

## Comparison of resection and ablation for hepatocellular carcinoma: A cohort study based on a Japanese nationwide survey

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**Background & Aims:** The treatment of choice for early or moderately advanced hepatocellular carcinoma (HCC) with good liver function remains controversial. We evaluated the therapeutic impacts of surgical resection (SR), percutaneous ethanol injection (PEI), and radiofrequency ablation (RFA) on long-term outcomes in patients with HCC.

**Methods:** A database constructed on the basis of a Japanese nationwide survey of 28,510 patients with HCC treated by SR, PEI, or RFA between 2000 and 2005 was used to identify 12,968 patients who had no more than 3 tumors ( $\leq 3$  cm) and liver damage of class A or B. The patients were divided into SR (n = 5361), RFA (n = 5548), and PEI groups (n = 2059). Overall survival and time to recurrence were compared among them.

**Results:** Median follow-up was 2.16 years. Overall survival at 3 and 5 years was respectively 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA, and 78.9%/56.3% in the PEI. Time to recurrence at 3 and 5 years was 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively. On multivariate analysis, the hazard ratio for death was significantly lower in the SR group than in the RFA (SR vs. RFA: 0.84, 95% confidence interval, 0.74–0.95;  $p = 0.006$ ) and PEI groups (SR vs. PEI: 0.75, 0.64–0.86;  $p = 0.0001$ ). The hazard ratios for recurrence were also lower in the SR group than in the RFA (SR vs. RFA: 0.74, 0.68–0.79;  $p = 0.0001$ ) and PEI groups (SR vs. PEI: 0.59, 0.54–0.65;  $p = 0.0001$ ).

**Conclusions:** Our findings suggest that surgical resection results in longer overall survival and shorter time to recurrence than either RFA or PEI in patients with HCC.

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### Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, worldwide [1]. Outcomes remain disappointing, despite recent progress in the techniques of diagnosis and therapy. Japanese [2], European [3] and American [4] clinical practice guidelines strongly recommend surgical resection (SR) and percutaneous ablation, including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI), for the management of early or moderately advanced HCC (i.e., up to 3 tumors 3 cm or less in diameter) in patients with adequately maintained liver function. Although comparative studies of these treatments have been conducted previously [5–7], the most suitable treatment strategy still remains controversial.

By nationwide surveys initiated in 1965, the Liver Cancer Study Group of Japan has prospectively collected data on patients with HCC in Japan. The Group conducted two retrospective analyses to define the treatment with the best outcomes [8,9]. However, each of the analyses was flawed, and had several problems: data on RFA were not included in the first report [8], and the follow-up period was short in the second one [9]. Although the second analysis demonstrated that surgical resection was superior to RFA and PEI for preventing recurrence [9], no apparent difference in the overall survival could be discerned between surgery and percutaneous ablation therapies (RFA and PEI). Thus, the treatment of choice for less advanced HCC still remains under debate.

Before starting this study, the results of 2 randomized controlled trials (RCT) were available [10,11]. As we pointed out in a previous report [12], however, the study designs of these 2

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**Abbreviations:** HCC, hepatocellular carcinoma; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; TACE, transcatheter hepatic arterial chemoembolization.



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trials were critically flawed by factors such as insufficient sample size, excessively optimistic hypotheses, and high conversion ratios. Because of these problems, the results of the two RCTs do not allow firm conclusions to be drawn concerning the important clinical question: is surgery or percutaneous ablation the treatment of choice for early or moderately advanced HCC? To answer this question, we conducted this cohort study based on the latest data available from a Japanese nationwide survey.

### Patients and methods

#### Patients and settings

The Liver Cancer Study Group of Japan has performed nationwide surveys of patients with primary liver cancer since 1965. Patients are registered and followed up, as reported previously [9]. Although this study protocol was not submitted to the Institutional Review Board of each institution participating in the nationwide survey, the collection and registration of data of patients with HCC were performed with the approval of each institution. Because RFA has been available for clinical use since 1999 in Japan, we set the study period from 2000 to 2005, to exclude preliminary experiences with RFA. During this period, a total of 28,510 patients with HCC were registered and received surgical resection, RFA or PEI as the primary treatment with curative intent for HCC. We identified 12,968 patients who met the following criteria: (1) liver function classified as liver damage A or B defined by the Liver cancer Study Group of Japan [13]; (2) number of tumors 3 or less; (3) maximum tumor diameter  $\leq 3$  cm. The 12,968 patients were divided into 3 groups according to the treatment received: SR group ( $n = 5361$ , 41.3%), RFA group ( $n = 5548$ , 42.8%), and PEI group ( $n = 2059$ , 15.9%). The diagnostic criteria and details of follow-up were described previously [8]. Because it has been unusual for biopsies to be performed in cases treated by percutaneous ablation in Japan, histological findings such as microscopic vascular invasion, tumor grading, and microscopic intrahepatic metastasis were not evaluated in this study. Relevant clinical data were collected and analyzed.

#### Statistical analyses

The baseline characteristics of the three groups (Table 1) were compared by analysis of variance for continuous variables and by Chi-square or Mantel-trend tests for categorical variables. Consistent with our preliminary report [9], the SR group had a higher proportion of younger patients and male patients than the RFA and PEI groups. Hepatitis C virus infection was less prevalent in the SR group than in the RFA and PEI groups. Based on the liver damage class, serum albumin and total bilirubin levels, platelet counts, and the indocyanine green retention rate at 15 min, liver function was better in the SR group than in the RFA and PEI groups, consistent with our previous report [9]. As for tumor-related factors, the number of tumors was smaller, and the maximum tumor diameter was larger in the SR group than in the RFA or PEI group. The SR group had the lowest proportion of patients with abnormally elevated alpha-fetoprotein levels ( $\geq 15$  ng/ml) and the highest proportion of patients with abnormally elevated des- $\gamma$ -carboxy prothrombin levels ( $\geq 40$  AU/ml).

Overall survival and time to recurrence curves were plotted using the Kaplan-Meier method and compared with the use of the log-rank test. Recurrence was diagnosed on the basis of imaging studies, clinical data, and/or histopathological studies at each institution [9].

The therapeutic impacts of surgical resection, RFA and PEI were estimated using a Cox proportional hazards model including the following 10 covariates: age, gender, liver damage class, hepatitis C virus antibody, hepatitis B surface antigen, platelet count, number of tumors, tumor size, and serum alpha-fetoprotein and des- $\gamma$ -carboxy prothrombin levels. The results of multivariate analysis were expressed as hazard ratios with 95% confidence intervals.  $p$  values of  $<0.05$  were considered to indicate statistical significance.

For the subgroup analyses, the study populations were classified into 8 subgroups according to the tumor size ( $<$  or  $\geq 2$  cm), tumor number (single or multiple), and liver damage class (A or B). Macroscopic vascular invasion was excluded from the subgroup analyses because its presence is a contraindication to percutaneous ablation therapies. The therapeutic impacts of the three treatments were evaluated in each of these subgroups, and hazard ratios with 95% confidence intervals and  $p$  values were calculated according to the above three factors (tumor size, number of tumors, and liver damage class).

### Results

The median follow-up after treatment was 2.16 years, and the 5th and 95th percentiles were 0.14 and 5.19 years, respectively. The overall survival rates at 3/5 years were 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA group, and 78.9%/56.3% in the PEI group (Fig. 1). The median survival times were 8.4, 5.9, and 5.6 years in the three groups, respectively. The time to recurrence rates at 3/5 years in the 3 groups were 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively (Fig. 2).

According to the results of the multivariate analysis, the hazard ratio for death in the SR group was 0.84 (0.74–0.95,  $p = 0.006$ ) relative to that in the RFA group, and 0.75 (0.64–0.86,  $p = 0.0001$ ) relative to that in the PEI group (Table 2A). The hazard ratios for recurrence in the SR group were 0.74 (0.68–0.79,  $p = 0.0001$ ) and 0.59 (0.54–0.65,  $p = 0.0001$ ) relative to those in the RFA and PEI groups, respectively (Table 2B). These results indicated that the overall survival and time to recurrence rates were both significantly better in the SR group than in the RFA and PEI groups.

The overall survival rates following surgical resection, RFA and PEI in the 4 subgroups with a single tumor are shown in Fig. 3A–D. The results of the subgroup analyses (summarized in Fig. 4A) showed that the overall survival was significantly longer in the SR group than in the RFA group in 2 subgroups of patients, namely, those who had a single tumor smaller than 2 cm in diameter with liver damage class A, and those who had a single tumor 2 cm or larger in diameter with liver damage class B.

As shown in Fig. 4B, the time to recurrence was shorter in the SR group than that in the RFA group in the 4 following subgroups: patients with a single tumor with liver damage class A (regardless of the tumor size), those with multiple tumors 2 cm or larger in diameter with liver damage class A, and those with a single tumor 2 cm or larger in diameter with liver damage class B.

### Discussion

Our study showed that surgical resection was associated with significantly lower risk of both death and recurrence as compared to RFA and PEI in patients with early or moderately advanced HCC. Our previous preliminary report [9] suggested that surgery reduces the risk of recurrence, but failed to demonstrate any difference in the overall survival between surgery and percutaneous ablation therapies in patients with early or moderately advanced HCC. The present study reconfirms that surgery is associated with a reduced recurrence rate and newly shows that surgery yields a longer overall survival than percutaneous ablation therapies.

Differences in the results between the present study and previous investigations are most likely related to the sample size and length of follow-up. The total number of subjects increased markedly from 7185 in our previous study to 12,968 in this study, and the median follow-up period increased from 10.4 months to 2.16 years (25.9 months). These factors are considered not only to have enhanced the reliability of our findings, but also to have strengthened our conclusions. We believe that our results, which are, of course, subject to the inherent drawbacks of the study design, are meaningful, given the current lack of credible data derived from well-designed RCTs.

The large sample size and prolonged follow-up period also allowed us to perform several subgroup analyses, which were not feasible in our previous study [9]. We classified the patients

Table 1. Baseline characteristics.

