Original Paper

Relationship Between Microvessel Count and Postoperative Survival in Patients with Intrahepatic Cholangiocarcinoma

Atsushi Nanashima, MD,[1] Kenichiro Shibata, MD, [12] Toshiyuki Nakayama, MD, [2]
Syuichi Tobinaga, MD, [1] Masato Araki, MD, [1] Masaki Kunizaki, MD, [1]
Hiroaki Takeshita, MD, [1] Shigekazu Hidaka, MD, [1] Terumitsu Sawai, MD, [1]
Tsutomu Tagawa, MD, [1] Takeshi Nagayasu, MD[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 Molecular Pathology, Atomic Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Address correspondence and reprint request to: Atsushi Nanashima, MD, 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-ac.jp

Abstract

Background: The present study aimed to elucidate the relationship between microvessel count (MVC) according to CD34 expression and prognosis in intrahepatic cholangiocarcinoma (ICC) patients who underwent hepatectomy based on our preliminary study.

Methods: Relationships between MVC and clinicopathological factors were examined in 37 ICC patients. CD34 expression was analyzed using immunohistochemical methods.

Results: Median MVC for ICC patients was 140/mm^2, which was applied as a cut-off value. Lower MVC was significantly associated with larger tumor size, periductal infiltrating type, and advanced Japanese Tumor-Node-Metastasis stage ($p<0.05$). Univariate survival analysis identified higher carcinoembryonic antigen level, peri-ductal infiltrating type, poor histological differentiation and lower MVC as significantly associated with lower 5-year survival rates. The 5-year survival rate in the higher MVC group was significantly greater than that in the lower MVC group (44 vs 7%, $p=0.048$). According to Cox multivariate survival analysis, only periductal infiltrating type on macroscopic examination was identified as a significant independent risk factor for poor survival after hepatectomy (risk ratio, 4.8; $p=0.006$), not MVC (1.1; $p=0.82$).

Conclusion: Tumor MVC might offer a useful prognostic marker of ICC patient survival after hepatectomy and further investigation in a larger series is warranted.

KEY WORDS: intrahepatic cholangiocarcinoma; hepatic resection; microvessel count; CD34

Synopsis

Microvessel counts according to CD34 expression representing tumor angiogenesis offer a candidate prognostic factor in intrahepatic cholangiocarcinomas to predict tumor recurrence and poorer patient survival, but not an independent factor in multivariate analysis.

Hepatic resection is currently the only curative option for radical treatment of intrahepatic cholangiocarcinoma (ICC). However, the recurrence rate after resection remains high and patient survival is thus unsatisfactory.[1,2] Although some conventional clinicopathological factors and surgical margins in ICC have been shown to be related to tumor relapse and shorter patient survival,[3-5] accurate prediction of prognosis for ICC is currently impossible. The examination of differences in biological characteristics of tumors may provide useful information on the activity of ICC. According to recent reports, candidates for tumor biological factors and molecular markers in ICC patients include chromosomal aberration,[6] lymphatic microvessels,[7] adhesion molecules,[8] mucin expression,[9] Expression of matrix metalloproteinase,[10] expression of cyclooxygenase-2,[11] and CD24 expression.[12] Combining the use of conventional clinicopathological factors and prognostic factors from tumor biology may improve the prediction of prognosis after hepatectomy for ICC and may contribute to a predictive staging classification. The recent American loint Committee on Cancer/Union Internatic nale Contre le Cancer (AICC/UICC) staging is inadequate for predicting patient survival.[13] A combination of conventional clinicopathologic factors and tumor biological factors may thus improve predictions of prognosis after hepatectomy for ICC.

Tumor angiogenesis seems likely to be important for supporting tumor growth in general,[14]and ICC 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 (MVD) is known to correlate with tumor aggressiveness and prognosis in ICC by Shirabe et al.[16] We have provided preliminary results of higher microvessel count (MVC), using CD34 antibody as a marker, associated with poor prognosis in ICC patients undergoing hepatic resection for 32

months. [17] However, the utility of this marker has yet to be fully elucidated. ICC is typically a hypovascular tumor, based on imaging diagnosis, and has very poor survival compared to other digestive diseases. [18] In such tumors, the above theory with respect to tumor angiogenesis does not always apply. Our recent study showed that hypovascular image findings on computed tomography were associated with patient outcomes after operation.[19] Thus, although few recent reports have shown relationships between MVD and postoperative outcomes in ICC patient, [20] the clinical impacts of these relationships have yet to be fully clarified. Based on our study of MVD in hepatocellular carcinoma and metastatic liver carcinoma, [21, 22] we have hypothesized that tumor vascularity in ICC represents a factor associated with tumor growth and invasion, thus causing poor prognosis that MVC may play a significant role in liver tumor progression.

