Published Ahead of Print on November 5, 2012 as 10.1200/JCO.2012.44.3234
The latest version is at http://jco.ascopubs.org/cgi/doi/10.1200/JCO.2012.44.3234

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

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

Junichi Shindoh, Kiyoshi Hasegawa, Yutaka Matsuyama, Yosuke Inoue, Takeaki Ishizawa, Taku Aoki, Yoshihiro Sakamoto, Yasuhiko Sugawara, Masatoshi Makuuchi, and Norihiro Kokudo

Junichi Shindoh, Kiyoshi Hasegawa, Yutaka Matsuyama, Yosuke Inoue, Takeaki Ishizawa, Taku Aoki, Yoshihiro Sakamoto, Yasuhiko Sugawara, and Norihiro Kokudo, University of Tokyo; Masatoshi Makuuchi, Japan Red Cross Medical Center, Tokyo, Japan.

Submitted May 17, 2012; accepted September 19, 2012; published online ahead of print at www.jco.org on November 5, 2012.

Supported by Health and Labor Sciences Research Grant No. H21-015 for Clinical Cancer Research and by a grant from the Japanese Foundation for Multidisciplinary Treatment of Cancer.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Junichi Shindoh, MD, PhD, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655 Japan; e-mail: shindou-tky@umin.ac.jp.

0732-183X/12/3099-1/$20.00

DOI: 10.1200/JCO.2012.44.3234

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.

J Clin Oncol 30. © 2012 by American Society of Clinical Oncology

INTRODUCTION

Recent developments in medical and surgical treatments have significantly improved the long-term outcomes of patients with hepatocellular carcinoma (HCC).[1] 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.[2-7]

Hepatitis C virus (HCV), a major cause of chronic hepatitis and liver cirrhosis, has been recognized as a potent risk factor of carcinogenesis[8] and/or the recurrence of HCC.[9,10] 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.[11-13]

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.[14,15]

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 (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.[16] 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.[17] 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.[18]

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

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 ($> 9.0 \times 10^4/\mu$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 α-fetoprotein (AFP) and des-γ-carboxyprothrombin every 1 to 2 months, with ultrasonography every 2 months, and with dynamic computed tomography every 4 months, as previously reported.[22] 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.[23,24]

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 χ^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[21]). 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

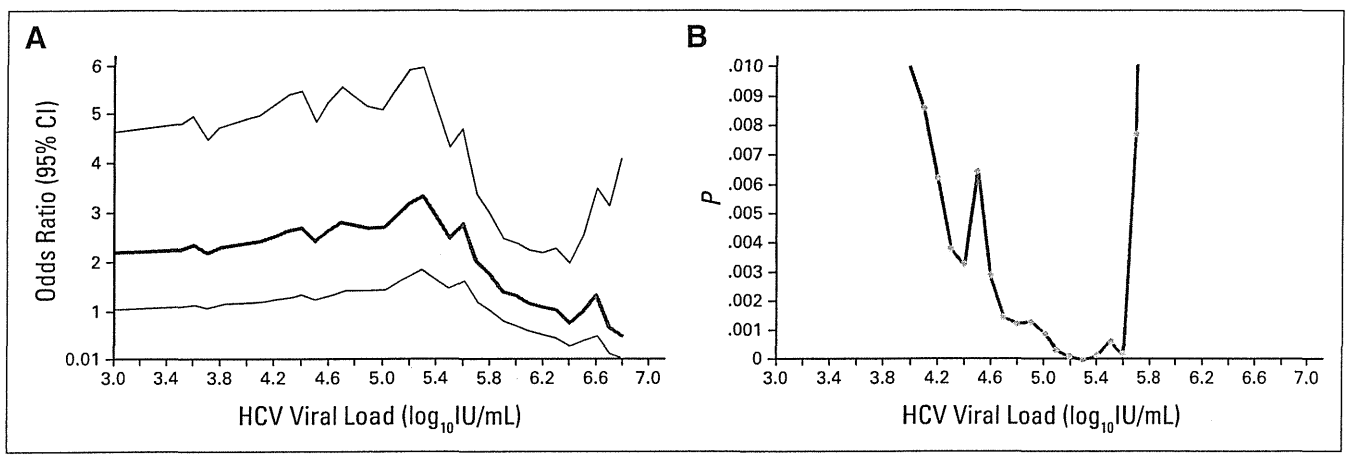

Fig 1. Optimal cutoff value of hepatitis C virus (HCV) RNA viral load to predict moderate to severe activity of hepatitis. (A) Plot of odds ratio. (B) Plot of P value in likelihood test (null hypothesis: odds ratio, 1).

Table 1. Baseline Demographics and Clinical Characteristics

Characteristic	Patients With Low Viral Load (n = 168) No.	%	Patients With High Viral Load (n = 202) No.	%	*P*
Age, years					.07
Median	69		70		
Range	47-83		39-85		
Sex					
Male	139	82.7	131	64.9	< .001
Female	29	17.3	71	35.1	
HBsAg					
Positive	7	4.2	4	2.0	.24
Negative	161	95.8	198	98.0	
HBcAb					
Positive	45	28.5	59	33.7	.30
Negative	113	71.5	116	66.3	
HCV genotype					
1b	40	69.0	87	81.3	0.07
Other	18	31.0	20	18.7	
HCV-RNA, log_{10}IU/mL					< .001
Mean	3.0		6.0		
Standard deviation	1.9		0.4		
History of IFN therapy					
Positive	69	41.6	48	24.0	< .001
Negative	97	58.4	152	76.0	
No. of tumors					
Solitary	107	63.7	127	63.2	.87
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, $10^4/\mu L$					.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 (n = 168) No.	%	Patients With High Viral Load (n = 202) No.	%	*P*
Surgical factors					
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					
Median	700		660		.77
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		
Histopathologic factors					
Tumor differentiation*					
Well	35	21.5	42	21.2	.86
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†					
F0-2	52	49.1	56	37.1	.06
F3-4	54	50.9	95	62.9	
Postoperative factors					
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, α-fetoprotein; DCP, des-γ-carboxyprothrombin; HCV, hepatitis C virus; HX, hepatectomy; IFN, interferon.
*Based on modification of the Edmondson grade.[19]
†Based on the classification by Desmet et al.[21]
‡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 ± 2.0 $\log_{10}$IU/mL before surgery *v* 4.6 ± 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 ≤ 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,[4,25-31] 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),