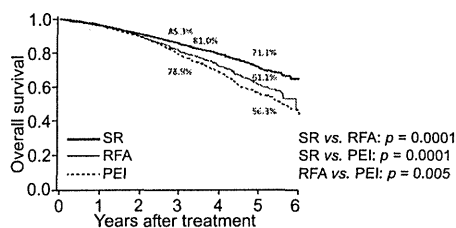
Variables	SR n = 5361	RFA n = 5548	PEI n = 2059	p value
Age, median (5, 95 percentile), yr	66 (48, 77)	69 (52, 80)	69 (52, 80)	<0.0001 <sup>a</sup>
Sex				<0.0001 <sup>b</sup>
Male, No. (%)	3967 (74.0)	3569 (64.3)	1303 (63.3)	
Female, No. (%)	1394 (26.0)	1979 (35.7)	756 (36.7)	
Hepatitis virus infection				<0.0001 <sup>b</sup>
HBs Ag(+)/HCV-Ab(-), No. (%)	908 (16.9)	462 (8.3)	141 (6.8)	
HBs Ag(-)/HCV-Ab(+), No. (%)	3393 (63.3)	4263 (76.8)	1632 (79.3)	
HBs Ag(+)/HCV-Ab(+), No. (%)	106 (2.0)	87 (1.6)	32 (1.6)	
HBs Ag(-)/HCV-Ab(-), No. (%)	760 (14.2)	512 (9.2)	160 (7.8)	
Unknown	194 (3.6)	224 (4.0)	94 (4.6)	
Liver damage				<0.0001 <sup>b</sup>
A, No. (%)	4000 (74.6)	3349 (60.4)	1204 (58.5)	
B, No. (%)	1361 (25.4)	2199 (39.6)	855 (41.5)	
Serum albumin, median (5, 95 percentile), g/dl	3.9 (3.1, 4.6)	3.7 (2.9, 4.4)	3.7 (2.8, 4.4)	<0.0001 <sup>a</sup>
Serum total bilirubin, median (5, 95 percentile), mg/dl	0.8 (0.4, 1.5)	0.9 (0.4, 1.9)	0.9 (0.4, 2.2)	<0.0001 <sup>a</sup>
Platelet count, median (5, 95 percentile), x 10 <sup>4</sup> /μl	12.6 (5.8, 24.0)	9.9 (4.5, 20.4)	9.5 (4.4, 19.6)	<0.0001 <sup>a</sup>
ICG R15, median (5, 95 percentile), %	15 (5, 35)	22 (7, 51)	24 (8, 51)	<0.0001 <sup>a</sup>
Tumor number				<0.0001 <sup>c</sup>
Single, No. (%)	4458 (83.2)	4068 (73.3)	1449 (70.4)	
Two, No. (%)	706 (13.2)	1096 (19.8)	443 (21.5)	
Three, No. (%)	197 (3.7)	384 (6.9)	167 (8.1)	
Tumor size, median (5, 95 percentile), mm	23 (12, 30)	20 (10, 30)	17 (10, 30)	<0.0001 <sup>a</sup>
Alpha-fetoprotein				<0.0001 <sup>b</sup>
≥15 ng/ml, No. (%)	2726 (50.9)	3028 (54.6)	1125 (54.6)	
<15 ng/ml, No. (%)	2457 (45.8)	2301 (41.5)	828 (40.2)	
Unknown, No. (%)	178 (3.3)	219 (3.9)	106 (5.2)	
Des-γ-carboxy prothrombin				<0.0001 <sup>b</sup>
≥40 AU/ml, No. (%)	2182 (40.7)	1593 (28.7)	541 (26.3)	
<40 AU/ml, No. (%)	2651 (49.5)	3322 (59.9)	1169 (56.8)	
Unknown, No. (%)	528 (9.9)	633 (11.4)	349 (17.0)	

HBsAg, hepatitis B virus antigen; HCV-Ab, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min.

<sup>a</sup>ANOVA.

<sup>b</sup>Chi-square.

<sup>c</sup>Mante-trend test.



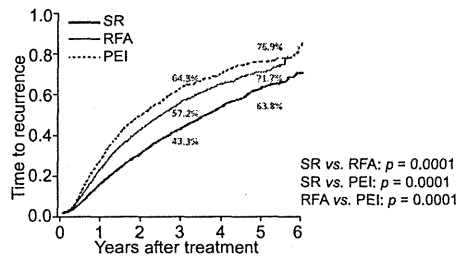
Patients at risk	SR	RFA	PEI
0	5361	5548	2059
1	3833	3780	1595
2	2570	2328	1112
3	1680	1264	718
4	894	569	444
5	400	160	247
6	29	5	58

Fig. 1. Overall survival curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

into 8 subgroups according to 3 factors (liver damage class, tumor size, and number of tumors), which have repeatedly been shown to be clinically relevant prognostic factors. The results of the sub-

group analyses indicated that surgical resection would effectively prevent recurrence in patients with relatively advanced HCC (2–3 cm in diameter) among the study populations, irrespective of liver damage class or number of tumors. This finding suggests that surgery might be superior to percutaneous ablation therapies in patients with a more advanced tumor stage. As for the subgroups with a single tumor, surgical resection yielded better overall survival and time to recurrence rates than RFA or PEI. Especially in the subgroup with a single tumor smaller than 2 cm in diameter, both the overall and time to recurrence rates were statistically significantly better after surgery than after RFA, whereas no such statistically significant differences in these two parameters between the two treatment groups were detected in a few subgroups with a single tumor, maybe due to the insufficient sample size of the subgroups. Thus, surgical resection would be considered as the treatment modality of first choice for a single HCC, as recommended by the Japanese clinical practice guideline [2]. Overall, there was a trend toward superior

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Patients at risk							
SR	5361	3265	1844	1039	451	189	15
RFA	5548	2954	1396	591	225	62	4
PEI	2059	1154	583	304	172	90	15

Fig. 2. Time to recurrence curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

overall and time to recurrence rates after surgery than after RFA and PEI.

The reason why the long-term outcomes of the SR group were better than those of the PEI and RFA groups cannot be definitely

clarified from the results of this study, however, in theory, surgical resection has the advantage of offering better local control of HCC over PEI and RFA, both of which have some potential risks of local recurrence associated with insufficient ablation. In addition, anatomic resection to remove minute tumor satellites [14] might have decreased the recurrence rate in the SR group, although this remains a speculation.

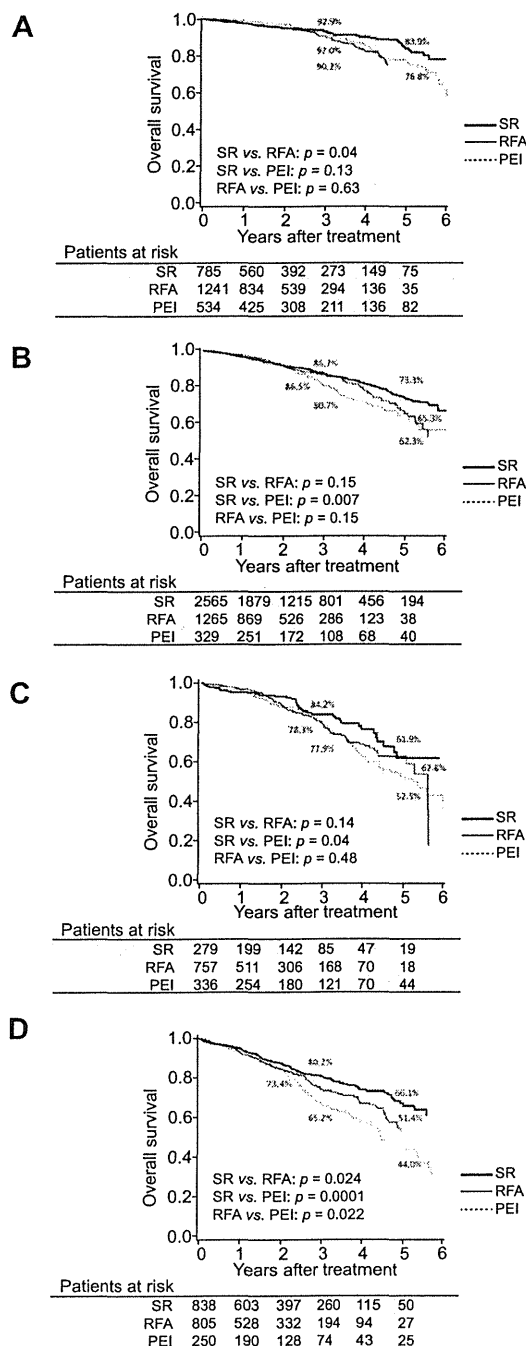
Recently, the latest trial from China [15], which had an adequate sample size (total 230 patients), reported that surgical resection yielded significantly better long-term outcomes than RFA. Although the study design was better than that of the two previously reported RCTs [10,11], it appeared to have limitations with respect to the results, such as drop in the overall survival in the RFA group as compared with that in the surgery group during the early period after treatment. The early deaths in the RFA group could have been treatment-related rather than cancer-related. Thus, no conclusion can be drawn from the three currently available RCTs.

One of the limitations of our study is the diversity of demographic factors in the study population, which would have been

Table 2. Hazard ratios for death and recurrence adjusted by multivariate analysis.

