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An Immunohistochemical Study of Tumor Vascularity and Proliferation Activity in Cholangiocellular Carcinoma: Relationship to Clinicopathologic Factors and Prognosis after Hepatic Resection

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This study was designed to provide an immunohistochemical analysis of tumor biological factors in 28 patients who underwent hepatectomy for cholangiocellular carcinoma (CCC). Analyzed factors were microvessel counts (stained by CD34) and proliferating cell nuclear antigen (PCNA). PCNA L.I. was correlated with serum level of CA19-9, which was correlated with a higher recurrence rate and shorter patient survivals. Microvessel counts were negatively correlated with tumor size. Furthermore, the microvessel count in CCC with mass-forming (MF) plus periductal infiltrating (PI) type associated with poorer survivals, was significantly lower compared to that of CCC with MF type or PI type. Neither microvessel counts nor PCNA L.I. were associated with any other clinicopathologic factors or cancer recurrence. The five-year overall and cancer-free survival rates were 26% and 13%, respectively. Patients with MF plus PI type, poorer differentiated carcinoma, stage 4A and higher CA19-9 level had shorter cancer-free and overall survivals after hepatectomy ($p<0.05$). Cancer-free and overall survivals in patients with lower microvessel counts tended to be slightly worse but were not significantly different. Although tumor microvessel count and proliferating activity were correlated with prognostic clinicopathologic parameters, both factors might not be prognostic markers for predicting CCC recurrence and patient survival.

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Key Words: cholangiocellular carcinoma, hepatic resection, microvessel count, proliferating cell nuclear antigen.

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Introduction

Hepatic resection is the most appropriate option for radical treatment of intrahepatic cholangiocellular carcinoma (CCC). However, the recurrence rate and the rate of cancer-related death after resection remain high.[1] Although the conventional clinicopathological factors such as a tumor morphology in CCC may be related to shorter patient survival,[2,3] accurate prediction of prognosis in CCC is not currently possible. The examination of differences in tumor biological characteristics may provide useful information about the activity of CCC. It has been previously reported that microvessel counts that show angiogenesis and the proliferation activity of cancer cells were significantly related with malignant behavior and poor prognosis in patients with liver cancers.[4－6] A combination of conventional clinicopathologic factors and such tumor biological factors may improve predictions of prognosis after hepatectomy for CCC.

In the present study, we examined both the expression of microvessel counts by using CD34 and the proliferative activity by using proliferating cell nuclear antigen (PCNA) labeling index in CCC using immunohistochemical stains. Using those figures, we investigated the relationship between their expression in CCC, the clinicopathological factors and patient prognosis after hepatic resection.

Materials and Methods

Patients

Specimens of CCC from 28 patients were obtained during surgery on patients who were admitted to the Division of Surgical Oncology, Department of Translational

Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences (the previous First Department of Surgery, Nagasaki University School of Medicine) between January 1994 and December 2002. The mean age of the patients at the time of surgery was 63.4±10.2 years (range: 44-82 years), and they comprised 16 males and 12 females. Prior to surgery for CCC, patients were treated with neither chemotherapy nor radiation. After surgery, two patients (7.1%) received adjuvant oral chemotherapy using 5-fluorouracil analog (UFT™: Tegafur and Uracil at a molar ratio of 1:4, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan). Twenty-three patients (82.1%) had tumor recurrence after hepatectomy. The operative procedures included lobectomy or extended lobectomy (n=20), segmentectomy or subsegmentectomy (n=5) and partial resection (n=3). The study design was approved by the Ethics Review Board of our department and a signed consent was obtained from each patient.

We used the classification system of the *General Rules for the Clinical and Pathological Study Of Primary Liver Cancer.*[7] This system provides a clinicopathological evaluation and macroscopic classification of CCC.

The minimum follow-up period after hepatic resection of CCC was 12 months. Radical hepatectomy was performed to remove the hepatic tumor without leaving any residual tumor. All hepatic tumors were completely resected without macroscopic exposure of the amputated section to the remaining liver.