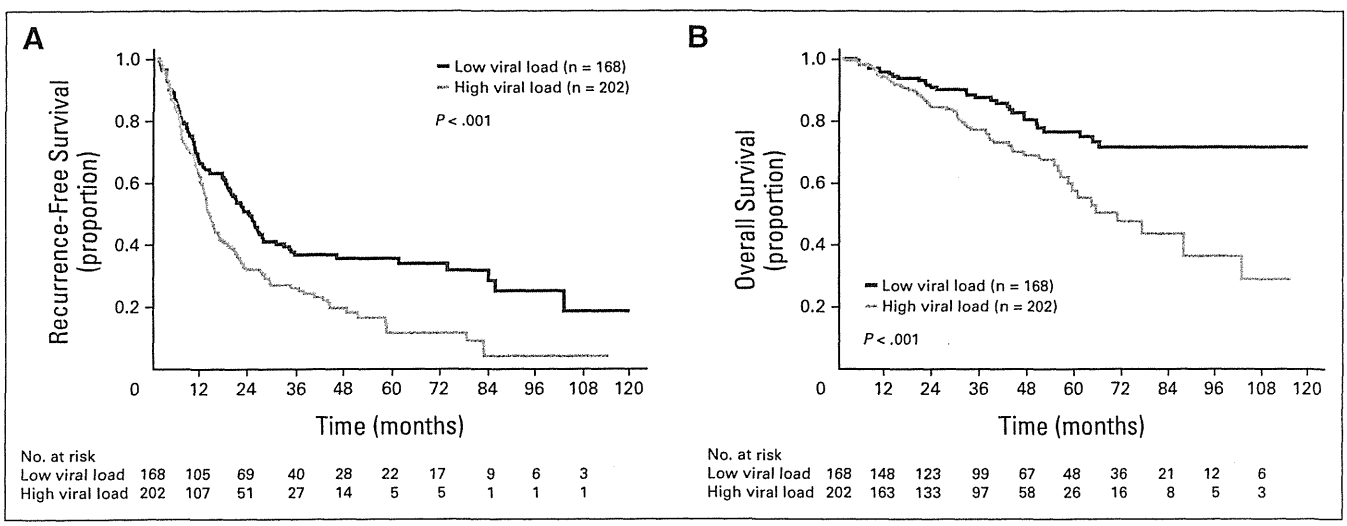

Fig 2. (A) Cumulative recurrence rate and (B) cumulative overall survival curves of low and high viral load groups.

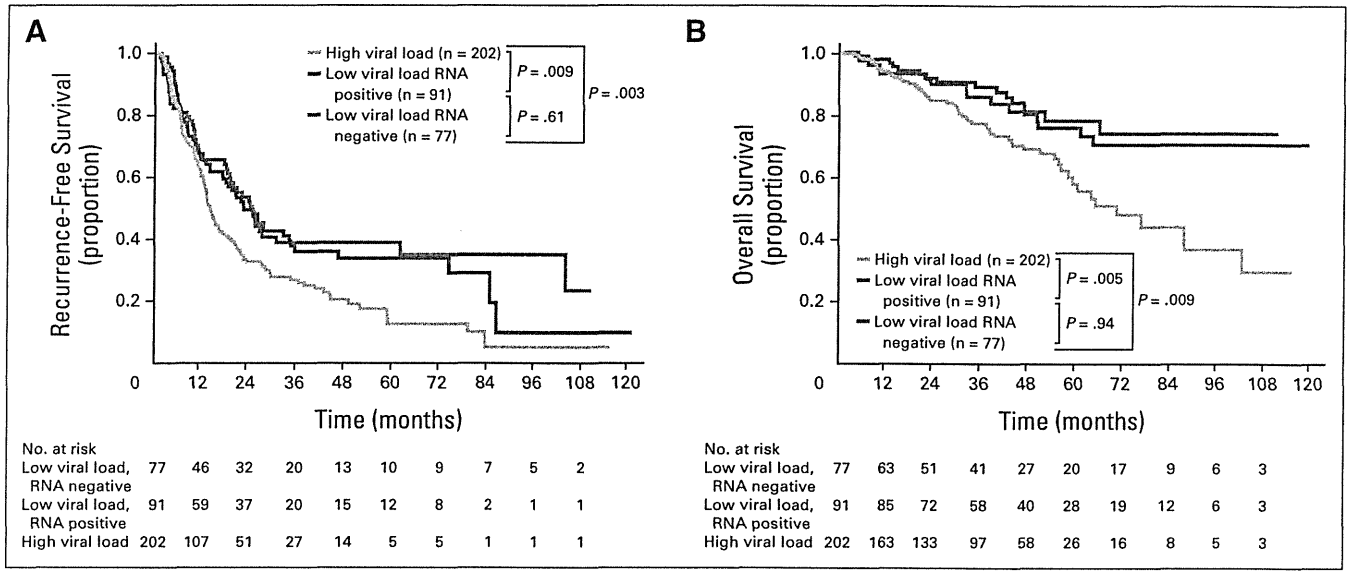

Fig 3. (A) Cumulative recurrence rate and (B) cumulative overall survival curves of low and high viral load groups stratified according to the results of hepatitis C virus RNA quantification.

and it improved to 0.627 (95% CI, 0.590 to 0.665) when including HCV viral load greater than 5.3 $\log_{10}$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.[9,10] 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.[11,32-34] However, the effectiveness of antiviral therapy has been discussed mainly from the view point of virus eradication,[11-13,34-36] and little is known about the significance of the viral load itself for tumor recurrence.

Akamatsu et al[37] 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 χ^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 α-fetoprotein level (> *v* $\leq$ 20 ng/mL), plasma des-γ-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[38,39] 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[14,15] and a recent meta-analysis[40] 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.[32,33] 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.[41-44] 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[45,46] or possibly a combination with protease inhibitors,[47-49] 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 micrometastases 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.
Employment or Leadership Position: None **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** None **Research Funding:** Norihiro Kokudo, Bayer, Dainippon Sumitomo **Expert Testimony:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Junichi Shindoh, Norihiro Kokudo
Collection and assembly of data: Junichi Shindoh, Kiyoshi Hasegawa, Yosuke Inoue, Takeaki Ishizawa, Taku Aoki, Yoshihiro Sakamoto, Yasuhiko Sugawara, Masatoshi Makuuchi, Norihiro Kokudo
Data analysis and interpretation: Junichi Shindoh, Kiyoshi Hasegawa, Yutaka Matsuyama, Norihiro Kokudo
Manuscript writing: All authors
Final approval of manuscript: All authors

REFERENCES

1. Rahbari NN, Mehrabi A, Mollberg NM, et al: Hepatocellular carcinoma: Current management and perspectives for the future. Ann Surg 253:453-469, 2011
2. Belghtiti J, Panis Y, Farges O, et al: Intrahepatic recurrence after resection of hepatocellular carcinoma complicating cirrhosis. Ann Surg 214: 114-117, 1991
3. Grazi GL, Ercolani G, Pierangeli F, et al: Improved results of liver resection for hepatocellular carcinoma on cirrhosis give the procedure added value. Ann Surg 234:71-78, 2001
4. Imamura H, Matsuyama Y, Miyagawa Y, et al: Prognostic significance of anatomical resection and des-gamma-carboxy prothrombin in patients with hepatocellular carcinoma. Br J Surg 86:1032-1038, 1999
5. Kakazu T, Makuuchi M, Kawasaki S, et al: Repeat hepatic resection for recurrent hepatocellular carcinoma. Hepatogastroenterology 40:337-341, 1993
6. Nakajima Y, Ko S, Kanamura T, et al: Repeat liver resection for hepatocellular carcinoma. J Am Coll Surg 192:339-344, 2001

7. Poon RT, Fan ST, Lo CM, et al: Long-term survival and pattern of recurrence after resection of small hepatocellular carcinoma in patients with preserved liver function: Implications for a strategy of salvage transplantation. Ann Surg 235:373-382, 2002