The present study examined relationships between MVC in ICC using immunohistochemical staining and conventional clinicopathological factors, and prognosis in ICC patients with a longer follow-up period (5 years) with 18 months of minimum follow-up period and 28 moths of median follow-up period to clarify our hypothesis.

MATERIALS AND METHODS

Patients

A total of 37 consecutive patients (20 men, 17 women) with ICC 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 January 2002 and April 2007 (minimum follow-up, 18 months) were analyzed retrospectively in this study. Data were retrieved from both anesthetic

and patient charts, plus the NUGSBS database, to cover the period of hospitalization following hepatectomy. 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, no patients routinely received adjuvant chemotherapy in the early postoperative period. Follow-up included measurement of serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) every 3 months and abdominal CT and chest X-ray every 3 to 6 months. When recurrence was detected, patients received chemotherapy (intravenous infusion or oral intake of anticancer drugs such as Gemcitabone (Gemzar®; Eli Lilly Co., IN) and Tegafur-Gimeracil - Oteracil Potassium (TS-1; TAIHO Phamaceutical Co., Ltd., Tokyo, Japan)).

Indocyanine green retention rate at 15 min ($ICGR_{15}$) was routinely examined to define the preoperatively functional liver reserve. The volume of liver to be resected was determined preoperatively based on the results of $ICGR_{15}$ and the estimated resected liver volume, excluding tumor volume, as measured by the computed tomography volumetry.[23] Liver activity as measured using technetium-99m galactosyl human serum albumin (^{99m}Tc-GSA) scintigraphy at 15 min after injection[23] and other liver functional parameters were also evaluated before surgery. All 37 patients underwent hepatic resection, with surgical procedures including segmentectomy (n=3), sectoriectomy (n=17), right or left hepatectomy (n=14), and extended hepatectomy (n=3). All hepatic tumors were resected without macroscopic exposure of the amputated section to the remaining liver. Pathological and morphological parameters, and Japanese Tumor-Node-Metastasis (TNM) stage were used as defined by the Liver Cancer Study Group of Japan.[24]

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, [25] 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 CCC examined under ×200 magnification. Each slide was assessed by 2 pathologists blinded to patient data. 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. Multivariate analysis was performed using proportional hazards regression modeling, despite the limited number of subjects. 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 37 patients in the present study, overall 1-, 3- and 5-year survival rates were 51%, 35% and 27%, respectively, and median overall survival was 48 months. Disease-free 1-, 3- and 5-year survival rates were 45%, 17% and 6%, respectively, and median disease-free survival was 29 months. A total of 26 patients (70.3%) displayed tumor recurrence after hepatectomy, which were liver metastasis in 9, local recurrence in 6, peritoneal dissemination in 8, lymph node metastasis in 4 and bone in one (dual recurrence in two patients).

Median MVC within the tumor area was 140/mm^2 (51-363/mm^2), and this value was applied as a cut-off value. Table I shows the relationship between MVC and clinicopathological features. Chronic viral hepatitis tended to be more frequent in the higher MVC group (≥140/mm^2). Size ≥5 cm, peri-ductal infiltrating type and advanced TNM stage (III) were significantly more frequent in the lower MVC group than in the higher MDC group. Postoperative tumor recurrence tended to be more frequent in the lower MDC group than in the higher MDC group but not significant. However, MVC was not different between types of recurrence. Table II shows the relationship between overall survival and clinicopathological factors including MVC. Higher carcinoembryonic antigen (CEA) level, peri-ductal infiltrating type on macroscopic examination, poor histological differentiation and lower MVC were significantly associated with lower 5-year survival rate. In addition, nodal status, tumor size, and intraoperative blood loss tended to be associated with overall survival. Figure 2 shows that survival was better in the higher MVC group than in the lower MVC group. By applying these prognostic parameters, multivariate analysis for overall survival after hepatectomy was performed even though the number of subjects was limited in the

present study (Table III). Only the macroscopic finding of periductal infiltrating type, not MVC, was identified as a significant independent risk factor for poor overall survival after hepatectomy.