Resected specimens were fixed in 10% formalin and embedded in paraffin. Thin sections (4μm) were deparaffinized twice by xylene and rehydrated in a series of ethanol solutions (100, 90 and 80%).

Immunohistochemical staining

Sections were placed in 0.01 M trisodium citrate dehydrate buffer (pH 6.0) and treated in a microwave oven for 10 min at 500 watts. For CD34 staining,[5] the tissue sections were digested with 0.2% trypsin in 0.01 M phosphate-buffered saline (PBS) for 20 min at 37℃. In the next step, the 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 either overnight at 4℃ with mouse anti-monoclonal CD34 antibody (1:25 dilution; QB-END/10, Novocastra Laboratories, Newcastle, UK), or for 1 hour at room temperature with monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:100; PC-10, Dako Co., Carpinteria, CA) as the primary antibody, respectively. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using labeled streptavidin-biotin (LSAB) kit® Peroxidase (Dako). The peroxidase reaction was visualized with 0.01% H_2O_2 and 3,3'-diaminobenzidine using a light microscope (x200).

Evaluation and statistical analysis

The average percentage of positive nuclear PCNA expression in 1,000 nuclei in five areas of the tumor was estimated. With the microvessel count using CD34 staining, we determined the average count in the five most vascular areas examined at x200 magnification.[5] CD34 expression was evaluated in the surrounding areas of tumor. In the periductal-infiltrating (PI), the mass forming (MF) and the intraductal growth (IG) type,[7] the stained cells were counted in the marginal area of tumor. In the MF plus PI type, the stained cells were counted in the marginal area of MF tumor.

For the univariate analysis, categorical data were analyzed by the chi-square test or Fisher's exact test. The disease-free interval and the overall survival were calculated according to the Kaplan-Meier method, and the differences between the groups were tested for significance using the log-rank test. The continuous variables, such as the microvessel count and PCNA expression, were classified into two groups based on the median values for analyzing patient survivals. A two-tailed P value of < 0.05 was considered significant. Statistical analyses were performed with using the computer software STATISTICA™ (StatSoft, Tulsa, OK).

Results

Relationship between expression of CD34 and PCNA labeling index (L.I.), and clinicopathological features

The median values of microvessel counts by using CD34 in surrounding tissues of the tumor area were 106±33/mm^3. The median values of PCNA in CCC cells were 14.9±7.8%. Table 1 shows the relationship between the expression of these molecules and the clinicopathological features in CCC. The microvessel count and PCNA L.I. were not correlated with patient age. The recurrence rate of patients with the higher CA19-9 levels (>100 U/ml) (14/14; 100%) was significantly higher than that of the patients with the lower CA19-9 levels (8/14; 57%) (p=0.021). PCNA L.I. was positively correlated with a serum level of CA19-9. The microvessel count was negatively correlated with tumor size. Both microvessel counts and PCNA L.I

were correlated with neither liver function tests nor the degree of hepatic fibrosis.

In these patients, the recurrence rate in patients with the PI type, and the MF plus PI type (3/3; 100% and 14/14; 100%, respectively) was significantly higher than that in patients with the MF type or the IG type (6/9; 67% and 0/2;0%, respectively) (p=0.0024). The microvessel count in CCC with MF plus PI type was significantly lower than that of CCC with MF type or PI type (Table 2). However, neither the microvessel count nor PCNA L.I. were associated with any other clinicopathologic factors and cancer recurrence.

Table 1. Correlation between microvessel counts and proliferating activity in the tumor , and clinicopathological factors in cholangiocellular carcinomas.