8. Tsukuma H, Hiyama T, Tanaka S, et al: Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 328:1797-1801, 1993

9. Kumada T, Nakano S, Takeda I, et al: Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology 25:87-92, 1997

10. Yamanaka N, Tanaka T, Tanaka W, et al: Correlation of hepatitis virus serologic status with clinicopathologic features in patients undergoing hepatectomy for hepatocellular carcinoma. Cancer 79:1509-1515, 1997

11. Kubo S, Nishiguchi S, Hirohashi K, et al: Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by postoperative interferon therapy. Br J Surg 89:418-422, 2002

12. Miyake Y, Takaki A, Iwasaki Y, et al: Meta-analysis: Interferon-alpha prevents the recurrence after curative treatment of hepatitis C virus-related hepatocellular carcinoma. J Viral Hepat 17:287-292, 2010

13. Singal AK, Freeman DH Jr, Anand BS: Meta-analysis: Interferon improves outcomes following ablation or resection of hepatocellular carcinoma. Aliment Pharmacol Ther 32:851-858, 2010

14. Kasahara A, Hayashi N, Mochizuki K, et al: Clinical characteristics of patients with chronic hepatitis C showing biochemical remission, without hepatitis C virus eradication, as a result of interferon therapy: The Osaka Liver Disease Study Group. J Viral Hepat 7:343-351, 2000

15. Yabuuchi I, Imai Y, Kawata S, et al: Long-term responders without eradication of hepatitis C virus after interferon therapy: Characterization of clinical profiles and incidence of hepatocellular carcinoma. Liver 20:290-295, 2000

16. Hasegawa K, Kokudo N, Imamura H, et al: Prognostic impact of anatomic resection for hepatocellular carcinoma. Ann Surg 242:252-259, 2005

17. Makuuchi M, Kosuge T, Takayama T, et al: Surgery for small liver cancers. Semin Surg Oncol 9:298-304, 1993

18. Nakashima T, Kojiro M: Pathologic characteristics of hepatocellular carcinoma. Semin Liver Dis 6:259-266, 1986

19. Liver Cancer Study Group of Japan: The General Rules for the Clinical and Pathological Study of Primary Liver Cancer (ed 5). Tokyo, Japan, Kanehara, 2008

20. Edmondson HA, Steiner PE: Primary carcinoma of the liver: A study of 100 cases among 48,900 necropsies. Cancer 7:462-503, 1954

21. Desmet VJ, Gerber M, Hoofnagle JH, et al: Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513-1520, 1994

22. Takayama T, Makuuchi M, Hirohashi S, et al: Early hepatocellular carcinoma as an entity with a high rate of surgical cure. Hepatology 28:1241-1246, 1998

23. Combes B, Carithers RL Jr, Maddrey WC, et al: Biliary bile acids in primary biliary cirrhosis: Effect of ursodeoxycholic acid. Hepatology 29:1649-1654, 1999

24. Yamamura Y, Kotaki H, Tanaka N, et al: The pharmacokinetics of glycyrrhizin and its restorative effect on hepatic function in patients with chronic hepatitis and in chronically carbon-tetrachloride-intoxicated rats. Biopharm Drug Dispos 18:717-725, 1997

25. Fan ST, Ng IO, Poon RT, et al: Hepatectomy for hepatocellular carcinoma: The surgeon's role in long-term survival. Arch Surg 134:1124-1130, 1999

26. Vauthey JN, Lauwers GY, Esnaola NF, et al: Simplified staging for hepatocellular carcinoma. J Clin Oncol 20:1527-1536, 2002

27. Ikeda K, Marusawa H, Osaki Y, et al: Antibody to hepatitis B core antigen and risk for hepatitis C-related hepatocellular carcinoma: A prospective study. Ann Intern Med 146:649-656, 2007

28. Fuster J, García-Valdecasas JC, Grande L, et al: Hepatocellular carcinoma and cirrhosis: Results of surgical treatment in a European series. Ann Surg 223:297-302, 1996

29. Lee CS, Sheu JC, Wang M, et al: Long-term outcome after surgery for asymptomatic small hepatocellular carcinoma. Br J Surg 83:330-333, 1996

30. Yamamoto J, Kosuge T, Takayama T, et al: Recurrence of hepatocellular carcinoma after surgery. Br J Surg 83:1219-1222, 1996

31. Yamanaka N, Okamoto E, Toyosaka A, et al: Prognostic factors after hepatectomy for hepatocellular carcinomas: A univariate and multivariate analysis. Cancer 65:1104-1110, 1990

32. Ikeda K, Arase Y, Saitoh S, et al: Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor: A prospective randomized study of hepatitis C virus-related liver cancer. Hepatology 32:228-232, 2000

33. Shiratori Y, Shiina S, Teratani T, et al: Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. Ann Intern Med 138:299-306, 2003

34. Suou T, Mitsuda A, Koda M, et al: Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: A pilot study. Hepatol Res 20:301-311, 2001

35. Uenishi T, Kubo S, Hirohashi K, et al: Relationship between response to previous interferon therapy and postoperative recurrence of hepatitis C virus-related hepatocellular carcinoma. Hepatol Res 24:404-412, 2002

36. Uenishi T, Nishiguchi S, Tamori A, et al: Influence of interferon therapy on outcome after surgery for hepatitis C virus-related hepatocellular carcinoma. Hepatol Res 36:195-200, 2006

37. Akamatsu M, Yoshida H, Shiina S, et al: Neither hepatitis C virus genotype nor virus load affects survival of patients with hepatocellular carcinoma. Eur J Gastroenterol Hepatol 16:459-466, 2004

38. Uenishi T, Nishiguchi S, Tanaka S, et al: Response to interferon therapy affects risk factors for postoperative recurrence of hepatitis C virus-related hepatocellular carcinoma. J Surg Oncol 98:358-362, 2008

39. Kubo S, Hirohashi K, Tanaka H, et al: Risk factors for recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. World J Surg 24:1559-1565, 2000

40. Miao RY, Zhao HT, Yang HY, et al: Postoperative adjuvant antiviral therapy for hepatitis B/C virus-related hepatocellular carcinoma: A meta-analysis. World J Gastroenterol 16:2931-2942, 2010

41. Kogure T, Ueno Y, Fukushima K, et al: Pegylated interferon plus ribavirin for genotype Ib chronic hepatitis C in Japan. World J Gastroenterol 14:7225-7230, 2008

42. Sezaki H, Suzuki F, Kawamura Y, et al: Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. Dig Dis Sci 54:1317-1324, 2009

43. Hadziyannis SJ, Sette H Jr, Morgan TR, et al: Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: A randomized study of treatment duration and ribavirin dose. Ann Intern Med 140:346-355, 2004

44. Tsubota A, Chayama K, Ikeda K, et al: Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology 19:1088-1094, 1994

45. Kudo M, Sakaguchi Y, Chung H, et al: Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation: A matched case-control study. Oncology 72:132-138, 2007 (suppl 1)

46. Shimomura S, Ikeda N, Saito M, et al: Long-term interferon therapy after radiofrequency ablation is effective in treating patients with HCV-associated hepatocellular carcinoma. Hepatol Int 5:559-566, 2010

47. Hayashi N, Okanoue T, Tsubouchi H, et al: Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. J Viral Hepat 19:e134-e142, 2012

