

Clinicopathological Criteria for Multicentricity of Hepatocellular Carcinoma and Risk Factors for Such Carcinogenesis

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Multicentric occurrence is an important characteristic of hepatocellular carcinoma. We evaluated clinicopathological criteria for multicentric hepatocellular carcinoma and identified risk factors for such carcinogenesis. Subjects were 251 consecutive patients undergoing liver resection for hepatocellular carcinoma. One kind of multicentric hepatocellular carcinoma had at least one tumor consisting of well-differentiated hepatocellular carcinoma, together with moderately or poorly differentiated hepatocellular carcinoma located in a separate region. The other kind had an area of well-differentiated component around hepatocellular carcinoma with less differentiation in all occurrences. The outcome of patients with tumors classified in this way was studied. Univariate and multivariate analyses were done to identify risk factors for multicentric hepatocellular carcinoma. The cumulative survival rate was significantly higher in patients with multicentric hepatocellular carcinoma than in patients with hepatocellular carcinoma associated with intrahepatic metastasis. Analysis by Cox's proportional hazard model showed that multicentricity was not a factor in the outcome. The risk of multicentric occurrence increases with progression of chronic liver disease. Univariate analysis showed hepatitis C virus marker and hepatitis B core antibody to be risk factors. By multivariate analysis, the odds ratio for multicentric occurrence in patients infected with hepatitis C virus and with serum hepatitis B virus core antibody compared with patients without either hepatitis C virus or hepatitis B virus was 10.86. This ratio in patients with hepatitis C virus alone was 4.30. These criteria for multicentric hepatocellular carcinoma seem to be clinically useful. Hepatitis C virus infection with or without former infection by hepatitis B virus is a strong risk factor for multicentric hepatocarcinogenesis.

Key words: Hepatocellular carcinoma — Hepatitis B virus — Hepatitis C virus — Multicentricity — Liver cirrhosis

Recent studies in molecular biology have shown that some hepatocellular carcinomas (HCCs) are multicentric in origin.^{1–5} It is important to know for individual patients whether multiple tumors in the liver are multicentric or metastases originated from a main tumor to decide the most appropriate treatment, because the results of treatment for single (unicentric) HCCs, multicentric HCCs, and HCCs with intrahepatic metastasis differ.^{6–8} Hepatitis C virus (HCV) and hepatitis B virus (HBV) are risk factors for HCC.⁹ In Japan^{10–12} and Europe,¹³ the incidence of HCC is higher in patients with HCV than in those with HBV. The difference in the incidence of HCC in patients with HCV or HBV affects decisions about treatment, because these viruses are factors in the outcome after surgery.^{14, 15} HCV-related HCCs tend to be multifocal,^{7, 9} but no large-scale studies on the incidence of and risk factors for multicentric occurrence of HCC have been reported.

We retrospectively evaluated criteria for identification of multicentric HCCs from clinicopathological findings,

examined the clinical differences among patients with single HCC, multicentric HCCs, and HCCs with intrahepatic metastasis, and investigated risk factors in multicentric carcinogenesis, especially the roles of HCV and HBV.

PATIENTS AND METHODS

Patients Subjects were all 251 patients who had undergone liver resection for HCC at our hospital during the past 6 years up to June 1996. Before surgery, patients underwent sonography, computed tomography, angiography, and (for most patients) magnetic resonance imaging. Intraoperative sonography also was done in all patients, and when a nodule(s) not detected earlier was found, it was biopsied. If the nodule was malignant or premalignant, it was resected, treated with microwaves, or injected with ethanol. This study was conducted in accordance with the Helsinki Declaration and the guidelines of the ethics committee of our institution. Informed consent was obtained from each patient.

