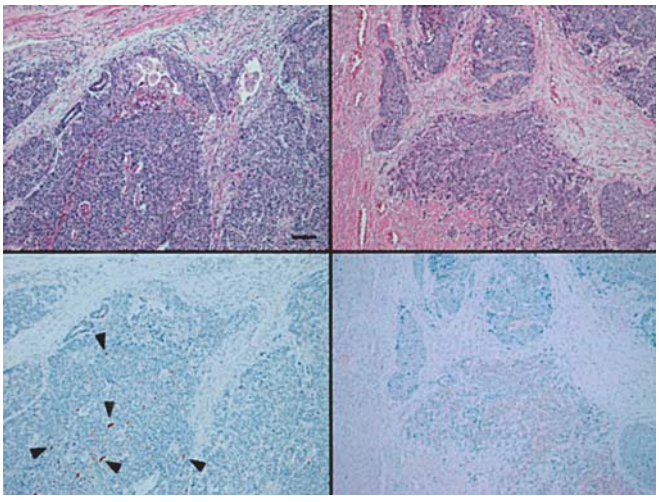

Figure 1. Histological characteristics and immunohistochemical staining properties of chromogranin A and synaptophysin in PD adenocarcinoma with NE cell differentiation. PD adenocarcinoma with NE cell differentiation showing common PD morphology without NE cellular features such as rosette or vascular rosette formations (top). Immunohistochemical staining of chromogranin A (bottom left, arrowheads) and synaptophysin (bottom right) showing cytoplasmic localization in PD adenocarcinoma cells with NE cell differentiation. Top, H&E staining; bottom, immunohistochemical staining. Bar =100 μm.

at 37˚C for 15 min with 100 μg/ml proteinase K. The sections were then postfixed for 5 min in PBS containing 4% paraformaldehyde (PFA), and incubated twice for 15 min each with PBS containing 2 mg/ml glycine at RT and once in 50% (V/V) formamide/2X SSC for 1 h at 42˚C prior to the initiation of the hybridization reaction. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 mg/ml yeast tRNA, lX Denhard's solution, 10% dextran sulfate, 40% formamide and the indicated digoxigenin-labeled riboprobe at 5 ng/ml. Hybridization was performed in a moist chamber for 16 h at 42˚C. The sections were then washed sequentially with 2X SSC for 20 min at 42˚C, and 0.2X SSC for 20 min at 42˚C. For immunological detection, the DIG nucleic acid detection kit was used. The sections were washed briefly with buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) and incubated with 1% (W/V) blocking reagent in buffer 1 solution for 60 min at RT, and thereafter with alkaline-phosphatase-conjugated polyclonal sheep anti-digoxigenin Fab fragment containing 0.2% Tween-20 at a l:2000 dilution for 60 min at RT. The sections were then washed twice for 15 min at RT with buffer 1 solution containing 0.2% Tween-20, equilibrated with buffer 3 solution (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min, and incubated with a staining solution containing nitroblue tetrazolium and X-phosphate in a dark box for 1 h. After the reaction was stopped with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), the sections were mounted with an aqueous mounting medium. The sections were observed using a differential interference contrast (DIC) system attached to a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

Statistical analyses. The chi-square test was used to analyze the correlation of clinicopathological factors between PD adenocarcinomas with NE cell differentiation and PD adenocarcinomas without NE cell differentiation, and the correlation of clinicopathological factors between VEGF-positive PD adenocarcinomas and VEGF-negative PD adenocarcinomas. The unpaired t-test was used to analyze the correlation of age between each group. The Mann Whitney U-test was used to compare MVD between PD adenocarcinoma with or without NE cell differentiation. The overall survival curves were computed according to the Kaplan-Meier method; differences in overall survival were computed using the log-rank test. A p value of <0.05 was considered significant. Statistical analyses were performed using the StatView J version 4.5 (SAS Institute, Inc., Cary, NC, USA) software package.

Table I. Summary of immunohistochemical staining of NE markers in PD adenocarcinoma.