DISCUSSION

Clear prognostic factors, other than pathological factors such as nodal status, tumor size or number and vascular involvement, have not been clarified for ICC and the significance of the present Japanese Tumor-Node-Metastasis staging remains controversial. Various angiogenic factors in ICC have been reported as candidate prognostic marker in recent years. [14-16, 20] In both the preliminary and present studies, we focused on MVC for liver malignancies, including ICC. [17] This parameter can be conventionally and easily examined using immunohistochemistry at any institute, and we propose the inclusion of this examination in conventional pathological diagnosis to predict tumor malignancy. Our preliminary study and other studies have revealed that tumor angiogenesis might be related to patient prognosis in ICC patients who undergo radical hepatectomy, although results have not been similar between investigators. [16,20] Our previous study examined the usefulness of MVC for predicting outcomes in ICC patients and we have thus waited for 5 years since the first report to determine the relationship with a longer period after treatment.

In the present study, MVC <140/mm^2 tended to be associated with the status of chronic viral hepatitis, primarily hepatitis C. According to previous reports, some ICCs occur in patients with chronic hepatitis, but not with biliary diseases. [26] Our previous report with respect to the enhancement pattern of the ICC, indicated that some ICC showed hypervascularity on computed tomography (CT) in patients with concomitant chronic viral hepatitis. [19] As MVC reflects tumor vascularity, the present result is plausible. Chronic viral hepatitis may be associated with carcinogenesis of ICC such as a mass-forming type.[27] Lower MVC, on the other hand, related to progression of tumor malignancy in the present results. Basically, ICC showed a characteristic of

hypovascularity on imaging analysis.[28] ICC is a devastating malignancy that is notoriously difficult to diagnose, but with increasing incidence worldwide.[29] Current imaging modalities such as CT and magnetic resonance imaging (MRI) provide useful diagnostic information, as imaging analysis is important in defining the indications for surgery.[30] The predominant radiological characteristic of ICC is hypovascular or marginal enhancement (ring enhancement) of the mass lesion, with or without peripheral biliary dilatation or segmental liver atrophy.[31] However, tumor morphology and vascularity show different characteristics in some cases.[32] Imaging findings on enhanced CT or positron emission tomography[33] were also related to patient outcomes. According to these findings, tumor vascularity may be increased according to tumor progression for limited growth of ICC. By reflecting a correlation between MVC and tumor malignancy, however, the lower MVC also tended to be related to postoperative tumor recurrence in the present study. Our present results demonstrate that a greater blood supply is not always associated with more aggressive tumor behavior. Even in hepatocellular carcinoma as a representative hypervascular tumor, patients with lower MVC carcinomas showed a poor prognosis.[22] Tumor angiogenesis is undoubtedly an important factor for tumor growth; however, other factors may be closely related to tumor invasiveness.[34] Lower tumor vascularity may lead to anti-cancer treatment resistance via intra-hepatic blood flow.[35] Further study needs to clarify relationships between angiogenic parameters and specific clinicopathological findings.

The goal of this study was to clarify the relationship between MVC and postoperative survival in ICC patients. In survival analysis, several associated parameters including MVC were revealed by univariate survival analysis. Predictive factors for patient prognosis have been proposed by many investigators, but no

consensus has yet been reached.[20,36] The present study showed that lower MVC was significantly related to shorter survival after surgery, unlike the results of previous reports. [16,20,36] However, our previous study of tumor enhancement pattern showed that a hypovascular pattern was related to poorer survival, [19] so the present result seems reasonable. In our preliminary report, a significant correlation between MVC and patient survival in ICC was not observed and, therefore, longer patient follow-up or a larger size study in collaboration with other institutions is necessary when dealing with rare tumors such as ICC. Higher MVC clearly does not always correlate with malignant behavior of carcinomas. A similar study we performed in hepatocellular carcinomas also showed that lower MVC was associated with poor patient outcomes. [22] Tumor angiogenesis may be necessary for the growth of liver tumors in earlier stages, [37] but this influence might be reduced during the acquisition of treatment resistances such as fibrosis in ICC. As shown in the survival curve, the drop in the survival curve was similar between higher and lower MVC within 1 year after hepatectomy. Only 6 ICC patients showed long-term survival exceeding 1 year after surgery. Given this result, tumor recurrence and failure of survival might occur regardless of whether ICC displays higher MVC. Some reports have shown that the expression of angiogenic factors or higher MVC are associated with tumor malignancy or poor patient survival, [16] while others have shown no relationship with survival in ICC patients. [20,36] The influences of angiogenic factors thus might not be particularly strong. Under multivariate analysis, only macroscopic findings were identified as a strong independent prognostic factor, with other candidate factors including MVC not showing any significance. We also performed the same survival analysis after excluding this strong contributing factor, but this failed to identify any significant independent factors predictive of poor survival.