Virus markers Serum samples obtained before surgery from all patients were assayed for HCV and HBV. Serum

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was examined for hepatitis B surface antigen (HBsAg) with an enzyme immunoassay (International Reagents Corp., Kobe), and for hepatitis B core antibody (HBcAb) with a radioimmunoassay (Dinabot, Tokyo). Samples were examined for HCVAb by second- or third-generation enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Tokyo). Serum was examined for HCV RNA by means of the polymerase chain reaction with reverse transcription (RT-PCR) and with primers derived from a conserved 5'-untranslated region of the viral genome.¹⁶⁾ Serum HBV DNA was assayed by nested PCR as reported previously.¹⁷⁾

Pathologic examination Resected specimens were cut into serial slices 5 mm thick and fixed in 10% formalin. Biopsy specimens were also fixed in 10% formalin. Sections from trimmed paraffin blocks containing at least one nodule were stained with hematoxylin and eosin. The number of sections examined was at least 10 for each patient and the maximum number was 302. A pathologic diagnosis was made based on the General Rules for the Clinical and Pathological Study of Primary Liver Cancer in Japan,¹⁸⁾ with some modifications.^{19, 20)} Well-differentiated HCC had high cell density, a high nucleus/cytoplasm ratio, strong cytoplasmic eosinophilia, and an irregular pattern with thin trabeculae; occasionally, the pattern was pseudoglandular. There was replacement growth at the boundary between the tumor and healthy tissue. Multicentric occurrence of HCC was classified into two categories by application of the criteria used in the General Rules,¹⁸⁾ with some modifications.⁴⁻⁷⁾ In one kind of occurrence (pattern 1), at least one tumor consisting of well-differentiated HCC grew in a replacement growth pattern, with hepatic structures maintained, and with moderately or poorly differentiated HCC elsewhere (near or far); a tumor embolus might be found in the portal vein of the main tumor. In the other kind (pattern 2), an area of well-differentiated HCC surrounded an area of HCC showing less differentiation in all occurrences. The validity of these criteria is suggested by metastases not being detected in other studies from well-differentiated HCC or from HCC of Edmondson-Steiner grade I,^{6, 19, 21-23)} and also by evidence that carcinogenesis occurs in steps in most tumors.^{21, 24-28)} Multiple HCCs that did not meet these criteria were assumed to be metastases originating from a main tumor. Noncancerous tissues also were examined histopathologically. At least two pathologists inspected each specimen.

Evaluation of criteria for multicentric HCCs To evaluate the clinical appropriateness of the criteria given above for multicentric HCCs, we compared the outcome after surgery among patients with single HCC, patients with only multicentric HCCs, patients with HCCs with intrahepatic metastasis, and patients with multicentric HCCs and intrahepatic metastasis.

Risk factors for multicentric HCCs We evaluated risk factors and calculated the relative risk of a number of variables for multicentric HCCs by univariate and multivariate analysis. The variables were selected for their potential relationship to multicentric occurrence as suggested by studies of the development of HCC in patients with chronic liver disease⁹⁻¹¹⁾ or by our own clinical experience. The variables chosen were the kind of virus(es) causing the infection, age, sex, history of heavy drinking [intake of at least 86 g of ethanol daily for at least 10 years²⁹⁾], history of blood transfusion, presence of liver cirrhosis, activity of hepatitis,³⁰⁾ and results of laboratory tests for α -fetoprotein concentration, indocyanine green retention rate at 15 min (ICGR₁₅), aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, total bilirubin concentration, platelet count, and albumin concentration.

Statistics Statistical analysis was done with SAS 6.11 (SAS Institute, Inc.). Analysis of variance followed by the Tukey method was used to compare the results of laboratory tests. If the results did not have a normal distribution, the analysis was done after logarithmic transformation. The χ^2 test followed by Bonferroni's method was used to compare categorical data among groups. Survival curves after surgery were calculated by the Kaplan-Meier method and the significance of differences was evaluated by the log-rank test. Cox's proportional hazards model was also used. Differences with $P < 0.05$ were considered to be statistically significant. The odds ratio was used to estimate relative risk for multicentric HCC. For univariate analysis, logistic regression analysis was used. For multivariate analysis, multiple logistic regression analysis with a stepwise procedure was used.