	Positive/total	PD adenocarcinoma with NE cell differentiation
Chromogranin A	5/48 (10.4%)	8/48 (16.7%)
Synaptophysin	5/48 (10.4%)	

PD, poorly differentiated; NE, neuroendocrine.

Results

NE markers in PD adenocarcinoma with NE cell differentiation. PD adenocarcinoma with NE cell differentiation did not show any characteristic NE morphology such as rosette or vascular rosette formations (Fig. 1, top). However, five of the 48 patients (10.4%) were positive for chromogranin A (Fig. 1, bottom left, arrowheads) and five of the 48 patients were positive for synaptophysin (Fig. 1, bottom right) by immunohistochemical staining. Two patients were positive both for chromogranin A and synaptophysin, thus eight of the 48 patients (16.7%) were diagnosed as having PD adenocarcinoma with NE cell differentiation (Table I). The clinicopathological findings of PD adenocarcinoma with NE cell differentiation are shown in Table II. The patients' ages ranged from 50 to 77 years (mean, 60.4); five patients were male and three patients were female. PD adenocarcinomas with NE cell differentiation were observed in the right colon (six patients) and in the rectum (two patients), but not in the left colon. Six of eight patients (75.0%) were diagnosed as having the advanced stage of PD adenocarcinoma with metastases.

Correlations between PD adenocarcinoma with NE cell differentiation and clinicopathological factors. No statistically significant correlations except with liver metastasis were observed between PD adenocarcinoma with NE cell differentiation and clinicopathologic factors (e.g. sex, age, location of tumor, depth of tumor, stage of tumor, lymphatic invasion, vascular invasion, lymph node metastasis, lung metastasis and peritoneal dissemination). The incidence rate of liver metastasis in the patients having PD adenocarcinoma with NE cell differentiation was 62.5% (five of eight patients), which was significantly higher than that in the patients having

Table II. Pathological findings of immunohistochemical staining of PD adenocarcinoma with NE cell differentiation.

Case no.	Age (years)	Gender	Site	Depth of invasion	Lymphatic invasion	Venous invasion	Lymph node metastasis	Liver metastasis	Lung metastasis	Peritoneal dissemination	Chromogranin A	Synaptophysin	VEGF
1	58	F	R	a2	2	2	2	+	+	-	+	-	2+
2	70	F	C	se	3	2	2	+	-	-	+	-	-
3	77	M	C	se	2	1	2	-	-	-	+	+	+
4	50	M	R	a2	3	3	3	+	-	-	-	+	+
5	64	F	A	si	3	3	2	-	-	-	+	-	-
6	57	M	C	ss	0	3	0	-	-	+	-	+	-
7	53	M	T	ss	3	3	1	+	-	-	+	+	-
8	54	M	A	si	2	3	2	+	-	+	-	+	2+

PD: poorly differentiated; NE: neuroendocrine.

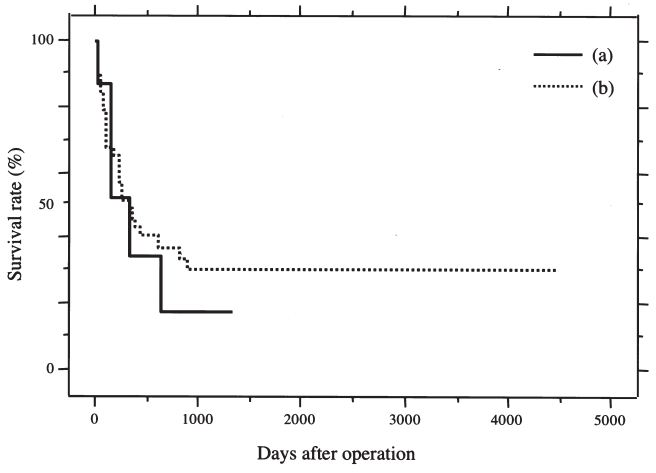

Figure 2. Kaplan-Meier overall survival curves of patients with PD colorectal adenocarcinoma with and without NE cell differentiation. The overall survival curves are for all patients who underwent surgery (n=48): eight patients with NE cell differentiation (a) and 40 patients without NE cell differentiation (b). p=0.6911 (log-rank test).

