Original Paper

Relationship Between Microvessel Count and Clinicopathological Characteristics and Postoperative Survival in Patients with Pancreatic Carcinoma

Atsushi Nanashima[1], Kenichirou Shibata,[1] Toshiyuki Nakayama,[2] Takafumi Abo[1], Takashi Nonaka[1], Daisuke Fukuda[1], Hidetoshi Fukuoka[1], Shigekazu Hidaka[1], Hiroaki Takeshita[1],Terumitsu Sawai[1], Toru Yasutake[1], Takeshi Nagayasu[1]

[1]Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

[2]Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Running title: Microvessel in pancreatic carcinoma

Corresponding and reprint requests to: Atsushi Nanashima, M.D.

Division of Surgical Oncology, Department of Translational Medical Sciences,

Nagasaki University Graduate School of Biomedical Sciences,

1-7-1 Sakamoto, Nagasaki 852-8501, JAPAN

Tel.: +81-95-819-7304 , Fax: +81-95-819-7306

E-mail: a-nanasm@nagasaki-u.ac.jp

ABSTRACT

Background/Aims: The present study aimed to elucidate the relationship between microvessel count (MVC) according to CD34 expression and clinicopathological characteristics or prognosis in pancreatic carcinoma (PC) patients who underwent hepatectomy.

Methodology: CD34 expression was analyzed using immunohistochemical methods. Mean MVC in 5 areas per specimen and clinicopathological factors were consecutively examined in 42 PC patients.

Results: Median MVC for PC patients was 123/mm^2, which was applied as a cut-off value. Higher MVC was significantly associated with the advanced Japanese Tumor-Node-Metastasis stage IVa and IVb (p=0.034). Univariate survival analysis identified higher carcinoembryonic antigen (CEA) and CA19-9 level, infiltrative type on macroscopic examination, invasive ductal carcinoma, node metastasis and higher tumor-node-metastasis classification were significantly associated with poor survival. The 5-year overall survival rate in the higher MVC group tended to be lower than that in the higher MVC group (37 vs 55%), but not statistically significant (p=0.15).

Conclusions: Tumor MVC might be a candidate prognostic marker of PC patient survival after pancreatectomy and further investigation in a larger series is warranted to clarify the significance of this marker.

KEYWORDS: pancreatic carcinoma; pancreatic resection; microvessel count; CD34 , survival.

ABBREVIATIONS: Pancreatic carcinomas (PC), pancreatoduodenectomy (PD), distal pancreatectomy (DP), microvessel count (MVC), intrahepatic cholangiocarcinomas (ICC)

INTRODUCTION

Surgical resection is the only curable treatment for pancreatic carcinomas (PC) and concurrent extended pancreatic resection is often necessary to accomplish complete (R0) resections, which may improve patient prognosis (1-3). However, the recurrence rate after resection remains high and patient survival is thus unsatisfactory (1-3). Although some conventional clinicopathological factors and surgical parameters in PC have been shown to be related to tumor recurrence and poor patient survival (4, 5), accurate prediction of prognosis except tumor stage for PC is currently impossible. The examination of differences in biological characteristics of tumors may provide useful information on the activity of PC. According to recent reports, candidates for tumor biological factors and molecular markers in PC patients include mucin expression (6), activating enhancer binding protein 2 (6), HER-2 expression (7), heparin-binding growth factor (8), and tumor angiogenesis (9-13). Combining the use of conventional clinicopathological factors and prognostic factors from tumor biology may improve the prediction of prognosis after hepatectomy for PC and may contribute to a predictive staging classification.

Tumor angiogenesis seems likely to be important for supporting tumor growth in general (14), and PC also expresses some angiogenic factors such as vascular endothelial growth factor (VEGF) (15). Levels of these angiogenic factors might affect patient survival and microvessel density is known to correlate with tumor aggressiveness and prognosis in PC (9-13). We have provided preliminary results of higher microvessel count (MVC), using CD34 antibody as a marker, associated with poor prognosis in patients with adenocarcinomas in the liver undergoing surgical resection (16, 17).

However, the utility of this marker has yet to be fully elucidated. PC is typically a hypovascular tumor, based on imaging diagnosis and, in such tumors, the above theory with respect to tumor angiogenesis does not always apply (18). However, some PC showed intra-tumorous vascularity (19); however, the clinical implications of these relationships have yet to be fully clarified. We have hypothesized that tumor vascularity in PC represents a factor associated with tumor growth and invasion, thus causing a poor prognosis, indicating that MVC may play a significant role in tumor progression.