48. Poordad F, McCone J Jr, Bacon BR, et al: Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 364:1195-1206, 2011

49. Zeuzem S, Andreone P, Pol S, et al: Telaprevir for retreatment of HCV infection. N Engl J Med 364:2417-2428, 2011

Appendix

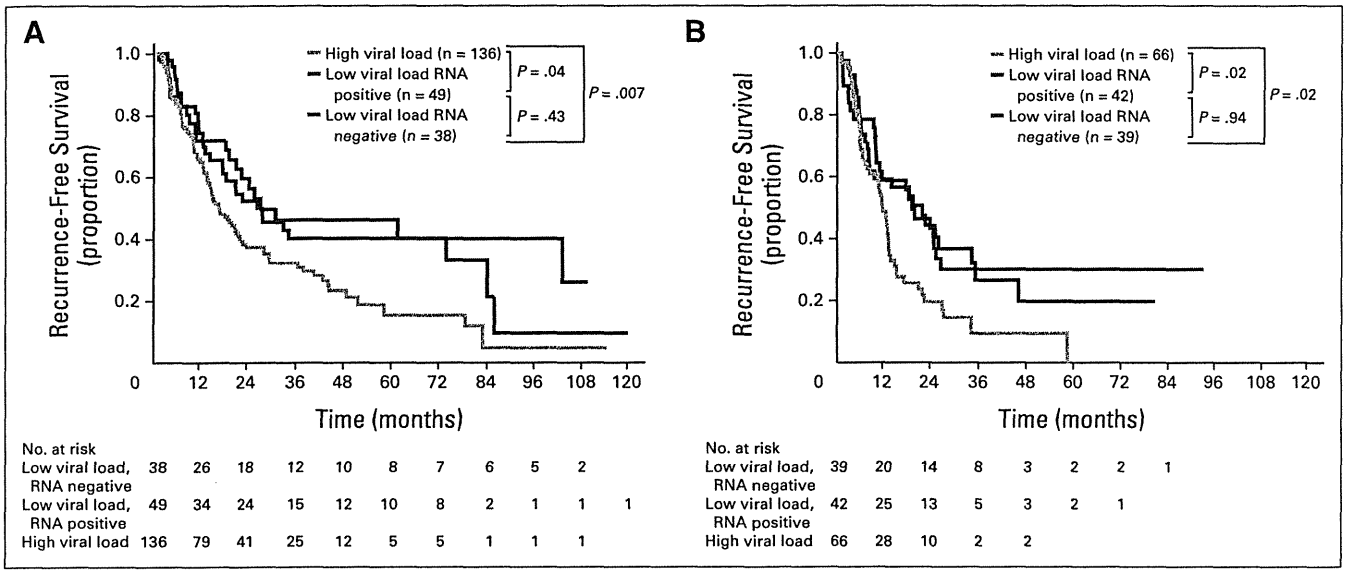

Fig A1. Recurrence rates in patients with primary lesions and recurrent lesions. (A) Hepatectomies for primary lesions. (B) Repeat hepatectomies for recurrent lesions.

J Gastroenterol (2013) 48:115–124
DOI 10.1007/s00535-012-0613-0

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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

Yasuji Seyama · Hiroshi Imamura · Yoshinori Inagaki · Yutaka Matsuyama · Wei Tang · Masatoshi Makuuchi · Norihiro Kokudo

Received: 27 December 2011 / Accepted: 16 May 2012 / Published online: 16 June 2012

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

Y. Seyama · H. Imamura · Y. Inagaki · W. Tang · M. Makuuchi · N. Kokudo
Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Present Address:
H. Imamura (✉)
Department of Hepatobiliary-Pancreatic Surgery, Juntendo School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
e-mail: himamura-tky@umin.ac.jp

Y. Matsuyama
Department of Biostatistics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

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 min × 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 min × 6) in which 10 min of clamping alternated with 3.3 min of reperfusion was repeated 6 times; (4) a long IC cycle group (15 min × 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.

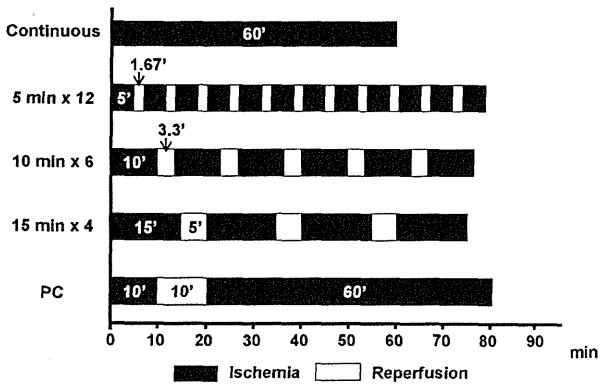

Fig. 1 Experimental protocols used for rat liver ischemia/reperfusion. Various parameters of liver injury were measured at 3 h after the termination of the respective protocols. In all groups, the total ischemia time was set at 60 min. In each intermittent inflow occlusion group, the time ratio of occlusion 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

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 °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 °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 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™, 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×*g* for 10 min at 4 °C. A reagent containing 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate was added to the supernatant to form substitution products (thioethers). Then, a β-elimination reaction was initiated by the addition of 30 % NaOH to transform the substitution product into a chromophoric thione. After incubation at 25 °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® 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 °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×) 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 °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 μm-thick), stained with hematoxylin and eosin, and evaluated semi-quantitatively in a blinded manner at 200× 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 hypereosinophilia, 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/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.

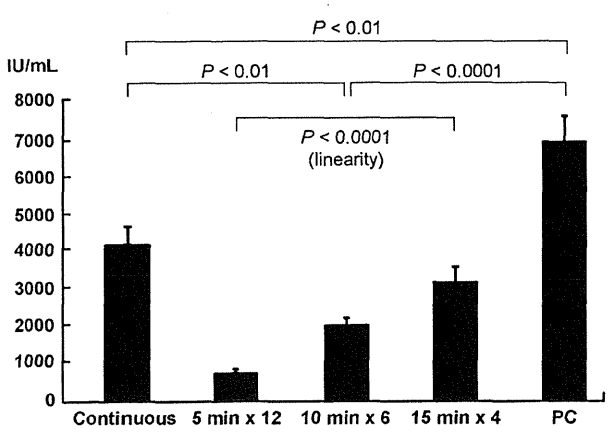

Fig. 2 Serum levels of alanine aminotransferase after 3 h of reperfusion in livers subjected to 60 min of ischemia. Data are presented as means with SEM. Multiple comparisons among groups were done by the Tukey–Kramer method. Linearity within the three intermittent inflow occlusion groups was checked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

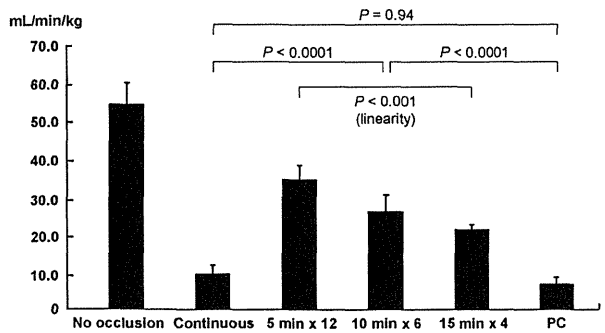

Fig. 3 Bile flow from the median and left lateral (ischemic) lobes after 3 h of reperfusion. In addition to the five experimental groups with differing liver ischemia/reperfusion protocols, a sham operation group was added as a reference for normal bile flow from the median and left lateral lobes under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

