TABLE 1. *Comparison of patients' characteristics based on microvascular invasion (MVI)*

Characteristic	MVI		*P* value
	Present (n = 49)	Absent (n = 61)	
Sex (M/F)	40/9	50/11	.964
Age (y)	61.5 ± 11.3	63.4 ± 9.87	.336
Cause (HCV/HBV/both negative)	26/23	31/30	.579
Background liver (normal or CH/cirrhosis)	26/23	31/30	.815
Bilirubin (mg/dL)	.8 ± .4	.9 ± .4	.302
Albumin (g/dL)	4.0 ± .4	3.9 ± .4	.286
Prothrombin time (%)	91.5 ± 11.8	88.0 ± 12.7	.149
Prothrombin time (%)	17.6 ± 10.9	19.5 ± 10.9	.333
AFP (ng/mL) (≤100/>100)	28/21	43/18	.210
DCP (AU/mL) (≤100/>100)	29/20	45/16	.156
Maximum tumor size (mm)	30.8 ± 9.56	26.0 ± 9.2	.009
No. tumors (single/≥2)	36/13	49/12	.394
Gross classification (VN + SN/SNEG + CMN)	12/37	50/11	<.0001
Histological grade (well/moderate/poor)	15/34	2/59	.033
Intrahepatic micrometastasis (present/absent)	15/34	2/59	<.0001
Cell encapsulation (present/absent)	35/14	42/19	.770
Type of surgical resection (lobectomy + segmentectomy/subsegmentectomy + partial hepatectomy)	27/22	36/35	.637

HCV, hepatitis C virus; HBV, hepatitis B virus; CH, chronic hepatitis; $ICGR_{15}$, indocyanine green retention at 15 minutes; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; VN, vaguely nodular type; SN, single nodular type; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

DCP levels, and Child-Pugh scores were measured, and ultrasonography was performed monthly. Contrast-enhanced dynamic CT was performed every 3 months until 6 months after treatment and every 6 months thereafter. MRI was performed as a supplemental examination. The closing date of this study was December 2006 or the date of the patient's death. Follow-up ranged from 8 to 130 months (median, 47 months). Of the 110 patients, 27 patients died during follow-up. Of these 27 patients, 23 patients died from HCC-related causes.

Statistical Analysis

All data are expressed as mean ± standard deviation. Comparisons between the two groups were performed by the Mann-Whitney *U*-test for continuous variables, and the χ^2 test or the Fisher exact test for discrete variables. The multiple logistic regression model was used to identify factors related to MVI. Survival curves were constructed by the Kaplan-Meier method, and curves were compared by the log rank test. A Cox proportional hazard stepwise model was used for univariate and multivariate analysis to identify any independent variables that were related to survival. All *P* values were two-tailed, and a level of <.05 was considered to be statistically significant. Statistical analysis was performed by SPSS software (SPSS, Chicago, IL).

TABLE 2. *Independent predictor of microvascular invasion*

Predictor	HR (95% CI)	*P* value
AFP (ng/mL) (>100)	1.25 (.44–3.57)	.679
DCP (AU/mL) (>100)	1.31 (.45–3.86)	.621
Maximum tumor size (mm) (>30)	1.77 (.57–5.43)	.321
No. of tumors (≥2)	2.19 (.67–7.18)	.195
Gross classification (SNEG + CMN)	11.81 (3.93–37.80)	<.0001
Histological grade (poor)	2.31 (.38–14.14)	.364
Intrahepatic micrometastasis (present)	3.32 (.58–18.97)	.178
Cell encapsulation (absent)	1.73 (.57–5.26)	.337

HR, hazard ratio; 95% CI, 95% confidence interval; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

RESULTS

Clinicopathological Characteristics Predictive of MVI

Of the 110 resected specimens, 49 (45%) had evidence of MVI. Comparisons of patient clinicopathological characteristics according to presence or absence of MVI are shown in Table 1. Baseline variables, including age, sex, etiology, background liver function, and serum AFP and DCP levels, were similar in both groups. In addition, there was no difference in surgical procedures between the two groups.

Of the pathological features, the present of cell encapsulation and the number of tumors were not

TABLE 3. *Univariate and multivariate analyses of recurrence-free survival for hepatocellular carcinoma*

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex (M)	.90 (.46–1.79)	.770		
Age (>65 years)	1.17 (.70–1.96)	.540		
HCV (positive)	1.15 (.66–1.98)	.622		
HBV (positive)	.96 (.54–1.73)	.902		
Background liver (cirrhosis)	1.65 (.99–2.76)	.056	1.79 (1.05–3.06)	.032
$ICGR_{15}$ (%)	1.26 (.75–2.13)	.380		
AFP (ng/mL) (>100)	1.17 (.69–2.21)	.556		
DCP (AU/mL) (>100)	1.28 (.75–2.19)	.359		
Maximum tumor size (mm) (>30)	1.28 (.76–2.15)	.362		
No. of tumors (≥2)	1.79 (1.02–3.16)	.044		
Gross classification (SNEG + CMN)	1.98 (1.19–3.32)	.009		
Histological grade (poor)	1.23 (.56–2.72)	.601		
Microvascular invasion (present)	2.75 (1.62–4.67)	.0002	2.97 (1.69–5.24)	.0002
Intrahepatic micrometastasis (present)	2.14 (1.13–4.04)	.020		
Cell encapsulation (absent)	1.02 (.59–1.74)	.958		
Type of surgical resection (subsegmentectomy + partial hepatectomy)	1.59 (.95–2.65)	.076		

HR, Hazard ratio; 95% CI, 95% confidence interval; HCV, hepatitis C virus; HBV, hepatitis B virus; CH, chronic hepatitis; $ICGR_{15}$, indocyanine green retention at 15 minutes; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

significantly associated with MVI. The maximum tumor size of patients with MVI was larger than those without MVI ($P = .009$). None of the patients with well-differentiated HCC had MVI, in contrast to the 41 (43%) and 8 (73%) patients with moderately and poorly differentiated HCC, respectively ($P = .033$). IM was evident in 17 (15%) of the 110 patients enrolled, and the presence of IM was significantly associated with MVI ($P < .0001$). In addition, the gross classifications of SNEG type (21 tumors, 72%) and CMN type (16 tumors, 84%) showed MVI more frequently than those of VN type (0%) and SN type (12 tumors, 20%) ($P < .0001$).

Multiple logistic regression analysis was performed to identify which of eight variables was independently associated with MVI (Table 2). The gross classifications of SNEG and CMN types were independent predictors of MVI (hazard ratio [HR], 11.81; 95% confidence interval [95% CI], 3.93–37.80; $P < .0001$).

Predictors of Recurrence-Free Survival After Hepatic Resection

Cox proportional hazard regression analysis was performed to identify independent predictors of recurrence-free survival (Table 3). The results of univariate analysis show that number of tumors (two or more; $P = .044$), gross classification (SNEG + CMN type; $P = .009$), presence of MVI ($P = .0002$), and presence of IM ($P = .02$) were found to be significant risk factors affecting recurrence-free survival. By multivariate analysis, liver cirrhosis (HR, 1.79; 95% CI, 1.05–3.06; $P = .032$) and presence of MVI (HR, 2.97; 95% CI, 1.69–5.24; $P = .0002$) were identified as independent predictors of recurrence-free survival. Recurrence-free survival curves of patients with and without MVI are shown in Fig. 1. The recurrence-free survival of patients with MVI was significantly shorter compared with survival of patients without MVI ($P = .0001$). During the follow-up period, tumor recurrence developed in 59 patients (54%), consisting of 31 patients (63%) with MVI and 28 patients (46%) without MVI. As the pattern of recurrence in 31 patients with MVI, 24 patients (77%) showed intrahepatic recurrence near

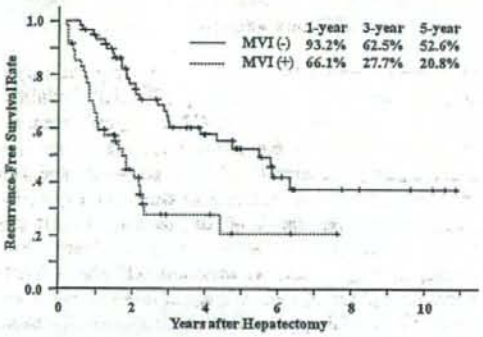

FIG. 1. Comparison of recurrence-free survival rates in patients with and without microvascular invasion (MVI). The 1-, 3-, and 5-year recurrence-free survival rates are shown. The recurrence-free survival of patients with MVI was significantly shorter compared with survival of patients without MVI ($P = .0001$).

TABLE 4. *Univariate and multivariate analyses of disease-specific survival for hepatocellular carcinoma*

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex (M)	1.08 (.39–2.95)	.885		
Age (>65 years)	.95 (.42–2.16)	.902		
HCV (positive)	1.07 (.45–2.52)	.881		
HBV (positive)	1.53 (.62–3.77)	.356		
Background liver (cirrhosis)	1.97 (.83–4.67)	.126		
$ICGR_{15}$ (%)	1.35 (.57–3.20)	.495		
AFP (ng/mL) (>100)	2.19 (.96–4.97)	.061		
DCP (AU/mL) (>100)	1.10 (.46–2.60)	.835		
Maximum tumor size (mm) (>30)	1.24 (.54–2.83)	.613	2.93 (1.03–8.29)	
No. of tumors (≥2)	2.19 (.89–5.40)	.088		.043
Gross classification (SNEG + CMN)	2.99 (1.23–7.26)	.016		
Histological grade (poor)	2.09 (.61–7.22)	.243		
Microvascular invasion (present)	3.88 (1.65–9.13)	.002	3.51 (1.27–9.74)	.016
Intrahepatic micrometastasis (present)	3.53 (1.36–9.16)	.009	3.28 (1.04–10.34)	.043
Cell encapsulation (absent)	1.00 (.41–2.47)	.997		
Type of surgical resection (subsegmentectomy + partial hepatectomy)	1.31 (.57–3.03)	.531		

HR, hazard ratio; 95% CI, 95% confidence interval; HCV, hepatitis C virus; HBV, hepatitis B virus; CH, chronic hepatitis; $ICGR_{15}$, indocyanine green retention at 15 minutes; AFP, alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin; SNEG, single nodular with extranodular growth type; CMN, confluent multinodular type.

to the resected tumor or in the same lobe of the resected tumor, and four patients (13%) had extrahepatic recurrence (two peritoneum, one lung, and one bone).

Predictors of Disease-Specific Survival After Hepatic Resection

Cox proportional hazard regression analysis was performed to identify independent predictors of recurrence-free survival (Table 4). The results of univariate analysis show that gross classifications (SNEG + CMN types; $P = .016$), presence of MVI ($P = .002$), and presence of IM ($P = .009$) were found to be significant risk factors affecting disease-specific survival. By multivariate analysis, number of tumors (two or more; HR, 2.93; 95% CI, 1.03–8.29; $P = .043$), presence of MVI (HR, 3.51; 95% CI, 1.27–9.74; $P = .016$), and presence of IM (HR, 3.28; 95% CI, 1.04–10.34; $P = .043$) were identified as independent predictors of disease-specific survival. Disease-specific survival curves of patients with and without MVI are shown in Fig. 2. The short-term survival of patients with MVI was significantly shorter compared with those of patients without MVI ($P = .001$).