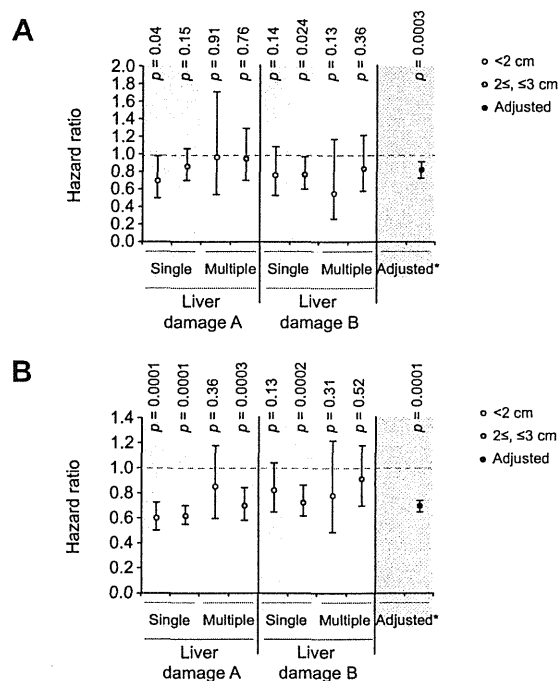
A For death				
Variables		Hazard ratio	95% CI	p value
Treatments	SR vs. RFA	0.84	0.74, 0.95	0.006
	SR vs. PEI	0.75	0.64, 0.86	0.0001
	RFA vs. PEI	0.88	0.77, 1.01	0.08
Age	<65 vs. ≥65	0.71	0.63, 0.79	0.0001
Sex	Female vs. male	0.87	0.78, 0.98	0.03
HBsAg	Positive vs. negative	0.91	0.74, 1.11	0.34
HCV Ab	Positive vs. negative	0.93	0.79, 1.10	0.40
Liver damage	A vs. B	0.62	0.56, 0.69	0.0001
Platelet count	≥10 <sup>4</sup> vs. <10 <sup>4</sup> /μl	0.76	0.68, 0.85	0.0001
Tumor size	<2 vs. ≥2 cm	0.82	0.73, 0.92	0.0007
Tumor number	Single vs. multiple	0.72	0.64, 0.80	0.0001
AFP	<15 vs. ≥15 ng/ml	0.66	0.59, 0.74	0.0001
DCP	<40 vs. ≥40 AU/ml	0.59	0.53, 0.66	0.0001
B For recurrence				
Variables		Hazard ratio	95% CI	p value
Treatments	SR vs. RFA	0.74	0.68, 0.79	0.0001
	SR vs. PEI	0.59	0.54, 0.65	0.0001
	RFA vs. PEI	0.81	0.74, 0.88	0.0001
Age	<65 vs. ≥65	0.83	0.78, 0.89	0.0001
Sex	Female vs. male	0.88	0.82, 0.95	0.0001
HBsAg	Positive vs. negative	1.04	0.92, 1.17	0.53
HCV Ab	Positive vs. negative	1.15	1.04, 1.27	0.007
Liver damage	A vs. B	0.87	0.81, 0.93	0.0001
Platelet count	≥10 <sup>4</sup> vs. <10 <sup>4</sup> /μl	0.92	0.86, 0.98	0.02
Tumor size	<2 vs. ≥2 cm	0.84	0.79, 0.90	0.0001
Tumor number	Single vs. multiple	0.69	0.64, 0.74	0.0001
AFP	<15 vs. ≥15 ng/ml	0.71	0.67, 0.76	0.0001
DCP	<40 vs. ≥40 AU/ml	0.72	0.67, 0.77	0.0001

HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; Ab, antibody; AFP, alpha-fetoprotein; DCP, des-γ-carboxy prothrombin; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; CI, confidence interval.



**Fig. 3.** Overall survival rates after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI) in the subgroup of cases with single tumor and liver damage class A and B. (A) Liver damage class A, a single tumor (<2 cm); (B) liver damage class A, a single tumor (2–3 cm); (C) liver damage class B, a single tumor (<2 cm); (D) liver damage class B, a single tumor (2–3 cm).

caused by the selection process of treatment modalities. As similar to the previous retrospective studies [5–9], the patients amenable to surgery had had younger age, less prevalence of hepatitis



**Fig. 4.** Hazard ratios for death and recurrence with 95% confidence intervals and  $p$  values after surgical resection relative to those after radiofrequency ablation in the 8 subgroups. \*The adjusted values for death and recurrence were calculated according to the three factors (tumor size, number of tumors, and liver damage class), as done in each subgroup. (A) Hazard ratios for death; (B) hazard ratios for recurrence.

C virus infection, better liver function, less association with portal hypertension, fewer number of tumors and lower alpha-fetoprotein level, whereas their tumor size was larger and their des- $\gamma$ -carboxy prothrombin level was higher. To minimize potential effects of confounding factors, we studied patients who had similar tumor-related and liver function-related factors and performed multivariate analysis using 10 clinically important factors, similar to our previous study [9]. Although it is impossible to completely eliminate potential negative impacts of demographic diversity, we believe that our results are clinically meaningful, because of the large sample size of our study. In Japan, a nationwide RCT in patients with HCC is now ongoing, and the results are expected to lead to more definitive conclusions [16].

Another potential limitation of our study is the lack of data on liver function during the follow-up, which precluded assessment of the relationship between the liver function status and the choice of treatment at recurrence. In HCC, the influence of the first treatment is considered to be smaller than that in other primary malignant diseases, because the liver function remarkably affects the recurrence rate. Further investigations, particularly prospective clinical trials, are needed to address these issues.

In conclusion, this large cohort study based on data obtained by a nationwide survey in Japan, suggests that surgical resection may offer some advantage over RFA and PEI in terms of both overall survival and time to recurrence in patients with less advanced HCC. Although our results are considered as being more reliable than those of previous studies comparing the treatment

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outcomes in HCC, our conclusions need to be confirmed by future RCTs.

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### Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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## Low Hepatitis C Viral Load Predicts Better Long-Term Outcomes in Patients Undergoing Resection of Hepatocellular Carcinoma Irrespective of Serologic Eradication of Hepatitis C Virus

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### ABSTRACT

#### Purpose

Hepatitis C virus (HCV) infection has been recognized as a potent risk factor for the postoperative recurrence of hepatocellular carcinoma (HCC). However, little is known about the impact of HCV viral load on surgical outcomes. The study objective was to investigate clinical significance of HCV viral load on long-term outcomes of HCC.

#### Patients and Methods

Three hundred seventy patients who were classified as Child-Pugh class A and underwent curative liver resections for HCV-related HCC were divided into low and high viral load groups ( $\leq$  or  $>$  5.3  $\log_{10}$  IU/mL) based on the results of a minimum *P* value approach to predict moderate to severe activity of hepatitis; the clinical outcomes were then compared.

#### Results

The 5-year recurrence-free survival rate was 36.1% in the low viral load group and 12.4% in the high viral load group ( $P < .001$ ). The 5-year overall survival rate was 76.6% in the low viral load group and 57.7% in the high viral load group ( $P < .001$ ). Multivariate analysis confirmed significant correlation between high viral load and tumor recurrence with a hazard ratio of 1.87 (95% CI, 1.41 to 2.48;  $P < .001$ ). Subanalysis revealed that the favorable results in the low viral load group were not attributed to whether or not serologic eradication of HCV was obtained both in primary and recurrent lesions.

#### Conclusion

Low HCV viral load predicts better long-term surgical outcomes in patients with HCC regardless of the serologic eradication of HCV.

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### INTRODUCTION

Recent developments in medical and surgical treatments have significantly improved the long-term outcomes of patients with hepatocellular carcinoma (HCC).<sup>1</sup> However, the cumulative recurrence rate remains as high as 50% to 60% at 3 years and 70% to 100% at 5 years, even after curative liver resection.<sup>2-7</sup>

Hepatitis C virus (HCV), a major cause of chronic hepatitis and liver cirrhosis, has been recognized as a potent risk factor of carcinogenesis<sup>8</sup> and/or the recurrence of HCC.<sup>9,10</sup> Because postoperative persistent viremia is thought to be the main cause of sustained liver dysfunction and the high tumor recurrence rate in patients with HCV, adjuvant antiviral therapy using interferon (IFN)

has recently been attempted, and favorable outcomes have been reported in several studies.<sup>11-13</sup>

Conventionally, the eradication of HCV and a sustained status of undetectable HCV-RNA have been regarded as the most important factors for obtaining better clinical results after IFN therapy. In recent studies, however, possibly favorable effects of a reduced viral load on long-term outcomes have been suggested in patients with chronic hepatitis.<sup>14,15</sup>

Our hypothesis was that a correlation existed between the HCV viral load and long-term surgical outcomes. Simply labeling patient as having viremia did not sufficiently stratify those who had low viral load versus those who had high viral load. In this study, we tested this hypothesis by examining patients who had undergone curative

liver resection for HCV-related HCC and analyzed the impact of the HCV viral load on postoperative outcomes.

## PATIENTS AND METHODS

### Study Population

This study was performed in accordance with the ethical guidelines for clinical studies at the University of Tokyo Hospital (Tokyo, Japan). The subject pool consisted of 508 consecutive patients who underwent curative liver resection for HCV-related HCC between January 2002 and December 2011. Patients classified as Child-Pugh class B ( $n = 49$ ) or patients missing preoperative viral load data ( $n = 89$ ) were excluded because the goal of this study was to reveal the prognostic impact of the HCV viral load in patients who were considered to be capable of tolerating antiviral therapies. The remaining 370 patients were included in the analysis.

### Serum HCV-RNA Quantification

Serum HCV-RNA was quantified within 4 weeks before surgery using a conventional reverse transcriptase polymerase chain reaction (PCR) assay before 2007 and a new commercially available real-time PCR assay (TaqMan PCR; Roche Molecular Systems, Pleasanton, CA) in 2007 and thereafter. In this study, the viral load unit was standardized to a logarithm style ( $\log_{10}$  IU/mL) for the statistical analysis according to the following equation:  $Y (\log_{10}$  IU/mL) =  $\log_{10} [X (\text{kIU/mL}) \times 10^3]$ .

### Surgical Treatment and Histopathologic Assessments

The indications for hepatic resection and the types of operative procedures were determined as previously described.<sup>16</sup> Briefly, operative decisions were based on an algorithm consisting of the presence of ascites, the serum total bilirubin level, and the results of an indocyanine green tolerance test.<sup>17</sup> Because HCC has a high propensity to invade the portal veins and because intrahepatic metastasis via vascular invasion is one of the major forms of recurrence, tumor-bearing portal regions (ie, the segment or subsegment of the liver) were systematically removed (ie, an anatomic resection) to reduce the risk of local recurrence as long as such resections were feasible given the functional reserve of the liver.<sup>18</sup>

The histologic classifications of the tumor and background liver were described based on the system of the Liver Cancer Study Group of Japan.<sup>19</sup> The histologic differentiation of HCC (well, moderate, or poor) was determined according to the Edmondson grade.<sup>19,20</sup> Both the fibrotic stage and the activity of the hepatitis in the background liver were also recorded according to the classification proposed by Desmet et al.<sup>21</sup>

### Postoperative Antiviral Therapy

Postoperative adjuvant IFN therapy was performed only in patients who had a good performance status and were capable of tolerating a standard

high-dose combination therapy with ribavirin. Specifically, patients who were younger than 65 years of age, had no evidence of cirrhosis, and had a sufficient platelet count ( $> 9.0 \times 10^4/\mu\text{L}$ ) were considered good candidates for postoperative antiviral therapy.