ICC is a malignant tumor originating in the intrahepatic bile duct, and comprises cells that mostly resemble those of the peripheral bile ducts.[38] However, ICC is not always uniform, and is classified into subgroups according to macroscopic findings, with the clinical and pathological characteristics and prognosis differing significantly among subgroups.[39] Diagnosis of ICC subtype would be useful in predicting prognosis or selecting adjuvant treatments with hepatic resection.

Further investigation in a larger population of ICC patients is needed to resolve the problem of whether tumor angiogenesis offers a useful predictor of patient survival. As ICC is not a common disease, a nationwide comprehensive study to investigate various candidate factors is necessary. When determining the specific prognostic factors of carcinoma cells, we must eventually consider strategies for additional anti-angiogenic treatments after hepatectomy. At this stage, some anti-angiogenic drugs have been developed in the field of ICC, but they are not yet clinically applicable.[20]

In conclusion, the present study examined the relationship between MVC according to CD34 expression and overall survival in ICC patients. As a tumor biological factor, MVC representing tumor angiogenesis might offer a candidate prognostic factor in ICC to predict tumor recurrence and poorer patient survival, but not an independent factor in multivariate analysis. Further study in a larger population of ICC patients undergoing surgical resection is warranted to clarify the role of tumor angiogenesis in this disease.

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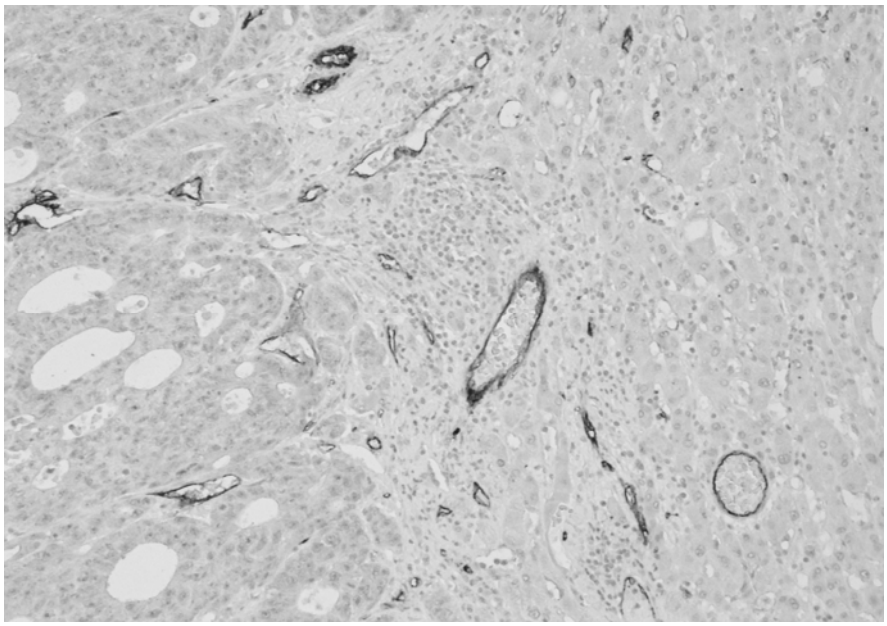
FIGURE LEGENDS

Fig.1. Representative figures of microvessels around the metastatic liver tumor. Cases of lower (A) and higher (B) MVC. Findings at 100× magnification.