The present study examined the relationship between MVC in PC using immunohistochemical staining and conventional clinicopathological factors, and prognosis in 42 PC patients with 12 months of minimum follow-up to elucidate our hypothesis.

METHODOLOGY

Patients

A total of 42 consecutive patients with PC who were admitted to the Division of Surgical Oncology in the Department of Translational Medical Sciences at Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 2001 and August 2009 (minimum follow-up, 12 months) were analyzed retrospectively in this study. Highly advanced PC such as a local extension to the supra-mesenteric artery (SMA), peritoneal dissemination or distant metastasis was found and radical operation was avoided during this period. Data were retrieved from both anesthetic and patient charts, plus the NUGSBS database, to cover the period of hospitalization following

pancreatectomy. The study design was approved by the Ethics Review Board of our institute and signed consent for clinical research using tissue or blood samples was obtained from each patient. After surgery, 14 patients (33%) received adjuvant chemotherapy in the early postoperative period. Follow-up included measurement of serum carbohydrate antigen 19-9 (CA19-9) every 3 months and abdominal CT every 3 to 6 months. In case of adjuvant chemotherapy or detection of tumor recurrence, patients received chemotherapy (intravenous infusion or oral intake of anticancer drugs such as gemcitabine [Gemzar®; Eli Lilly Co., IN] and tegafur- gimeracil - oteracil potassium [TS-1; TAIHO Pharmaceutical Co., Ltd., Tokyo, Japan]).

Tumor stage and curability was followed by the *Classification of Pancreatic Carcinoma* (20).

Operative procedures and follow-up

Pancreaticoduodenectomy (PD) is a basic surgical option for PC located in the pancreatic head (n=28) and distal pancreatectomy (DP) is selected for PC in the body or tail of pancreas (n=14). Lymphadenectomy was basically performed in Group 2 lymph nodes and lymph nodes at the para-aortic lesion (station number 16a2 and 16b1). Extrapancreatic nerve plexus was also resected in half of the cases around the SMA. In case of PD, Child's intestinal reconstruction with end-to-side anastomosis of pancreatojejunostomy or pancreato-gastrostomy was routinely selected. In case of DP, pancreatic stump was closed by suturing in a fish mouth shape. In case of tumor

involvement to the portal vein or supra-mesenteric vein (SMV), splenic artery or vein, and common hepatic artery, radical operation was considered. When combined resection of the portal vein or SMV was performed, end-to-end anastomosis of vessels was applied in 5 patients.

Immunohistochemical staining

Resected specimens were fixed in 10% formalin and embedded in paraffin. Thin sections (4 μm) were deparaffinized twice using xylene and rehydrated in ethanol series (100%, 90% and 80%). Sections were placed in 0.01 mol/L of trisodium citrate dehydrate buffer (pH 6.0), then treated in a microwave oven for 10 min at 500 W. For CD34 staining (21), tissue sections were digested with 0.2% trypsin in 0.01 mol/L phosphate-buffered saline (PBS) for 20 min at 37°C. In the next step, tissues were immersed in 3% H_2O_2 with distilled water for 10 min to inactivate endogenous peroxidases. After blocking non-specific binding by normal goat serum, sections were incubated overnight at 4°C with mouse anti-monoclonal CD34 antibody (1:25, QB-END/10; Novocastra Laboratories, Newcastle, UK) as the primary antibody. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using labeled streptavidin-biotin (LSAB) kit peroxidase (Dako, Carpinteria, CA). The peroxidase reaction was visualized with 0.01% H_2O_2 and 3,3'-diaminobenzidine under light microscopy (×200). For MVC using CD34 staining, average count was determined for the 5 most-vascular areas in the PC examined under ×200 magnification. **Figure 1A and B** show the high and low MVC, respectively.

Statistical analysis

Continuous data are expressed as mean ± standard deviation. Data from different groups were compared using one-way analysis of variance and examined with Student t-test or the Dunnett multiple comparison test. For univariate analysis, categorical data were analyzed using the Fisher exact test. Disease-free and overall survival rates were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test. Two-tailed values of $p<0.05$ were considered statistically significant. All statistical analyses were performed using SAS software (Statistical Analysis System, Cary, NC).