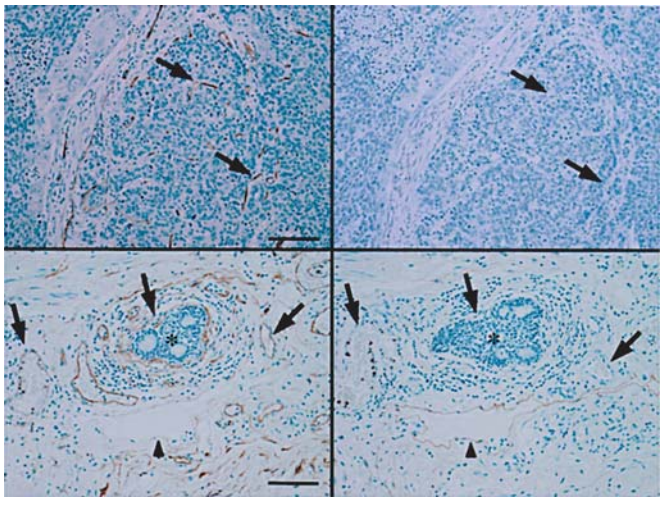

Figure 3. Immunohistochemical analyses for CD34 and D2-40 in PD adenocarcinoma with NE cell differentiation. Tumor vascular endothelial cells were positively stained for CD34 (top left and bottom left, arrows), but were not positively stained for D2-40 (top right and bottom right, arrows). In contrast, lymphatic endothelial cells were not positively stained for CD34 (bottom left, arrowhead), but were positively stained for D2-40 (bottom right, arrowhead). Immunohistochemistry, *adenocarcinoma cell nest. Bar =100 μm.

PD adenocarcinoma without NE cell differentiation [20.0% (eight of 40 patients), p=0.03] (Table III). The overall survival rate of the patients having PD adenocarcinoma with NE cell differentiation was lower than that of the patients having PD adenocarcinoma without NE cell differentiation; however, it was not statistically significant (Fig. 2). In the patients having PD adenocarcinoma with NE cell differentiation, the one-year survival rate was 35.0% and the three-year survival rate was 17.5%, whereas the one-year survival rate was 46.3% and the three-year survival rate was 30.3% in the patients having PD adenocarcinoma without NE cell differentiation.

MVD of PD adenocarcinoma with and without NE cell differentiation. To differentiate vascular endothelial cells and lymphatic endothelial cells in the tumors, immunohistochemistry using CD34 and D2-40 antibodies was performed. Vascular endothelial cells in colorectal cancer cell nests were positively stained for CD34 (Fig. 3, top left and bottom left, arrows), but not for D2-40 (Fig. 3, top right and bottom right, arrows). In contrast, lymphatic endothelial cells were not positively stained for CD34 (Fig. 3, bottom left, arrowhead), but were positively stained for D2-40 (Fig. 3, bottom right, arrowhead). MVD determined with CD34 antibody ranged from 18.0 to 84.6, with a mean value ± standard deviation of 43.1±16.2. MVD tended to be higher in PD adenocarcinoma with NE cell differentiation than in PD adenocarcinoma without NE cell differentiation (51.0±7.1 and 41.5±2.4, p=0.13, respectively) (Fig. 4).

Relationship between NE cell differentiation and VEGF expression. In our study, PD adenocarcinomas with NE cell differentiation have a higher ratio of liver metastasis and higher MVD than PD adenocarcinomas without NE cell differentiation. Therefore, we compared the VEGF expression rate between PD adenocarcinoma with NE cell differentiation and PD adenocarcinoma without NE cell differentiation. VEGF immunoreactivity was localized mostly in the cytoplasm of cancer cells, and strongly localized in the cancer cells at the

Table III. Correlation between clinicopathological findings and NE cell differentiation of PD adenocarcinoma.