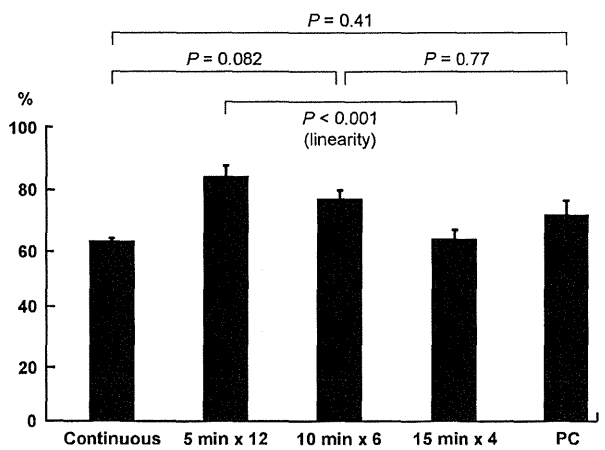

Fig. 4 Glutathione alteration from the baseline values after 3 h of reperfusion. The content of glutathione was measured in liver biopsy specimens taken from the caudate lobe before inflow occlusion, and from the median and left lateral lobes after 3 h of reperfusion. Although the levels appeared to 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

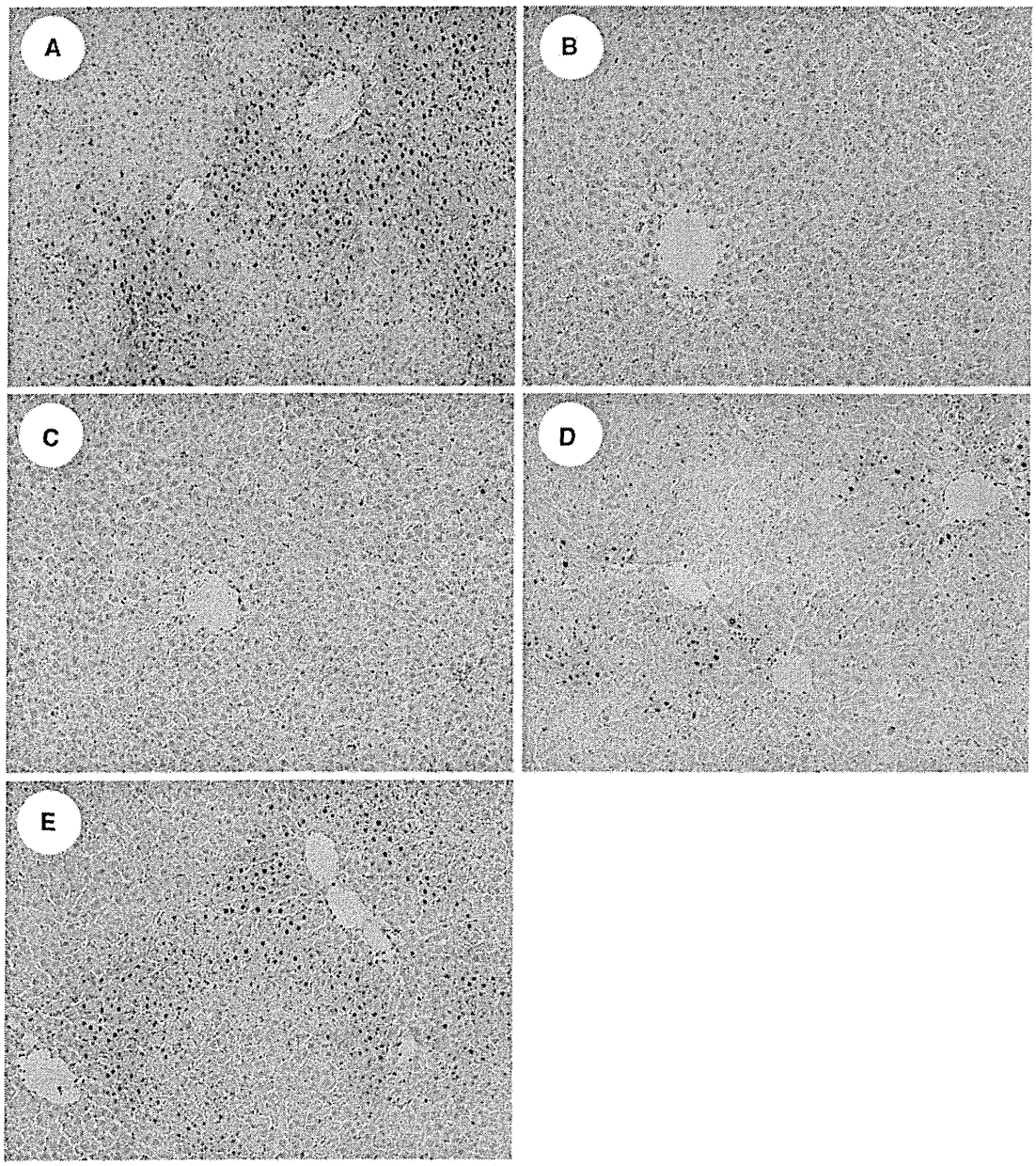

Fig. 5 Representative micrographs of terminal deoxynucleotidyl transferase-mediated biotin nick end-labeling (TUNEL) stained sections after 3 h of reperfusion are shown. **a** Continuous inflow occlusion for 60 min, **b** intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion (5 min × 12), **c** intermittent inflow occlusion for 10 min alternated with 3.3 min of reperfusion (10 min × 6), **d** intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion (15 min × 4), **e** ischemic preconditioning for 10 min followed by 10 min of reperfusion (PC) (Original magnification ×200)

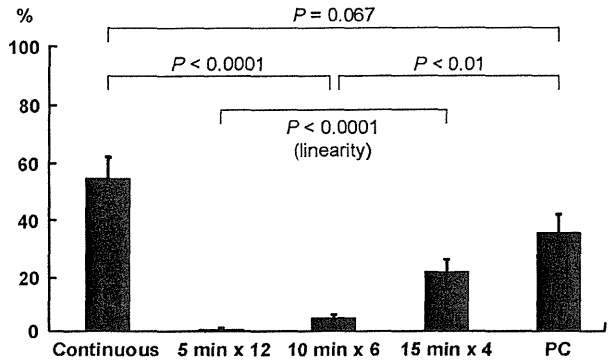

Fig. 6 Degree of hepatocyte apoptosis induction after 3 h of reperfusion. Data (means with SEM) are expressed as the proportions (%) of hepatocytes with TUNEL-positive staining. Data are presented as means with SEM. $N = 8$ in each group

characteristic biochemical marker of apoptosis, was not detected in the groups with shorter IC cycles, but it gradually increased with longer IC cycles and was also increased in the Continuous group. On the other hand, a smear of DNA, but not a DNA ladder, was detected in the PC group, indicating non-specific degradation, i.e., necrosis (Fig. 7).