RESULTS

Among the 42 patients in the present study, overall 1-, 3- and 5-year survival rates were 73%, 49% and 44%, respectively, and median overall survival was 28.3 months. Disease-free 1-, 3- and 5-year survival rates were 54%, 44% and 44%, respectively, and median disease-free survival was 14.1 months. A total of 21 patients (50%) displayed tumor recurrence after pancreatectomy, which were liver metastasis in 11, local recurrence in 4, peritoneal dissemination in 4, lymph node metastasis in 3 and lung in two.

Median MVC within the tumor area was 123/mm^2 (16-208/mm^2), and this value was applied as a cut-off value. **Table 1** shows the relationship between MVC and clinicopathological features. Gender, age, tumor marker, tumor size, macroscopic findings, histological differentiation, vascular and nerve invasion were not associated with MVC. Advanced TNM stage (III) was significantly more frequent in the higher MVC group than in the lower MVC group. Prevalence of postoperative tumor recurrence was similar between both groups. **Table 2** shows the relationship between survival and clinicopathological factors including MVC. Higher carcinoembryonic antigen (CEA) and CA19-9 level, infiltrative type on macroscopic examination, invasive ductal carcinoma, node metastasis and higher tumor-node-metastasis classification were significantly associated with poor survival. However, MVC was not associated with survival. **Figure 2** shows that overall survival tended to be better in the lower MVC group than in the higher MVC group but not statistically significant (p=0.15). MVC was not associated with disease-free survival.

DISCUSSION

Recently, aggressive surgical exploration with or without vascular resections for PC is usually performed and survival has been remarkably improved (1-3). As the techniques and perioperative management have remarkably improved, we have actively performed extended resections for complete tumor resections (R0) during the last 15 years as well. Clear prognostic factors, other than conventional pathological factors such as nodal status, tumor size and vascular involvement, have not been clarified for PC yet. Most invasive ductal PC is clinically observed in the advanced stage as III, IVa and IVb and, therefore, other biological prognostic factors are not related to findings of the conventional tumor stage system. Various angiogenic factors in PC have been previously reported as candidate prognostic marker (9-13). In previous studies, we focused on MVC for liver malignancies, particularly hypovascular diseases because such tumors sometimes showed tumor vascularity using the recently advanced imaging modalities such as multi-detector enhanced computed tomography (16, 17, 19). We have wanted to clarify the relationship between tumor angiogenesis and malignant behavior influencing patient prognosis. This parameter can be analyzed conventionally and is easily examined using immunohistochemistry at any institute, and we propose the inclusion of this examination in conventional pathological diagnosis to predict tumor malignancy. Other studies have revealed that tumor angiogenesis might be related to patient prognosis in PC patients who undergo radical resection (12, 13). Our previous study showed that higher MVC was significantly associated with advanced tumor stages and poor patient survival in hypovascular liver malignancies as intrahepatic cholangiocarcinomas (ICC) and metastatic liver carcinoma from colorectal carcinoma (16, 17). Therefore, in such hypovascular malignancies, tumor angiogenesis may play an important role for tumor

aggressiveness

In the present study, MVC over 123/mm^2 was set as a cut-off value by the median value in all specimens in the present study, which was relatively lower in comparison with that in the ICC (16) and, therefore, low vascularity of PC was indicated. In the present study, MVC was not associated with various clinicopathological parameters except invasion of portal trunk. A previous study showed that tumor angiogenesis was closely associated with tumor invasion in adenocarcinomas of many organs including PC (22). Although statistical significance was not shown, MVC tended to be related to portal invasion based on our present results. Invasion to the portal trunk or supra-mesenteric vein is a specific characteristic in PC and is a key factor influencing patient prognosis (23). Therefore, tumor angiogenesis may play a role when the PC tumor infiltrates surrounding vessels.

With respect to macroscopic finding and histological findings, MVC was not associated with these characteristics although a relationship of MVC was expected. The intraductal papillary mucin producing neoplasm (IPMN) often showed tumor vascularity (24). In the present series, we selected IPMN or solid pseudo-papillary neoplasm with carcinoma components. In the early stage or low grade malignancies, MVC might not be associated with carcinogenesis or tumor development. MVC in PC was significantly associated with only tumor stage defined by Japanese classification (20). Higher MVC was frequently observed in stage IVa and IVb, which was a highly advanced stage in the present study. As described above, the relationship between higher MVC and portal invasion might be a factor of advanced stages. This result showed that tumor angiogenesis was associated with tumor infiltration or development, as was reported in previous studies (10-13).