(A)



(B)

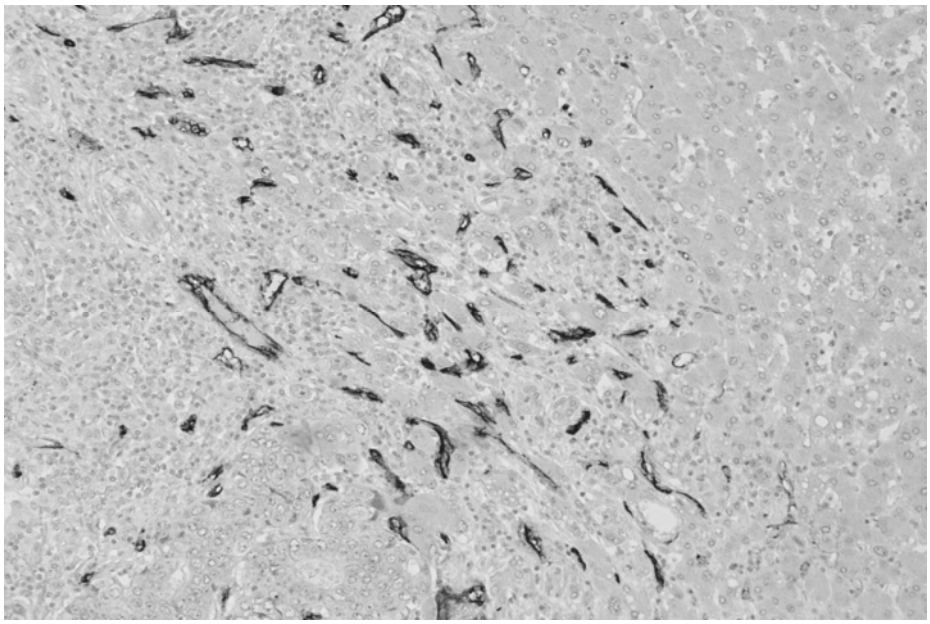


Fig. 2. Relationship between MVC and overall survival in ICC patients who underwent hepatic resection. The solid line shows survival in patients with higher MVC and the dotted line shows survival in patients with lower MVC.

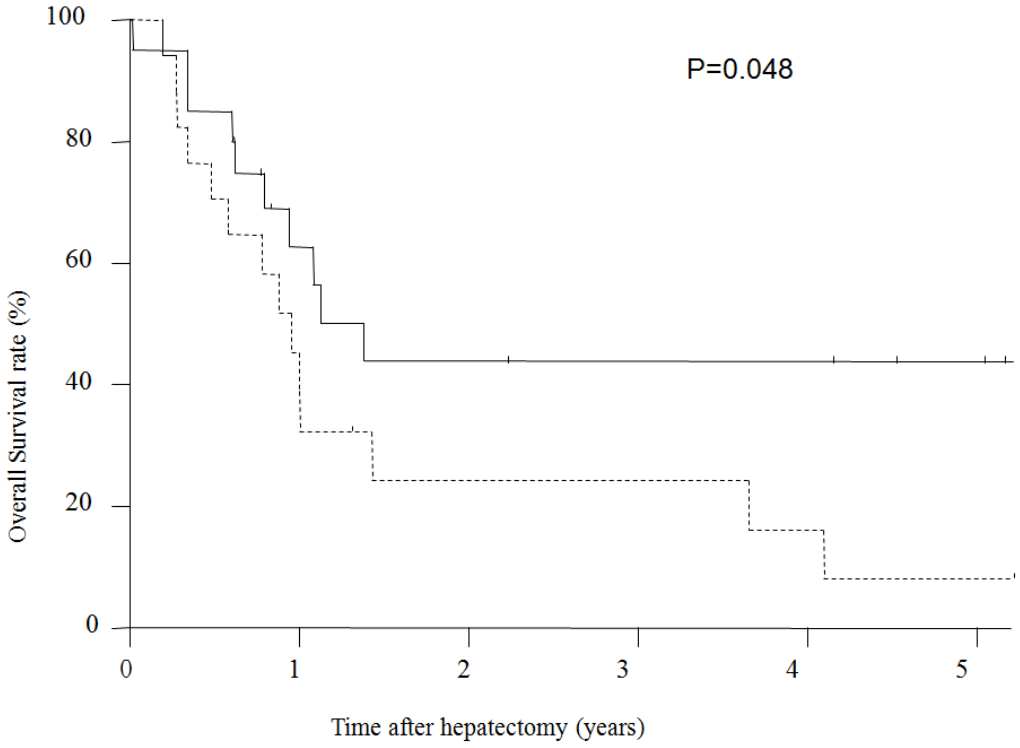


TABLE 1. *Correlations between tumor microvessel counts and clinicopathologic parameters or postoperative recurrence rate in intrahepatic cholangiocarcinomas*