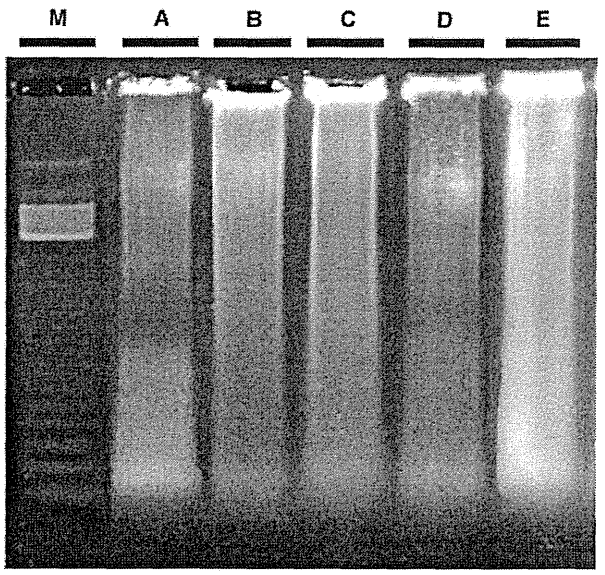

Fig. 7 DNA agarose gel electrophoresis of livers after 3 h of reperfusion. Although significant DNA laddering was not detected for shorter cycles of intermittent inflow occlusion, the level of apoptotic DNA laddering gradually increased with longer cycles of intermittent occlusion, and the level also increased in the continuous group, indicating increasing DNA fragmentation or apoptosis. On the other hand, a smear of DNA, but not DNA laddering, was detected in the preconditioning group. *Lane A* Continuous inflow occlusion for 60 min, *lane B* intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion (5 min × 12), *lane C* intermittent inflow occlusion for 10 min alternated with 3.3 min of reperfusion (10 min × 6), *lane D* intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion (15 min × 4), *lane E* ischemic preconditioning for 10 min followed by 10 min of reperfusion (PC)

Histological assessment of the degree of liver necrosis

Figure 8 shows the representative histological appearance of the liver in the five experimental groups. Necrosis occurred typically in the pericentral and midzonal regions of the hepatic lobule and usually in confluent areas of adjacent cells. We counted the areas of grade 2 and grade 3 necrosis semi-quantitatively (Fig. 9). The areas of both grade 2 and 3 necrosis were significantly smaller in the IC groups than in the Continuous group, and these areas became further reduced when the duration of the clamping/reperfusion cycles became shorter. In contrast, the areas of necrosis in the PC group were similar to those in the Continuous group.

Discussion

In the present study, we adopted 15 min of clamping alternated with 5 min of reperfusion as the control IC condition, because this is the most widely used clinical protocol for IC at experienced centers [4, 5, 10, 12, 13], and thus the time ratio of clamping to reperfusion was set at 3:1 in all IC groups. We adopted 10/10 min of preconditioning/reperfusion as the PC protocol because this is the one most widely used clinically [11, 13] and was reportedly most effective in a previous experimental study [23]. We excluded 10 min of PC from the duration of total ischemia on the basis of previous clinical and experimental studies [11–13, 19, 23].

Bile flow is reportedly a reliable indicator of liver and/or graft function, and of viability in hepatic warm as well as cold I/R injury [19, 22, 24]. Koeppel et al. [25] reported that decreased bile flow after cold I/R liver injury was explained by the impaired biliary excretion of GSH, a primary osmotic driving force in the bile flow. Reactive oxygen species are thought to play pivotal roles in liver I/R injury [26]. GSH is an endogenous radical scavenger that reacts spontaneously with nearly all oxidants formed during inflammation [27]. Previous studies have shown that the administration of GSH precursors attenuated liver I/R injury by increasing intracellular GSH [28, 29]. Likewise, Peralta's group (Serafin et al. [30]) have reported that PC conferred resistance against the liver damage induced by reactive oxygen species by preventing the depletion of GSH [30]. In line with these findings, liver GSH content was reported to be a valid indicator of the degree of I/R injury [30–32] and therefore we measured its alteration.

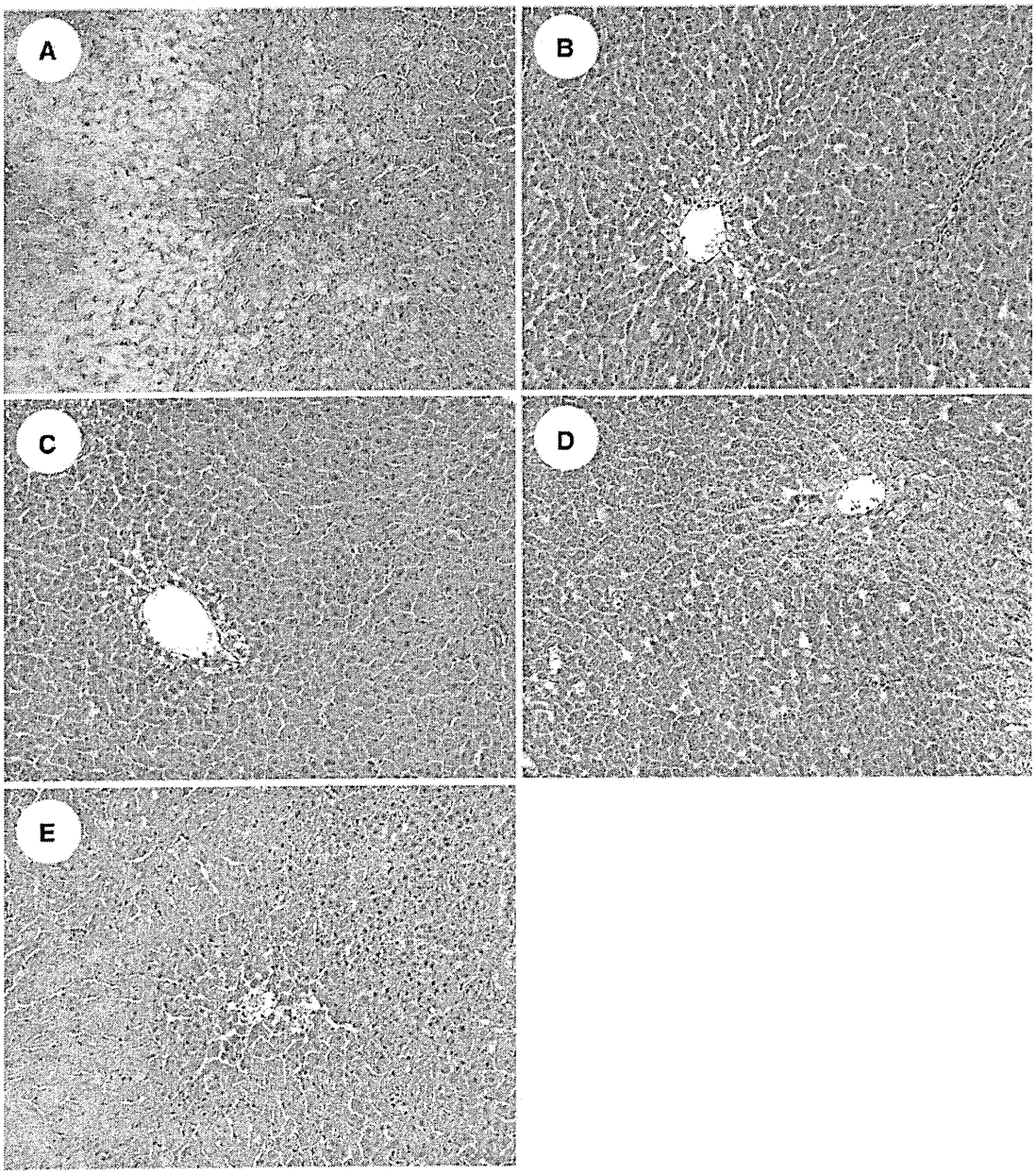

Fig. 8 Tissue sections stained with hematoxylin and eosin. Representative histological appearances after 3 h of reperfusion are shown. **a** Continuous inflow occlusion for 60 min, **b** intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion (5 min × 12), **c** intermittent inflow occlusion for 10 min alternated with 3.3 min of reperfusion (10 min × 6), **d** intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion (15 min × 4), **e** ischemic preconditioning for 10 min followed by 10 min of reperfusion (PC) (Original magnification ×200)