Variables	Poorly differentiated adenocarcinoma		
	NE group (n=8)		Non-NE group (n=40)
Sex		p=0.24	
Male	5		14
Female	3		26
Age			
mean ± 1SD	60.4±9.3	p=0.29	65.5±12.6
range	50-77		32-86
Location		p=0.36	
C/A/T	3/2/1		6/8/7
D/S	0/0		1/6
R	2		12
Depth of tumor invasion		p>0.99	
m/sm	0/0		0/1
mp/ss (al)/se (a2)/si (ai)	0/2/4/2		2/12/17/8
p-stage		p=0.66	
0/I/II	0/0/1		0/3/7
IIIa/IIIb/IV	0/2/5		10/7/13
Lymphatic invasion		p>0.99	
Positive	7		36
Negative	1		4
Vascular invasion		p=0.17	
Positive	8		29
Negative	0		11
Lymph node metastasis		p=0.42	
Positive	7		28
Negative	1		12
Liver metastasis		p=0.03[a]	
Positive	5		8
Negative	3		32
Lung metastasis		p=0.31	
Positive	1		1
Negative	7		39
Peritoneal dissemination		p=0.67	
Positive	2		8
Negative	6		32

NE, neuroendocrine; PD, poorly differentiated; NE group, group of PD adenocarcinoma patients with NE cell differentiation; non-NE group, group of PD adenocarcinoma patients without NE cell differentiation; [a]statistically significant (P<0.05).

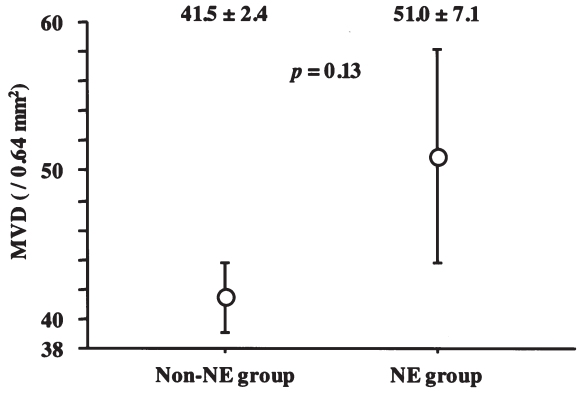

Figure 4. MVD of PD adenocarcinoma with and without NE cell differentiation. MVD determined by immunohistochemical staining with CD34 antibody tended to be higher in PD adenocarcinoma with NE cell differentiation than in PD adenocarcinoma without NE cell differentiation (51.0±7.1 and 41.5±2.4, mean ± SD, respectively, p=0.13).

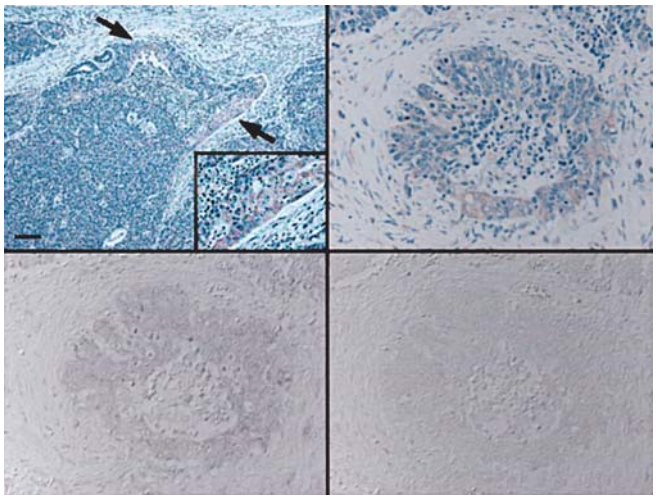

Figure 5. Immunohistochemical staining and *in situ* hybridization analysis for VEGF in PD adenocarcinoma with NE cell differentiation. VEGF immunoreactivity was observed in the cytoplasm of most cancer cells and it was strongly detected at the invasive front of the tumor (top left, arrows and inset). Serial tissue sections from the immunohistochemistry and *in situ* hybridization analyses showed VEGF and its mRNA expression in the cancer cells (top right and bottom left, respectively). VEGF mRNA was strongly expressed in the cytoplasm of PD adenocarcinoma cells (bottom left). The sense probe analysis did not yield any positive signals in the PD adenocarcinoma cells (bottom right). Top, immunohistochemistry; bottom left, *in situ* hybridization, anti-sense; bottom right, *in situ* hybridization, sense. Bar =100 μm.

