J Cancer Res Clin Oncol (1999) 125:419–426

ORIGINAL PAPER

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Microvessel density of hepatocellular carcinoma: its relationship with prognosis

Received: 27 November 1998 / Accepted: 5 January 1999

Abstract ***Purpose:*** To elucidate the relationship between angiogenesis and prognosis after curative resection of hepatocellular carcinoma (HCC). ***Methods:*** An immunohistochemical study using anti-CD34 monoclonal antibody was carried out on surgical specimens from 78 HCC patients who had undergone curative resection; microvessel density (MVD) was counted and the overall survival and disease-free survival were analyzed retrospectively. ***Results:*** Blood vessels in the tumor were strongly stained by anti-CD34 antibody, but not those in the surrounding liver parenchyma. There were three types of tumor vessels: capillary-like (n = 59), sinusoid-like (n = 16) and mixed-type (n = 3). The median MVD count was 100 per field. The HCC were designated as hypovascular (n = 36) with an MVD count below 100, and hypervascular (n = 42) with an MVD count of 100 or more per field. The 5-year survival and disease-free survival rates were 49.7% and 42.8% respectively, and statistical analysis showed that the MVD level was not correlated with tumor size, capsule status, Edmondson's grade, α-fetoprotein level, associated cirrhosis, γ-glutamyltransferase, and serum HBsAg status. The sinusoid-like tumor vessels appeared more frequently in the more differentiated tumors ($P < 0.05$). No statistical difference in overall and disease-free survival between different MVD levels and microvessel types was found. Tumor size was the only predicting factor in the entire series. In patients with small HCC (≤ 5 cm, n = 40), 5-year survival and disease-free survival rates were 58.9% and 52.7% respectively, higher than the values in large HCC (39.8% and 32.0% respectively, $P < 0.05$). The MVD level was an independent predicting factor of disease-free survival, 5-year disease-free survival in the hypovascular group (74.6%) being better than that in the hypervascular group (34.7%, $P < 0.05$). ***Conclusions:*** The MVD level was not related to tumor size, capsule statuo, Edmondson's grade, α-fetoprotein level, associated cirrhosis, γ-glutamyltransferase and serum HBsAg status. In the entire series, tumor size was the only factor influencing survival after curative resection. However, in patients with small HCC, the MVD level was an independent factor of disease-free survival. The pathological and clinical implications of different types of tumor vessels in HCC remain to be studied.

Key words Angiogenesis · Hepatocellular carcinoma
Prognosis · Microvessel density

Abbreviations *HCC* hepatocellular carcinoma · *MVD* microvessel density

Introduction

Hepatocellular carcinoma (HCC) is one of the major medical problems in China; about 10^5 people die of HCC in China every year. Surgical operation is the most effective method of treatment; however, even after curative resection of HCC, the recurrence rate is as high as 45% (Tang et al. 1995), 54.9% (Zhou et al. 1994) and 57% (Shuto et al. 1996) in the 5 years after operation, and is the major reason for the death of patients after operation (Ikeda et al. 1993). It has been demonstrated that there are both unicentric and multicentric origins for HCC recurrence (Liang et al. 1991; He et al. 1996), intrahepatic metastatic lesions beyond the surgical

This work was supported by China Medical Board of New York, USA, Grant 93-583 and the Leading Specialty of Shanghai Metropolitan Bureau of Public Health

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margin being one of the main sources of recurrence. Because re-resection of recurrent disease has proved effective in prolonging survival further (Ikeda et al. 1993; Tang et al. 1997), adjuvant treatments, such as transcatheter artery chemoembolization, interferon, and other biotherapeutic agents, are being tested as means of killing residual cancer cells or multicentric HCC lesions to minimize the chances of recurrence, a practical parameter is therefore needed to identify the group at high-risk of recurrence.

Many researchers have studied the relationship between patient outcome and clinicopathological factors, such as the number of tumor nodules, tumor size, capsule status, cirrhosis, portal vein involvement, DNA ploidy and serum α-fetoprotein mRNA etc. However, the results differed because the patients selected were not comparable (Noguchi et al. 1997; Terris et al. 1997; Chiu et al. 1997; Balsells et al. 1996; Ko et al. 1996; Sasaki et al. 1992; Shirabe et al. 1997).