	CD34 (/mm^3) [a]	PCNA (%) [b]
Age	-0.184	0.386
CEA (ng/ml) [c]	-0.177	-0.210
CA19-9 (U/ml) [d]	0.066	0.502 [i]
Tumor size (cm)	-0.582 [h]	0.262
ICG R15 (%) [e]	0.320	-0.078
LHL 15 [f]	-0.179	0.279
Fibrotic score [g]	0.301	-0.300

[a]Microvessel counts in the surrounding tissue of CCC, [b]Proliferating activity in CCC, [c] carcinoembryonic antigen, [d]sialyl Lewis[a] antigen, [e]indocyanine green retention rate at 15 minutes, [f]liver activity at 15 minutes by technetium-99m galactosyl human serum albumin scintigraphy, [g] Staging score for hepatic fibrosis defined by Knodell et al.,[24] [h] p=0.0014, [i]p=0.034

Table 2. Relationship between microvessel counts and proliferating activity, and clinicopathologic factors in cholangiocarcinoma.

	CD34 (/mm^3)	PCNA (%)
Gender		
Male (n=16)	114±32	18.3±5.1
Female (n=12)	92±29	14.7±4.6
Macroscopic classification [a]		
Intraductal growth type (n=2)	113±33	16.5±2.7
Mass-forming type (MF) (n=9)	125±97	14.9±6.4
Periductal infiltrating type (PI) (n=3)	139±35	10.4±8.1
MF+PI type (n=14)	86±25 [c]	15.6±9.1
Intrahepatic metastases		
No (n=18)	103±29	16.3±7.3
Yes (n=10)	103±34	11.8±9.3
Lymph ode metastases		
No (n=15)	102±32	15.0±8.9
Yes (n=13)	108±34	14.9±7.4
Serosal invasion		
No (n=18)	106±30	13.2±8.6
Yes (n=10)	104±38	17.2±6.3
Histopathological differentiation		
Well (n=6)	116±31	13.1±8.1
Moderate-poor (n=22)	103±36	15.8±8.2
Pathological TNM stage [b]		
I, II (n=4)	125± 7	15.1± 4.8
III (n=8)	100±32	14.5± 9.1
IVA (n=3)	69±13	16.8± 5.5
IVB (n=13)	108±33	14.9± 7.4
Recurrence		
No (n=5)	119±23	13.7± 8.4
Yes (n=23)	103±34	15.3± 7.9

[a] *Classification of Primary Liver Cancer* [7], [b] the *General Rules for the Clinical and Pathological Study Of Primary Liver Cancer* [7] [c] p=0.008 vs. MF or PI type

Relationship between biological factors in CCC and survival after hepatic resection

Among 28 patients in the present study, the cancer-free 1-, 3- and 5-year cancer-free survival rates were 41%, 26% and 26%, respectively, and the median cancer-free survival period was 2.1 years. The overall 1-, 3- and 5-year survival rates were 48%, 29% and 13%, respectively, and the median overall survival period was 1.8 years. By the univariate analysis, patients with PI type and MF plus PI type by the macroscopic classification, poorer differentiated carcinoma, stage 4A and higher CA19-9 level (>100 U/ml) had the shorter cancer-free and overall survivals after hepatectomy in the present series (p<0.05). Figure 1 shows the cancer-free and overall patient survivals after hepatectomy for each parameter. The cancer-free and overall survivals in patients with lower microvessel counts tended to be slightly worse but were not significant