In the present study, the alterations in bile flow and liver GSH content showed similar trends among the experimental groups. Of note, the magnitude of the alterations in bile flow was more marked than the alterations in liver GSH content. Although biliary GSH content was not measured, assuming that the decrease in the bile flow in the present work was attributable to the decreased biliary GSH excretion, it could be speculated that the alteration in the GSH content was more profound in the bile than in the liver. As support for this consideration, Accatino et al. [33] have reported that, while bile flow and biliary excretion of GSH was markedly decreased after liver I/R injury, liver GSH content was unchanged.

We measured the serum level of ALT at 3 h after liver I/R to assess the degree of hepatocyte injury. Moreover, we conducted both qualitative and semi-quantitative

histological evaluation of liver specimens obtained at identical time points, paying particular attention to the emergence of necrotic areas. In addition, the induction of hepatocyte apoptosis was assessed in terms of the TUNEL assay and DNA fragmentation. Although hepatocyte apoptosis reportedly plays a central role in models of liver injury induced by FAS [34] and tumor necrosis factor (TNF)-alpha [35], its role in hepatic I/R injury has been questioned [36]. Most previous studies supporting a major role of hepatocyte apoptosis in liver I/R injury used a quantified TUNEL assay [37, 38], but these studies have been criticized on the grounds that the TUNEL assay also detects cells that have succumbed to oncotic death, and is not necessarily specific for apoptosis [26, 36]. For this reason, we also studied DNA laddering by agarose gel electrophoresis and confirmed the results of the TUNEL assay [39]. On the other hand, it has been reported that apoptosis and oncosis may share the same underlying mechanism, and that the outcome depends on the strength of the stimulus, or that excessive parenchymal cell apoptosis itself is a signal for the necrotic reaction, including that in models of liver I/R injury [34, 40]. Indeed, agarose gel electrophoresis revealed signs suggestive of the presence of both apoptosis and oncosis according to the experimental conditions employed (Fig. 7). Overall, the results of the TUNEL assay and DNA laddering are thought to be at least comprehensive indicators of the cell death caused by liver I/R injury.

Necrosis occurred in the pericentral and midzonal regions of the hepatic lobule, probably because these regions are furthest removed from the oxygen supply (Fig. 8). In contrast, TUNEL-positive cells were spread over a wider area, including areas of necrosis and parts of the periportal region. In addition, individual cells, rather than groups of contiguous cells, showed TUNEL positivity, especially in the peripheral midozonal and perioral regions (Fig. 5). Taking into account the non-specific TUNEL staining of necrotic cells, these findings suggested that, at least under the present experimental conditions, the main mode of cell death during warm I/R liver injury was oncotic necrosis and lent support for the contention that pathways leading to oncosis and apoptosis were shared [34, 41]. A warm I/R injury may culminate in either apoptosis or necrosis, depending on other variables such as ATP supply or the extent of hypoxia.

The most straightforward results of the present study are that all of the parameters of liver I/R injury were universally ameliorated in the IC groups in comparison with findings in the continuous inflow occlusion group. Moreover, it was noteworthy that when the time ratio of clamping to alternated reperfusion was kept constant, shorter cycles of IC were more effective. Several studies have examined whether the degree of I/R injury is affected by the duration of the IC cycles, but all them focused on the optimal duration of alternated reperfusion under a fixed inflow occlusion time [21], or vice versa [20, 39]. Horiuchi et al. reported that the extent of liver injury was reduced as the duration of reperfusion increased (15 min compared with 5 and 10 min) if the period of clamping was fixed at 15 min [21]. In contrast, Clavien's group (Jang et al. [20, 39] and Kang et al. [20, 39]) documented that the protective effect of IC was similar for both 15 and 30 min of intermittent inflow occlusion, compared with continuous inflow occlusion, when the duration of alternated reperfusion was fixed at 5 min [20, 39]. However, scrutinization of their data showed that within the IC groups, the magnitude of liver injury appeared to be more marked for 30 min of occlusion, and hence their results were in line with those of Horiuchi et al. [20, 39]. In the present investigation, we studied and clarified, for the first time, the separate effect of IC cycle length on protection against I/R.

In the clinical setting of liver resection, however, 15 min of clamping alternated with 5 min of reperfusion is the most popular and well accepted IC condition, and it may be argued that it is not practical to shorten the cycle length further. Here, we have to bear in mind the allometric law in animals, known as Kleiber's 3/4-power scaling law, which states that an animal's basal metabolic rate is proportional to 3/4 power of its body weight, and thus the metabolic time or physiological time of a species is proportional to 1/4 power of its body weight [42]. If we apply this law to extrapolate the present results to a human setting, the values of 15/5, 10/3.3, and 5/1.7 min in rats weighing 250 g correspond to 60/20, 40/13, and 20/7 min, respectively, for a human weighing 50 kg. The currently most popular human IC protocol (15/5 min) may be optimal, and a longer cycle might induce liver damage, albeit at a subclinical level.

Unexpectedly, PC did not confer a protective effect against liver I/R injury, except for the prevention of apoptosis induction, although all of the experiments were conducted under identical conditions. This result showed a clear contrast to those for IC, which demonstrated universal attenuation of every aspect of liver I/R injury, irrespective of cycle length, and with constant linearity between cycle length and the strength of the effect. Also, these results were contrary to those of previous studies indicating that PC suppressed liver I/R injury to an extent equal to that of IC [8, 19, 20, 39]. A possible explanation for this apparent discrepancy is that, under the present experimental conditions, 10 min of ischemia followed by 10 min of reperfusion did not have a preconditioning effect but worked as an additive ischemia. Peralta et al. [23] evaluated the effects of various types of PC in a rat model of 90 min of warm liver I/R injury. They reported that, for 10 min of reperfusion, 10–15 min of ischemic preconditioning was most