In our series, most cases underwent PD and DP with D2 lymphadenectomy. Complete lymphadenectomy at station number 162a and 16b1 are considered to be necessary because of a high rate of node metastasis (25). Combined resections of the portal vein or SMV were aggressively performed to accomplish R0 resection, as in other reports (6, 23, 25). The goal of this study was to clarify the relationship between MVC and postoperative survival in PC patients. Predictive factors for patient prognosis other than conventional histological findings have been proposed by many investigators, but no consensus has yet been reached (6-8). In the present survival analysis, several associated parameters were revealed by univariate survival analysis. Serum level of CEA and CA19-9 were significantly associated with poor survival in PC patients, which are important prognostic factors in tumor aggressiveness, as found in a previous report (26). However, the present study showed that MVC was not significantly related to survival after surgery, unlike the results of previous reports (12, 13). Patients with higher MVC tended to have poorer survival and, therefore, tumor angiogenesis might influence poor prognosis based on our results. In the present series, survival in stage III was very poor contrary to expectation and this result might influence this survival analysis because lower MVC was frequently observed in stage III. To clarify this relationship, longer patient follow-up or a larger size study in collaboration with other institutions is necessary. With respect to stage of tumor, most PC patients except non-invasive IPMC showed advanced stages, which led to a poor prognosis in general. The five year-survival in the present study was still low regardless of improvement of surgical techniques or

perioperative management, which was lower than that in previous reports (1-6, 27, 28). Cystic type or ductectatic type is a typical macroscopic finding of IPMN (29, 30) and this type showed good outcomes in the present study as well as previous reports (30). Non-invasive IPMC is a proper indication for radical operation expecting long-term survival (30).

Further investigation in a larger population of PC patients is needed to resolve the problem of whether tumor angiogenesis offers a useful predictor of patient survival based on this preliminary analysis. When determining the specific prognostic factors of carcinoma cells, we must eventually consider strategies for additional anti-angiogenic treatments after resection. In case of PC with poor prognostic factors, adjuvant chemotherapy in addition to the conventional chemotherapy as gemcitabine or S-1 (31). At this stage, some anti-angiogenic drugs have been developed in the field of PC, but they are not yet clinically applicable (32).

In conclusion, the present study examined the relationship between MVC according to CD34 expression and clinicopathological parameters or survival in 42 PC patients who underwent aggressive surgical pancreatectomy. As a tumor biological factor, MVC representing tumor angiogenesis might offer a candidate prognostic factor in PC to predict poorer patient survival, but is not a significant factor at this stage. Further study in a larger population of PC patients undergoing surgical resection is warranted to clarify the role of tumor angiogenesis in this disease.

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Figure legend

Figure 1. Microvessel counts using CD34 staining was determined for the 5 most-vascular areas in the PC examined under ×200 magnification. The 1A and B show the high and low MVC, respectively.

Figure 2. Comparison between high or low MVC and cumulative cancer-free (a) and overall (b) survival after hepatectomy.