invasive front of the tumor (Fig. 5, top left, arrows and inset). Serial sections from the immunohistochemistry and *in situ* hybridization analyses showed VEGF and its mRNA expression in the cancer cells (Fig. 5, top right and bottom left, respectively). Sense probe analysis did not show any positive signals (Fig. 5, bottom right). VEGF expression tended to be higher in PD adenocarcinoma with NE cell differentiation than in PD adenocarcinoma without NE cell differentiation [50.0% (four of eight patients) and 17.5% (seven of 40 patients), respectively, p=0.068] (Table IV).

Correlations between VEGF expression and clinicopathological factors. Positivity for VEGF was observed in 11 of the 48 patients (22.9%). No statistically significant correlations except for tumor site, vascular invasion and lung metastasis were identified between VEGF expression and clinicopathological factors (sex, age, depth of tumor, stage of tumor, lymphatic invasion, lymph node metastasis, liver metastasis and peritoneal dissemination). Vascular invasion in the patients with VEGF-positive tumors was 100% (11 of 11 patients), which was significantly higher than that in patients with VEGF-negative tumors [70.3% (26 of 37 patients), p=0.048]. The incidence rate of lung metastasis in patients with VEGF-

Table IV. Relationship between NE cell differentiation and VEGF expression.

	VEGF expression		
	VEGF positive (n=11)		VEGF negative (n=37)
PD adenocarcinoma			
with NE cell differentiation	4 (50.0%)	p=0.068	4 (50.0%)
without NE cell differentiation	7 (17.5%)		33 (82.5%)

PD, poorly differentiated; NE, neuroendocrine.

positive tumors was 18.2% (two of 11 patients), which was significantly higher than that in patients with VEGF-negative tumors [0% (none of 37 patients), p=0.049] and the incidence rate of liver metastasis tended to be higher in patients with VEGF-positive tumors (p=0.14) (Table V).

Discussion

Cancer cells with NE cell differentiation have been observed in gastrointestinal carcinomas (29,30). NE cell differentiation in colorectal carcinomas is often difficult to diagnose by routine H&E staining. The immunohistochemical studies of undifferentiated and PD colorectal adenocarcinomas have also shown NE cell differentiation (7,31,32). These studies were conducted employing a number of monoclonal antibodies that recognize antigens indicative of NE and exocrine features.

In this study, we examined PD adenocarcinoma with NE cell differentiation by immunohistochemistry using two NE markers. However, the classification between NE carcinoma and adenocarcinoma with NE cell differentiation remains unclear and their definitions have not yet been determined. In this study, we excluded typical NE carcinomas and defined PD adenocarcinoma with NE cell differentiation according to a previous report as having >2% of cancer cell population expressing chromogranin A or synaptophysin or both (10,22), because a normal colorectal epithelium contains up to 2% NE cells (33).

PD adenocarcinoma is a rare histological type, so extensive analysis of a sufficient number of samples for statistical evaluation has been difficult. In this study, we collected clinical information on 48 patients having PD adenocarcinoma from 2204 colorectal cancer patients. Similar to previous reports (0.1-3.9%) (7-9,31,34-36), the frequency of PD adenocarcinoma with NE cell differentiation was 0.36% and all of the PD adenocarcinomas with NE cell differentiation were localized only in the right colon and rectum. The overall survival rate was not significantly different between patients having PD adenocarcinoma with NE cell differentiation and patients having common PD adenocarcinoma; however, the frequency of liver metastasis at the time of diagnosis was significantly higher in patients having PD adenocarcinoma with NE cell differentiation. Grabowski *et al* also showed a high frequency of liver metastasis in patients having undifferentiated carcinoma with NE cell differentiation (22). These findings provide an interesting hypothesis that NE cell differentiation of tumors may be related to their increased liver metastatic activity and a more aggressive course of the disease.

Table V. Correlation between clinicopathological findings and VEGF expression in PD adenocarcinoma patients.