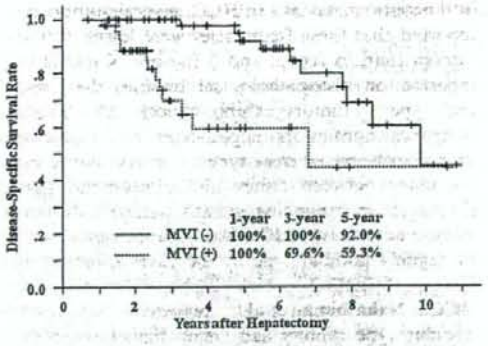

FIG. 2. Comparison of disease-specific survival rates in patients with and without microvascular invasion (MVI). The 1-, 3-, and 5-year disease-specific survival rates are shown. The short-term survival of patients with MVI was significantly shorter compared with those of patients without MVI ($P = .001$).

DISCUSSION

In this study, we show that the presence of MVI is the most statistically significant independent risk factor affecting recurrence-free and disease-specific survival in patients with HCC after curative resection. Therefore, our results suggest that when determining a treatment plan for a patient with HCC, it is important to detect the presence of MVI as soon as possible before hepatic resection. However, it is difficult to diagnose MVI even with the most recent diagnostic imaging procedures, such as ultrasonography, CT, and MRI because it is a histopathological finding.

Several studies of HCC patients who underwent orthotopic liver transplantation have shown that MVI was strongly associated with tumor size and histological grade.[22,23] Adachi et al.[24] reported that tumors >3 cm and high histological grades were

strong predictors of microscopic portal venous invasion by histological analysis of resected HCC. Similarly, in our study, univariate analysis showed that tumor size and histological grade were associated with MVI. However, in the small HCCs (<3 cm) that are frequently detected by recent advanced imaging procedures, it is difficult to predict the presence of MVI by imaging findings before hepatic resection.

We showed by univariate and multivariate analyses that gross classification of HCC was a strongly independent predictor of MVI. Kanai et al.[21] proposed a new gross classification that divides the Eggel's nodular type HCC into the three groups on the basis of patterns of tumor growth and extension, as follows: single nodular type (type 1), single nodular with extranodular growth type (type 2), and confluent multinodular type (type 3). This classification of nodular type HCC has been accepted by the Liver Cancer Study Group of Japan[20] and is widely used in Japan. As to the incidence of portal vein invasion and intrahepatic metastasis in HCC, several authors have reported that these frequencies were lower in type 1 tumors than in type 2 and 3 tumors. Kanai et al.[21] reported on histopathological findings that type 2 and type 3 tumors exhibit cancer cells invading peripheral nontumoral hepatocytes in a replacement growth pattern, whereas type 1 tumors show a clear boundary between tumor and nontumoral parenchyma in an expanding growth pattern. Moreover, single nodular type HCC has a subcategory defined as vaguely nodular type by the Liver Cancer Study Group of Japan,[20] which is characterized as early HCC. Nakashima et al.[25] reported that vaguely nodular type tumors had small diameters, most of them consisted solely of well-differentiated HCC tissues, and did not have portal vein invasion or intrahepatic metastasis. Similarly, in this study, all three cases of VN type HCC were histologically well-differentiated HCC and did not have MVI. Thus, our results supported the correlation between MVI and gross classification of HCC. Gross classification can be mostly determined by combining preoperative imaging modalities such as contrast-enhanced ultrasonography and dynamic or angio-CT. Therefore, gross classification may be helpful in predicting MVI even if the tumor is small.

Although surgical resection has been one of the mainstays in the curative treatment for HCC, the incidence of fatal recurrence is high.[3,9] After curative resection for HCC, two patterns of recurrence are primarily observed: multicentric occurrence and intrahepatic metastasis. Poon et al.[5] reported that early recurrences seem to arise mainly from intrahepatic metastasis, whereas late recurrences are more likely to be multicentric in origin. In addition, they showed by multivariate analysis that vascular invasion is an independent risk factor for early recurrence, and cirrhosis is an important risk factor for late recurrence. Similarly, in this study, cirrhosis and MVI were independent risk factors associated with disease-free survival after curative resection of HCC.

The presence of vascular invasion is consistently reported as strongly predictive of intrahepatic metastasis,[26,27] as also shown in this study. Moreover, our results showed that intrahepatic micrometastases occurred in approximately 31% of HCC patients with MVI. These findings suggest that cancer cell spreading via the portal vein is main mechanism for such intrahepatic metastasis. Several other authors have also demonstrated that early recurrence after curative resection is observed with high frequency in HCC with MVI.[6-8,18,28] Therefore, before treating a patient with newly diagnosed HCC, it is important to predict whether MVI and IM are present because of their highly malignant potential.

Irrespective of factors such as MVI in the resected primary tumor, multicentric occurrence of HCC is also caused by the background condition of the liver. Cirrhosis, in which premalignant lesions such as adenomatous hyperplasia frequently occur, is known to have high carcinogenic potential.[29,30] In addition, previous studies have shown that the incidence of multicentric HCC is higher in liver cirrhosis patients with HCV infection than in cirrhosis patients with other factors, including hepatitis B virus infection.[31,32] Our study included 74 patients (67%) with HCV infection.

In this study, patients with MVI experienced disease recurrence far more frequently after hepatic resection for HCC than patients without MVI. However, it has been suggested that our results may include detection bias: it is possible that both the factors of multicentric occurrence and intrahepatic metastasis caused the recurrence of HCC.

It has been generally known that recurrence of HCC is the major risk factor affecting survival after hepatic resection.[33,34] Especially, it is thought that the survival in the HCC patients with early recurrence is influenced by primary tumor factors that affect intrahepatic metastasis.[5,35] In this study, factors with high malignant potential, such as number of tumors, IM, and MVI, were independent risk factors affecting survival. Moreover, among these factors, the presence of MVI was found to be the most strongly prognostic factor. Several authors have reported an association between MVI and survival,[16,17] and

Kondo et al.[18] demonstrated that MVI was a statistically significant predictor for early recurrence and early death within 2 years after hepatectomy in patients with a solitary HCC. Similarly, in this study, the 3-year recurrence-free and disease-specific survivals of patients with MVI were far shorter compared with those of patients without MVI. Thus, the presence of MVI may play an important role in short-term survival after hepatic resection.

In conclusion, our study found that the presence of MVI was the strongest predictor for recurrence and survival in HCC patients after curative resection. This result suggests that it is important to predict the presence of MVI before hepatic resection for help in determining treatment strategies. Furthermore, gross classification of HCC can be mostly helpful in predicting the presence of MVI.

REFERENCES

1. Takayama T, Makuuchi M, Hirohashi S, et al. Early hepatocellular carcinoma as an entity with a high rate of surgical cure. *Hepatology* 1998; 28:1241–6.
2. Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2004; 130:417–22.
3. Nagasue N, Uchida M, Makino Y, et al. Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma. *Gastroenterology* 1993; 105:488–94.
4. Yang Y, Nagano H, Ota H, et al. Patterns and clinicopathologic features of extrahepatic recurrence of hepatocellular carcinoma after curative resection. *Surgery* 2007; 141:196–202.
5. Poon RT, Fan ST, Ng IO, et al. Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. *Cancer* 2000; 89:500–7.
6. Shah SA, Greig PD, Gallinger S, et al. Factors associated with early recurrence after resection for hepatocellular carcinoma and outcomes. *J Am Coll Surg* 2006; 202:275–83.
7. Portolani N, Coniglio A, Ghidoni S, et al. Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. *Ann Surg* 2006; 243:229–35.
8. Shirabe K, Kanematsu T, Matsumata T, et al. Factors linked to early recurrence of small hepatocellular carcinoma after hepatectomy: univariate and multivariate analyses. *Hepatology* 1991; 14:802–5.
9. Harada T, Shigemura T, Kodama S, et al. Hepatic resection is not enough for hepatocellular carcinoma. A follow-up study of 92 patients. *J Clin Gastroenterol* 1992; 14:245–50.
10. Park JH, Koh KC, Choi MS, et al. Analysis of risk factors associated with early multinodular recurrences after hepatic resection for hepatocellular carcinoma. *Am J Surg* 2006; 192:29–33.
11. Liver Cancer Study Group of Japan Primary liver cancer in Japan. Clinicopathologic features and results of surgical treatment. *Ann Surg* 1990; 211:277–87.
12. Izumi R, Shimizu K, Ii T, et al. Prognostic factors of hepatocellular carcinoma in patients undergoing hepatic resection. *Gastroenterology* 1994;106:720–7.
13. Otto G, Heuschen U, Hofmann WJ, et al. Survival and recurrence after liver transplantation versus liver resection for hepatocellular carcinoma: a retrospective analysis. *Ann Surg* 1998; 227:424–32.
14. Jonas S, Bechstein WO, Steinmuller T, et al. Vascular invasion and histopathologic grading determine outcome after liver transplantation for hepatocellular carcinoma in cirrhosis. *Hepatology* 2001; 33:1080–6.
15. Llovet JM, Bustamante J, Castells A, et al. Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. *Hepatology* 1999; 29:62–7.
16. Tsai TJ, Chau GY, Lui WY, et al. Clinical significance of microscopic tumor venous invasion in patients with resectable hepatocellular carcinoma. *Surgery* 2000; 127:603–8.
17. Cha C, Fong Y, Jarnagin WR, et al. Predictors and patterns of recurrence after resection of hepatocellular carcinoma. *J Am Coll Surg* 2003; 197:753–8.
18. Kondo K, Chijiiwa K, Makino I, et al. Risk factors for early death after liver resection in patients with solitary hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2005; 12:399–404.
19. Pugh RN, Murray-Lyon IM, Dawson JL, et al. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; 60:646–9.
20. Liver Cancer Study Group of Japan. General Rules for the Clinical and Pathological Study of Primary Liver Cancer. 2nd English ed. Tokyo: Kanehara, 2003.
21. Kanai T, Hirohashi S, Upton MP, et al. Pathology of small hepatocellular carcinoma. A proposal for a new gross classification. *Cancer* 1987; 60:810–9.
22. Esnaola NF, Lauwers GY, Mirza NQ, et al. Predictors of microvascular invasion in patients with hepatocellular carcinoma who are candidates for orthotopic liver transplantation. *J Gastrointest Surg* 2002; 6:224–32.
23. Shirabe K, Itoh S, Yoshizumi T, et al. The predictors of microvascular invasion in candidates for liver transplantation with hepatocellular carcinoma-with special reference to the serum levels of des-gamma-carboxy prothrombin. *J Surg Oncol* 2007; 95:235–40.
24. Adachi E, Maeda T, Kajiyama K, et al. Factors correlated with portal venous invasion by hepatocellular carcinoma: univariate and multivariate analyses of 232 resected cases without preoperative treatments. *Cancer* 1996; 77:2022–31.
25. Nakashima Y, Nakashima O, Tanaka M, et al. Portal vein invasion and intrahepatic micrometastasis in small hepatocellular carcinoma by gross type. *Hepatol Res* 2003; 26:142–7.
26. Matsumata T, Kanematsu T, Takenaka K, et al. Patterns of intrahepatic recurrence after curative resection of hepatocellular carcinoma. *Hepatology* 1989; 9:457–60.
27. Yamamoto J, Kosuge T, Takayama T, et al. Recurrence of hepatocellular carcinoma after surgery. *Br J Surg* 1996; 83:1219–22.
28. Imamura H, Matsuyama Y, Tanaka E, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J Hepatol* 2003; 38:200–7.
29. Kojiro M. Premalignant lesions of hepatocellular carcinoma: pathologic viewpoint. *J Hepatobiliary Pancreat Surg* 2000; 7:535–41.
30. Eguchi A, Nakashima O, Okudaira S, et al. Adenomatous hyperplasia in the vicinity of small hepatocellular carcinoma. *Hepatology* 1992; 15:843–8.
31. Oikawa T, Ojima H, Yamasaki S, et al. Multistep and multicentric development of hepatocellular carcinoma: histological analysis of 980 resected nodules. *J Hepatol* 2005; 42:225–9.
32. Hanazaki K, Wakabayashi M, Sodeyama H, et al. Surgical outcome in cirrhotic patients with hepatitis C-related hepatocellular carcinoma. *Hepatogastroenterology* 2000; 47:204–10.
33. Shimada K, Sano T, Sakamoto Y, et al. A long-term follow-up and management study of hepatocellular carcinoma patients surviving for 10 years or longer after curative hepatectomy. *Cancer* 2005; 104:1939–47.