Figure 1A

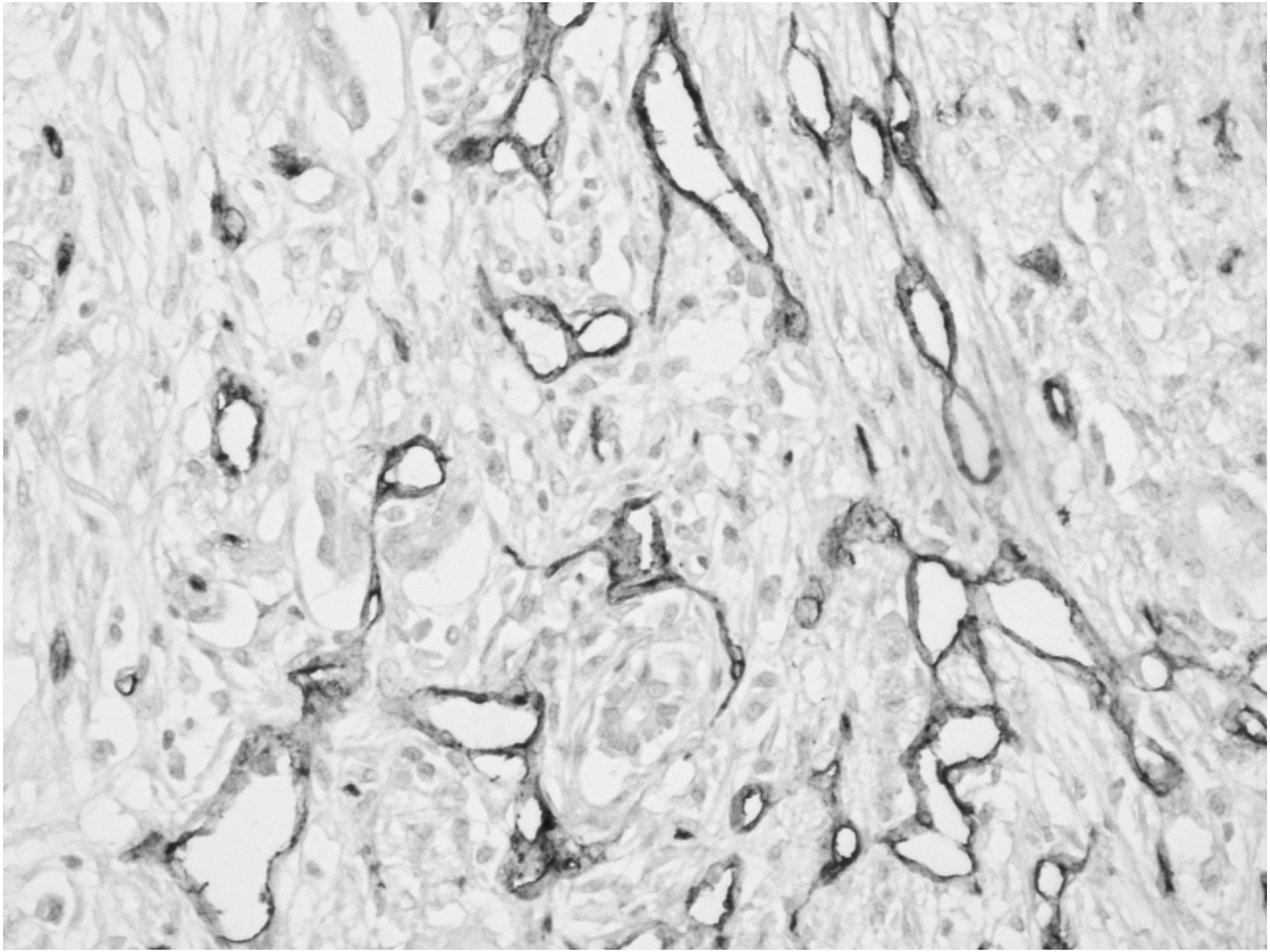

Figure 1B

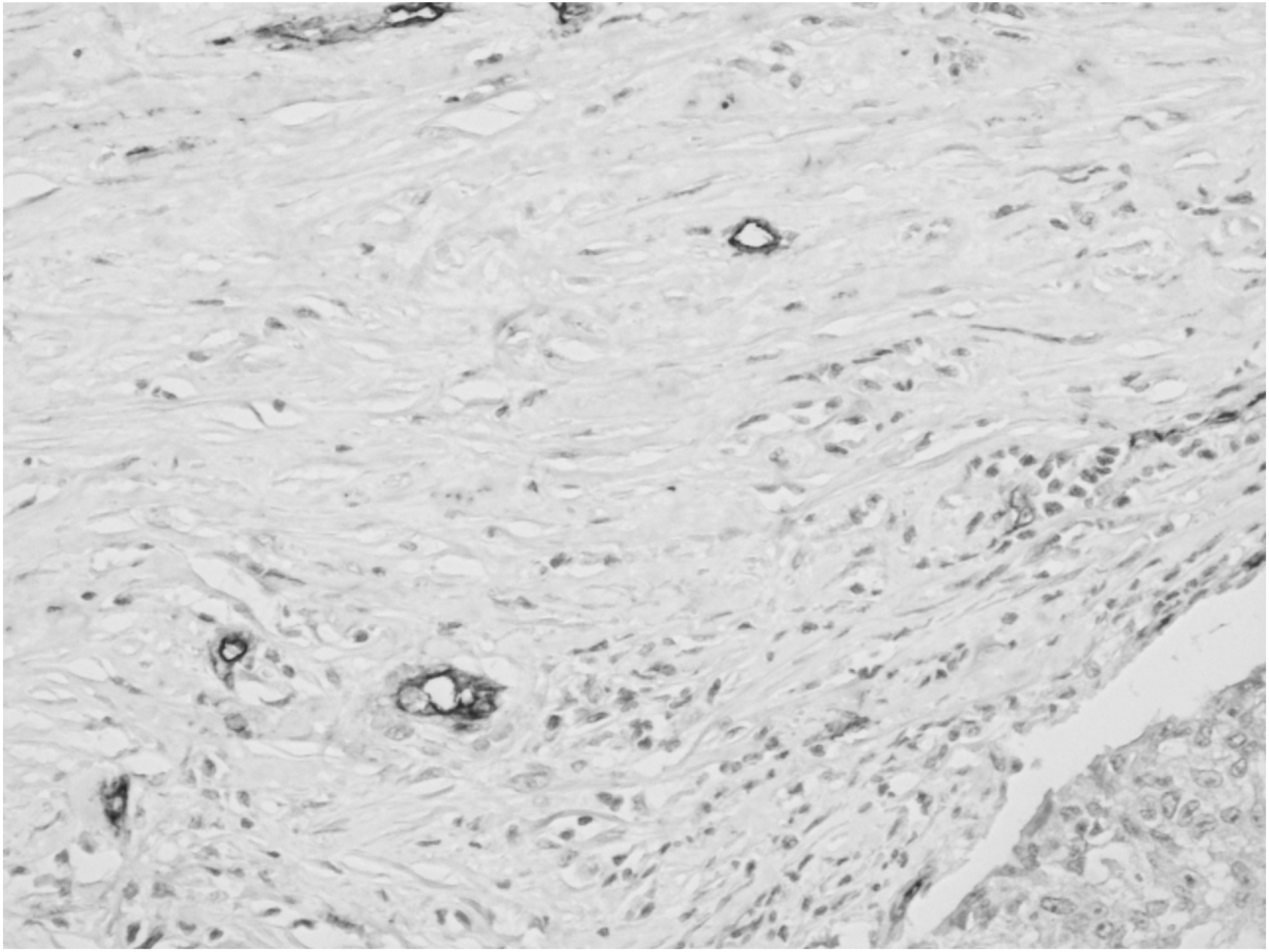

Figure 2A

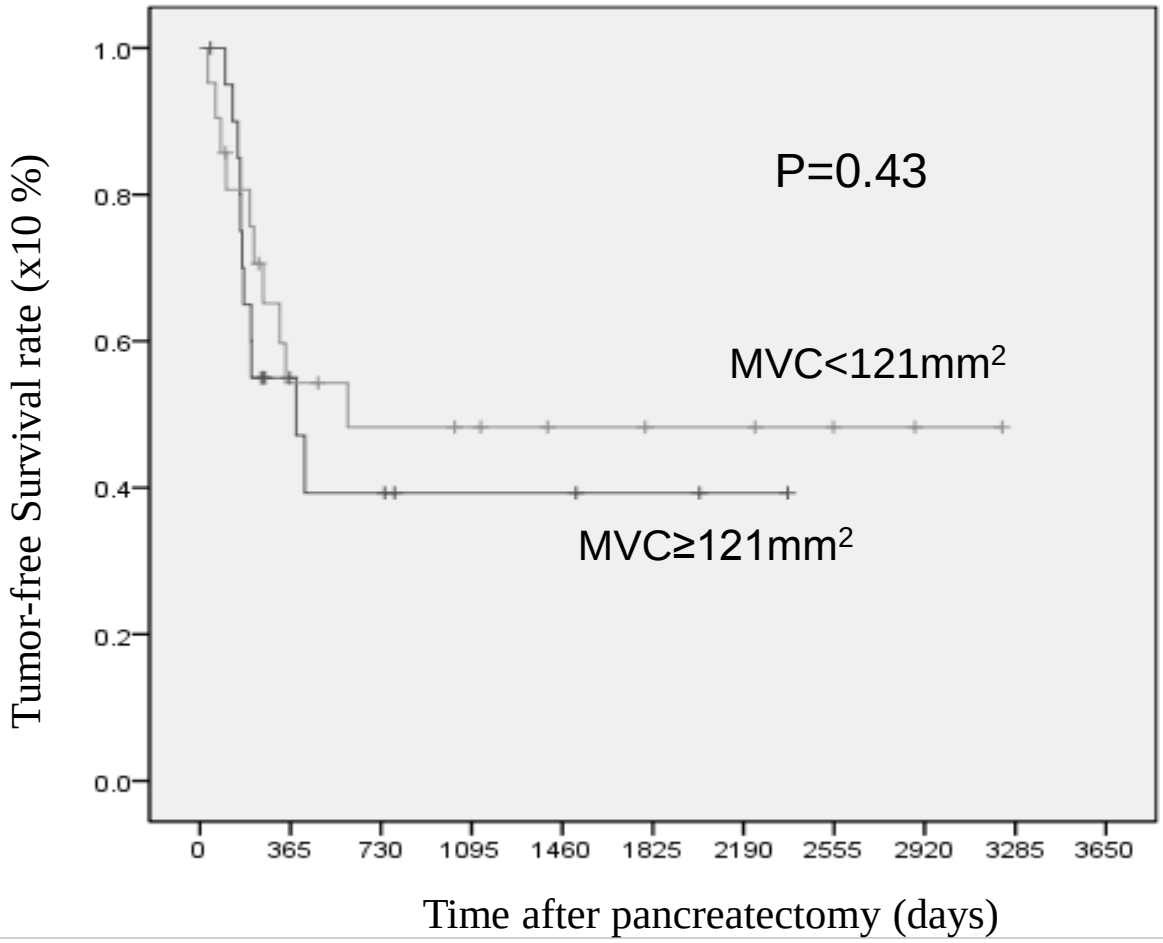
Tumor-free Survival rate (x10 %)
1.0
0.8
0.6
0.4
0.2
0.0
P=0.43
MVC<121mm2
MVC≥121mm2
0
365
730
1095
1460
1825
2190
2555
2920
3285
3650
Time after pancreatectomy (days)