Tumor growth and metastasis are angiogenesis-dependent (Folkman et al. 1996). Tumor microvessel density (MVD), reflecting angiogenesis in tumor areas, was proven to be an independent factor of prognosis and metastasis in many tumors (Weidner et al. 1993; Hollingsworth et al. 1995; Tanigawa et al. 1997a; Araya et al. 1997). HCC is a typical hypervascular tumor; intrahepatic and lung metastases suggest its hematogenous dissemination, and angiogenesis may be an important factor identifying those patients who are at high risk of recurrence or distant metastasis. This study aims to elucidate the relationship between MVD and prognosis of HCC patients after curative resection, attempting to provide a useful parameter for evaluating the risk of recurrence and survival.

Materials and methods

Patients

A group of 78 consecutive patients with HCC received curative resection in the Liver Cancer Institute, Shanghai Medical University, from January 1992 to May 1993, including lobectomy, segmentectomy, and local resection. There were 71 men and 7 women. The median age was 47.5 years (48.8 ± 12.18 years). In 72 of these patients (92.3%) disease was associated with liver cirrhosis, and 61 patients (78.2%) were positive for serum HBsAg. The median size of the HCC in this series was 5 cm. There were 38 patients with large HCC (> 5 cm), and 40 with small HCC (≤ 5 cm).

Before operation, no patient was found to have extrahepatic metastasis or multiple tumors (more than two nodules). The criteria for curative resection were no gross residual tumor in the remaining liver and no cancerous thrombi in the main trunk and secondary branches of the portal vein and hepatic veins. All patients were followed-up by monitoring serum α-fetoprotein and ultrasonography every 2 months after resection; for suspicious cases, computed tomography and/or magnetic resonance imaging were used to verify the recurrence. No chemotherapy or other treatment was given before the operation. Three patients were given transcatheter artery chemoembolization treatment after curative resection, including 1 who survived for 62 months and 2 who developed recurrence 22 and 26 months after operation respectively. Patients who survived less than 6 months because of liver failure have been excluded from this study. No patient underwent re-resection for recurrence in this series. Five cases of hemangioma and 2 cases of hyperplasia were also included in this study to examine the microvessels in the lesions.

Immunohistochemical study

Formalin-fixed, paraffin-embedded, pathologically proven HCC tissue blocks were collected from the tissue bank of the Department of Pathology in Zhong Shan Hospital. Immunohistochemical studies were performed on sections (4 μm thick) by the avidin-biotin immunoperoxidase method. Briefly, these sections were dewaxed in xylene, dehydrated in ethanol, then incubated with 3% hydrogen peroxidase for 30 min to block endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the sections were incubated in 10% normal mouse serum for 20 min to reduce the non-specific antibody binding. The sections were then incubated with a 1:50 diluted mouse CD34 monoclonal antibody (Clone QBEnd/10, NeoMarkers, Fremont, Calif. USA), which recognizes an endothelial cell surface marker CD34, at room temperature for 45 min. After three washes with PBS, the sections were incubated with 10 μg/ml biotinylated rabbit anti-(mouse IgG) for 30 min. They were then treated with peroxidase-conjugated streptavidin for 30 min at a concentration of 100 μg/ml followed by three washes with PBS. Finally, 0.02% diaminobenzidine and 1% hydrogen peroxidase were reacted for 10 min. Because CD34 is a proliferating endothelial cell surface marker, the blood vessels located in the normal liver served as the negative control, and tumor vessels served as a positive internal control.

Microvessel counting

The slides were examined under 100× magnification to identify the highest vascular density area within the tumor, and five areas of highest MVD were selected for counting under 200× magnification (0.708 mm^2/field). The average of the five areas was recorded as the MVD level of this case. Every single brown-stained cell and cell cluster was calculated as a blood vessel, no matter with or without the vessel lumen structure.

Because we found a kind of tumor vessel with large lumens, the regular counting method was not suitable to reflect the vascular density in this case. A modified method introduced by Tanigawa was used (Tanigawa et al. 1997b). Briefly, every 40-μm length of lumen was calculated as one point, as in the case of vessels of the regular type.

Statistical analysis

A two-sample t-test was used to compare the mean MVD counts of groups of patients. Survival curves and disease-free survival curves were estimated by the Kaplan-Meier method. Log-rank tests were used to compare the distribution of survival times of groups of patients. The χ^2-test was used to analyze the relationship between microvessel type and other pathological parameters. A multivariate Cox stepwise-regression model was used to examine the values of tumor size, tumor capsule statuo, Edmondson's grades, α-fetoprotein levels, γ-glutamyltransferase, serum HBsAg status and MVD levels for predicting survival times. Data analysis was performed using SPSS 7.5 for Windows (SPSS Inc., Chicago, Ill., USA). When analyzing disease-free status, the patients who died of liver failure without sign of recurrence were treated as censored cases.