	Microvessel counts		P value
	<140/mm^2 [n=17]	≥140/mm^2 [n=20]	
Gender			
Male	8 (47)	12 (60)	0.65
Female	9 (53)	8 (40)	
Age, ≥60 years	13 (77)	14 (70)	0.73
Chronic viral hepatitis, Yes	4 (24)	10 (50)	0.14
Serum tumor marker			
CEA ≥5 ng/ml	7 (42)	6 (30)	0.73
CA19-9 ≥37 U/ml	11 (65)	13 (65)	0.99
AFP ≥100 ng/ml	1 (6)	0 (0)	0.99
Tumor size, ≥5 cm	15 (88)	9 (45)	0.016
Macroscopic finding [§]			
Mass-forming type	1 (6)	8 (40)	
Peri-ductal infiltrating type	14 (82)	10 (50)	0.049
Intra-ductal growth	2 (12)	2 (10)	
Histological differentiation			
Well	4 (24)	6 (30)	
Moderately	12 (71)	13 (65)	0.67
Poorly	1 (5)	1 (5)	
Intrahepatic metastasis, Yes	1 (5)	3 (15)	0.62
Lymph node metastasis, Yes	8 (47)	10 (50)	0.65
Tumor-node-metastasis stage [¶]			
I	0 (0)	4 (20)	
II	7 (41)	2 (10)	0.027
III	10 (59)	14 (70)	
IV	0 (0)	0 (0)	
Postoperative tumor recurrence, Yes	15 (88)	11 (55)	0.065

Parenthesis shows a percentage.

CEA: carcinoembryonic antigen, CA19-9: carbohydrate antigen19-9, AFP: alpha-feto protein,

[§]Macroscopic classification of intrahepatic cholangiocarcinoma,[24] [¶] Japanese TNM stage by classification of intrahepatic cholangiocarcinoma[24]

TABLE 2. *Relationship between clinicopathological factors and survival rates in intrahepatic cholangiocarcinoma*

	Overall survival rates (5years:%)	P value
Gender		
Male	22	0.27
Female	34	
Age (years)		
>60	20	0.37
≤60	34	
Chronic viral hepatitis		
No	20	0.29
Yes	44	
Preoperative CEA (ng/ml)		
≤10	32	0.008
>10	0	
Macroscopic finding		
MF	39	0.021
PDI	18	
IG	100	
Node status of primary cancer		
No	38	0.11
Yes	0	
Intrahepatic metastasis		
No	26	0.49
Yes	38	
Size of tumor (mm)		
<50	47	0.14
≥50	12	
Histological differentiation		
Well	67	<0.001
Moderately	21	
Poorly	0	
Microvessel counts, CD34(/mm^2)		
<140	7	0.048
≥140	44	
Intraoperative blood loss (ml) ¶		
<1000	38	0.06
≥1000	14	

CEA: carcinoembryonic antigen

MF, mass-forming; IG, intraductal growth; PDI, peri-ductal infiltrating

¶ n=19 in case of blood loss≥1000ml

TABLE 3. Multivariate analysis by Cox's proportional hazard test of prognostic factors influencing overall survival in intrahepatic cholangiocarcinomas after hepatectomy

	Overall survival		
	Risk ratio	95%CI	p
Preoperative CEA level			
<10 ng/ml vs ≥10 ng/ml	1.05	0.36-3.03	0.72
Tumor size			
<5cm vs ≥5cm	1.50	0.63-3.05	0.27
Macroscopic finding			
MF/IG vs PDI	4.77	1.56-14.57	0.006
Pathological differentiation			
Well vs Moderately/Poorly	1.81	0.52-6.34	0.35
Node metastasis of primary cancer			
Negative vs Positive	1.25	0.56-2.78	0.58
Blood loss			
<1000ml vs ≥1000ml	2.07	0.87-4.93	0.10
Microvessel counts by CD34			
≥140mm^2 vs <140mm^2	1.12	0.44-2.82	0.82

MF, mass-forming; IG, intraductal growth; PDI, peri-ductal infiltrating