Figure 2B

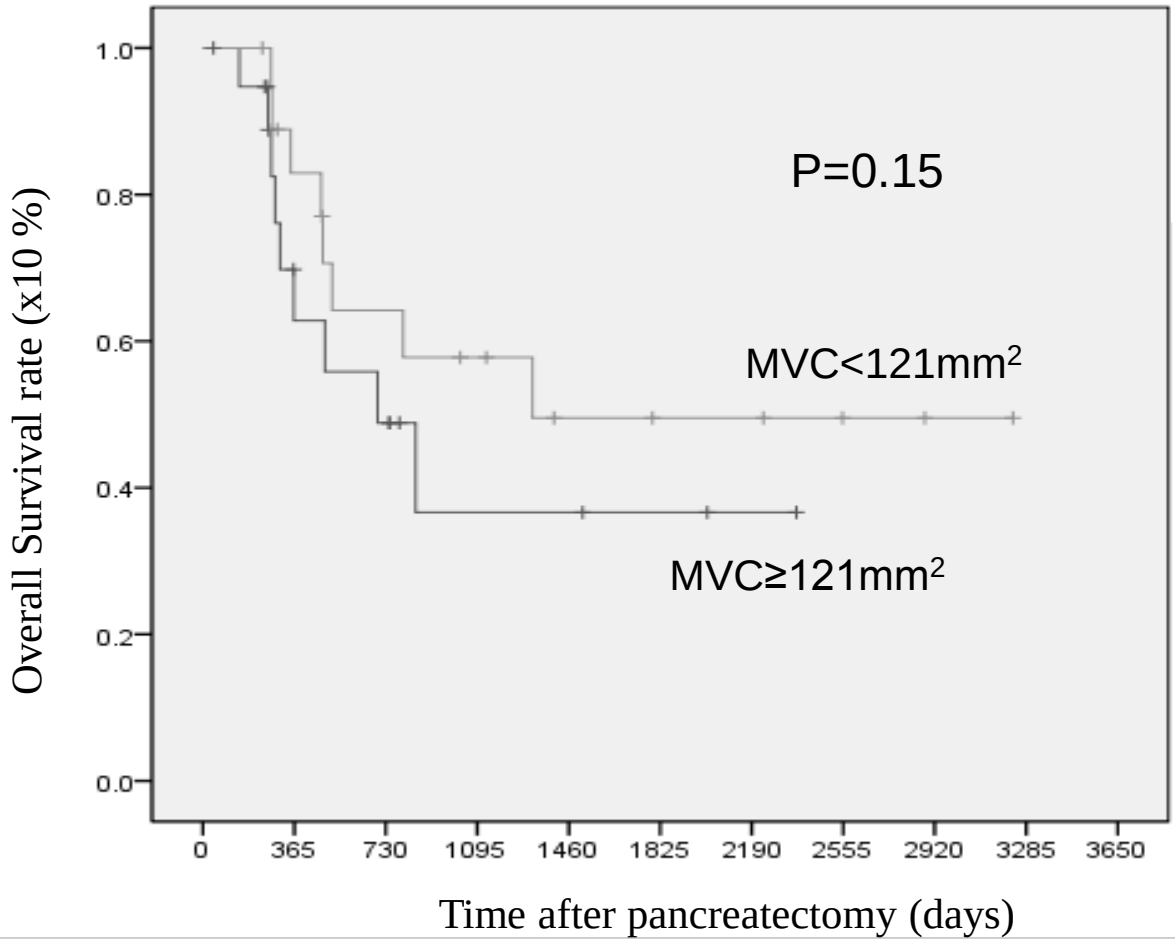
P=0.15
MVC<121mm2
MVC≥121mm2
Overall Survival rate (x10 %)
1.0
0.8
0.6
0.4
0.2
0.0
0
365
730
1095
1460
1825
2190
2555
2920
3285
3650
Time after pancreatectomy (days)