### Patient Follow-Up

All patients were regularly screened for recurrences through the evaluation of the HCC-specific tumor markers  $\alpha$ -fetoprotein (AFP) and des- $\gamma$ -carboxyprothrombin every 1 to 2 months, with ultrasonography every 2 months, and with dynamic computed tomography every 4 months, as previously reported.<sup>22</sup> The HCV viral load was re-examined after surgery in possible candidates for adjuvant antiviral therapy. The function of the background liver was monitored using the serum ALT levels. If the ALT levels increased beyond 100 IU/L, an appropriate dose of ursodeoxycholic acid and/or monoammonium glycyrrhizinate was administered expecting their liver protective effects.<sup>23,24</sup>

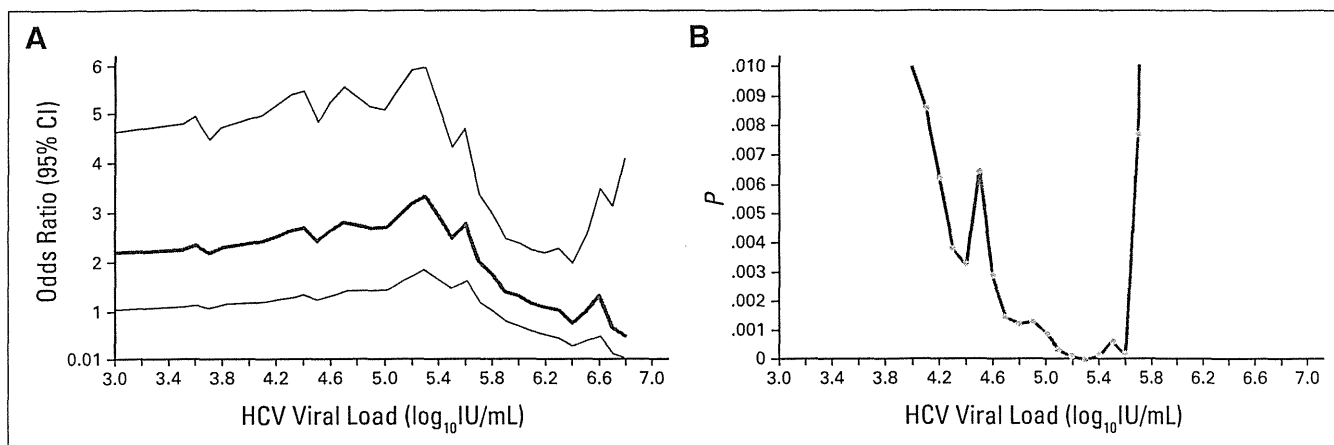
Recurrence was defined as the appearance of a new lesion with radiologic features compatible with HCC, as confirmed using at least two imaging modalities. When a recurrence was detected, the patient received further treatment using a repeated hepatectomy, radiofrequency ablation, transcatheter arterial chemoembolization (TACE), or other treatment options, as indicated. In the present study, recurrence-free survival (RFS) was defined as the interval between the operation and the date of the diagnosis of the first recurrence or the last follow-up examination, and overall survival (OS) was calculated based on the time from surgery to death or last follow-up.

### Data Analysis

Statistical analysis was performed using SAS software, version 9.3 (SAS Institute, Cary, NC). Medians and ranges of continuous data were compared using the Mann-Whitney  $U$  test. Categorical data were compared using Pearson's  $\chi^2$  test or Fisher's exact test as appropriate.  $P < .05$  was considered statistically significant.

High viral load was defined as HCV viral load to predict moderate to severe activity of hepatitis (grade 2 or 3 in Desmet classification<sup>21</sup>). The cutoff value was determined using the minimum  $P$  value approach, and clinical outcomes were compared between the patients with a high viral load and those with a low viral load. In addition, the low viral load group was further subclassified according to whether or not HCV-RNA was detectable, and clinical outcomes between these subgroups were also compared.

Survival curves for OS and RFS were generated using the Kaplan-Meier method and were compared using the log-rank test. To identify risk factors for tumor recurrence, multivariate regression analysis was performed with the Cox proportional hazards model using a backward elimination procedure. To prevent overfitting, only factors that showed statistically significant association with tumor recurrence with  $P < .10$  were included in the final model. Prognostic value of HCV viral load was



**Fig 1.** Optimal cutoff value of hepatitis C virus (HCV) RNA viral load to predict moderate to severe activity of hepatitis. (A) Plot of odds ratio. (B) Plot of  $P$  value in likelihood test (null hypothesis: odds ratio, 1).



Impact of HCV Viral Load on Surgical Outcomes of HCC

Table 1. Baseline Demographics and Clinical Characteristics

Characteristic	Patients With Low Viral Load (n = 168)		Patients With High Viral Load (n = 202)		P
	No.	%	No.	%	
Age, years					.07
Median	69		70		
Range	47-83		39-85		
Sex					< .001
Male	139	82.7	131	64.9	
Female	29	17.3	71	35.1	
HBsAg					.24
Positive	7	4.2	4	2.0	
Negative	161	95.8	198	98.0	
HBcAb					.30
Positive	45	28.5	59	33.7	
Negative	113	71.5	116	66.3	
HCV genotype					0.07
1b	40	69.0	87	81.3	
Other	18	31.0	20	18.7	
HCV-RNA, log <sub>10</sub> IU/mL					< .001
Mean	3.0		6.0		
Standard deviation	1.9		0.4		
History of IFN therapy					< .001
Positive	69	41.6	48	24.0	
Negative	97	58.4	152	76.0	
No. of tumors					.87
Solitary	107	63.7	127	63.2	
Multiple	61	36.3	75	37.1	
Maximum diameter of the tumor, mm					.45
Median	24		25		
Range	8-130		6-200		
AST, IU/L					< .001
Median	34		49		
Interquartile range	24-56		36-63		
ALT, IU/L					< .001
Median	32		47		
Interquartile range	20-52		29-67		
Total bilirubin, mg/dL					.12
Median	0.7		0.7		
Interquartile range	0.5-0.9		0.6-0.9		
PT, %					.18
Median	82.9		82.9		
Interquartile range	73.1-94.9		75.6-98.3		
ICG-R15, %					< .001
Median	13.8		16.9		
Interquartile range	8.8-20.8		12.1-24.3		
Platelets, 10 <sup>4</sup> /μL					.11
Median	14.4		14.0		
Interquartile range	11.4-19.0		10.1-17.1		
AFP, ng/mL					< .001
Median	9		15		
Interquartile range	4-42		7-99		
DCP, mAU/mL					.49
Median	32		35		
Interquartile range	17-177		19-146		

Abbreviations: AFP, α-fetoprotein; DCP, des-γ-carboxyprothrombin; HBcAb, hepatitis B core antibody; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green retention rate at 15 minutes; IFN, interferon; LC, liver cirrhosis; PT, prothrombin time.

quantified by comparing Harrell's concordance statistics of prognostic models based on the results of the multivariate analysis.

## RESULTS

### Characteristics of High and Low HCV Viral Load Groups

The best cutoff value of HCV viral load to predict moderate to severe activity of hepatitis was more than  $5.3 \log_{10}$  IU/mL in both the plots of odds ratio and *P* value in the likelihood test (Fig 1). The background characteristics are compared between the high viral load (*n* = 202) and low viral load (*n* = 168) groups in Table 1. Female sex was more

frequent in the high viral load group than in the low viral load group. The rates of coinfection with hepatitis B were not significantly different between the two groups. A history of IFN therapy was more common in the low viral load group. Number and maximum diameter of lesions were comparable between the two groups. The serum ALT and AST levels, indocyanine green retention rate at 15 minutes, and AFP levels were significantly higher in the high viral load group, whereas the platelet count was almost the same between the groups.

As for surgical factors (Table 2), the initial hepatectomy rates were 51.8% and 67.3% in the low and high viral load groups, respectively (*P* < .001). The remaining patients had repeat hepatectomies for

**Table 2.** Surgical, Histopathologic, and Postoperative Factors

Factor	Patients With Low Viral Load ( <i>n</i> = 168)		Patients With High Viral Load ( <i>n</i> = 202)		<i>P</i>
	No.	%	No.	%	
<b>Surgical factors</b>					
Liver resection					< .001
First HX	87	51.8	136	67.3	
≥ Second HX	81	48.2	66	32.7	
Operation time, minutes					.06
Median	359		330		
Interquartile range	279-461		263-443		
Blood loss, g					.77
Median	700		660		
Interquartile range	370-1,059		350-1,050		
Transfusion	7	4.2	7	3.5	.79
Anatomic resection	73	43.5	80	39.6	.45
Surgical margin, mm					.20
Mean	3.1		3.6		
Standard deviation	5.4		4.9		
<b>Histopathologic factors</b>					
Tumor differentiation*					.86
Well	35	21.5	42	21.2	
Moderate	106	65.0	134	67.7	
Poor	22	13.5	22	11.1	
Major vascular invasion	10	5.9	5	2.6	.39
Microvascular invasion	59	35.3	64	32.0	.50
Fibrosis score†					.06
F0-2	52	49.1	56	37.1	
F3-4	54	50.9	95	62.9	
<b>Postoperative factors</b>					
Adjuvant IFN therapy	6	3.6	7	3.5	1.00
HCV-RNA at 1 year, $\log_{10}$ IU/mL‡					< .001
Mean	3.3		6.0		
Standard deviation	2.0		0.4		
ALT after surgery, IU/L					.004
Median	33		42		
Interquartile range	20-56		28-64		
AFP at 1 month, ng/mL					< .001
Median	4		7		
Interquartile range	3-7		4-13		
DCP at 1 month, mAU/mL					.08
Median	14		15		
Interquartile range	10-16		10-19		

Abbreviations: AFP,  $\alpha$ -fetoprotein; DCP, des- $\gamma$ -carboxyprothrombin; HCV, hepatitis C virus; HX, hepatectomy; IFN, interferon.

\*Based on modification of the Edmondson grade.<sup>19</sup>

†Based on the classification by Desmet et al.<sup>21</sup>

‡Based on data from 31 and 28 patients with low viral load and high viral load, respectively.

recurrent lesions. Type of surgery (anatomic *v* nonanatomic), operating time, blood loss, and surgical margins were comparable between the groups. Histopathologically, no significant difference was observed in histologic grade of tumor or presence of vascular invasions. Fibrotic scores tended to be higher in the high viral load group.

Postoperative IFN therapy was performed in only six patients (3.6%) and seven patients (3.5%) in the low and high viral load groups, respectively. Because the median age of the patients in this study was 70 years and approximately 50% of the patients exhibited marked thrombocytopenia and/or cirrhotic changes in their background livers, the standard combination therapy of IFN with ribavirin was difficult to apply in most of the patients.