Results

Immunohistochemistry

Immunohistochemical studies showed the tumor microvessels to be highlighted by the antibody against

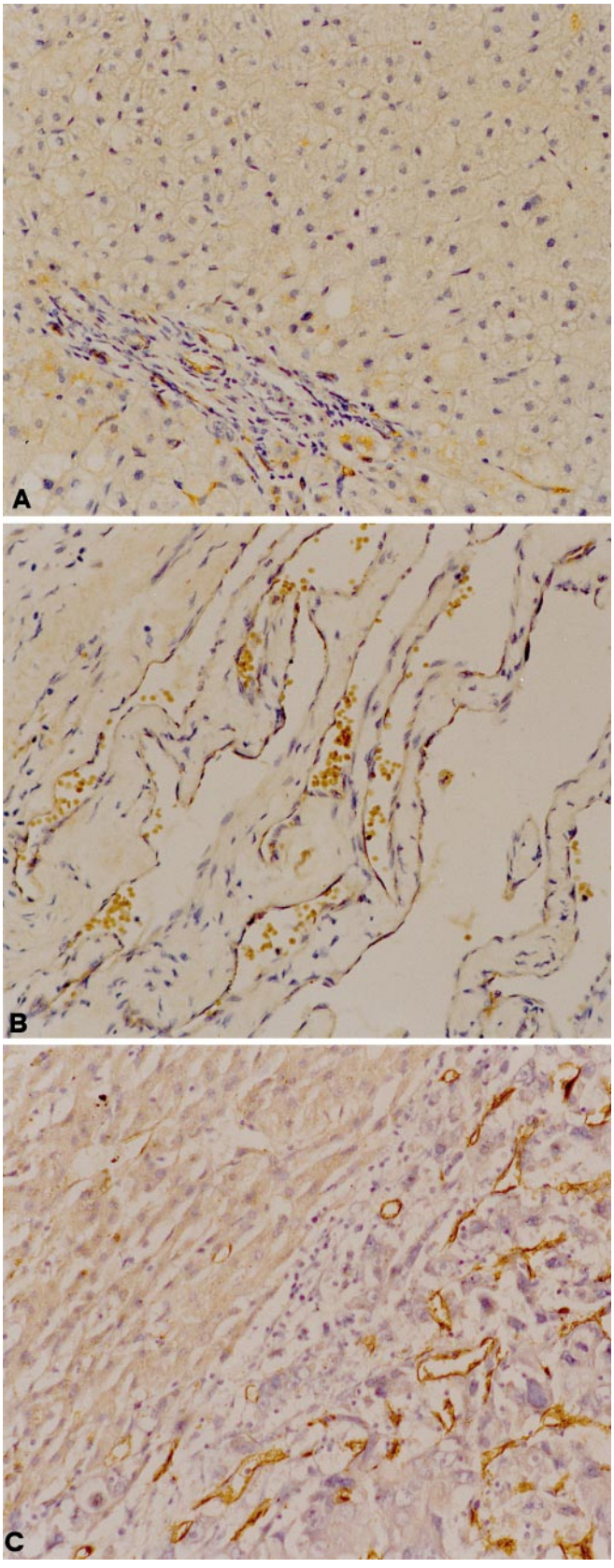

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Fig. 1A–C Immunohistochemical study of microvessels in different tissues using anti-CD34 antibody (200×). **A** Normal liver; the stained vessels are located only in the trabecular area. **B** Hemangioma; the endothelial cells were not stained. **C** Hepatocellular carcinoma (HCC) tissue surrounded by cirrhotic liver; CD34 is expressed on the endothelial cells in the tumor, but not on sinusoid endothelial cells in the surrounding cirrhotic liver (×400)