Variables	VEGF expression		
	Positive group (n=11)		Negative group (n=37)
Sex		p=0.30	
Male	6		13
Female	5		24
Age		p=0.68	
mean ± 1SD	63.3±13.7		65.0±11.9
range	41-83		32-86
Location		p=0.02[a]	
C/A/T	2/1/0		7/9/8
D/S	0/1		1/5
R	7		7
Depth of tumor invasion		p>0.99	
m/sm	0/0		0/1
mp/ss (al)/se (a2)/si (ai)	0/2/8/1		2/12/13/9
p-stage		p=0.42	
O/I/II	0/0/1		0/3/7
IIIa/IIIb/IV	2/2/6		8/7/12
Lymphatic invasion		p=0.58	
Positive	11		32
Negative	0		5
Vascular invasion		p=0.048[a]	
Positive	11		26
Negative	0		11
Lymph node metastasis		p=0.25	
Positive	10		25
Negative	1		12
Liver metastasis		p=0.14	
Positive	5		8
Negative	6		29
Lung metastasis		p=0.049[a]	
Positive	2		0
Negative	9		37
Peritoneal dissemination		p=0.68	
Positive	3		7
Negative	8		30

PD, poorly differentiated; [a]statistically significant (P<0.05).

The biological mechanisms of PD adenocarcinoma with NE cell differentiation and distant metastasis remain unclarified. Biogenic amines and polypeptide hormones play a role in the growth regulation of normal and neoplastic intestinal epithelia (37). Hypothetically, NE tumor cells can stimulate growth and metastatic capacity through the secretion of neurohormonal substances by the autocrine or paracrine loop. In several human cancers, tumor angiogenesis has been regarded as an important factor for tumor growth and metastasis (38-40). MVD has

been reported to be a prognostic factor in colon cancer (21). In our study, MVD determined by immunohistochemistry using CD34 antibody tended to be higher in PD adenocarcinoma with NE cell differentiation than in patients having common PD adenocarcinoma. The high MVD of PD adenocarcinoma with NE cell differentiation may indicate an increased metastatic capacity.

VEGF has been demonstrated to induce endothelial cell migration, proliferation, and invasion (41-44). VEGF expression is useful for predicting distant recurrence in patients with stage II colon cancer (21,45). VEGF expression in colorectal cancer has been shown to be associated with various clinicopathological factors such as vascular invasion and distant metastasis (17,19,20). In our study, PD adenocarcinoma with NE cell differentiation tended to have a high VEGF expression level. Interestingly, three of four patients who had VEGF-positive tumors with NE cell differentiation had liver metastasis. On the other hand, VEGF expression was significantly correlated with vascular invasion and lung metastasis, and tended to be correlated with liver metastasis in all PD adenocarcinoma patients. The number of cases studied are limited; however, VEGF may play a partial role in the liver metastasis of PD adenocarcinoma with NE cell differentiation. Previous reports have shown that VEGF expression can be used as a prognostic factor for colorectal cancer (19-21); however, it had no correlation with the survival rate of our colorectal PD adenocarcinoma patients. We presumed that PD adenocarcinoma has a very poor prognosis; thus, the overall survival rate may not significantly differ between patients having PD adenocarcinoma with VEGF expression and those having PD adenocarcinoma without VEGF expression.

The prognosis of NE carcinoma is poor; however, there is no established therapy yet. In *in vivo* studies, neutralizing VEGF antibodies administered to mice bearing human colon cancer xenografts decrease tumor growth and inhibit experimental metastasis (46). Human monoclonal antibody to VEGF (rhuMab VEGF), in combination with a conventional chemotherapy, has been shown to increase the time for tumors to develop and even the survival rates of patients with colorectal cancer producing distant metastases (47,48). In addition to cisplatin-based chemotherapy for NE carcinoma, there is a possibility that patients may benefit from VEGF-inhibiting agents (49).

In summary, NE cell differentiation of PD colorectal adenocarcinoma was correlated with liver metastasis and tended to be associated with a higher MVD and VEGF expression. Additional studies of immunohistochemical staining with NE markers for PD adenocarcinoma are necessary to evaluate the correlation between NE cell differentiation of PD adenocarcinoma and vascular invasion, and the effectiveness of anti-angiogenic therapy against the tumor.