In 59 patients in whom postoperative viral load data were available, the HCV-RNA levels did not significantly change from baseline to 1 year after surgery ( $4.5 \pm 2.0 \log_{10}$ IU/mL before surgery *v*  $4.6 \pm 1.9 \log_{10}$ IU/mL after surgery;  $P = .78$ ). HCV-RNA viral load at 1 year and postoperative mean ALT levels were higher in the high viral load group. Postoperative AFP levels were also higher in the high viral load group even after curative resection.

**Patient Survival**

The median follow-up time of the studied population was 38.4 months (range, 1 to 120 months), and no hospital deaths occurred. During the study period, recurrence was observed in 108 patients (60.7%) and 137 patients (71.4%) in the low and high viral load groups, respectively.

The 1-, 3-, and 5-year RFS rates were 66.1%, 37.4%, and 36.1% in the low viral load group and 60.2%, 25.8%, and 14.9% in the high viral load group, respectively ( $P < .001$ ; log-rank test). The 3- and 5-year OS rates were 87.6% and 76.6% in the low viral load group and 77.2% and 57.7% in the high viral load group, respectively ( $P < .001$ ; log-rank test; Fig 2). At the time of the first recurrence, multiple intrahepatic recurrences were more frequent in the high viral load group (47.5%) than in the low viral load group (33.8%;  $P = .05$ ). Repeat hepatectomy, radiofrequency ablation, and TACE were performed for intrahepatic recurrence in 26.0% ( $n = 33$ ), 19.7% ( $n = 25$ ), and 42.5% ( $n = 54$ ) of patients in the high viral load group, respectively,

and 41.0% ( $n = 34$ ), 20.5% ( $n = 17$ ), and 30.1% ( $n = 25$ ) of patients in the low viral load group, respectively ( $P = .06$ ).

The median HCV viral load of the positive HCV-RNA subgroup in the patients with low viral load was  $4.9 \log_{10}$ IU/mL (range, 2.3 to 5.3  $\log_{10}$ IU/mL), and it was significantly lower than that in the high viral load group ( $P < .001$ ). Clinicopathologic parameters were almost comparable between the two subgroups in the low viral group except that HCV-RNA titers and serum AST and ALT levels were significantly higher in positive HCV-RNA patients ( $P < .001$ ). The 1- and 3-year RFS rates were similar between the two subgroups (65.6% and 38.8% for the negative HCV-RNA patients and 66.5% and 35.9% for the positive HCV-RNA patients, respectively;  $P = .61$ ; Fig 3A). The RFS rate among the low viral load group with positive HCV-RNA was superior to that of the high viral load group ( $P = .009$ ). A similar tendency was also observed in the OS rates. The positive HCV-RNA patients had relatively favorable results, similar to the negative HCV-RNA patients when the viral load was  $\leq 5.3 \log_{10}$ IU/mL. The 3- and 5-year OS rates were 85.8% and 78.1% for the negative HCV-RNA patients, respectively, and 89.0% and 75.8% for the positive HCV-RNA patients, respectively ( $P = .94$ ; Fig 3B). The OS rate of the low viral load group with positive HCV-RNA was superior to that of the high viral load group ( $P = .005$ ). These observations were constant when stratifying the study population according to hepatectomies for primary or recurrent lesions (Appendix Fig A1, online only).

**Risk Factors for Postoperative Recurrence**

Risk factors for postoperative recurrence were investigated in 357 patients without postoperative antiviral therapy. In the multivariate analysis, we chose 17 potential confounders considering their clinical significance and reported evidences,<sup>4,25-31</sup> as indicated in Table 3. There were no specific combinations of factors suggesting multicollinearity in scatter plots. In multivariate analysis, high HCV viral load ( $> 5.3 \log_{10}$ IU/mL), macroscopic vascular invasion, repeat resection for recurrent tumor, tumor exposure, and tumor size greater than 2 cm were selected in the final model. The concordance statistic of the four-factor model (macroscopic vascular invasion + repeat resection + tumor exposure + size  $> 2$  cm) was 0.603 (95% CI, 0.559 to 0.647),

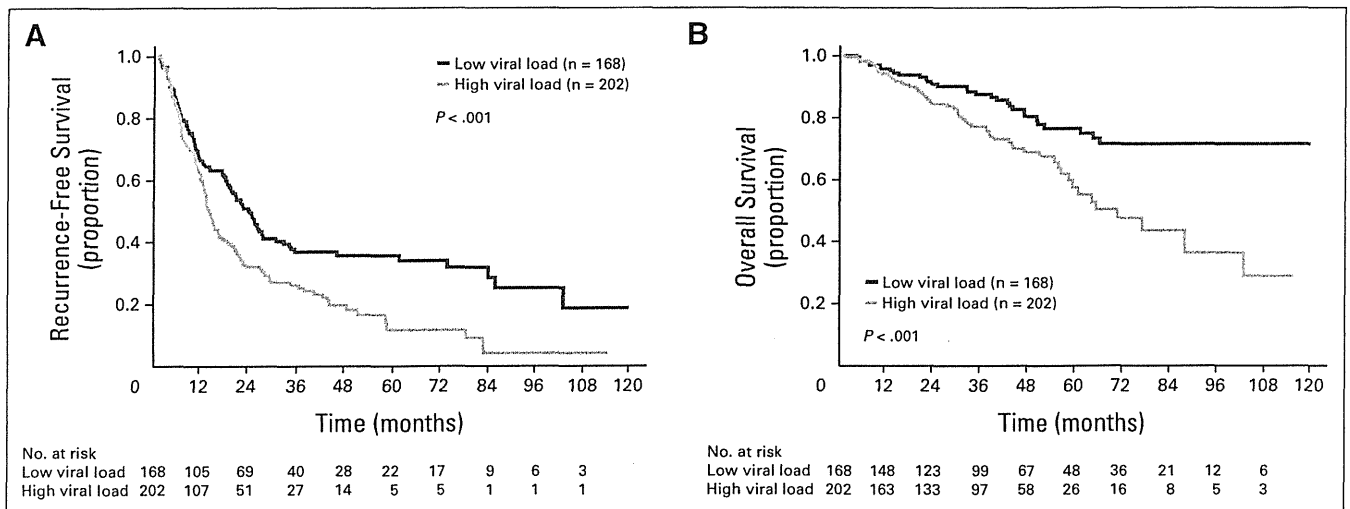
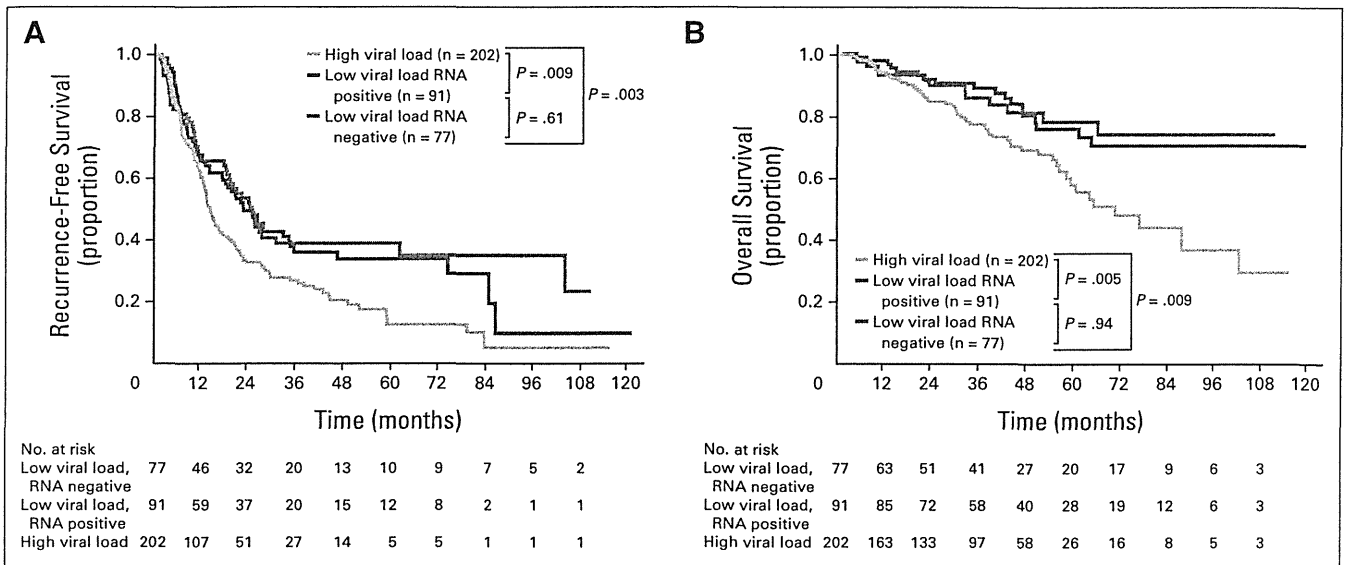


Fig 2. (A) Cumulative recurrence rate and (B) cumulative overall survival curves of low and high viral load groups.



**Fig 3.** (A) Cumulative recurrence rate and (B) cumulative overall survival curves of low and high viral load groups stratified according to the results of hepatitis C virus RNA quantification.

and it improved to 0.627 (95% CI, 0.590 to 0.665) when including HCV viral load greater than 5.3 log<sub>10</sub>IU/mL in the prognostic model (Table 3).

**DISCUSSION**

In this study, we analyzed 370 patients who underwent curative liver resection for HCV-related HCC. The current study indicates that a low viral load ( $\leq 5.3 \log_{10}$ IU/mL) is strongly associated with lower recurrence rate and better OS regardless of the serologic eradication of HCV. These observations were constant both in initial hepatectomy for primary lesions and repeat hepatectomy for recurrent lesions. Multivariate analysis confirms that a high HCV viral load ( $> 5.3 \log_{10}$ IU/mL) is an independent factor associated with a 1.84-fold greater risk of tumor recurrence after curative resection of HCC.

In patients with HCV-related HCC, virologic status of HCV has been thought to be a prognostic factor associated with high tumor recurrence rate.<sup>9,10</sup> Adjuvant antiviral therapy is performed with the aim of preventing tumor recurrence by improving the fibrotic status and/or activity of inflammation in the background liver through the reduction of the viral load. Several studies have shown favorable long-term outcomes of adjuvant IFN therapy after locoregional treatments or surgical resections.<sup>11,32-34</sup> However, the effectiveness of antiviral therapy has been discussed mainly from the view point of virus eradication,<sup>11-13,34-36</sup> and little is known about the significance of the viral load itself for tumor recurrence.