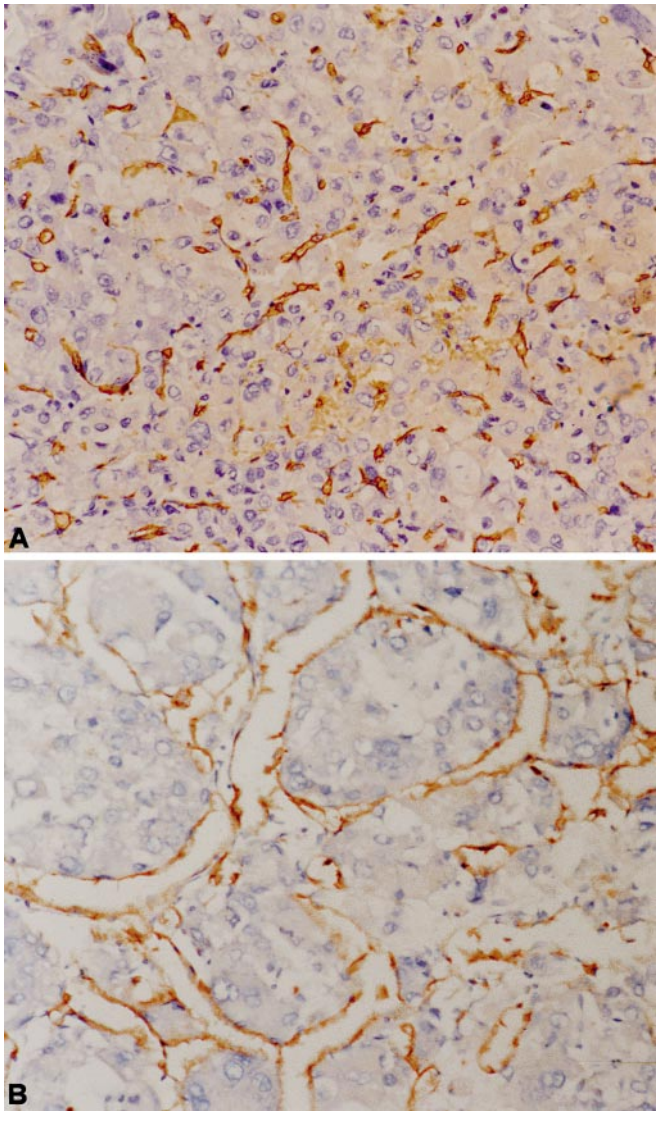

Fig. 2A,B Capillary-like and sinusoid-like microvessels in HCC highlighted by anti-CD34 antibody (200×). **A** capillary-like microvessels. **B** Sinusoid-like microvessels, with wide and long lumen structure; a modified microvessel counting method was used (×400)

CD34. Large blood vessels were faintly stained in some cases. In cirrhotic liver, and other focal liver diseases, like hemangioma and focal nodular hyperplasia, microvessels were not stained by this antibody, except in some trabecular areas.

There were three types of tumor vessels in this series: capillary (n = 59), the most frequently found type of microvessel, sinusoid (n = 16) with wide and long lumen structure, which was less frequently observed, and a mixed type (n = 3) (Figs. 1, 2).

Table 1 Microvessel density (*MVD*) count was not related to other pathological factors (*t*-test)

Pathological factor	*n*	MVD (mean ± SD)	*P*
α-Fetoprotein			
⩽20 μg/ml	28	109.0 ± 50.3	
>20 μg/ml	50	106.8 ± 60.3	0.866
Capsule status[a]			
Intact	50	111.4 ± 50.3	
Not intact	26	100.9 ± 68.1	0.476
Cirrhotic nodules			
⩽3 mm	55	101.4 ± 54.1	
>3 mm	23	122.5 ± 60.8	0.135
Edmondson' s grade			
I–II	7	120.6 ± 60.1	0.635 (1 versus 2)
II–III	64	108.8 ± 56.5	0.260 (1 versus 3)
III–IV	7	84.0 ± 55.6	0.229 (2 versus 3)
Tumor size			
⩽5 cm	40	111.8 ± 53.4	
>5 cm	38	103.3 ± 60.2	0.513
Serum γ-glutamyltransferase[b]			
⩽10	48	114.5 ± 59.2	
>10	30	96.6 ± 51.2	0.177
Serum HBsAg			
−	61	107.9 ± 41.0	
+	17	106.4 ± 60.5	0.937

[a] Only 76 patients had tumor capsule status recorded
[b] Modified Orlowski method

Table 2 The relationship between types of tumor vessel and other pathological parameters. (χ^2 method)