34. Poon RT, Fan ST, Lo CM, et al. Intrahepatic recurrence after curative resection of hepatocellular carcinoma: long-term results of treatment and prognostic factors. *Ann Surg* 1999; 229:216–22.

35. Shah SA, Cleary SP, Wei AC, et al. Recurrence after liver resection for hepatocellular carcinoma: risk factors, treatment, and outcomes. *Surgery* 2007; 141:330–9.

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Sca-1+ endothelial cells (SPECs) reside in the portal area of the liver and contribute to rapid recovery from acute liver disease ☆

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Abstract

The liver has a high potential to regenerate however, the relation between oval cells and endothelial cells in the portal area during liver regeneration has not been adequately described. We have focused on sca-1+ endothelial cells (SPEC: sca-1+CD31+CD45− cells) and analyzed their localization, growth potential, and the role of these cells in damaged liver. SPECs are localized in the portal area and comprise approximately 20–30% of CD31+CD45− cells. These cells have higher growth potential than sca-1− endothelial cells and grow aggressively when the liver is severely damaged on the lateral side of the oval cells. In an *in vivo* study we show that when the liver is severely damaged in the presence of a VEGF (vascular endothelial growth factor)-inhibitor, the frequency of SPECs decreased and the recovery of liver volume was also delayed. These results strongly suggest that SPECs play important roles in the recovery of severely damaged liver.

Keywords: Sca-1; Endothelial cells; Liver regeneration; Oval cells; VEGF; Portal area; Mouse; Cytokeratin; CD31; Acute liver disease

The liver exhibits a great regenerative potential after acute or chronic liver damage. When liver damage occurs more rapidly than regeneration, liver failure will occur and thus management of liver function will be necessary in order to prolong life. Researches focused on hepatocytes and hepatic stem/progenitor cells have been progressing rapidly. In addition, Kupffer cells, stellate cells and sinusoidal endothelial cells are known to be important cells for liver regeneration [1,2] and HGF (hepatocytes growth factor) [3], EGF (epidermal growth factor) [4], insulin [4], IL-6 (interleukin) [5], and TNF-α (tumor necrosis factor) [6,7], have been shown to accelerate the regeneration [8]. Endothelial cells in the portal area during liver damage however, have not been aggressively investigated. This investigation may contribute to the efficient angiogenesis and liver regeneration.

Previously, we have reported a serum-free culture system for the expansion of fetal and postnatal hepatic stem/progenitor cells [9,10]. In these studies we found that although sca-1 expressing hepatic progenitor cells were present in our culture, the main population of sca-1 expressing cells in the liver is the endothelial cells. Sca-1 is known as a stem cell marker for hematopoietic cells [11], mesenchymal cells [12], heart [13], mammary gland

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[14], testis [15], and prostate gland [16]; however, few reports describe the function of sca-1. A study using sca-1 null mice revealed that the hematopoietic stem cells in these mice are defective in function, and their progenitor cells exhibit abnormal differentiation [17–20]. Furthermore, sca-1 null mice have a higher risk of osteoporosis than control mice [12]. These results revealed that the function of sca-1 is important for stem cell and progenitor cell self-renewal or differentiation. In this study, we have investigated sca-1+ cells in the liver, with a particular focus on the predominant population of sca-1+ endothelial cells. Matsumoto et al. have previously reported that in the developing liver, flk-1+ immature endothelial cells that do not form blood vessels are important for the extensive expansion of fetal hepatic cells [21]. We therefore predicted that sca-1+ endothelial cells are immature and necessary for rapid liver regeneration.

Reports studying sca-1+ endothelial cells are very rare and to our knowledge only two groups have described these cells. Cherqui et al. showed that sca-1+ endothelial cells have a highly angiogenic capacity and are present in the fetal liver [22]; however, the function and relationship of these cells to liver development remains unclear. In a second study, Luna et al. reported that sca-1+ endothelial cells are present in the liver as sca-1+CD31+ cells [23]. We now know however, that there are two populations of sca-1+CD31+ cells; sca-1+CD31+CD45+ cells and sca-1+CD31+CD45− cells, although the localization and frequency of these cells remains to be determined.

In this study we have determined that sca-1+ endothelial cells are indeed present in the liver. We speculated that sca-1+ endothelial cells are immature and have an important role in the remodeling of damaged livers. We examined the frequency and distribution of sca-1+ endothelial cells in the normal and damaged liver, as well as their growth potential and their role in liver regeneration, especially from oval cells to hepatocytes.

Materials and methods

Mice and liver dissociation. C57BL/6 mice (4-, 8-, 12-weeks old: 5 mice per each group) were obtained from SLC (Hamamatsu, Japan). Mice were maintained according to the Animal Protection Guidelines of Kyoto University. The livers from these mice were dissociated using a modified two step collagenase method. The mice were initially anesthetized with diethylether. A celiotomy was performed and a 24G indwelling needle was inserted into the abdominal inferior vena cava. After the insertion, thoracotomy, chest inferior vena cava ligation, and portal vein dissection were performed as swiftly as possible. Then, 30 ml Liver perfusion Medium (Gibco BRL, Grand Island, NY) and 50 ml Liver Digest Medium (Gibco) at 37 °C were perfused for 3–4 min each. The perfused livers were placed into 6 cm-diameter culture dishes and incubated at 37 °C for 8 min before they were dissociated in the DMEM/F12 (1:1; Sigma, St. Louis, MO) with 10% fetal bovine serum (Sigma), 10 mM HEPES (Nakalai Tesque, Inc. Kyoto, Japan), and antibiotics. Dissociated cells were filtered once with a 70 μm mesh and twice with a 40 μm mesh, and single cells were obtained. The single cell suspension was separated into 15 ml conical tubes and centrifuged at 250 rpm for 1 min. The supernatant was collected and centrifuged at 500 rpm for 1 min before the supernatant was again centrifuged at 500 rpm for 1 min. Finally, the supernatant was centrifuged at 1000 rpm for 3 min and the collected cells were cultured or analyzed.

Acute liver damaged mouse model. We used 4-weeks old mice to generate a mouse model with acute liver damaged. Liver damage was induced by the intraperitoneal injection of 12 μl (0.5 mg/ml) anti-Fas antibody (BD PharMingen, San Diego, CA) dissolved in 200 μl PBS (Phosphate Buffered Saline) three times (at days 1, 4, 7: 5 mice per each group). Livers were analyzed by flow cytometry or immunohistochemistry 2-, 4-, and 6-days after the last injection of anti-Fas antibody.

Flow cytometry. Dissociated normal and damaged liver cells were used to analyze sca-1+ cells in the liver. The resulting cells were incubated with anti-mouse CD16/32 antibodies (PharMingen) for 15 min on ice to block non-specific binding and then incubated with fluorescein isothiocyanate (FITC)-conjugated sca-1 antibodies, phycoerythrin (PE)-conjugated CD31, or allophycocyanin (APC)-conjugated CD45 antibodies (PharMingen) for 30 min on ice. FITC-conjugated rat IgG2a antibodies, PE-conjugated rat IgG2a antibodies or APC-conjugated rat IgG2b antibodies (PharMingen) served as isotype controls. After washing, the cells were analyzed or sorted using FACSCalibur or FACSVantage.

Immunochemical staining. Immunocytochemistry; cells were washed in PBS, fixed with methanol (Wako, Osaka, Japan) at −20 °C for 10 min, incubated with primary antibody overnight at 4 °C and then the appropriate secondary antibody for 30 min at room temperature. Antibodies were diluted to their optimal concentrations in PBS containing 0.1% saponin. The primary antibody used was rat anti-CD31 (PharMingen) and the secondary antibody was Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunohistochemistry; livers were collected, fixed in 10% formalin, processed using a standard protocol, and embedded in paraffin blocks. 4 micrometer sections were cut and placed on silane-coated slides. After removing the paraffin, antigen retrieval was performed using proteinase K (Dako, Kyoto, Japan) for 3 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (Wako, Osaka, Japan) for 10 min at room temperature, and sections were incubated with primary antibodies diluted with PBS containing 0.1% saponin. The primary antibodies used were rat anti-mouse sca-1 (Pharmingen), and rabbit anti-cow cytokeratin (CK; Dako, Z0622), which detects bile ducts and oval cells. For DAB (3,3′-diaminobenzidine tetrahydrochloride) staining, slides were then stained using Histofine Simple Stain Max-PO (Nichirei Bioscience Inc, Tokyo, Japan) and DAB Substrate Kit (Vector Laboratories, Burlingame, CA) for sca-1, and the Vectastain. ABC Kit and DAB Substrate Kit (Vector Laboratories) for anti-cow CK. For the immunofluorescence double staining of sca-1 and CK, FITC-conjugated donkey anti-rat IgG and Cy3-conjugated donkey anti-rabbit IgG were used as the secondary antibodies. Normal IgG of the species from which the primary antibodies had been obtained served as primary antibodies in the negative controls. Nuclei were stained using Mayer's hematoxylin solution (Wako).

Endothelial cell culture. We employed two methods to culture endothelial cells using the CS-C Complete Medium Kit (Cell Systems, Kirkland, WA). The medium was changed every 3 days. In the first method, 2×10^5 dissociated cells from normal or damaged livers were plated on 6 cm-diameter collagen coated dishes for 6 days. At day 6, cultured cells were stained with anti-CD31 and the area of the dish covered by CD31+ cells was determined in order to analyze the growth potential of SPECs and SNECs. The second method used dissociated cells that were sorted using FACS Vantage. 15,000 cells from normal or damaged livers were plated on 6 cm collagen coated dishes. Five days after plating, the number of cell clusters that contained more than 4 cells was counted in order to analyze the growth potential of SPECs and SNECs.