Akamatsu et al<sup>37</sup> previously reviewed 371 patients who had undergone locoregional treatments for HCV-related HCC and denied a correlation between the viral load and the recurrence rate of HCC. However, their study contained a heterogeneous population that underwent

**Table 3.** Factors Associated With Recurrence of Hepatocellular Carcinoma

Factor	P	Coefficient†	SE	Wald $\chi^2$	HR	95% CI
HCV-RNA $> 5.3 \log_{10}$ IU/mL	$< .001$	0.627	0.144	19.1	1.87	1.41 to 2.48
Macrovascular invasion	$< .001$	1.384	0.327	17.9	3.99	2.10 to 7.57
Repeat resection for recurrence	$< .001$	0.505	0.150	11.4	1.66	1.24 to 2.22
Tumor exposure	.013	0.337	0.136	6.1	1.40	1.07 to 1.83
Tumor size $> 2$ cm	.093	0.252	0.150	2.8	1.29	0.96 to 1.83

NOTE. The concordance statistic for the four-factor model (macrovascular invasion + repeat resection + tumor exposure + size  $> 2$  cm) was 0.603 (95% CI, 0.559 to 0.647). The concordance statistic for the full model (the four-factor model + HCV-RNA  $> 5.3 \log_{10}$ IU/mL) was 0.627 (95% CI, 0.590 to 0.665). Multivariate Cox regression was applied with stepwise backward selection. Initially, all factors were included in the model. Then factors that showed no or limited statistically significant association ( $P > .01$ ) with tumor recurrence adjusted for the remaining factors in the model were deleted from the model in a stepwise fashion. The 17 factors tested were as follows: sex, primary versus repeat resection, tumor size ( $> v \leq 2$  cm), number of tumors (solitary v multiple), hepatitis B core antibody (yes v no), HCV viral load ( $> v \leq 5.3 \log_{10}$ IU/mL), fibrotic status of the underlying liver (F3-4 v F0-2), serum ALT level ( $> v \leq 40$  IU/L), indocyanine green retention rate at 15 minutes ( $> v \leq 15\%$ ), serum  $\alpha$ -fetoprotein level ( $> v \leq 20$  ng/mL), plasma des- $\gamma$ -carboxyprothrombin level ( $> v \leq 40$  mAU/mL), type of hepatectomy (anatomic v nonanatomic), perioperative transfusion (yes v no), tumor exposure (yes v no), microvascular invasion (yes v no), macrovascular invasion (yes v no), and tumor differentiation (well/moderate v poor).

Abbreviations: HCV, hepatitis C virus; HR, hazard ratio.  
 †Based on likelihood test adjusted for the other factors in the final model.  
 ‡Estimated coefficient for the variable and the associated SE.

various types of treatments including surgery, ablation, and TACE. Therefore, the true clinical influence of HCV viral load on long-term outcomes of HCV-related HCC is still unclear. In the current study, we carefully reviewed patients who underwent curative surgical resections under a consistent treatment strategy in a single high-volume hepatobiliary center. Major prognostic improvements were observed both in recurrence and survival when a low viral load was obtained according to the cutoff value ( $5.3 \log_{10}$  IU/mL) that was determined by the minimum *P* value approach to predict moderate to severe activity of hepatitis. Comparison of clinicopathologic factors revealed that high viral load was associated with higher serum ALT and AST levels (both before and after surgery) and higher fibrotic status. These correlations are consistent with previous reports<sup>38,39</sup> and suggest the higher carcinogenic potential in the background liver in patients with high HCV viral load.

Another noteworthy result is that the preferable outcomes in the low viral load group are not significantly influenced by whether or not the serologic eradication of HCV is obtained. As shown in Figure 3, when the survival curves were compared between the RNA-positive and RNA-negative patients, no significant difference was observed, although both curves represented apparently better outcomes than that for the high viral load group. We also confirmed a similar tendency both in initial hepatectomy and repeat hepatectomy in a subset analysis (Appendix Figure 1, online only). These results suggest that a lower viral load might be preferable even if the serologic eradication of HCV is not obtained, supporting the outcomes of previous studies<sup>14,15</sup> and a recent meta-analysis<sup>40</sup> studying the effectiveness of IFN therapy.

Recent introduction of combination therapy consisting of pegylated IFN and ribavirin has dramatically improved the sustained viral response rate in patients with HCV.<sup>32,33</sup> However, the postoperative use of IFN remains a major concern because HCC usually emerges in the liver that has been damaged over the course of decades, and accordingly, patients tend to be elderly and to exhibit cirrhotic changes. Therefore, a high-dose standard combination therapy is not always applicable because of the issue of tolerability. Furthermore, even if IFN therapy is available, a sustained viral response may not always be achievable, especially in female patients or patients infected with HCV genotype 1b, both of which have been reported as factors refractory to antiviral therapy.<sup>41-44</sup> In fact, the median age of the current population was 70 years, and 47.8% of the patients were clinically diagnosed with cirrhosis. The proportion of women was higher in the high viral load group, and 71.4% of the patients had genotype 1b.

Given the current results, a low HCV viral load can be a new clinical end point in adjuvant therapy for HCV-related HCC. In this context, a more tolerable antiviral therapy, including low-dose IFN therapy with prolonged therapeutic duration<sup>45,46</sup> or possibly a combination with protease inhibitors,<sup>47-49</sup> may be a therapeutic option for elderly patients or patients with liver cirrhosis. Given the fact that anatomic resection of the liver was also an independent predictor of

recurrence in the multivariate analysis, combination of anatomic resection and adjuvant IFN therapy may enhance the postoperative outcomes in patients with HCV-related HCC by eradicating micro-metastases and reducing the carcinogenic potential in the underlying liver.

Because this study was retrospective, prospective/randomized trials are needed to confirm the true influence of the HCV viral load and the effectiveness of adjuvant antiviral therapy on postoperative outcomes. In addition, the results of the Sorafenib as Adjuvant Treatment in the Prevention of Recurrence of Hepatocellular Carcinoma (STORM) trial, if sorafenib is found to be of benefit, will impact the selection of postoperative therapy in HCC in the near future. Given the possibility of drug interactions and competing toxicity between sorafenib and antiviral agents, further investigation on the selection of adjuvant treatment is needed, especially in patients with HCV-associated HCC.

In conclusion, a low viral load may predict lower recurrence and better survival in patients undergoing hepatic resection for HCV-related HCC irrespective of the serologic eradication of HCV. Postoperative antiviral therapy with individually adjusted intensity and incorporation of direct antiviral agents may warrant prospective study to characterize safety and impact on recurrence risk in patients undergoing surgical resection for HCV-associated HCC.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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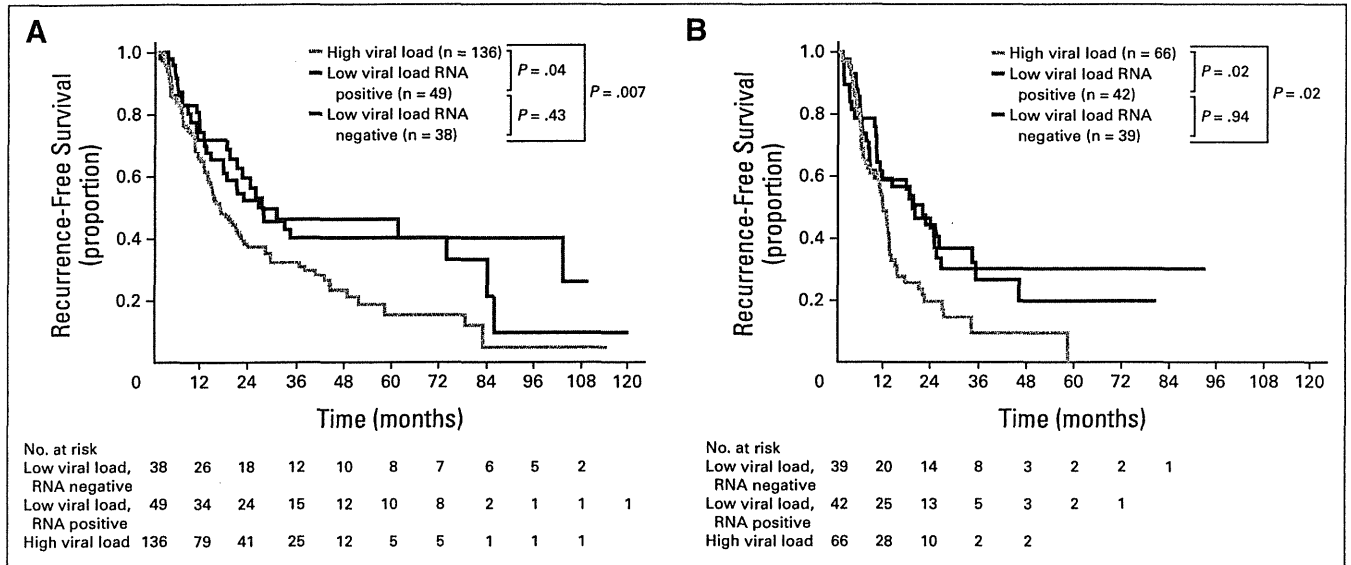
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Appendix



**Fig A1.** Recurrence rates in patients with primary lesions and recurrent lesions. (A) Hepatectomies for primary lesions. (B) Repeat hepatectomies for recurrent lesions.

# Intermittent clamping is superior to ischemic preconditioning and its effect is more marked with shorter clamping cycles in the rat liver

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## Abstract

**Background** Intermittent clamping (IC) and ischemic preconditioning (PC) reportedly protect the liver against the ischemia/reperfusion (I/R) injury induced by inflow occlusion during hepatectomy. While IC cycles consisting of 15 min of clamping with 5 min of reperfusion are used empirically, the optimal IC cycle has not been established. We compared the effects of various cycles of IC and PC in the rat liver.

**Methods** Rats subjected to 60 min of inflow occlusion were assigned to the following five groups ( $n = 8$  each): 60 min of continuous ischemia; 4 cycles comprising 15 min of ischemia/5 min of reperfusion; 6 cycles comprising 10 min of ischemia/3.3 min of reperfusion; 12 cycles comprising 5 min of ischemia/1.7 min of reperfusion (the time ratio of ischemia to reperfusion in the IC groups was 3:1); and PC (10/10 min of ischemia/reperfusion) prior to 60 min of ischemia. The severity of liver injury was assessed by determining the serum alanine aminotransferase (ALT) level, bile flow, tissue glutathione

content, and induction of apoptosis (terminal deoxynucleotidyl transferase-mediated biotin nick end-labeling [TUNEL] staining and DNA laddering), and by histological examination of areas of severe necrosis.