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References

1. Taniyama K, Suzuki H, Matsumoto M, Hakamada K, Toyama K and Tahara E: Flow cytometric DNA analysis of poorly differentiated adenocarcinoma of the colorectum. Jpn J Clin Oncol 21: 406-411, 1991.
2. Cass AW, Million RR and Pfaff WW: Patterns of recurrence following surgery alone for adenocarcinoma of the colon and rectum. Cancer 37: 2861-2865, 1976.
3. Copeland EM, Miller LD and Jones RS: Prognostic factors in carcinoma of the colon and rectum. Am J Surg 116: 875-881, 1968.
4. Chung CK, Zaino RJ and Stryker JA: Colorectal carcinoma: evaluation of histologic grade and factors influencing prognosis. J Surg Oncol 21: 143-148, 1982.
5. Kulke MH and Mayer RJ: Carcinoid tumors. N Engl J Med 340: 858-868, 1999.
6. Shinji S, Tajiri T, Ishiwata T, Seya T, Tanaka N and Naito Z: Different expression levels of lumican in human carcinoid tumor and neuroendocrine cell carcinoma. Int J Oncol 26: 873-880, 2005.
7. Staren ED, Gould VE, Jansson DS, Hyser M, Gooch GT and Economou SG: Neuroendocrine differentiation in 'poorly differentiated' colon carcinomas. Am Surg 56: 412-419, 1990.
8. Gaffey MJ, Mills SE and Lack EE: Neuroendocrine carcinoma of the colon and rectum. A clinicopathologic, ultrastructural, and immunohistochemical study of 24 cases. Am J Surg Pathol 14: 1010-1023, 1990.
9. Saclarides TJ, Szeluga D and Staren ED: Neuroendocrine cancers of the colon and rectum. Results of a ten-year experience. Dis Colon Rectum 37: 635-642, 1994.
10. Grabowski P, Schindler I, Anagnostopoulos I, *et al*: Neuroendocrine differentiation is a relevant prognostic factor in stage III-IV colorectal cancer. Eur J Gastroenterol Hepatol 13: 405-411, 2001.
11. Moertel CG, Kvols LK, O'Connell MJ and Rubin J: Treatment of neuroendocrine carcinomas with combined etoposide and cisplatin. Evidence of major therapeutic activity in the anaplastic variants of these neoplasms. Cancer 68: 227-232, 1991.
12. Dor Y, Porat R and Keshet E: Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. Am J Physiol Cell Physiol 280: C1367-C1374, 2001.
13. Ferrara N and Davis-Smyth T: The biology of vascular endothelial growth factor. Endocr Rev 18: 4-25, 1997.
14. Neufeld G, Cohen T, Gengrinovitch S and Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13: 9-22, 1999.
15. Becker C, Fantini MC, Schramm C, *et al*: TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. Immunity 21: 491-501, 2004.
16. Reinmuth N, Fan F, Liu W, *et al*: Impact of insulin-like growth factor receptor-I function on angiogenesis, growth, and metastasis of colon cancer. Lab Invest 82: 1377-1389, 2002.
17. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR and Ellis LM: Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res 55: 3964-3968, 1995.
18. Takahashi Y, Bucana CD, Cleary KR and Ellis LM: p53, vessel count, and vascular endothelial growth factor expression in human colon cancer. Int J Cancer 79: 34-38, 1998.
19. Tokunaga T, Oshika Y, Abe Y, *et al*: Vascular endothelial growth factor (VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. Br J Cancer 77: 998-1002, 1998.
20. Ishigami SI, Arii S, Furutani M, *et al*: Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. Br J Cancer 78: 1379-1384, 1998.
21. Takahashi Y, Tucker SL, Kitadai Y, *et al*: Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. Arch Surg 132: 541-546, 1997.