Functional analysis of SPECs. To analyze the function of SPECs, the mice with anti-Fas-induced liver damage were separated by two groups (5 mice per each group), and were treated with or without VEGF-inhibitor. VEGF inhibitor (CBO-P11, Sigma, 0.2 mg/mice) was dissolved in DMSO (dimethylsulfoxide), and used to fill Osmotic Pumps (DURECT Corp., Cupertino, CA) that were subsequently implanted intraperitoneally into the mice. Osmotic Pumps released the VEGF-inhibitor continuously throughout the experiments. To analyze the effect of the VEGF-inhibitor on SPECs and liver regeneration, the frequency of sca-1+ endothelial cells

in the CD31+CD45− cells as well as the liver volume as the percentage of the total body weight was analyzed 2-, 4-days after the last injection of anti-Fas antibody.

Statistical analysis. The data are presented as means ± SD. The Student's *t* test was used to determine the statistical significance of observed differences.

Results

SPECs reside in the portal area

In order to determine which cells expressed sca-1 in the liver, we analyzed dissociated liver cells by flow cytometry. After liver dissociation, concentrated non-parenchymal cells were analyzed by flow cytometry. A fraction of small cells were gated to exclude parenchymal cells and dead cells (**Fig. 1**A). To determine whether the frequency of sca-1-expressing cells varied with age we analyzed liver cells from 4weeks, 8 weeks, and 12 weeks old mice (5 mice per each group). We identified four populations of sca-1+ cells in non-parenchymal population; (1) sca-1+CD31+CD45− cells, (2) sca-1+CD31+CD45+ cells, (3) sca-1+CD31−CD45+ cells, and (4) sca-1+CD31−CD45− cells (**Fig. 1**B–E). CD45 is a hematopoietic marker, therefore populations (2) and (3) were hematopoietic cells. The main population of sca-1+ cells was sca-1+CD31+CD45−

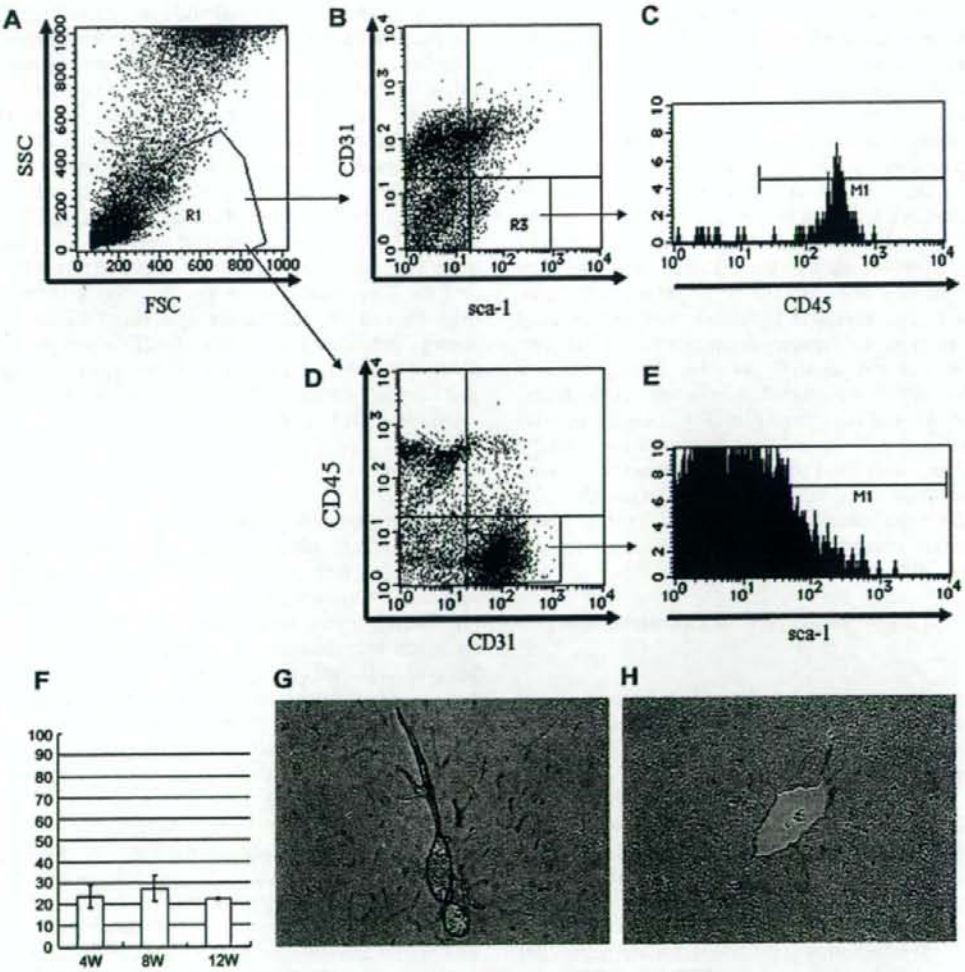

Fig. 1. Flow cytometric analysis and immunocytochemistry of SPECs in the normal liver. (A–F) Flow cytometric analysis of the dissociated normal liver cells. (A) Dissociated cells were gated according to forward scatter (FCS) and side scatter (SSC). Parenchymal cells and dead cells were excluded as much as possible. (B–E) Most of the sca-1+CD31− cells were CD45+ hematopoietic cells. Of the CD31+CD45− cells, approximately 20–30% were sca-1+. (F) The frequency of SPECs did not change with age. (G,H) Immunocytochemistric analysis of normal liver. Sca-1+ endothelial cells reside in the portal area (G) and not in the central vein (H).

endothelial cells (population 1: Fig. 1E). The frequency of sca-1+CD31+CD45− within the CD31+CD45− cell population was approximately 20–30% and the frequency did not change with the age of the mice (Fig. 1F). Surprisingly, immunohistochemistry for sca-1 revealed that sca-1+ cells reside in or around the portal area but not around the central vein (Fig. 1G and H). We named the sca-1+CD31+CD45− cells SPECs (sca-1 positive endothelial cells) and sca-1-CD31+CD45− cells SNECs (sca-1 negative endothelial cells).

SPECs expanded along the lateral side of oval cells during acute liver damage

SPECs were localized in the portal area of the liver, therefore we analyzed the frequency of SPECs during acute liver damage. In order to induce severe liver damage, 4-weeks old mice were injected three times with anti-Fas (Jo-2) antibody (days 1, 4, and 7). A large number of oval cells were detected by immunohistochemistry using an anti-cytokeratin antibody in the liver samples from treated mice (Fig. 2D–G). 2-, 4-, and 6-days after the last anti-Fas antibody injection, we analyzed the frequency of SPECs in the CD31+CD45− cells. On day 2, the frequency of SPECs in the CD31+CD45− cells had increased by approximately 60%; this number then gradually decreased with time (Fig. 2A–C). These results indicated that SPECs play important roles in the recovery from acute liver damage. Immunocytochemical analysis of the damaged livers revealed that SPECs were localized in or around the portal area and not around the central vein (Fig. 2A–C). The relationship between SPECs and oval cells was analyzed by double staining with sca-1 and anti-CK (oval cells). Interestingly, this staining revealed that sca-1+ endothelial cells extended along the lateral side of oval cells (Fig. 2G). These results strongly suggest that SPECs are necessary for the expansion or differentiation of oval cells.

SPECs exhibit higher growth potential comparing with SNECs

Two experiments were performed to compare the growth potential of SPECs and SNECs. Firstly, crude endothelial cells were obtained from normal livers and from livers damaged using an anti-Fas antibody. The frequency of SPECs in crude endothelial cells prepared from damaged livers was more than twofold higher than those obtained from normal livers. Six days after the culture started, the area of endothelial cells was analyzed by immunocytochemistry using an anti-CD31 antibody. While crude endothelial cells from normal livers formed small sheets of CD31+ endothelial cells, crude endothelial cells from damaged livers formed more than fivefold larger sheets of endothelial cells than those from normal livers (Fig. 3A–D). In the second experiment, we cultured 15,000 sorted SPECs and SNECs for 6 days on 6 cm collagen-coated dishes, and subsequently counted the cell groups that consisted of more than 4 cells. It is difficult to maintain and grow sorted endothelial cells; however counted cell groups of SPECs were approximately fivefold higher than those of SNECs (Fig. 3E–G). These two experiments revealed that SPECs have higher growth potential than SNECs.

VEGF-inhibitor decreased the frequency of SPECs and also delayed the recovery from acute liver damage

As SPECs have a higher growth potential than SNECs and grow along the lateral side of oval cells, we speculated that SPECs have an important role in liver regeneration in response to acute liver damage. To test this hypothesis, mice with anti-Fas antibody-induced acute liver damage were separated into two groups. We implanted an osmotic pump containing either the VEGF-inhibitor (group 1: dissolved in DMSO) or no VEGF-inhibitor (group 2: DMSO only as a vehicle control) into the peritoneum of each mouse. Osmotic Pumps released VEGF-inhibitor (CBO-P11, 0.2 mg/mice) or DMSO continuously throughout the experiments. At 2 or 4 days after the last anti-Fas antibody injection, we analyzed the frequency of SPECs in the CD31+CD45− cells and the liver weight as a percentage of the total body weight. At day 4, the frequency of SPECs and the liver weight was lower in group 1 than group 2 (Fig. 4A and B). Continuous injection of VEGF-inhibitor strongly inhibited the growth of SPECs and furthermore delayed the liver regeneration. These results strongly suggest that SPECs play an important role in the early recovery from acute liver damage.

Discussion

The liver exhibits the potential to regenerate in response to acute and chronic damage [1]. For example, after partial hepatectomy preexisting hepatocytes continued to divide and thus contributed to the recovery of the liver volume [1]. During severe acute liver injury, this system cannot maintain liver function. In these cases, it is commonly believed that oval cells contribute to the recovery of hepatocytes. Most of the cases of acute liver damage can be overcome by bed rest. However, during fulminant hepatic failure, a therapeutic margin still exists even after combined modality therapy. In these cases, liver transplantation is often the only the way to rescue the patient. The degree of liver damage may be the important deciding factor; however, we think that appropriate liver regeneration from oval cells to hepatocytes could have the potential to rescue some patients from fulminant hepatic failure.