Pathological factor	Number of patients		*P*
	Capillary-like	Sinusoid-like[b]	
α-Fetoprotein			
⩽20 μg/ml	19	9	
>20 μg/ml	40	10	0.231
Capsule status[a]			
Intact	41	9	
Not intact	16	10	0.083
Cirrhotic nodules			
⩽3 mm	42	13	
>3 mm	17	6	0.818
Edmondson's grade			
I–II	2	5	
II–III	53	11	
III–IV	4	5	0.003*
Tumor size			
⩽5 cm	33	7	
>5 cm	26	12	0.148
Serum γ-glutamyltransferase			
⩽10	38	10	
>10	21	9	0.359
Serum HBsAg			
−	14	3	
+	45	16	0.466

* $P < 0.05$
[a] Only 76 patients had records of tumor capsules
[b] When calculated, the mixed type was combined with the sinusoid-like type

The relationship between MVD level and other clinicopathological factors

The median MVD count of the entire series was 100/field under 200× magnification (0.708 mm^2), therefore cases with MVD less than 100 were designated as being of the hypovascular type, and the rest were hypervascular. In this series, there were 36 cases of the hypervascular type, and 42 cases of the hypovascular type. As shown as in Table 1, The MVD level was not related to other clinicopathological factors.

The relationship between types of microvessel and other pathological factors

As shown in Table 2, the types of microvessels were not related to other clinicopathological factors except Edmondson's grade.

Patient outcome

The 5-year survival rate for the entire series was 49.7% with a median survival time of 40 months; for the small HCC ($n = 40$), it was 58.9% with a median survival time of 51.5 months.

Analysis of overall survival and disease-free survival

When comparing the survival curves of patients with different tumor sizes, we found both the overall and disease-free survival of patients with small HCC (58.9% and 52.7% at 5 years respectively) to be better than that of patients with large HCC (39.8% and 32.0% at 5 years respectively, $P < 0.05$) (Fig. 3). For the entire series, the Cox stepwise-regression model showed that tumor size

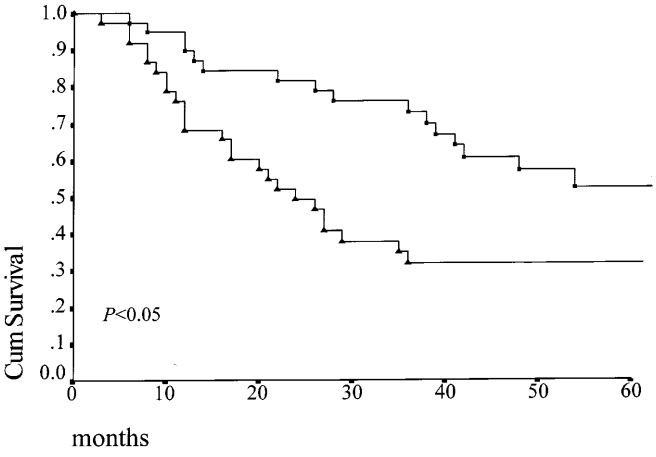

Fig. 3 Disease-free survival curves in the entire series with different tumor sizes. *Cum* Cumulative. Tumor size: ▲ > 5 cm ($n = 38$); ■ ⩽ 5 cm ($n = 40$)

Table 3 Comparison of 5-year survival rate (*OS*) and 5-year disease-free survival (*DFS*) rate in the entire series (Kaplan Meier method)

Factors	No. cases	5-year OS (%)	*P*	5-year DFS (%)	*P*
α-Fetoprotein					
⩽20 μg/ml	28	59.8		48.2	
>20 μg/ml	50	43.3	0.025	39.5	0.147
Tumor capsule[a]					
Intact	50	55.1		45.8	
Not intact	26	43.4	0.211	39.4	0.283
Edmondson's grade					
I–II	7	53.6		71.4	
II–III	64	48.2		38.8	
III–IV	7	57.1	0.894	45.7	0.317
Cirrhotic nodules					
⩽3 mm	56	50.9		42.8	
>3 mm	22	48.2	0.475	42.2	0.943
MVD count					
<100	36	56.2		52.8	
⩾100	42	41.9	0.322	33.5	0.174
Tumor size					
⩽5 cm	40	58.9		52.7	
>5 cm	38	39.8	0.048*	32.0	0.011*
Serum γ-glutamyltransferase					
⩽10	48	55.4		49.9	
>10	30	40.4	0.179	31.1	0.131
Serum HBsAg					
−	17	52.3		49.6	
+	61	48.9	0.296	41.3	0.267
Total	78	49.7		42.8	