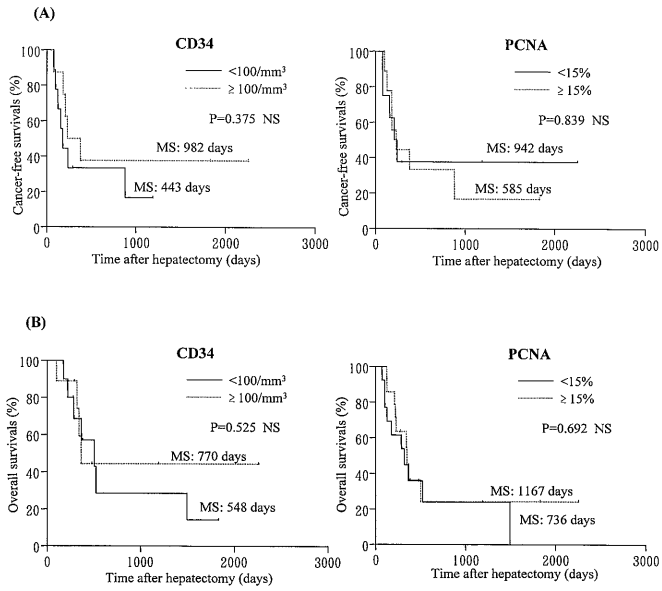

Figure 1. Cancer-free (A) and overall (B) survivals for patients who underwent hepatic resection for cholangiocellular carcinomas. Relationship between microvessel counts and proliferation activity of the tumor, and survivals. MS: Mean survival period.

differences. PCNA L.I. was associated with neither cancer-free nor overall survivals.

Discussion

Patient prognosis in cholangiocarcinoma is quite poor because of the tumor aggressiveness and high incidence of lymph node metastases.[1,8,9] The rate of curative surgical resection is low and patient survival after hepatectomy is still poor.[2,7,8,10] By questionnaire investigation at the conference of Nagasaki Hepato-Biliary-Pancreas surgery (Nagasaki Kan-Tan-Sui Geka Kenkyukai, Nagasaki, 1997), the median survival period after hepatic resection in 56 Nagasaki patients with CCC was 18.2 months and the 3- and 5-year cumulative survival rates were 10% and 4%, respectively. According to the recent reports, the UICC's tumor-node-metastasis (TNM) classification system, surgical margin, serum CA19-9 level, histological differentiation and macroscopic classification are related with prognosis in CCC patients who undergo radical hepatectomy at present.[10-13]

The recent studies of CCC have revealed the candidate tumor biological factors by using advanced methods for detecting genetic abnormalities such as DNA ploidy pattern,[14] the reduced expression of keratin 903[15] or p27[16], and the overexpression of MUC-1[17], MMP-7[18] or cyclin D1.[19] We believe that clarifying the relationship between such factors and patient prognosis should contribute to future classification criteria for CCC. In the present study, we performed an immunohistochemical study using two markers: microvessel count (as a marker of angiogenesis) and PCNA labeling index (as a marker of proliferation) on the basis of previous studies on other solid tumors.[4-6,20,21] These markers were closely associated with tumor aggressiveness. From the results of metastatic liver tumors in our previous studies, the microvessel count was very a reliable parameter in predicting patient prognosis.[5] Aggressive cancers may have higher proliferating activities. To our knowledge, these parameters concerning patient survival have not been fully examined in CCC.

In the present study, the microvessel counts of CCC in larger tumors were lower. CCC usually shows marginal enhancement and central hypo-vascularity on enhanced imaging. With an increase in tumor size, the fibrous tissue components become increased in the tumor and, therefore, vascularity may reduce. The microvessel counts in CCC with MF plus PI type were lower compared to other types. Previous reports and the present results showed that patient survivals with CCC showing MF plus PI type are significantly poor.[10] Therefore, despite the fact that its mechanism remains unknown, the reduced vascularity of CCC may be associated with tumor aggressiveness. We speculate that hypovascular tumors may be resistant to the anticancer drugs or host immune responses. In our preliminary study, hepatocellular carcinoma with lower microvessel count was also associated with poor patient survival by multivariate analysis (submitted but unpublished). The PCNA labeling index (L.I.) of carcinomas was correlated with serum CA19-9 level in the present study. CA19-9 is a sensitive marker for pancreatic and biliary carcinomas, which reflect the malignant behavior of tumors.[22] Serum CA19-9 level in patients with CCC may be correlated with tumor proliferation activity. Ohashi et al. reported that PCNA L.I. in periductal- or specula-forming type of CCC was significantly higher than that in mass-forming type.[23] They suggested that macroscopic classification of CCC reflect the proliferating activity or invasiveness of the tumor. In the present study, there was no significant difference of PCNA L.I. between each macroscopic type. PCNA L.I. in CCC remained the same from the early to the advanced stages of tumor and, therefore, tumor cells of CCC might already have malignant potential at the early stage.