We found that the population of sca-1+ cells in the liver was predominantly composed of endothelial cells (SPECs), and these were the focus of our study. Endothelial cells in the liver can be divided into 2 groups; SPECs (sca-1+ endothelial cells) and SNECs (sca-1− endothelial cells). SPECs have some intriguing aspects, the most intriguing of which is their localization. In this study we found that

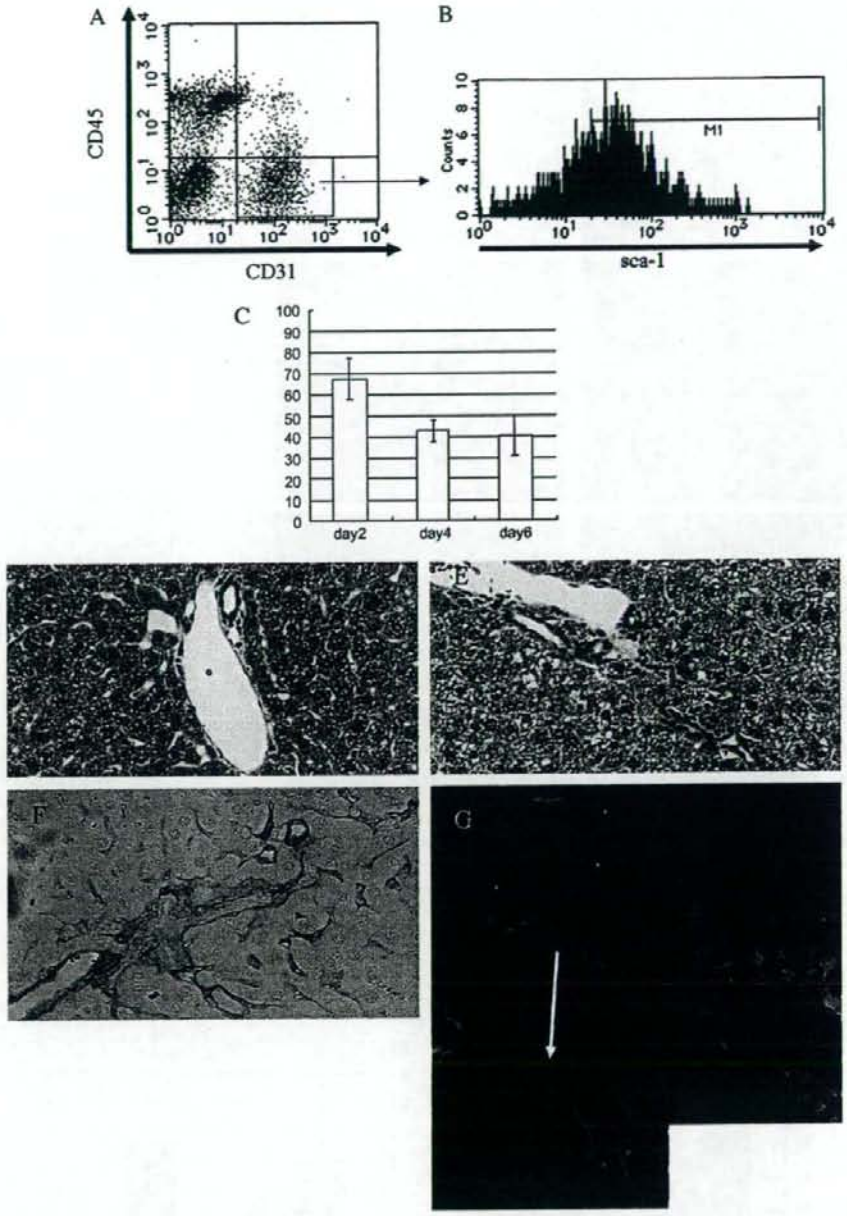

Fig. 2. Flow cytometric analysis and immunocytochemistry of SPECs in the damaged liver. (A–C) Flow cytometric analysis of the dissociated damaged liver cells. (B,C) after the damage, the frequency of sca-1+ cells within the CD31+CD45− cell population increased rapidly and then gradually decreased(B,C; at day 2 after the last anti-Fas antibody injection). Hematoxylin-eosin staining of normal (D) and damaged livers (E) at day 4 after the last anti-Fas injection. Immunocytochemistry of sca-1+ cells and oval cells revealed that SPECs grow at the lateral side of oval cells (F,G; green: oval cells, red: SPECs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

the localization of SPECs was limited to the vicinity of the portal area, not in the vicinity of the central vein. Interestingly, their frequency was elevated in response to acute liver damage. Furthermore, when the liver was damaged severely, the localization of SPECs extended along the lateral side of oval cells. In addition, continuous administra-

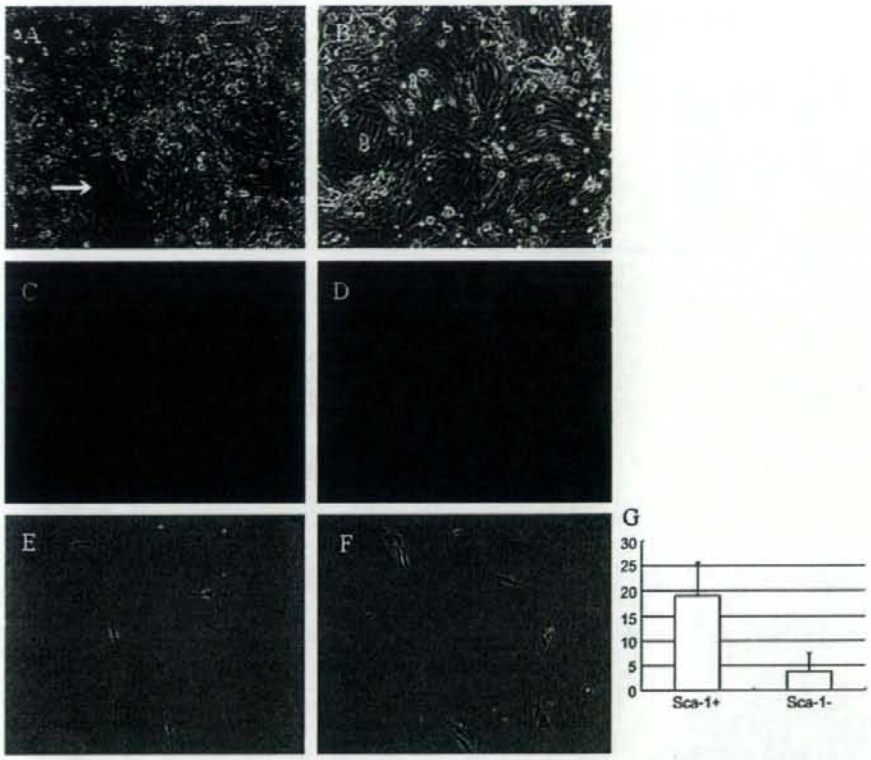

Fig. 3. Growth potential of SPECs and SNECs. (A–G) crude cell culture and sorted cell culture. (A–D) crude dissociated cell culture from normal and damaged livers revealed that the area of endothelial cells from damaged liver cells (A,B; spindle shaped cells indicated by white arrow: endothelial cells, C; red: CD31+ endothelial cells) was wider than those from normal livers (B, D). (E–G) Cell culture of sorted SNECs (E) and SPECs (F) revealed that SPECs have a greater growth potential than SNECs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

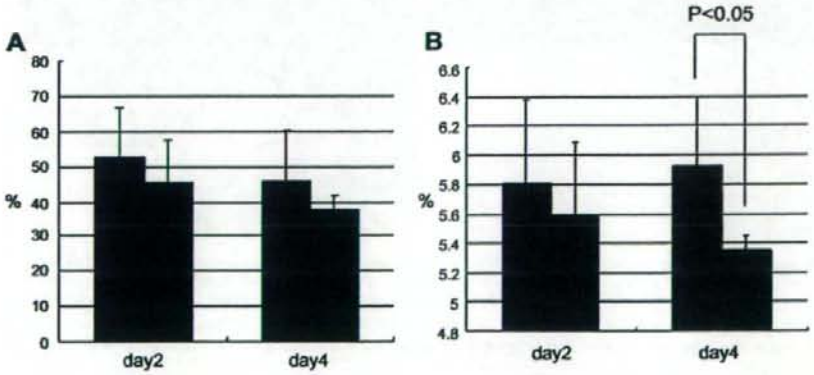

Fig. 4. Role of SPECs in *in vivo* model. (A,B) Analysis of SPECs in an *in vivo* model. (A) The frequency of SPECs from damaged livers treated with no VEGF-inhibitor (A, red) was higher than that in damaged livers treated with VEGF inhibitor (A, blue). In contrast, liver weight per body weight (%) of damaged mice treated with no VEGF inhibitor (B, red) was higher than that in damaged mice treated with VEGF-inhibitor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

tion of a VEGF-inhibitor to mice with severe liver damage decreased the frequency of SPECs and prolonged the recovery of the liver size. Indeed, Namisaki et al. previously reported that VEGF had a salvage effect on chemically induced acute severe liver injury in rats [24], and this result supports our conclusions. SPECs function to form a passage leading from bile duct to hepatocytes, and may also directly or indirectly influence the growth and differentiation of oval cells. We believe that sca-1+ endothelial cells play an important role in the remodeling of damaged liver. Sca-1 is a marker unique to mice and thus cannot be used in human studies. In the future we believe that a common system will allow the analysis of SPECs in mice and in humans, and will thus help to elucidate the role of oval cells in human disease. In addition, our study implies that effective angiogenesis therapy may improve the ability of the liver to regenerate, and hence improve the therapeutic outcome for patients.

References

[1] N. Fausto, J.S. Campbell, K.J. Riehle, Liver regeneration, Hepatology 43 (2006) S45–S53.

[2] R. Malik, C. Selden, H. Hodgson, The role of non-parenchymal cells in liver growth, Semin. Cell. Dev. Biol. 13 (2002) 425–431.

[3] K. Matsumoto, T. Nakamura, Hepatocyte growth factor: molecular structure, roles in liver regeneration, and other biological functions, Crit. Rev. Oncog. 3 (1992) 27–54.

[4] H.S. Lai, Y.C. Chung, W.J. Chen, K.M. Chen, Rat liver regeneration after partial hepatectomy: effects of insulin, glucagon and epidermal growth factor, J. Formos. Med. Assoc. 91 (1992) 685–690.

[5] A. Blindenbacher, X. Wang, I. Langer, R. Savino, L. Terracciano, M.H. Heim, Interleukin 6 is important for survival after partial hepatectomy in mice, Hepatology 38 (2003) 674–682.

[6] H. Hayashi, M. Nagaki, M. Imose, Y. Osawa, K. Kimura, S. Takai, M. Imao, T. Naiki, T. Kato, H. Moriwaki, Normal liver regeneration and liver cell apoptosis after partial hepatectomy in tumor necrosis factor-alpha-deficient mice, Liver Int. 25 (2005) 162–170.

[7] P.A. Gruppuso, J.E. Mead, N. Fausto, Transforming growth factor receptors in liver regeneration following partial hepatectomy in the rat, Cancer Res. 50 (1990) 1464–1469.

[8] R. Taub, Liver regeneration: from myth to mechanism, Nat. Rev. Mol. Cell Biol. 5 (2004) 836–847.

[9] A. Tsuchiya, T. Heike, S. Baba, H. Fujino, K. Umeda, Y. Matsuda, M. Nomoto, T. Ichida, Y. Aoyagi, T. Nakahata, Long-term culture of postnatal mouse hepatic stem/progenitor cells and their relative developmental hierarchy, Stem Cells (2007).

[10] A. Tsuchiya, T. Heike, H. Fujino, M. Shiota, K. Umeda, M. Yoshimoto, Y. Matsuda, T. Ichida, Y. Aoyagi, T. Nakahata, Long-term extensive expansion of mouse hepatic stem/progenitor cells in a novel serum-free culture system, Gastroenterology 128 (2005) 2089–2104.

[11] S. Okada, H. Nakauchi, K. Nagayoshi, S. Nishikawa, Y. Miura, T. Suda, In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells, Blood 80 (1992) 3044–3050.

[12] M. Bonyadi, S.D. Waldman, D. Liu, J.E. Aubin, M.D. Grynpas, W.L. Stanford, Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice, Proc. Natl. Acad. Sci. USA 100 (2003) 5840–5845.