Table 1. Correlations between tumor microvessel counts and clinicopathologic parameters or postoperative recurrence rate in pancreatic carcinomas

	Microvessel counts		P value
	$<123/mm^2$ [n=19]	$\geq 123/mm^2$ [n=23]	
Gender			
Male	9 (47)	14 (61)	0.55
Female	10 (53)	9 (39)	
Age, ≥60 years	13 (68)	20 (86)	0.25
Serum tumor marker			
CEA ≥5 ng/ml	2 (11)	5 (22)	0.43
CA19-9 ≥37 U/ml	10 (53)	11 (48)	0.99
DUPAN-II ≥150 U/ml	3 (16)	5 (22)	0.87
Tumor size, ≥3 cm	17 (89)	18 (78)	0.78
Macroscopic finding [§]			
Nodular type	10 (53)	10 (44)	
Infiltrative type	5 (26)	5 (22)	0.52
Cystic type	3(16)	7 (30)	
Ductectatic type	1 (5)	0	
Mixed type	0	1 (4)	
Histological differentiation			
Well	5 (26)	6 (26)	
Moderately	4 (21)	3 (13)	
Poorly	4 (21)	6 (26)	0.84
IPMC	4 (21)	7 (30)	
SPN	2 (11)	1 (5)	
Lymphatic invasion, Yes	12 (63)	15 (65)	0.44
Venous invasion, Yes	12 (63)	15 (65)	0.44
Nerve invasion, Yes	11 (58)	15 (65)	0.38
Lymph node metastasis, Yes	10 (53)	11 (48)	0.99
Portal trunk invasion, Yes	5 (25)	11 (47)	0.17
Tumor-node-metastasis stage [¶]			
I	2 (11)	1 (5)	
II	2 (11)	4 (17)	
III	8 (42)	1 (5)	0.034
IVa	5 (25)	11 (47)	

IVb	2 (11)	6 (26)	
Postoperative tumor recurrence, Yes	12 (63)	12 52)	0.89
Local or node recurrence	5	2	0.37
Distant metastasis or peritoneal dissemination	7	10	

Parenthesis shows a percentage.

CEA: carcinoembryonic antigen, CA19-9: carbohydrate antigen19-9, DUPAN-II: Duke-Pancreas-II

IPMC; intraductal papillary mucin producing carcinoma, SPN; Solid pseudo-papillary neoplasm

[§]Macroscopic classification of pancreatic carcinoma (20), #; findings according to *the Classification of Pancreatic Carcinoma* (20)

Table 2. Relationship between clinicopathological factors and survival rates in pancreatic carcinoma

	Tumor-free survival rates (3years:%)	P value	Overall survival rates (5years:%)	P value
Gender				
Male	45	0.74	49	0.27
Female	38		34	
Age (years)				
>60	44	0.45	45	0.63
≤60	45		39	
CEA (ng/ml)				
≤10	54	0.002	49	0.019
>10	0		0	
CA19-9 (U/ml)				
≤37	76	<0.001	83	<0.001
>37	8		0	
Tumor size				
<3cm	80	0.21	75	0.22
≥3 cm	39		35	
Macroscopic finding				
Nodular type	36		26	
Infiltrative type	0	<0.001	0	0.001
Cystic type	100		100	
Ductectatic type	100		100	
Mixed type	0		0	
Histological differentiation				
Invasive ductal carcinoma	11	<0.001	8	<0.001
IPMC	100		100	
SPN	100		100	
Lymph node metastasis,				
No	85	<0.001	79	<0.001
Yes	6		12	
Tumor-node-metastasis stage				
I	100	0.001	100	0.001
II	100		100	

III	0		0	
IVa	32		27	
IVb	0		0	
Microvessel counts, CD34(/mm^2)				
<123	49	0.43	55	0.15
≥123	39		37	

Parameters; See Table 1.