With respect to patient survival after hepatectomy,[1,2,6,7] the results of our series were similar. The previous studies showed higher TNM stage,[1,12,13] tumor except intraductal growth type,[2,5,13] higher CA19-9 level,[11] less differentiation[12] and less curability of surgical resection[5,7,11,12] to be associated with poor survival in patients with CCC, in agreement with our present series. Many tumors showing MF or PI type revealed the higher tumor stage, although some patients with stage IV had longer survival.[2,7] It is thus necessary to clarify cellular characteristics showing tumor aggressiveness except conventional clinicopathologic parameters by the new approach. Our results showed that the mean survival period in patients with lower microvessel counts tended to be slightly shorter but the difference was not statistically significant. Although this factor might have been slightly associated with tumor aggressiveness and patient survival, the roles of tumor vascularity were not predominant. The pathogenesis of CCC is complicated and the various biological factors could have important relations with malignant behavior of CCC. A future study using other biological markers should be expected to clarify the aggressiveness of CCC.

In conclusion, we have demonstrated that hypovascularity of CCC was associated with tumor size and macroscopically invasive type while, on the other hand, proliferation

activity of CCC was correlated with serum CA19-9 level. Neither vascularity nor proliferation activity of CCC were significantly associated with the prognosis of patients undergoing hepatic resection. Determination of other biological factors will be necessary for evaluating the prognosis of patients who undergo surgical resection of cholangiocarcinoma in the next step.