* $P < 0.05$
[a] Only 76 patients had records of tumor capsules

Table 4 Comparison of 5-year survival rate (*OS*) and 5-year disease-free survival (*DFS*) rate in small HCC (Kaplan Meier method)

Factors	No. cases	5-year OS (%)	*P*	5-year DFS (%)	*P*
α-Fetoprotein					
⩽20 μg/ml	16	54.7		44.0	
>20 μg/ml	24	59.6	0.637	57.4	0.669
Tumor capsule					
Intact	29	52.3		45.9	
Not intact	11	71.6	0.479	68.2	0.345
Edmondson's grade					
I–II	6	62.5		66.7	
II–III	29	61.8		54.6	
III–IV	5	40.0	0.155	0	0.163
Cirrhotic nodules					
⩽3 mm	27	65.7		60.0	
>3 mm	13	46.4	0.052	36.3	0.047*
MVD count					
<100	18	70.5*		74.6[a]	
⩾100	22	47.3	0.163	34.7	0.020*
Serum γ-glutamyltransferase					
⩽10	27	65.1		56.2	
>10	13	44.4	0.149	39.5	0.512
Serum HbsAg status					
−	13	64.7		56.6	
+	27	56.8	0.369	52.0	0.607
Total	40	58.9		52.7	

* $P < 0.05$
[a] Because the deaths from liver failure were calculated as censored at the time of recurrence, the 5-year DFS rate was higher than the 5-year OS rate

was an independent factor for both overall and disease-free survival. In the group with small HCC, the Cox stepwise-regression model showed that MVD level was the only independent prognostic factor for disease-free survival, the 5-year rate being 74.6% for those patients with MVD less than 100, compared with 34.7% for the patients with an MVD count more than 100 ($P < 0.05$). (Tables 3, 4; Fig. 4).

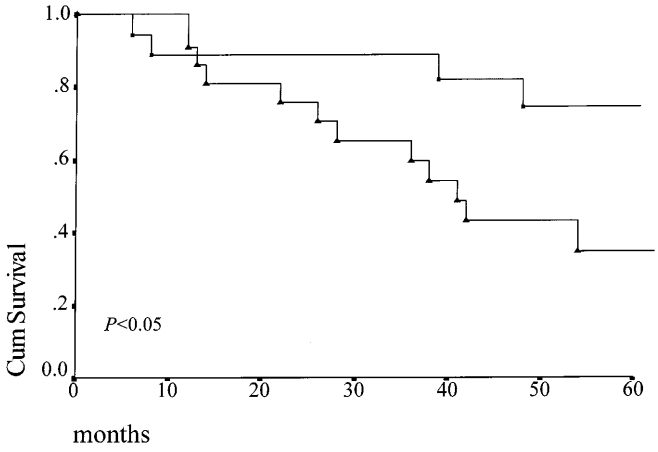

Fig. 4 Disease-free survival curves with different microvessel density (*MVD*) levels in patients with small HCC. MVD count: ▲ $\geqslant 100$ ($n = 42$); ■ < 100 ($n = 36$)

Discussion

Several endothelial cell markers, including antibodies against CD31, factor VIII, CD34, and UEA-1 have been tested for localization of endothelial cells in HCC tissues (Tanigawa et al. 1997b; Ruck et al. 1995; Cui et al. 1996; Terada et al. 1991). CD34, an L-selectin ligand located on the surface of endothelial cells at the growing sprouts during angiogenesis, was reported to be a more sensitive and specific marker for identifying the tumor vessels than other endothelial cell markers (Anthony and Ramani 1991; Hollingsworth et al. 1995; Tanigawa et al. 1997b). In this study CD34 was strongly expressed on the tumor endothelial cells; by contrast, very weak and spare expression of CD34 was detected in the surrounding cirrhotic liver parenchyma, mostly located in some big vessels at the trabecular area. In the hemangioma and focal nodular hyperplastic tissues, CD34 expression was not detected. This result was consistent with previous studies. Though several studies have reported that the intensity of CD34 expression was correlated with tumor progression (Nakamura et al. 1997), we failed to find any relationship with α-fetoprotein level, tumor size, Edmondson's grade or tumor capsule status.