22. Grabowski P, Schonfelder J, Ahnert-Hilger G, *et al*: Expression of neuroendocrine markers: a signature of human undifferentiated carcinoma of the colon and rectum. Virchows Arch 441: 256-263, 2002.
23. Vermeulen PB, Gasparini G, Fox SB, *et al*: Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. Eur J Cancer 32A: 2474-2484, 1996.
24. Kahn HJ and Marks A: A new monoclonal antibody, D2-40, for detection of lymphatic invasion in primary tumors. Lab Invest 82: 1255-1257, 2002.
25. Kahn HJ, Bailey D and Marks A: Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. Mod Pathol 15: 434-440, 2002.
26. Inoue K, Ozeki Y, Suganuma T, Sugiura Y and Tanaka S: Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma. Association with angiogenesis and tumor progression. Cancer 79: 206-213, 1997.
27. Ishiwata T: Immunohistochemical and *in situ* hybridization analysis of lumican in colorectal carcinoma. Burlington: Elsevier Academic Press, 2005.
28. Ishiwata T, Kornmann M, Beger HG and Korc M: Enhanced fibroblast growth factor 5 expression in stromal and exocrine elements of the pancreas in chronic pancreatitis. Gut 43: 134-139, 1998.
29. Kubo T and Watanabe H: Neoplastic argentaffin cells in gastric and intestinal carcinomas. Cancer 27: 447-454, 1971.
30. Smith DM Jr and Haggitt RC: The prevalence and prognostic significance of argyrophil cells in colorectal carcinomas. Am J Surg Pathol 8: 123-128, 1984.
31. Staren ED, Gould VE, Warren WH, *et al*: Neuroendocrine carcinomas of the colon and rectum: a clinicopathologic evaluation. Surgery 104: 1080-1089, 1988.
32. Jansson D, Gould VE, Gooch GT, *et al*: Immunohistochemical analysis of colon carcinomas applying exocrine and neuroendocrine markers. APMIS 96: 1129-1139, 1988.
33. Lewin KJ: The endocrine cells of the gastrointestinal tract. The normal endocrine cells and their hyperplasias. Part I. Pathol Annu 21: 1-27, 1986.
34. Yaziji H and Broghamer WL Jr: Primary small cell undifferentiated carcinoma of the rectum associated with ulcerative colitis. South Med J 89: 921-924, 1996.
35. Thomas RM and Sobin LH: Gastrointestinal cancer. Cancer 75: 154-170, 1995.
36. DiSario JA, Burt RW, Kendrick ML and McWhorter WP: Colorectal cancers of rare histologic types compared with adenocarcinomas. Dis Colon Rectum 37: 1277-1280, 1994.
37. Johnson LR: Regulation of gastrointestinal mucosal growth. Physiol Rev 68: 456-502, 1988.
38. Folkman J: Tumor angiogenesis: therapeutic implications. N Engl J Med 285: 1182-1186, 1971.
39. Folkman J, Watson K, Ingber D and Hanahan D: Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339: 58-61, 1989.
40. Rak J, Filmus J and Kerbel RS: Reciprocal paracrine interactions between tumour cells and endothelial cells: the 'angiogenesis progression' hypothesis. Eur J Cancer 32A: 2438-2450, 1996.
41. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS and Dvorak HF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219: 983-985, 1983.
42. Keck PJ, Hauser SD, Krivi G, *et al*: Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246: 1309-1312, 1989.
43. Leung DW, Cachianes G, Kuang WJ, Goeddel DV and Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246: 1306-1309, 1989.
44. Plouet J, Schilling J and Gospodarowicz D: Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. EMBO J 8: 3801-3806, 1989.
45. Cascinu S, Staccioli MP, Gasparini G, *et al*: Expression of vascular endothelial growth factor can predict event-free survival in stage II colon cancer. Clin Cancer Res 6: 2803-2807, 2000.
46. Warren RS, Yuan H, Matli MR, Gillett NA and Ferrara N: Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J Clin Invest 95: 1789-1797, 1995.
47. Kabbinavar F, Hurwitz HI, Fehrenbacher L, *et al*: Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol 21: 60-65, 2003.
48. Ferrara N, Hillan KJ and Novotny W: Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 333: 328-335, 2005.
49. Glade-Bender J, Kandel JJ and Yamashiro DJ: VEGF blocking therapy in the treatment of cancer. Expert Opin Biol Ther 3: 263-276, 2003.