RESULTS

Profile of the subjects The profiles of the patients are summarized in Table I. Patients who received blood transfusions after 1989 when the screening of donated blood for HBcAb and HCVAb began are not listed here as having had transfusions, because the chance of infection with HCV and HBV became negligible. Twenty-six of the 38 patients with serum HBsAg had a family history of liver disease and 24 of the 123 patients with serum HBcAb, but not HBsAg had such a history. In one patient without serum HCVAb, HCV RNA was detected by RT-PCR. In 8 of the 182 patients with serum HCVAb, HCV was not detected by RT-PCR. The 183 patients in whom HCV was detected by RT-PCR or with serum HCVAb were assumed to be infected with HCV. Serum HBV DNA was not detected by PCR in any patient whose serum had neither HBsAg nor HBcAb.

Of the 251 patients, 95 had multiple HCCs. Of the 95 patients, 28 patients had multicentric HCCs alone, 58

Table I. Profile of the Patients

	Single HCC <i>n</i> =156	Multiple HCC		
		Multicentric HCC alone <i>n</i> =28	HCC with intrahepatic metastasis <i>n</i> =58	Multicentric HCC with intrahepatic metastasis <i>n</i> =9
Age, years (mean±SD)	59±8	63±4	61±10	63±4
Sex ratio (M:F) ^{a)}	122:34	22:6	48:10	9:0
Heavy drinker ^{a)}	45	6	16	5
History of blood transfusion ^{a)}	29	11	12	2
Family history of liver disease ^{a)}	42	5	15	2
Viral markers ^{a)}				
HCVAb or RNA	108	28 ^{c)}	40	8
HBsAg	28	0	9	1
HBcAb	69	20	25	7
α-Fetoprotein (≥20 ng/ml) ^{a)}	76	12	34	4
ICGR ₁₅ (%) ^{b)}	17.3 (8.5, 31.3)	20.8 (9.9, 31.2)	15.3 (6.2, 26.4)	18.4 (8.5, 23.8)
AST (IU/liter) ^{b)}	54 (31, 93)	65 (49, 123) ^{d)}	67 (30, 137)	62 (26, 154)
ALT (IU/liter) ^{b)}	61 (27, 125)	98 (41, 124) ^{e)}	50 (24, 121)	69 (27, 137)
Total bilirubin (mg/dl) ^{b)}	0.8 (0.5, 1.3)	0.9 (0.6, 1.4)	0.9 (0.5, 1.5)	0.9 (0.5, 1.8)
Albumin (g/dl) ^{b)}	3.7 (3.2, 4.2)	3.5 (3.1, 4.0)	3.6 (3.1, 4.1)	3.8 (2.8, 4.2)
Platelet count (×10 ⁴ /mm ³) ^{b)}	13.4 (7.2, 20.9)	10.3 (7.3, 18.3)	14.3 (8.3, 26.1)	12.1 (4.8, 33.4)
Cirrhosis ^{a)}	105	24	42	7

a) Numbers of patients.

b) Median with 10th and 90th percentiles.

c) $P < 0.001$ compared with patients with single HCC and $P < 0.005$ compared with patients with intrahepatic metastasis.

d) $P < 0.05$ compared with patients with single HCC.

e) $P < 0.05$ compared with patients with single HCC or with intrahepatic metastasis.

patients had HCCs with intrahepatic metastasis but without multicentric HCCs, and 9 patients had multicentric HCCs and intrahepatic metastasis; 156 patients had single HCC. Therefore, 37 patients had multicentric HCCs with or without intrahepatic metastasis. HCCs in 35 of the 37 patients were judged as multicentric HCCs by the criteria pattern 1 and HCCs in 2 other patients were judged as such HCCs by the criteria pattern 2.