[13] K. Matsuura, T. Nagai, N. Nishigaki, T. Oyama, J. Nishi, H. Wada, M. Sano, H. Toko, H. Akazawa, T. Sato, H. Nakaya, H. Kasanuki, I. Komuro, Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes, J. Biol. Chem. 279 (2004) 11384–11391.

[14] B.E. Welm, S.B. Tepera, T. Venezia, T.A. Graubert, J.M. Rosen, M.A. Goodell, Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population, Dev. Biol. 245 (2002) 42–56.

[15] M.P. van Bragt, N. Ciliberti, W.L. Stanford, D.G. de Rooij, A.M. van Pelt, LY6A/E (SCA-1) expression in the mouse testis, Biol. Reprod. 73 (2005) 634–638.

[16] L. Xin, D.A. Lawson, O.N. Witte, The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis, Proc. Natl. Acad. Sci. USA 102 (2005) 6942–6947.

[17] S.B. Bradfute, T.A. Graubert, M.A. Goodell, Roles of Sca-1 in hematopoietic stem/progenitor cell function, Exp. Hematol. 33 (2005) 836–843.

[18] C.Y. Ito, C.Y. Li, A. Bernstein, J.E. Dick, W.L. Stanford, Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice, Blood 101 (2003) 517–523.

[19] W.L. Stanford, S. Haque, R. Alexander, X. Liu, A.M. Latour, H.R. Snodgrass, B.H. Koller, P.M. Flood, Altered proliferative response by T lymphocytes of Ly-6A (Sca-1) null mice, J. Exp. Med. 186 (1997) 705–717.

[20] Z.X. Zhang, W.L. Stanford, L. Zhang, Ly-6A is critical for the function of double negative regulatory T cells, Eur. J. Immunol. 32 (2002) 1584–1592.

[21] K. Matsumoto, H. Yoshitomi, J. Rossant, K.S. Zaret, Liver organogenesis promoted by endothelial cells prior to vascular function, Science 294 (2001) 559–563.

[22] S. Cherqui, S.M. Kurian, O. Schussler, J.A. Hewel, J.R. Yates 3rd, D.R. Salomon, Isolation and angiogenesis by endothelial progenitors in the fetal liver, Stem Cells 24 (2006) 44–54.

[23] G. Luna, J. Paez, J.E. Cardier, Expression of the hematopoietic stem cell antigen Sca-1 (LY-6A/E) in liver sinusoidal endothelial cells: possible function of Sca-1 in endothelial cells, Stem Cells Dev. 13 (2004) 528–535.

[24] T. Namisaki, H. Yoshiji, H. Kojima, J. Yoshii, Y. Ikenaka, R. Noguchi, S. Sakurai, K. Yanase, M. Kitade, M. Yamazaki, K. Asada, M. Uemura, M. Nakamura, H. Fukui, Salvage effect of the vascular endothelial growth factor on chemically induced acute severe liver injury in rats, J. Hepatol. 44 (2006) 568–575.

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Features of hepatocellular carcinoma in cases with autoimmune hepatitis and primary biliary cirrhosis

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Abstract

AIM: To characterize the clinical features of hepatocellular carcinoma (HCC) associated with autoimmune liver disease, we critically evaluated the literature on HCC associated with autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC).

METHODS: A systematic review of the literature was conducted using the Japana Centra Revuo Medicina database which produced 38 cases of HCC with AIH (AIH-series) and 50 cases of HCC with PBC (PBC-series). We compared the clinical features of these two sets of patients with the general Japanese HCC population.

RESULTS: On average, HCC was more common in men than in women with AIH or PBC. While many patients underwent chemolipiodolization (CL) or transcatheter arterial embolization (TAE) (AIH-series: $P = 0.048$ (*vs* operation), $P = 0.018$ (*vs* RFA, PEIT); PBC-series: $P = 0.027$ (*vs* RFA, PEIT), others refused therapeutic interventions [AIH-series: $P = 0.038$ (*vs* RFA, PEIT); PBC-series: $P = 0.003$ (*vs* RFA, PEIT)]. Liver failure was the primary cause of death among patients in this study, followed by tumor rupture. The survival interval between diagnosis and death was fairly short, averaging 14 ± 12 mo in AIH patients and 8.4 ± 14 mo in PBC patients.

CONCLUSION: We demonstrated common clinical features among Japanese cases of HCC arising from AIH and PBC.

Key words: Autoimmune hepatitis; Autoimmune liver disease; Hepatocellular carcinoma; Literature review; Primary biliary cirrhosis

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INTRODUCTION

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerotic cholangitis (PSC) form the triad of autoimmune liver diseases. As defined by Mackay *et al*[1], AIH is a chronic active hepatitis resulting from several distinct autoimmune phenomena. While the anti-inflammatory effects of steroid therapy for this disease may inhibit the promotion of liver carcinogenesis, hepatocellular carcinoma (HCC) does occur rarely in patients with this condition (in about 0.5% of AIH cases)[2,3].

In contrast to AIH, PBC results from an autoimmune mechanism causing chronic cholestasis and chronic non-suppurative destructive cholangitis in medium sized intrahepatic bile ducts[4]. Rare cases of HCC arising from PBC have been reported to date. However,

this association is rare (affecting between 0.3% and 4.22% of cases)[5-11], because a PBC patient's ability to produce regenerative nodules is weak[5-9,12]. Additionally, PBC is pathologically characterized in chronic non-suppurative destructive cholangitis (CNSDC), and the main inflammatory lesions associated with PBC are not hepatocytes, but cholangiocytes, which may be one of the reasons why the incidence of HCC with PBC is low, especially at the early stage when cirrhotic and fibrotic changes do not progress. Recently, reports have suggested that the prevalence of HCC arising from both AIH and PBC is higher than previously believed. In 2001, Caballeria *et al*[13] found that the incidence of HCC in patients with advanced PBC (Scheuer histological stage Ⅲ or Ⅳ) was 11.1%, approximating the 15% incidence in patients with HCV-related cirrhosis (RR 0.812, 95% CI 0.229-2.883). The clinical features of HCC associated with AIH and PBC, however, have not yet been extensively described. Here, we performed a systematic literature review of HCC cases associated with AIH and PBC in Japan, a country with a high burden of autoimmune liver disease. We conducted a critical analysis of case reports to find common themes in the demographic and clinical histories of patients with HCC associated with AIH and PBC.

MATERIALS AND METHODS

We performed a systematic literature review of case reports published in Japan and listed in the Japana Centra Revuo Medicina database, version 3 (systematic literature search system through a computer web site for Japanese literature), using the keywords "hepatocellular carcinoma", "autoimmune hepatitis", and "primary biliary cirrhosis". The database search was limited to the period between 1990 (when the hepatitis C virus was first detected) to the present. The quality of this database available for analysis is thoroughly well-documented. In total, 38 cases of HCC associated with AIH, and 50 cases of HCC associated with PBC were identified. No cases were duplicated, and patients were identified across multiple Japanese medical centers. Most patients in the series had been diagnosed with autoimmune liver disease before HCC was identified. Several cases also presented with co-factors of liver damage and HCC development other than AIH or PBC, such as excessive alcohol intake, HBV, or HCV infection. However, no cases had evidence of hemochromatosis or α1-antitrypsin deficiency. The demographics of these two groups were recorded based on gender, age, period of medical observation, and history of blood transfusion or excessive alcohol intake. Clinical data was also recorded to determine noncancerous pathologies of the liver, HBV or HCV infection status, serum α-fetoprotein (AFP) level, maximal tumor size, history of HCC therapy, clinical outcomes, and cause of death. Cases that did not include a description of alcohol intake were assumed not to have histories of excessive alcohol intake.

We confirmed that all 38 identified cases of HCC associated with AIH met generally accepted international criteria for diagnosis of AIH[14]. Scoring was performed prior to AIH therapy initiation; all scores were greater than 10, and thereby classified as either "probable AIH" or "definite AIH".

Because no internationally accepted diagnostic criteria yet exists for PBC, we utilized the Japanese standard criteria for PBC diagnosis, a standard first proposed in 1992 by a clinical study group supported by the Japanese Ministry of Welfare. According to this standard, PBC diagnosis requires that cases meet at least one of the following criteria: (1) pathologic evidence of CNSDC and positive anti-mitochondrial antibody (AMA) or anti-PDH antibody titers, (2) positive AMA or anti-PDH antibody titers and non-CNSDC pathology compatible with PBC, or (3) no liver biopsy, but, positive AMA or anti-PDH antibody titers and a clinical picture and clinical course compatible with PBC. We confirmed that all 50 identified cases of HCC associated with PBC met the above diagnostic criteria. Six of 50 (12.0%) HCC cases with PBC met the third criteria for PBC, and 44 of 50 (88.0%) cases met the first or second criteria for PBC. The third criteria for PBC remain ambiguous, and it is really hoped that internationally accepted criteria will be determined for PBC diagnosis.

If a case met both generally accepted international criteria for diagnosis of AIH, and the Japanese standard criteria for PBC diagnosis, we diagnosed the case as overlap syndrome. We had two cases of overlap syndrome, and excluded these cases from our analysis.

We did not include a control group, but used the general HCC population in Japan for comparison[15].

Statistical analysis

Intention-to-treat analyses were used throughout, and statistical analysis for categorical comparisons of the data was performed using the program ystat2006.xls for Windows/Macintosh (Igaku Tosho Shuppan Corporation, Tokyo, Japan). We used the χ^2 test and Fisher's exact test for categorical comparisons between patients with HCC associated with AIH or PBC and HCC patients without associated autoimmune disease[15]. The following variables were assessed: gender, HBV or HCV co-infection, history of blood transfusions, history of excessive alcohol intake, positivity for serum-AFP and clinical outcomes. Because the baseline male to female ratio of AIH and PBC was 1:7 and 1:9, respectively, we performed the χ^2 test for males and females separately. We also used the χ^2 test with or without the Yates correction for categorical comparisons of pathological findings of noncancerous lesions of the liver, HCC therapy choices, and cause of death. Where significant differences were noted, χ^2 tests or Fisher's exact tests were repeated with all categorical combinations, using Bonferroni corrections for multiple comparisons. Two tailed Mann-Whitney *U*-tests and *F*-tests were performed at the 5% significance level only for comparisons between HCC patients with AIH and PBC, as the following variables were unavailable for the general HCC

Table 1 Developement period of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Observation period (mean ± SD)	Total: 38 1 yr 1 mo-23 yr (10 yr 6 mo ± 6 yr 7 mo)	Total: 49 3 mo-24 yr (9 yr 4 mo ± 6 yr 4 mo)	NA	NA	NA	$P = 0.307$ ($P = 0.815$)
Interval between liver damage and HCC diagnosis (mean ± SD)	Total: 34 0-22 yr 9 mo (10 yr 2 mo ± 6y 5 mo)	Total: 40 0-24 yr (9 yr 9 mo ± 7 yr 0 mo)	NA	NA	NA	$P = 0.740$ ($P = 0.688$)
Period from HCC development to death (mean ± SD)	Total: 18 2 mo-3 yr (1 yr 2 mo ± 12 mo)	Total: 16 0-5 yr (8.4 ± 14 mo)	NA	NA	NA	$P = 0.047$* ($P = 0.401$)

The *P*-value above was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. *$P < 0.05$, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; SD: Standard deviation; NA: Not available.