**Results** All the parameters indicated that liver injury was attenuated in the three IC groups compared with the continuous group; furthermore, this effect became increasingly marked with shorter cycles of IC. PC did not exert a protective effect under the present experimental conditions.

**Conclusion** Various cycles of IC consistently conferred protection against I/R injury, and IC with shorter cycles of ischemia and reperfusion was more effective. No protective effect of PC was evident. IC is a more robust strategy than the PC protocol for liver protection.

**Keywords** Ischemia/reperfusion injury · Inflow occlusion · Hepatectomy · Apoptosis · Bile flow · Intermittent clamping · Ischemic preconditioning

## Introduction

Blood loss during liver transection, and the resulting need for blood transfusion, remains a risk factor for postoperative morbidity and poor long-term outcome [1, 2]. Inflow occlusion produced by clamping of the portal triad, i.e., the Pringle maneuver [3], is a widely used technique for reducing blood loss during liver parenchymal transection [4–6] and its efficacy has been proven in a human randomized controlled trial (RCT) [6]. This technique, however, may lead to ischemia/reperfusion (I/R) injury of the remaining liver, the severity of which has been reported to increase with longer inflow occlusion duration [7–9].

To date, two techniques have been reported and applied clinically to minimize the liver I/R injury that may be

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caused by the Pringle maneuver. The first one is intermittent clamping (IC), devised by Makuuchi et al. [4–6, 10], where successive cycles of 15–20 min of inflow occlusion are alternated with 5 min of reperfusion [4–6, 10]; the second one is ischemic preconditioning (PC), in which a short period of ischemia (10 min) and reperfusion (10–15 min) is applied before sustained inflow occlusion for liver parenchymal transection [11–13]. Several animal studies [14–18] and human RCTs [10, 11] have reported that both techniques decrease the degree of liver injury compared with comparable periods of continuous inflow occlusion; however, studies to compare these two maneuvers are limited, and have yielded conflicting results [8, 12, 13, 19, 20]. Intuitively, a longer clamping time with a fixed duration of reperfusion in the IC protocol would increase the grade of injury, and this hypothesis has been verified by an animal study [21]. On the other hand, provided that the time ratio of clamping to alternated reperfusion is constant, e.g., 3:1, as in the case of 15 min of clamping alternated with 5 min of reperfusion, it remains unclear whether shorter or longer cycles of clamping/reperfusion are preferable.

In the present study, using model rats subjected to 60 min of warm I/R injury, we first compared IC and PC from the viewpoint of protection against liver I/R injury, and then investigated whether the protective effect of IC was related to the length of the clamping/reperfusion cycle.

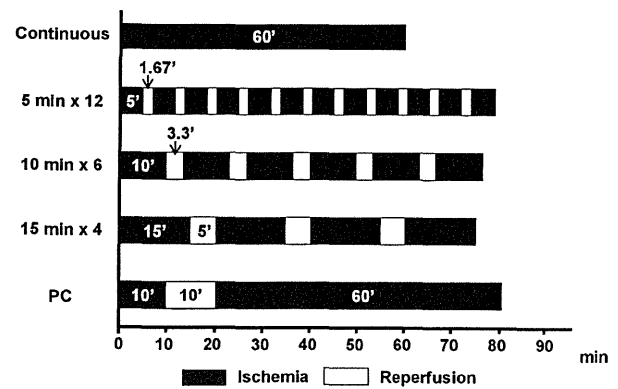
## Materials and methods

### Animals

Male Wistar rats (Charles River Japan, Yokohama, Japan) weighing 250–290 g (age 7–8 weeks) at the time of surgery were used in all experiments. The animals were allowed free access to food and water until 12 h preceding the surgery, after which they had free access to water only. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH.

### Experimental design

The animals were randomly assigned to five experimental inflow occlusion groups ( $n = 8$  in each group), as follows (Fig. 1): (1) a continuous clamping group (Continuous) in which the portal triad was clamped continuously for 60 min; (2) a short IC cycle group ( $5 \text{ min} \times 12$ ) in which 5 min of clamping alternated with 1.67 min of reperfusion was repeated 12 times; (3) a medium IC cycle group ( $10 \text{ min} \times 6$ ) in which 10 min of clamping alternated with 3.3 min of reperfusion was repeated 6 times; (4) a long IC



**Fig. 1** Experimental protocols used for rat liver ischemia/reperfusion. Various parameters of liver injury were measured at 3 h after the termination of the respective protocols. In all groups, the total ischemia time was set at 60 min. In each intermittent inflow occlusion group, the time ratio of occlusion to reperfusion was set at 3:1. *Continuous* continuous inflow occlusion for 60 min, *5 min × 12*: intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion, *10 min × 6*: intermittent inflow occlusion for 10 min alternated with 3.3 min of reperfusion, *15 min × 4*: intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion, *PC*: ischemic preconditioning for 10 min followed by 10 min of reperfusion

cycle group ( $15 \text{ min} \times 4$ ) in which 15 min of clamping alternated with 5 min of reperfusion was repeated 4 times; and (5) a preconditioning (PC) group in which 10 min of portal triad clamping followed by 10 min of reperfusion preceded 60 min of continuous clamping. In each experimental group, the total ischemic time was set at 60 min, and the ratio of the clamping to reperfusion time in each IC group was set at 3:1.

### Surgery

A model of segmental (70 %) hepatic ischemia was used [9]. All surgical procedures were performed with the animals under general anesthesia using halothane inhalation. After a midline laparotomy, a liver biopsy specimen was obtained from the caudate lobe, snap-frozen in liquid nitrogen and kept at  $-80^\circ\text{C}$  for the measurement of glutathione. Then, all structures in the portal triad supplying the left and median lobes were occluded by a microvascular clamp. This method prevented mesenteric venous congestion by permitting portal decompression through the right and the remaining caudate lobes [9]. After the reperfusion, the wound was closed with 3-0 silk, and the animal was allowed to recover from anesthesia and given access to water ad libitum during 3 h of reperfusion.

The animals were then anesthetized again and subjected to re-laparotomy. Initially, the common bile duct was cannulated, and a polyethylene (PE) size 10 tube was inserted up to the location of the bile duct bifurcation

draining the median and the left lateral lobes. At this level, the hepatic bile duct was ligated around the cannula. Thus, only bile juice from the ischemic lobes, i.e., the median and the left lateral lobes, was collected. After blood sampling from the inferior vena cava, liver biopsy specimens were obtained from the median and left lateral lobes. These specimens were prepared for use as paraformaldehyde-fixed paraffin-embedded tissues for histological examination and terminal deoxynucleotidyl transferase-mediated biotin nick end-labeling (TUNEL) staining; as well, we prepared frozen ( $-80^{\circ}\text{C}$ ) tissue samples for glutathione measurement and a DNA laddering assay.

#### Serum enzyme analysis

Evaluation of hepatocyte injury was performed by enzymatic determination of the serum alanine aminotransferase (ALT) level, using a commercial kit from Boehringer Mannheim (Munich, Germany).

#### Measurement of bile flow

Hepatocyte function was assessed by measuring bile flow collected for 15 min after 3 h of reperfusion [19, 22]. Bile volume was determined gravimetrically, assuming a density of  $1.0\text{ g/mL}$ , and the results were expressed as milliliters per minute per kilogram body weight.

#### Glutathione (GSH) content

The GSH content of the liver was measured using a commercially available kit (BIOTECH GSH-400; OxisResearch<sup>TM</sup>, Manhattan Beach, CA, USA). Liver tissue samples from each group were homogenized in 5 % metaphosphoric acid (1:13) for 30 s on ice. The homogenate was centrifuged at  $3000\times g$  for 10 min at  $4^{\circ}\text{C}$ . A reagent containing 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate was added to the supernatant to form substitution products (thioethers). Then, a  $\beta$ -elimination reaction was initiated by the addition of 30 % NaOH to transform the substitution product into a chromophoric thione. After incubation at  $25^{\circ}\text{C}$  for 10 min, absorbance was measured at 400 nm.

#### TUNEL staining

Apoptosis of hepatocytes was identified by determining DNA fragmentation in situ in serial sections adjacent to those used for histological examination, using a commercial TUNEL staining kit (ApopTag<sup>®</sup> Peroxidase in Situ Apoptosis Detection; Chemicon International, Temecula, CA, USA). Briefly, tissue sections were deparaffinized and rehydrated, and then pretreated with proteinase K for 15 min at room temperature. After endogenous peroxidase

had been quenched, the specimen was incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h at  $37^{\circ}\text{C}$ . Diaminobenzidine was used as the chromogen and methyl green as a counterstain. Morphometric analysis of the stained cells was performed under high-power magnification ( $400\times$ ) in a blinded manner. A total of 20 random sections per slide were investigated to determine the percentage of TUNEL-positive cells.

#### Assessment of DNA laddering as an indicator of DNA fragmentation

Frozen liver tissues were immediately minced, and portions of about 0.04 g were used for the determination of DNA fragmentation using an Apoptosis Ladder Detection Kit (Wako, Osaka, Japan). Briefly, each liver tissue sample was suspended in lysis buffer containing RNase and proteinases, followed by incubation for 30 min at  $50^{\circ}\text{C}$ . After extraction of DNA and ethanol precipitation, the resulting DNA was gently dissolved in Tris–ethylenediaminetetraacetic acid (EDTA) (TE) buffer and electrophoresed on a 1.5 % agarose gel. The gel was stained with SYBR Green and visualized under a UV illuminator.

#### Histological assessment

Paraffin-embedded liver tissues were sectioned ( $6\text{ }\mu\text{m}$ -thick), stained with hematoxylin and eosin, and evaluated semi-quantitatively in a blinded manner at  $200\times$  magnification according to the scale proposed by Camargo et al., as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hyper-eosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil inflammation [9]. Twenty random sections per slide were investigated.