Appropriateness of the clinicopathological criteria for multicentricity of HCCs Curves of survival rates after surgery are shown in Fig. 1. The rate was significantly different among groups ($P < 0.001$). The rate was higher in patients with multicentric HCCs than in patients with intrahepatic metastasis but without multicentric HCCs ($P < 0.005$). The rate was not different between patients with multicentric HCCs and with single HCCs. Analysis by Cox's proportional hazards model showed that intrahepatic metastasis was a factor in the outcome ($P < 0.0001$); the relative risk was 3.94 (CI, 2.13–7.29). Multicentric occurrence was not a risk factor ($P = 0.435$). Therefore, the clinicopathological criteria that we describe for multicentric occurrence of HCC appear to be of practical use.

Clinical characteristics of multicentric HCC The prevalence of HCV was significantly higher in the patients with multicentric HCCs alone than in patients with single

HCC ($P < 0.001$) or with intrahepatic metastasis ($P < 0.005$; Table I). The prevalence of HBcAb was significantly different in the different groups overall by the χ^2 test ($P < 0.05$), but no significant difference was found between groups by Bonferroni's method. AST activity was significantly higher in patients with multicentric HCCs than in patients with single HCC ($P < 0.05$). ALT activity was significantly higher in patients with multicentric HCC alone than in patients with single HCC ($P < 0.05$) or with intrahepatic metastasis ($P < 0.05$).

Risk factors for multicentric HCCs Table II shows the odds ratios of possible risk factors for multicentric HCC calculated by univariate analysis. By univariate analysis, HCV, HBcAb, history of blood transfusion, ICGR₁₅, ALT activity, total bilirubin, and platelet count were significant factors. The odds ratios for the categories of HCV, HBcAb, male sex, history of blood transfusion, and liver cirrhosis were each more than 2.0. The odds ratios for ICGR₁₅, AST, ALT, and total bilirubin were more than 1.0 and the odds ratios for the platelet count and albumin were less than 1.0, indicating that the risk of multicentric carcinogenesis increases as ICGR₁₅, AST and ALT activities, and the total bilirubin level increase and as the platelet count and the albumin level decrease. Adenomatous hyperplasia was not a risk factor (data not shown). Table

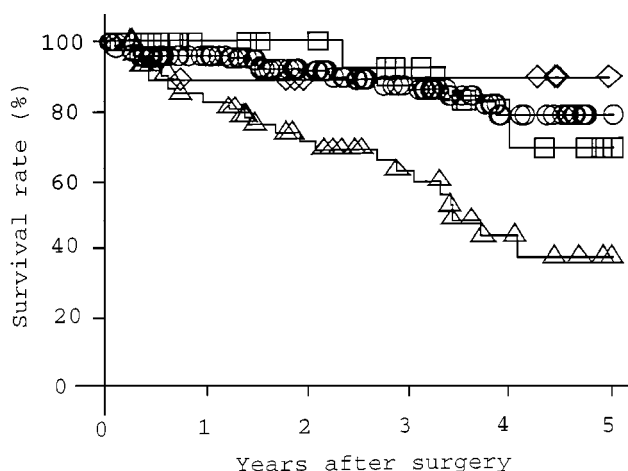


Fig. 1. Cumulative survival rates (Kaplan-Meier) after liver resection for hepatocellular carcinoma. ○, 156 patients with single HCC; □, 28 patients with multicentric HCCs; △, 58 patients with intrahepatic metastasis; ◇, 9 patients with multicentric HCC and intrahepatic metastasis. The rate was higher in patients with multicentric HCCs than in patients with intrahepatic metastasis ($P < 0.005$). The rate was not different between patients with multicentric HCCs and with single HCCs.

III shows adjusted odds ratios of selected variables calculated by multivariate analysis. The adjusted odds ratio of HCV was 4.455 and the ratio of HBcAb was 2.809. The risk increased as the total bilirubin level increased. These results indicate that the state of infection by hepatitis viruses was important. Therefore, odds ratios of various viral states were studied (Table IV). Compared with the ratio for patients without HCV infection and without serum HBsAg or HBcAb, the ratio for patients infected with HCV and with serum HBcAb was 10.86; the ratio in patients infected with HCV alone was 4.30. The odds ratio for the patients with serum HBsAg and HBcAb was 0.42. The results suggest that some interaction between current HCV infection and prior infection with HBV is involved in the development of multicentric HCCs.