References

1. Valverde A, Bonhomme N, Farges O, Sauvanet A, Flejou JF, Belghiti J: Resection of intrahepatic cholangiocarcinoma: a Western experience. *J Hepatobiliary Pancreat Surg* 6: 122-7, 1999
2. Isaji S, Kawarada Y, Taoka H, Tabata M, Suzuki H, Yokoi H: Clinicopathological features and outcome of hepatic resection for intrahepatic cholangiocarcinoma in Japan. *J Hepatobiliary Pancreat Surg* 6: 108-16, 1999
3. Hirohashi K, Uenishi T, Kubo S, Yamamoto T, Tanaka H, Shuto T, Kinoshita H: Macroscopic types of intrahepatic cholangiocarcinoma: clinicopathologic features and surgical outcomes. *Hepatogastroenterology* 49: 326-9, 2002
4. Sun HC, Tang ZY, Li XM, Zhou YN, Sun BR, Ma ZC: Microvessel density of hepatocellular carcinoma: its relationship with prognosis. *J Cancer Res Clin Oncol* 125: 419-26, 1999
5. Nanashima A, Yamaguchi H, Sawai T, Yamaguchi E, Kidogawa H, Matsuo S, Yasutake T, Tsuji T, Jibiki M, Nakagoe T, Ayabe H: Prognostic factors in hepatic metastases of colorectal carcinoma: immunohistochemical analysis of tumor biological factors. *Dig Dis Sci* 46: 1623-8, 2001
6. Suehiro T, Matsumata T, Itasaka H, Yamamoto K, Kawahara N, Sugimachi K: Clinicopathologic features and prognosis of resected hepatocellular carcinomas of varied sizes with special reference to proliferating cell nuclear antigen. *Cancer 1*; 76: 399-405, 1995
7. The General Rules for the Clinical and Pathological Study of Primary Liver Cancer (2000) Liver cancer study group of Japan. The Fourth ed. Kanehara & Co., Ltd., Tokyo 11-34
8. Shimada M, Yamashita Y, Aishima S, Shirabe K, Takenaka K, Sugimachi K: Value of lymph node dissection during resection of intrahepatic cholangiocarcinoma. *Br J Surg* 88: 1463-6, 2001
9. Madariaga JR, Iwatsuki S, Todo S, Lee RG, Irish W, Starzl TE: Liver resection for hilar and peripheral cholangiocarcinomas: a study of 62 cases. *Ann Surg* 227: 70-9, 1998
10. Yamamoto M, Takasaki K, Yoshikawa T, Ueno K, Nakano M: Does gross appearance indicate prognosis in intrahepatic cholangiocarcinoma? *J Surg Oncol* 69: 162-7, 1998
11. Ohtsuka M, Ito H, Kimura F, Shimizu H, Togawa A, Yoshidome H, Miyazaki M: Results of surgical treatment for intrahepatic cholangiocarcinoma and clinicopathological factors influencing survival. *Br J Surg* 89: 1525-31, 2002
12. Kawarada Y, Yamagiwa K, Das BC: Analysis of the relationships between clinicopathologic factors and survival time in intrahepatic cholangiocarcinoma. *Am J Surg* 183: 679-85, 2002
13. Hanazaki K, Kajikawa S, Shimozawa N, Shimada K, Hiraguri M, Koide N, Adachi W, Amano J: Prognostic factors of intrahepatic cholangiocarcinoma after hepatic resection: univariate and multivariate analysis. *Hepatogastroenterology* 49: 311-6, 2002
14. Abou-Rebyeh H, Al-Abadi H, Jonas S, Rotter I, Bechstein WO, Neuhaus P: DNA analysis of cholangiocarcinoma cells: prognostic and clinical importance. *Cancer Detect Prev* 26: 313-9, 2002
15. Aishima S, Asayama Y, Taguchi K, Sugimachi K, Shirabe K, Shimada M, Sugimachi K, Tsuneyoshi M: The utility of keratin 903 as a new prognostic marker in mass-forming-type intrahepatic cholangiocarcinoma. *Mod Pathol* 15: 1181-90, 2002
16. Taguchi K, Aishima S, Asayama Y, Kajiyama K, Kinukawa N, Shimada M, Sugimachi K, Tsuneyoshi M: The role of p27kip1 protein expression on the biological behavior of intrahepatic cholangiocarcinoma. *Hepatology* 33: 1118-23, 2001
17. Matsumura N, Yamamoto M, Aruga A, Takasaki K, Nakano M: Correlation between expression of MUC1 core protein and outcome after surgery in mass-forming intrahepatic cholangiocarcinoma. *Cancer* 94: 1770-6, 2002
18. Miwa S, Miyagawa S, Soeda J, Kawasaki S: Matrix metalloproteinase-7 expression and biologic aggressiveness of cholangiocellular carcinoma. *Cancer* 94: 428-34, 2002
19. Sugimachi K, Aishima S, Taguchi K, Tanaka S, Shimada M, Kajiyama K, Sugimachi K, Tsuneyoshi M: The role of overexpression and gene amplification of cyclin D1 in intrahepatic cholangiocarcinoma. *J Hepatol* 35: 74-9, 2001
20. Sternfeld T, Foss HD, Kruschewski M, Runkel N: The prognostic significance of tumor vascularization in patients with localized colorectal cancer. *Int J Colorectal Dis* 14: 272-6, 1999
21. Rugge M, Sonego F, Pollice L, Perilongo G, Guido M, Basso G, Ninfo V, Pennelli N, Gambini C, Guglielmi M, Fabiano A, Leandro G, Keeling JW: Hepatoblastoma: DNA nuclear content, proliferative indices, and pathology. *Liver* 18: 128-33, 1998
22. Yamaguchi K, Enjoji M, Tsuneyoshi M: Pancreatoduodenal carcinoma: a clinicopathologic study of 304 patients and immunohistochemical observation for CEA and CA19-9. *Surg Oncol* 47: 148-54, 1991
23. Ohashi K, Nakajima Y, Kanehiro H, Nakano H: Evaluation of proliferating activity of intrahepatic cholangiocarcinoma using proliferating cell nuclear antigen staining. (Short communication in Japanese) *Geka Chiryou* 70: 352-353, 1994
24. Knodell RG, Ishak KG, Black WC: Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1: 431-5, 1981