Much research has focused on identifying the association between patients' outcome and clinicopathological characteristics of HCC, such as the number of tumor nodules, tumor size, capsule status, involvement of blood vessels, tumor ploidy, hepatitis B virus infection, chronic active hepatitis, age, preoperative liver function, etc. However, the results of different studies were inconsistent, as the selection of patients was incompatible. MVD studies in HCC are still conflicting. Two recent papers have demonstrated that factor VIII staining is correlated with the prognosis of HCC after curative resection (Yamamoto et al. 1998; El-Assal et al. 1998) and, in another paper (Kimura et al. 1998), the authors have reported that CD34 staining did not provide any prognostic information for HCC patients. However, the study of Tanigawa et al. indicated that MVD using anti-CD34 antibody was a predictive factor for the prognosis of HCC.

In this study, when preoperative α-fetoprotein level, Edmondson's grade, tumor capsule status, tumor size, serum γ-glutamyltransferase and HBsAg status and MVD level were included in a univariate analysis, tumor size was the only predicting factor of overall and disease-free survival. Because more than 80% of oriental HCC cases are associated with cirrhosis, the extent of hepatectomy was limited to retain enough liver and avoid damage to the big vessels; the so-called sufficient surgical margin may not be applied in liver surgery, particularly for a large tumor. Therefore, the chance of intrahepatic metastasis in a large tumor was higher than that in a small tumor and tumor size became the primary factor affecting whether resection could be curative and the prognosis.

In the patients with small HCC, we found that only MVD level was an independent factor determining the outcome for patients, according to the Cox stepwise-regression model, and the chance of disease-free survival of patients with hypovascular tumor was better than that of patients with hypervascular tumor (74.6% compared to 34.7%, $P = 0.020$). It has been well established that tumor angiogenesis is an important step in the progression of solid tumor from dormancy to aggressiveness. Not only is the growth of the primary tumor dependent on angiogenesis, but the development of metastatic lesions is as well. The onset of angiogenesis marks a period of rapid growth, local invasion and ultimately metastasis (Folkman et al. 1995). Hence, hypervascularity is a characteristic of tumors with a high possibility of metastasis. As shown in this study, although tumor size was the only prognostic factor for the entire series, MVD was the prognostic factor for overall and disease-free survival of the patients with small HCC, indicating that the patients with hypervascular tumors were at higher risk of developing a recurrence than those with hypovascular tumors. Tanigawa et al. also studied a series of HCC patient ($n = 43$) including 36 with small HCC (< 5 cm), and reported MVD levels that correlated with patient survival; MVD level was an independent factor of overall survival and disease-free survival (Tanigawa et al. 1997b). Because there were more patients with small HCC in Tanigawa's study, our result was slightly different from theirs because, in our series, there were more patients with larger HCC (48.7% compared to 16.3%), the influence of tumor size on the prognosis outweights that of MVD levels.

Sasaki et al. reported that coexisting cirrhosis influenced the long-term prognosis after surgery in patients with HCC (Sasaki et al. 1992). However, in this study, though liver cirrhosis was related to disease-free survival after curative resection of small HCC in the univariate analysis, it was not proved in the multivariate analysis.

We found that HCC-induced microvessels were of three different types: capillary-like, sinusoid-like and of

a mixed type. While the first appeared to possess phenotype-like capillaries, in the second the endothelial lining appeared to surround the tumor cell clusters, forming large lumens between tumor areas; this was similar to the sinusoid in normal liver. A χ^2-test showed that microvessel types were correlated with Edmondson's grade ($P = 0.003$). Sinusoid-like microvessels appeared more frequently in the differentiated HCC tissues. Consistent with previous reports (Nakamura et al. 1997; Kin et al. 1994), endothelial cells in some differentiated HCC that remained similar to the hepatocytes were also similar to those in normal liver; when tumor advanced to the less-differentiated stage, endothelial cells acquired the characteristics of capillaries. However, different vascular types were not statistically correlated to survival. The underlying implication of this phenomenon therefore remains to be investigated.

In conclusion, tumor size was the only factor influencing the overall survival and disease-free survival of patients with HCC. In the group with small HCC, univariate analysis showed that cirrhosis and MVD level were related to patient survival, but only MVD proved to be an independent predicting factor of overall and disease-free survival in multivariate analysis. The type of tumor vessels correlated with Edmondson's grade, but not with other pathological factors or survival, and was not a predictive factor for survival.

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