DISCUSSION

Whether tumors in a particular patient are multicentric in origin can be found by examination of integrated HBV DNAs¹⁻³⁾ in HBV-related HCC, the pattern of loss of heterozygosity on chromosome 16,⁴⁾ and the mutation pattern of the *p53* gene.³¹⁾ HCV is unlikely to be integrated into the genome,⁹⁾ and clonal analysis cannot be used to investigate the multicentricity of tumors in patients infected with HCV. Examination of heterozygosity on chromosome 16 and of mutation of the *p53* gene is not possible routinely. For clinical use, a simpler method to decide

Table II. Odds Ratios of Possible Risk Factors for Multicentric HCC, Calculated by Univariate Analysis

Variables	Odds ratio	95% CI
Without HCVAb or RNA	1.000	
With HCVAb or RNA	7.804	1.823-33.409
Without HBsAg or HBcAb	1.000	
Without HBsAg but with HBcAb	2.653	1.141-6.165
With HBsAg and HBcAb	0.191	0.023-1.581
Female	1.000	
Male	2.950	0.866-10.10
Not heavy drinker	1.000	
Heavy drinker ^{a)}	1.061	0.494-2.280
Without history of blood transfusion	1.000	
With history of blood transfusion	2.286	1.073-4.867
Without liver cirrhosis	1.000	
With liver cirrhosis	2.355	0.938-5.913
Without active hepatitis	1.000	
With active hepatitis	1.316	0.654-2.651
Age (per year)	1.026	0.980-1.075
α -Fetoprotein (per 1 ng/ml)	1.000	1.000
ICGR ₁₅ ^{b)} (per percentage point)	1.040	1.002-1.079
Aspartate aminotransferase (per 1 IU/liter)	1.005	0.998-1.013
Alanine aminotransferase (per 1 IU/liter)	1.011	1.003-1.020
Total bilirubin (per 0.1 mg/dl)	1.329	1.026-1.759
Platelet count (per 10 ⁴ /mm ³)	0.930	0.869-0.995
Albumin (per 1 g/dl)	0.580	0.236-1.425

a) Heavy drinking was as defined by the Liver Cancer Study Group of Japan²⁸⁾ (intake of at least 86 g of ethanol daily for at least 10 years).

b) The 15-min indocyanine green retention rate.

Table III. Adjusted Odds Ratios of Possible Risk Factors for Multicentric HCC, Calculated by Multivariate Analysis

Variables	Adjusted odds ratio	95% CI
Without HCVAb or RNA	1.000	
With HCVAb or RNA	4.455	0.928-21.381
Without HBsAg or HBcAb	1.000	
Without HBsAg but with HBcAb	2.809	1.169-6.752
With HBsAg and HBcAb	0.447	0.047-4.243
Total bilirubin (per 0.1 mg/dl)	1.396	1.043-1.996

The stepwise procedure was done with variables other than viral markers.

whether HCC is probably multicentric is needed. In Japan, various clinicopathological criteria described above (see "Pathologic examination") are currently being used

Table IV. Odds Ratios of Various Patterns of Viral Infection

Viral state			No. of patients	No. of patients with multicentric tumors (%)	Odds ratio	95% CI
HCV	HBsAg	HBcAb				
+	-	-	65	7 (11)	4.30	0.91-20.26
+	-	+	108	28 (26)	10.86	0.99-119.66
+	+	+	10	0 (0)	1.80	0.04-78.39
-	-	-	15	1 (7)	1.00	1.00
-	-	+	15	0 (0)	2.53	1.08-5.91
-	+	+	38	1 (3)	0.42	0.05-3.87
Total			251	37 ^{a)}		

a) Nine of the 37 patients also had HCCs other than multicentric HCCs.