Table 2 Analysis on gender and age of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Gender						
Actual number	Total: 38	Total: 50	Total: 16743			
Male	7 (18.4)	13 (26.0)	12025 (71.8)			
Female	31 (81.6)	37 (74.0)	4718 (28.2)	$P = 0.149$[1]	$P = 0.512$[1]	$P = 0.244$
Relative number	Total: 38	Total: 50				
Male	23.3 (61.3)	38.0 (76.0)				
Female	14.7 (38.7)	12.0 (24.0%)				
Age at HCC diagnosis	Total: 38	Total: 50	Total: 16743			
(mean ± SD)	(67.61 ± 8.58)	(68.54 ± 9.30)	NA			
< 40 s	0 (0)	2 (4.0)	761 (4.6)	NA	NA	$P = 0.410$
50 s	8 (21.0)	6 (12.0)	2818 (16.8)			($P = 0.614$)
60 s	16 (42.1)	21 (42.0)	6179 (36.9)			
70 s	9 (23.7)	14 (28.0)	5976 (35.7)			
< 80 s	5 (13.2)	7 (14.0)	1009 (6.0)			

The *P*-value above was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. [1]The *P*-value was calculated from the relative numbers. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; SD: Standard deviation; NA: Not available.

population: interval between liver damage and HCC diagnosis, interval from HCC diagnosis to death, age at HCC diagnosis, serum-AFP levels, maximum tumor size and number of HCC loci. Because the patient sample size in each group was greater than 20, we chose to use *P*-values calculated from the asymptotic distribution. The total number of cases in each patient group did not include cases for which categorical data were unknown (Table 1).

The statistical analysis for survival among HCC patients with AIH and PBC was performed on a personal computer with the statistical package SPSS for Windows (version II, SPSS Inc., Chicago, IL, USA). Because there were too few published cases of HCC arising from AIH or PBC, however, differences in survival between patient groups could not be calculated.

RESULTS

The intervals between HCC diagnosis and death for HCC patients with AIH (14 ± 12 mo) and PBC (8.4 ± 14 mo) was notably shorter than among general HCC patients in Japan (77.5% 1-year survival, 52.5% 3-year survival, and 35.4% 5-year survival)[15]. As shown in Table 1, the survival interval for HCC patients with PBC was also significantly shorter than that for patients with AIH ($P = 0.047$).

Among HCC cases associated with AIH, the actual male to female ratio was 7:31. Because AIH patients in Japan are predominantly female (7:1), the corrected risk ratio for HCC among male AIH patients was 1.6:1 relative to females, and the male to female ratio of the relative numbers was 23.3:14.7 (Table 2). The majority of Japanese PBC patients are also female, outnumbering

Table 3 Clinical status of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/General-HCC patients	PBC-series/General-HCC patients	AIH-series/PBC-series
History of blood transfusion	Total: 29	Total: 38	Total: 12602	$P = 0.040^a$	$P = 0.581$	$P = 0.041^a$
+	3 (10.3)	13 (34.2)	3633 (28.8)			
-	26 (89.7)	25 (65.8)	8969 (71.2)			
History of excessive alcohol intake	Total: 38	Total: 50	Total: 14694	$P = 0.812$	$P = 0.056$	$P = 0.352$
+	1 (2.6)	5 (10.0)	3271 (22.3)			
-	37 (97.4)	45 (90.0)	11423 (77.7)			
Co-infection	Total: 33	Total: 40	Total: 4121			
HBV (prior) +	2 (6.1)	10 (25.0)	2138 (51.9)	$P < 0.001$	$P < 0.001$	$P = 0.025$
HBV (prior) -	31 (93.9)	30 (75.0)	1983 (48.1)			
Co-infection	Total: 38	Total: 49	Total:16492			
HCV +	3 (7.9)	10 (20.4)	11488 (69.7)	$P < 0.001$	$P < 0.001$	$P = 0.044$
HCV -	35 (92.1)	39 (79.6)	5004 (30.3)			
Pathological findings of noncancerous lesion of the liver	Total: 31	Total: 44	Total: 4941	$P = 0.163$	$P = 0.007^b$	$P = 0.489$
NL, CH, LF	13 (41.9)	15 (34.1)	2691 (54.5)			
LC	18 (58.1)	29 (65.9)	2250 (45.5)			

HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NL: Normal liver; CH: Chronic hepatitis; LF: Liver fibrosis; LC: Liver cirrhosis.

males by 9:1. The relative risk ratio for HCC among males with PBC was 3.2:1 relative to females, and the male to female ratio of the relative numbers was 38:12 (Table 2). No significant differences in male to female ratios were noted between the three patient groups ($P = 0.149$, $P = 0.512$, $P = 0.244$, respectively).

Among the HCC cases associated with AIH, only three (10.3%) had a history of blood transfusions, while 13 (34.2%) of the cases with PBC had such a history. Among all Japanese patients with HCC, 3633 (28.8%) had a history of blood transfusions[15]. The proportion of HCC cases associated with AIH having a history of blood transfusions was significantly lower than that of the general HCC cases in Japan ($P = 0.040$), and the proportion of HCC cases associated with PBC having a history of blood transfusions was significantly greater than that of the HCC cases associated with AIH ($P = 0.041$, Table 3).

Similarly, only one case (3.1%) of HCC associated with AIH had a history of excessive alcohol intake, while five (20.0%) cases associated with PBC had such a history ($P = 0.352$, Table 3). Among all Japanese patients with HCC, 3271 (22.3%) had a history of excessive alcohol intake[15].

While prior infection with HBV was relatively rare among AIH patients (6.1%), it was much more prevalent among patients with PBC (25.0%, $P = 0.025$). Similarly, 7.9% of AIH patients tested positive for HCV, as compared to 20.4% of PBC patients ($P = 0.044$). The population of Japanese HCC patients without autoimmune liver disease had significantly higher rates of both HBV and HCV co-infection ($P < 0.001$, Table 3).

Among the HCC cases associated with AIH, 18/31 (58.1%) were found to have cirrhosis on examination of liver biopsy samples or resected samples at operation. In contrast, 29/44 (65.9%) of the HCC cases associated with PBC were found to have cirrhotic liver tissue. Within the general HCC population in Japan, 2250 of the 4941 cases for which liver specimens were available (45.5%) showed evidence of cirrhosis[15]. While the proportion of liver cirrhosis among HCC cases associated with PBC was significantly greater than that in the general HCC population in Japan ($P = 0.007$), no statistical significance in the prevalence of cirrhosis was found between AIH-associated HCC and general HCC patients ($P = 0.163$, Table 3).

The numbers and positive ratios of the AIH-series, PBC-series and general-HCC patients were 22/37 (59.5%), 34/47 (72.4%) and 10075/15831 (63.6%), respectively. No significant differences in positive ratios of serum-AFP were noted between the three patient groups ($P = 0.597$, $P = 0.216$, $P = 0.214$, respectively, Table 4). AFP levels at diagnosis were 2340.2 ng/mL (range 1-49100 ng/mL) among patients with AIH, and 854.2 ng/mL (range 4.2-14646 ng/mL) among patients with PBC. The maximum size of the primary hepatic tumor at diagnosis was 3.97 cm (range 1.0-10.0 cm) among patients with AIH and 3.51 cm (range 1.0-8.8 cm) among PBC patients (Table 4). Due to lack of available data, we could not compare serum AFP levels, tumor sizes and numbers of HCC loci between the autoimmune-associated HCC cases and the general HCC cases in Japan. However, we found that serum AFP level did not vary widely, and that maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4).

Among both the AIH and PBC patient groups,

Table 4 Serum AFP levels, tumor sizes and number of HCC loci of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/PBC-series
Serum-AFP	Total: 37	Total: 47	Total: 15831			
AFP – (< 15 ng/mL)	15 (40.5)	13 (27.7)	5756 (36.4)	$P = 0.597$	$P = 0.216$	$P = 0.214$
AFP + (≥ 15 ng/mL)	22 (59.5)	34 (72.3)	10075 (63.6)			
Serum-AFP (ng/mL)	Total: 37	Total: 47	Total: 15831			
(mean ± SD)	(2340.21 ± 8823.45)	(854.18 ± 2263.83)	NA			
< 15	15 (40.5)	13 (27.6)	5756 (36.4)			
15-199	12 (32.5)	16 (34.0)	5786 (36.5)	NA	NA	$P = 0.106$ ($P < 0.001^b$)
200-399	1 (2.7)	6 (12.8)	902 (5.7)			
400-999	3 (8.1)	2 (4.3)	907 (5.7)			
≥ 1000	6 (16.2)	10 (21.3)	2480 (15.7)			
Maximum tumor size of HCC	Total: 36	Total: 48	Total: 15788	NA	NA	$P = 0.744$ ($P = 0.028^a$)
(mean ± SD) (cm)	(3.75 ± 2.42)	(3.51 ± 1.69)	NA			
< 2	14 (38.9)	11 (22.9)	5123 (32.4)			
2.1-5.0	16 (44.4)	29 (60.4)	7434 (47.1)			
≥ 5.1	6 (16.7)	8 (16.7)	3231 (20.5)			
Number of HCC loci	Total: 38	Total: 49	Total: 16187			
(mean ± SD)	(1.58 ± 2.05)	(1.74 ± 1.97)	NA	NA	NA	$P = 0.418$ ($P = 0.805$)
Single	33 (86.8)	38 (77.6)	9365 (57.9)			
Double	2 (5.3)	7 (14.3)	2850 (17.6)			
Multiple	3 (7.9)	4 (8.1)	3972 (24.5)			

The *P*-value in the first row was calculated from the χ^2 test and Fisher's exact test. The *P*-value in the following row was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. $^aP < 0.05$, $^bP < 0.01$, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; AFP: α-fetoprotein; SD: Standard deviation; NA: Not available.

the most commonly selected forms of treatment were chemolipiodolization (CL) and transcatheter arterial embolization (TAE); other options included percutaneous ethanol injection therapy (PEIT) and radiofrequency ablation (RFA). Differences in the choice of therapeutic procedures were noted as follows, although no comparisons reached statistical significance following the Bonferroni correction: (1) The rate of CL or TAE among HCC patients with AIH was greater than the rate of operations among general HCC patients ($P = 0.048$), (2) The rate of CL or TAE among HCC patients with AIH was greater than the rate of PEIT and RFA among general HCC patients ($P = 0.018$), and (3) The rate of CL or TAE in HCC patients with PBC was greater than the rate of PEIT and RFA among general HCC patients ($P = 0.027$). Additionally, the frequency with which HCC patients with PBC chose to forgo treatment was significantly higher than the frequency with which general HCC patients chose to undergo PEIT or RFA ($P = 0.003$). Although not statistically significant, the frequency with which HCC patients with AIH refused therapeutic interventions was also higher than the frequency of PEIT or RFA in the general HCC population ($P = 0.038$, Table 5). Ideally, data on survival by treatment modality should be presented. However, the number of patients receiving each treatment modality who were able to be followed up to death was small. Hence, the mean period from HCC development to death was calculated from patient survival following all treatment options. Future prospective studies are needed to further analyze mean survival for each treatment alternative.