#### Analytical procedures

We first investigated whether IC and/or PC attenuated the degree of liver I/R injury, and compared the effects of these two maneuvers. For this purpose, three IC groups with various clamping/reperfusion cycles were investigated together as a single IC group and multiple comparisons among the Continuous, IC, and PC groups were conducted using the Tukey–Kramer method. We then examined whether the protective effect of IC was related to the length of the clamping/reperfusion cycle. To this end, we separated the three IC groups and examined whether the attenuation effect of IC against I/R injury increased or decreased linearly within these three IC groups, using the

linear trend test. Differences at  $P < 0.05$  were accepted as statistically significant. Data are presented as means with SEM.

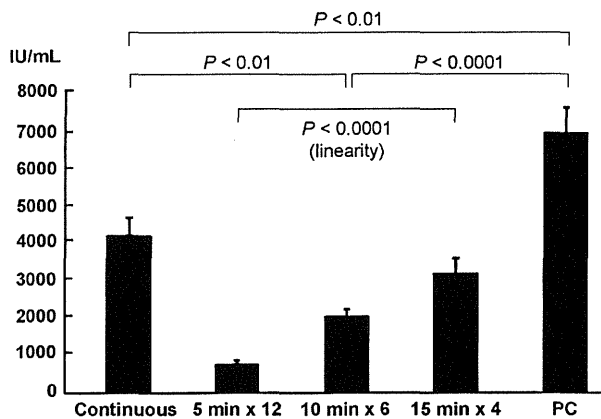
**Results**

**Serum ALT levels**

In the preliminary experiments, serum ALT concentration reached the peak at 2–3 h after liver I/R reperfusion and remained at a plateau level for several hours thereafter. Levels of ALT in the three IC groups at 3 h after reperfusion were significantly lower than that in the Continuous group as a whole, and decreased linearly with shorter cycles of clamping/reperfusion; while the ALT level in the PC group was higher than the levels in the Continuous group and the IC groups (Fig. 2).

**Bile flow**

In addition to the five experimental groups with different liver ischemia/reperfusion protocols, we used another group of animals, which underwent a sham operation without inflow occlusion, as a reference for normal bile flow from the left and median lobes, although no statistical comparison was done with this group (Fig. 3). It appeared that bile flow at 3 h after reperfusion was decreased in each I/R group as compared with that in the group without ischemia. Bile flow in the three IC groups as a whole was significantly higher than that in the Continuous group, and increased linearly with shorter cycles of clamping/

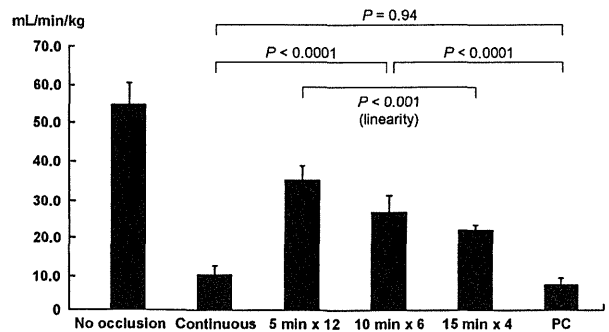


**Fig. 2** Serum levels of alanine aminotransferase after 3 h of reperfusion in livers subjected to 60 min of ischemia. Data are presented as means with SEM. Multiple comparisons among groups were done by the Tukey–Kramer method. Linearity within the three intermittent inflow occlusion groups was checked by linear trend test.  $N = 8$  in each group

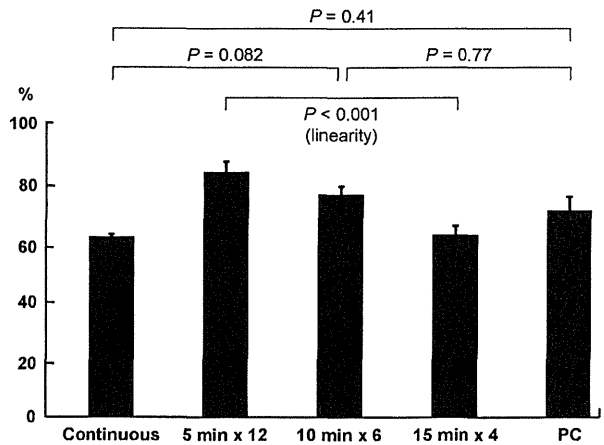
reperfusion, whereas bile flow in the PC group was not different from that in the Continuous group.

**GSH alteration**

The liver GSH content after I/R appeared to decrease from the baseline value, i.e., it was  $<100\%$ , in all experimental groups (Fig. 4). This decrease was weaker in the three IC groups as a whole than that in the Continuous group ( $P = 0.082$ ); moreover, this attenuation effect became more marked with shorter cycles of clamping/reperfusion.



**Fig. 3** Bile flow from the median and left lateral (ischemic) lobes after 3 h of reperfusion. In addition to the five experimental groups with differing liver ischemia/reperfusion protocols, a sham operation group was added as a reference for normal bile flow from the median and left lateral lobes under identical experimental conditions without inflow occlusion (*No occlusion*). Data (means with SEM) are expressed as  $\mu\text{g}/\text{min}$  per head.  $N = 8$  in each group



**Fig. 4** Glutathione alteration from the baseline values after 3 h of reperfusion. The content of glutathione was measured in liver biopsy specimens taken from the caudate lobe before inflow occlusion, and from the median and left lateral lobes after 3 h of reperfusion. Although the levels appeared to decrease from the baseline in all groups, i.e., all were  $<100\%$ , the extent of the decrease differed depending on the experimental protocol employed. Data (means with SEM) are expressed as percentages of the baseline value.  $N = 8$  in each group

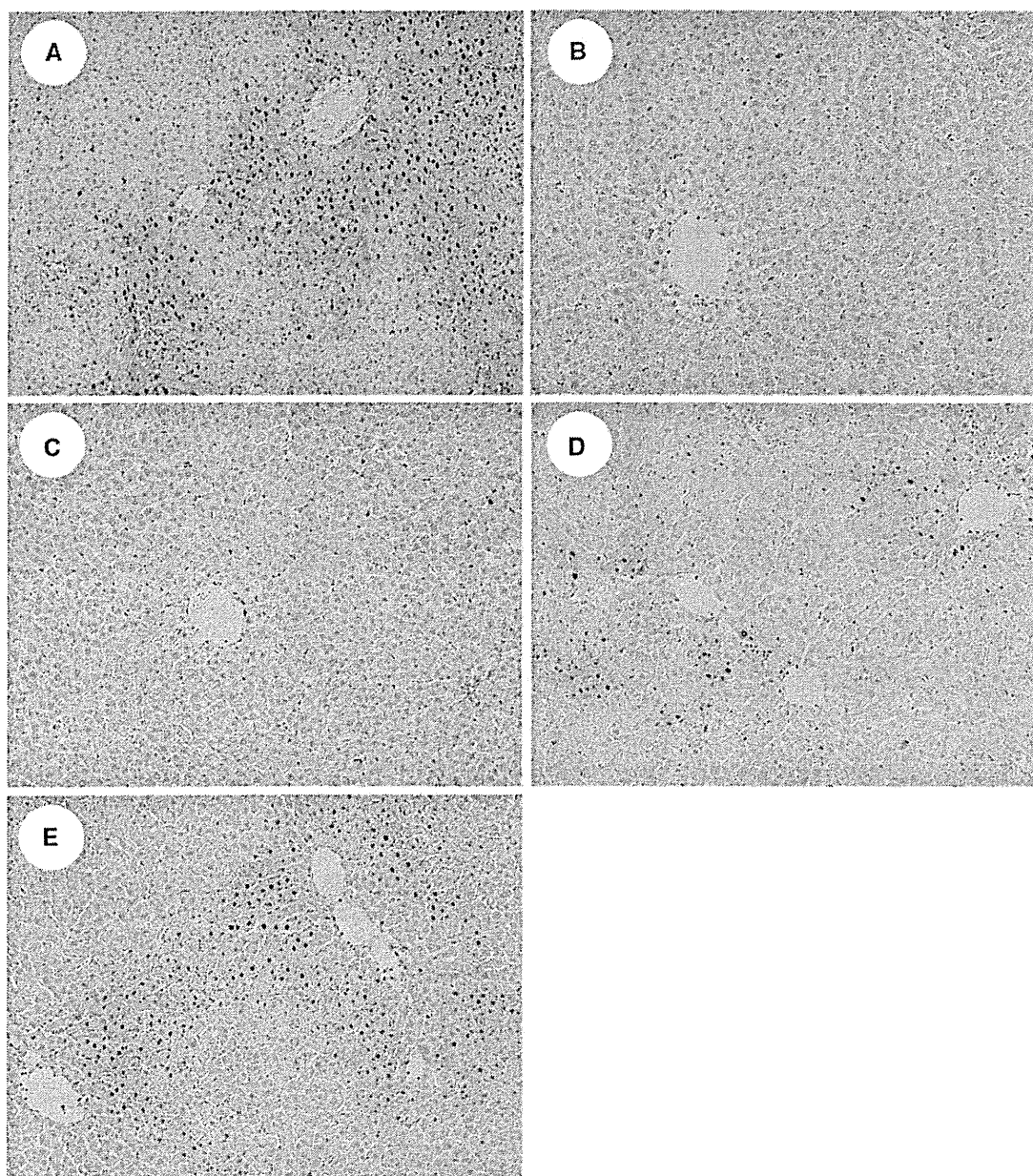
However, no such attenuation effect was observed in the PC group.

#### Evaluation of hepatocyte apoptosis by TUNEL staining and DNA laddering

Representative micrographs of TUNEL staining are shown in Fig. 5 and results of quantitative assessment of TUNEL-

positive hepatocytes are depicted in Fig. 6. The occurrence of apoptosis was suppressed in the IC groups as a whole compared with the Continuous group, and this suppression effect became stronger with shorter cycles of clamping/reperfusion; while the suppression of apoptosis in the PC groups was at borderline significant level ( $P = 0.067$ , Fig. 6).

The results of DNA ladder detection roughly coincided with those of TUNEL staining. DNA laddering, which is a



**Fig. 5** Representative micrographs of terminal deoxynucleotidyl transferase-mediated biotin nick end-labeling (TUNEL) stained sections after 3 h of reperfusion are shown. **a** Continuous inflow occlusion for 60 min, **b** intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion (5 min  $\times$  12), **c** intermittent

inflow occlusion for 10 min alternated with 3.3 min of reperfusion (10 min  $\times$  6), **d** intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion (15 min  $\times$  4), **e** ischemic preconditioning for 10 min followed by 10 min of reperfusion (PC) (Original magnification  $\times$ 200)