in decisions about treatment. The significant difference in the outcome of patients with multicentric HCCs and patients with HCCs with intrahepatic metastasis, and the lack of a significant difference in the outcome of patients with multicentric HCCs and patients with single HCC were confirmed again in the results reported here. In addition, the cumulative survival rate in patients with intrahepatic metastasis reported previously^{8, 32-34)} seems to be worse than the rates in this study. The findings indicate that the criteria used here to distinguish between multicentric HCC and metastasis originating from a main tumor are of clinical value, although there is a possibility that all tumors consisting of moderately or poorly differentiated HCC will be taken to be metastatic.

Many risk factors for the development of HCC have been reported: age, male sex, alcohol abuse, history of blood transfusion, liver cirrhosis, and infection by a hepatitis virus.^{9-11, 13)} In our study, HCV, HBcAb, and history of blood transfusion were significant risk factors. The odds ratios for the viral state, male sex, history of blood transfusion, and liver cirrhosis were all more than 2.0. We found an odds ratio for liver cirrhosis of 2.355 and one for active hepatitis of 1.316 by univariate analysis. We also found that AST and ALT activities were significantly higher in patients with multicentric HCC alone than in patients with single HCC and that the risk of multicentric carcinogenesis increased as ICGR₁₅, AST, ALT, and total bilirubin increased and as the platelet count and albumin level decreased. These results indicate that the potential of multicentric carcinogenesis increased with the progression of chronic liver disease and that continuous hepatitis is responsible for such carcinogenesis.

In this study, the prevalence of HCV was significantly higher in the patients with multicentric HCCs alone than in patients with single HCC or in patients with intrahepatic metastasis. HCV seems to be a stronger risk factor than HBV in multicentric HCCs. The difference may be related to the higher incidence of HCC development in patients with HCV (about 7% per year in Japan^{10, 16)}) than

in patients with HBV.^{10, 11)} The high odds ratio for having HBcAb with or without HCV suggests that HBV infection in the past or present may be important for HCC development, especially in the presence of HCV. The mechanism of the interaction, if any, of HCV and HBV during multicentric carcinogenesis is unknown. Some investigators have found that co-infection of HCV and HBV has a synergistic effect on HCC development.^{35, 36)} We have reported that HCC developed a mean of 5.6 years earlier after blood transfusion in patients with HCV and a history of HBV infection than in patients with HCV alone.³⁷⁾ Recent analysis of HBV DNA sequences showed that the *HBx* gene is sometimes detected in HCC and noncancerous tissues in patients without HBsAg.^{12, 38-41)} The HBV DNA is integrated into the cellular genome, after which rearrangements of the cellular DNA, insertional mutagenesis, production of truncated HBV proteins such as preS2/S and X proteins, activation of cellular proto-oncogenes, and inhibition of the function of *p53* may lead to carcinogenesis.^{9, 42-48)} In one possible mechanism, genetic changes or carcinogenesis caused by integration of *HBV* gene could be worsened in the presence of the continuous hepatitis caused by HCV.

The odds ratio for having both HCV and HBsAg was 1.80, but because there were only 10 such patients, we cannot interpret this result. On the other hand, the odds ratio for having HBsAg alone was low. The mean age at detection of HCC is lower for patients with HBsAg alone than for patients with HCV infection.^{7, 14, 15, 49)} The likelihood of multicentric carcinogenesis is low in patients with HBsAg, because the HCC often develops before cirrhosis.^{11, 49)}

The results in this study indicate that the clinicopathological criteria for multicentric HCC are of practical value and that HCV infection and a combination of current HCV infection and prior infection of HBV (perhaps with an integrated *HBV* gene or genes) are strong risk factors for multicentric HCCs. When treatment for HCC is being selected, the viral state of the patient must be taken into

consideration. Different states carry different risks of multicentric HCCs, even after resection of HCC.

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