Across all three patient groups, we found that liver failure was the leading cause of death, followed by rupture of HCC. Among general HCC patients, neoplastic death was most common (1487/2700, 55.1%), although differences between causes of death did not reach statistical significance. Comparisons between patient groups showed that: (1) The rate of neoplastic death in general HCC patients was higher than the rate of variceal rupture in HCC patients with AIH ($P = 0.050$), (2) The rate of neoplastic death in general HCC patients was higher than the rate of gastrointestinal bleeding in HCC patients with AIH ($P = 0.013$), and (3) The rate of neoplastic death in general HCC patients was greater than the rate of variceal rupture in HCC patients with PBC ($P = 0.050$, Table 5).

DISCUSSION

While autoimmune liver disease is more common among women than men in Japan, HCC in our group of patients with autoimmune liver disease was more common in men than women (Table 2). Men with AIH had a 1.6-fold greater risk of HCC than women, while men with PBC had a 3.2-fold greater risk of HCC than women with PBC. Moreover, when we followed AIH and PBC patients during HCC surveillance, we noted that the rate of HCC development was higher in male patients with autoimmune liver disease than in female patients with autoimmune liver disease.

Cirrhosis was found in only 18/31 (58.1%) of HCC

Table 5 Therapy and outcome of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			*P*-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Therapy choices for HCC	Total: 38	Total: 47	Total: 17005	Operation *vs* CL or TAE $P = 0.048$[1]	RFA, PEIT *vs* CL or TAE	
CL or TAE	18 (47.4)	17 (36.2)	4636 (27.2)	RFA,PEIT,MCT *vs*	$P = 0.027$[1]	
Operation	8 (21.0)	16 (34.0)	5268 (31.0)	CL or TAE	RFA, PEIT *vs*	
RFA, PEIT, MCT	6 (15.8)	6 (12.8)	4890 (28.8)	$P = 0.018$[1]	No therapy	
Chemotherapy	0 (0)	0 (0)	765 (4.5)	RFA,PEIT,MCT *vs*	$P = 0.003$[1]	
Others	0 (0)	0 (0)	122 (0.7)	No therapy		
No therapy	6 (15.8)	8 (17.0)	1324 (7.8)	$P = 0.038$[1]		
Clinical outcome	Total: 37	Total: 49	Total: 16646			
Alive	20 (54.1)	31 (63.3)	13946 (83.8)	$P < 0.001$[b]	$P < 0.001$[b]	$P = 0.389$
Dead	17 (45.9)	18 (36.7)	2700 (16.2)			
Cause of death	Total: 16	Total: 18	Total: 2700	Neoplastic death	Neoplastic death	
Liver failure	8 (50.0)	8 (44.4)	581 (21.5)	*vs* variceal rupture	*vs* variceal rupture	
HCC rupture	3 (18.8)	4 (22.2)	172 (6.4)	$P = 0.050$[1]	$P = 0.050$[1]	
Variceal rupture	1 (6.2)	1 (5.6)	85 (3.1)	Cancer death *vs* GI		
GI bleeding	1 (6.2)	2 (11.1)	55 (2.0)	bleeding		
Neoplastic death	0 (0)	0 (0)	1487 (55.1)	$P = 0.013$[1]		
Others	3 (18.8)	3 (16.7)	320 (11.9)			

[b]$P < 0.01$, Statistically significant. [1]The calculated *P*-values did not reach statistical significance with Bonferroni correction; without the correction, however, *P*-values were below 0.05. [2]The calculated *P*-values reached statistical significance with Bonferroni correction. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; CL: Chemolipiodolization; TAE: Transcatheter arterial embolization; RFA: Radiofrequency ablation therapy; PEIT: Percutaneous ethanol injection therapy; MCT: Microwave coagulation therapy; GI: Gastrointestinal.

patients with AIH, 29/44 (65.9%) of HCC patients with PBC, and in only 2250/4941 (45.5%) of the general Japanese HCC population. We did not add cases with liver fibrosis (LF) to the incidence of liver cirrhosis (LC) in the general Japanese HCC population, which may be one of the reasons why the incidence of liver cirrhosis was surprisingly low. Additionally, we think that the HCC cases with PBC and AIH and with non-cirrhotic liver, in which sufficient examinations and successful treatments were performed because of their higher hepatic reserve, were likely to be reported and submitted for publication. The possibility of bias in the selection of the reported cases should be raised.

Another interesting finding was that the interval between HCC diagnosis and death was shorter for patients with autoimmune liver disease than for the general HCC population of Japan[15]. Furthermore, although we found that serum AFP level did not vary widely, the maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4). One explanation for this finding may be a selection bias, as cases which were detected earlier and treated successfully were more likely to be submitted for publication. Despite a smaller tumor size and a lower number of HCC loci in patients with HCC arising in the setting of autoimmune liver disease at the time of HCC diagnosis, a shorter reported survival was not attributed to late detection of HCC and failure to survey patients with autoimmune liver disease for HCC, but was more likely to be due to advanced liver disease and cirrhosis. Future prospective studies will be needed to verify or refute these findings.

Although CL and TAE were the most frequently selected treatment modalities across all patient groups (Table 5), many patients ultimately refused treatment due to advanced age or social circumstances. Medical treatments using CL or TAE may be common because HCC cases are often inoperable due to cirrhotic liver disease in these patients. While survival may be related to the choice of therapeutic options, inconsistencies in data reporting over multiple decades and across multiple medical centers made the calculation of survival data difficult.

Several mechanisms explaining the development of HCC from autoimmune liver diseases have been proposed: enhanced progression to cirrhosis through progressive autoimmune hepatitis, decreased antitumor immune responses caused by long-term administration of steroids and immunosuppressants, or virus-mediated hepatitis[16,17]. In this study, we found significantly higher rates of HBV and HCV among PBC patients with HCC than among AIH patients with HCC. This finding may be attributable to the higher rates of blood transfusion in HCC patients with PBC ($P = 0.041$, Table 3). This result is supported by the findings of Shimizu *et al*[18], who reported that 3/16 (19%) HCC patients with PBC tested positive for prior HBV and present HCV infections. Given the high rates of prior HBV infections among HCC patients with PBC, it is possible that prior HBV infection predisposes patients to HCC through HBV-DNA becoming integrated into hepatocyte DNA. It has been reported that even in patients who test negative for serum HCV-RNA and serum HBV-DNA (less

than the sensitivity of HBV-DNA), liver tissue samples frequently test positive for HCV-RNA or HBV-DNA. This suggests a possible role for positive HCV-RNA or HBV-DNA in hepatic tissue in the development of HCC[19-23]. In the present study, however, only six HCC patients with AIH and two HCC patients with PBC were found to have detectable HBV-DNA and HCV-RNA in liver tissue samples. Aggressive liver biopsies should be taken to allow genetic analysis for HBV and HCV in liver tissue, in order to further study HCC cases with non-B, non-C hepatitis.

We reported high rates of HBV or HCV infection among HCC patients with PBC (Table 3); however, this is less surprising because international diagnostic criteria for AIH allocate negative points for positive HBV or HCV diagnostic tests[14]. Furthermore there are no definitive histological features that allow a clinician or pathologist to distinguish AIH from chronic viral hepatitis. Thus, HBV or HCV infected patients are rarely classified as having AIH in the modern era. In contrast, the unique histological features of PBC and the relative specificity of AMA tests allow clinicians to diagnosis and report cases of concurrent PBC and chronic viral hepatitis with a greater degree of confidence.

It has been reported that HCC develops significantly more often in patients with concurrent PBC and HCV infection than in patients with AMA-positive PBC[9]. The incidence of HCC associated with PBC has been suggested to have increased recently due to prolonged periods of liver cirrhosis resulting from longer survival on steroid therapy, concurrence of the hepatitis virus or alcohol intake with HCC, and the administration of immunosuppressants which may disturb immunoregulatory function[24,25]. In a proportional hazards analysis of patients with PBC, Shibuya *et al*[11] found 3 factors to be independently associated with the development of HCC: age at time of diagnosis, male gender, and a history of blood transfusion. Our findings showed that HCC cases arising from PBC were more common in men and those with liver cirrhosis.

While the number of HCC cases arising from PBC is stated to be small, it has been reported that the calculated crude incidence of HCC was 492.4/100 000 person years, and that HCC has a relatively high prevalence in PBC[7]. Furthermore, there is a dramatically increased risk for development of hepatobiliary malignancies in patients with PBC, with a relative risk of 46 ($P < 0.0001$) in women and 55 ($P < 0.0001$) in men[26].

Finally, several questions remain for the clinician. Namely, should AIH and PBC patients be screened for HCC? Should screening be limited to cirrhosis? Does the clinical course after diagnosis differ from other HCC patients? Late-stage AIH and PBC patients should be screened for HCC just as in HCV-related cirrhosis, given the similar reported incidence of HCC development in late-stage PBC[13]. Furthermore, Suzuki *et al*[27], reported very recently that patients of older age, male sex, history of blood transfusion, and any signs of portal hypertension or cirrhosis should be considered for HCC screening. A prospective study or a case control study for AIH patients is needed similar to that conducted for PBC patients.

At present, HCC transformation in early-stage pre-cirrhotic AIH and PBC were thought to be very rare. However, a high incidence of HCC development was observed in AIH and PBC patients with overlapping HCV and HBV infection, including occult HBV infection[9,19,20]. These patients should be closely followed using ultrasonography, CT-scanning and MRI of the abdomen, as well as tumor markers for HCC. Reports of HCC cases arising from "pure" AIH and PBC (with no history of blood transfusion, excessive alcohol intake, immunosuppressant administration, and with negative HBV and HCV serotyping) are rare[2,27-31]. El-Serag *et al*[32], in a multivariate analysis reported that AIH itself is not significant; however, our study indicates that early-stage AIH and PBC patients also have the potential to develop HCC. We advocate that "pure" or "early-stage" AIH and PBC cases should also be regularly screened for HCC.

Our data also indicate that the clinical course after diagnosis of HCC with AIH and PBC differs from virus-associated HCC, although prospective studies are needed to confirm these results. Clinicians should note the common clinical features of HCC cases with AIH and PBC at diagnosis, treatment, and follow-up of these patients.

Lastly, our findings also beg the question of why HCC rupture is the second most common cause of death in both groups of patients examined. We have recently reported a pelioid-type HCC patient with PBC, who died from rupture of HCC[33]. A peliotic change was observed more frequency in large poorly-differentiated and encapsuled HCC[34], and the features of pelioid-type HCC were high blood flow into the HCC, high pressure in the tumor and fibrous capsular formation. It is unknown whether the ruptured HCCs in the present study had these features, as this study had severe limitations because it was retrospective. Tumors in such patients may grow rapidly, and pathophysiological factors shared by both patient groups may trigger the rupture of HCC. A prospective study on the cause of death and a pathologic study of ruptured HCC with AIH and PBC is awaited with great interest.

Further clinical and laboratory studies are needed to describe which pathological, biological and genetic features are common among HCC cases arising from AIH and PBC. How HCC in these patients relates to viral hepatitis also requires further clarification. The present study was retrospective; however, this is the first study to date that highlights the importance of these future research topics. Future prospective studies on these important subjects are required.

COMMENTS

Background

Hepatocellular carcinoma (HCC) development in autoimmune hepatitis (AIH) as well as in primary biliary cirrhosis (PBC) is a rare event. The common clinical features of HCC associated with AIH and PBC have not yet been extensively