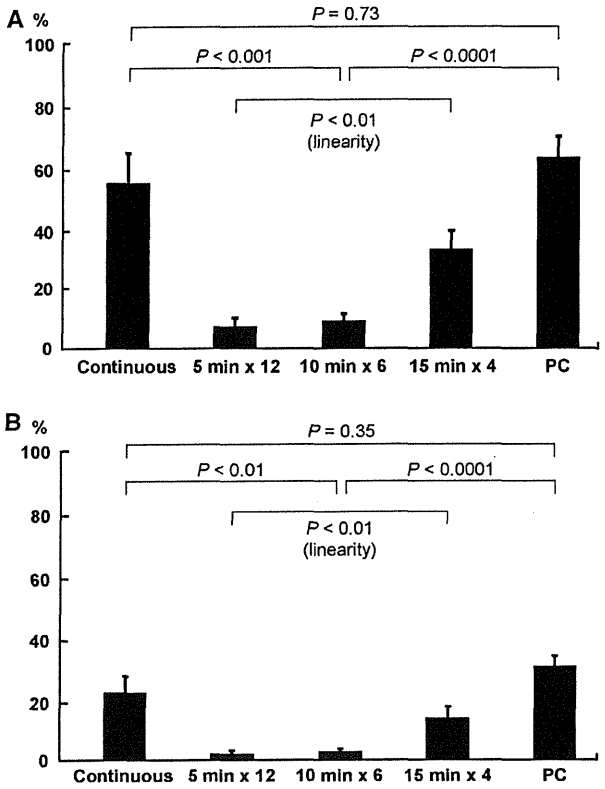

Fig. 9 Percentages of grade 2 (**a**) and grade 3 (**b**) necrosis after 3 h of reperfusion. Data (%) are expressed as means with SEM. $N = 8$ in each group

effective, and that either a shorter or longer period of preconditioning was associated with diminished protection [23]. On the other hand, their group (Serafin et al. [30]) compared three different PC methods in a rat model involving 60 min of liver I/R injury, i.e., 5 min of preconditioning followed by 10 min of reperfusion (5/10), 10 min of preconditioning followed by 15 min of reperfusion (10/15), and 10 min of preconditioning followed by 10 min of reperfusion (10/10) [30]. The group reported that the protective effect became weaker in this order, and was null in the 10/10 group [30]. Therefore, the results in their two studies ([23] and [30]) were contradictory in regard to the optimal length of preconditioning and following reperfusion in PC protocol. Likewise, it has been reported that the effect of preconditioning was obscure in aged [11] patients. Taking all these issues into account, it is possible to conclude that preconditioning may attenuate liver I/R injury, but that the degree of attenuation or the presence/absence of the effect itself is largely dependent on the particular conditions of individuals or ischemia, including the preconditioning protocol.

In conclusion, we have shown, using a rat model of liver warm I/R injury, that IC exerts a universal protective effect against I/R injury. In addition, if the time ratio of clamping to alternated reperfusion is constant, the protective effect increases as the cycle length becomes shorter. By contrast, preconditioning did not work effectively against I/R injury, at least under the present experimental conditions. Overall, IC is a robust method for reducing liver I/R injury that can be applied widely under various conditions; by contrast, preconditioning is a less robust protocol for liver protection.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research (B) (20390352) from Ministry of Education, Culture, Sports, Science and Technology and by Mitsui Life Social Welfare Foundation.

Conflict of interest The authors do not have a financial relationship with any organization that sponsored the research. The authors also have full control of all primary data and that they agree to allow the journal to review their data if requested.

References

1. Kooby DA, Stockman J, Ben-Porat L, Gonen M, Jarnagin WR, Dematteo RP, et al. Influence of transfusions on perioperative and long-term outcome in patients following hepatic resection for colorectal metastases. Ann Surg. 2003;237:860–9.
2. Tsao JI, Loftus JP, Nagomey DM, Adson MA, Ilstrup DM. Trends in morbidity and mortality of hepatic resection for malignancy: a matched comparative analysis. Ann Surg. 1994; 220:199–205.
3. Pringle J. Note on the arrest of hepatic hemorrhage due to trauma. Ann Surg. 1908;48:501.
4. Makuuchi M, Mori T, Gunven P, Yamazaki S, Hasegawa H. Safety of hemihepatic vascular occlusion during resection of the liver. Surg Gynecol Obstet. 1987;164:155–8.
5. Imamura H, Seyama Y, Kokudo N, Maema A, Sugawara Y, Sano K, et al. One thousand fifty-six hepatectomies without mortality in 8 years. Arch Surg. 2003;138:1198–206.
6. Man K, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Prospective evaluation of Pringle maneuver in hepatectomy for liver tumors by a randomized study. Ann Surg. 1997;226:704–13.
7. Kim YI, Nakashima K, Tada I, Kawano K, Kobayashi M. Prolonged normothermic ischaemia of human cirrhotic liver during hepatectomy: a preliminary report. Br J Surg. 1993;80:1566–70.
8. Rudiger HA, Kang KJ, Sindram D, Riehle HM, Clavien PA. Comparison of ischemic preconditioning and intermittent and continuous inflow occlusion in the murine liver. Ann Surg. 2002;235:400–7.
9. Camargo CA Jr, Madden JF, Gao W, Selvan RS, Clavien PA. Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in therodent. Hepatology. 1997;26:1513–20.
10. Belghiti J, Noun R, Malafosse R, Jagot P, Sauvanet A, Pierangeli F, et al. Continuous versus intermittent portal triad clamping for liver resection: a controlled study. Ann Surg. 1999;229:369–75.
11. Clavien PA, Selzner M, Rudiger HA, Graf R, Kadry Z, Rousson V, et al. A prospective randomized study in 100 consecutive patients undergoing major liver resection with versus without ischemic preconditioning. Ann Surg. 2003;238:843–52.
12. Petrowsky H, McCormack L, Trujillo M, Selzner M, Jochum W, Clavien PA. A prospective, randomized, controlled trial comparing intermittent portal triad clamping versus ischemic

preconditioning with continuous clamping for major liver resection. Ann Surg. 2006;244:921–30.
13. Smymiotis V, Theodoraki K, Arkadopoulos N, Fragulidis G, Condi-Pafiti A, Plemenou-Fragou M, et al. Ischemic preconditioning versus intermittent vascular occlusion in liver resections performed under selective vascular exclusion: a prospective randomized study. Am J Surg. 2006;192:669–74.
14. Peralta C, Closa D, Hotter G, Gelpí E, Prats N, Roselló-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. Biochem Biopyhs Res Commun. 1996;22:264–70.
15. Peralta C, Hotter G, Closa D, Gelpí E, Roselló-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia–reperfusion in the rat: role of nitric oxide and adenosine. Hepatology. 1997;30:1223–31.
16. Hardy KJ, Tancheroen S, Syulkes A. Comparison of continuous versus intermittent ischemia–reperfusion during liver resection in an experimental model. Br J Surg. 1995;82:833–6.
17. Isozaki H, Adam R, Gigou M, Szekely AM, Shen M, Bismuth H. Experimental study of the protective effect of intermittent hepatic pedicle clamping in the rat. Br J Surg. 1992;79:310–3.
18. van Wagensveld BA, van Gulik TM, Gelderblom HC, Scheepers JJ, Bosma A, Endert E, et al. Prolonged continuous or intermittent vascular inflow occlusion during hemihepatectomy in pigs. Ann Surg. 1999;229:376–84.
19. Nieuwenhuijs VB, de Bruijin MT, Schiesser M, Morphett A, Padbury RT, Barritt GJ. Ischemic preconditioning and intermittent ischemia preserve bile flow in a rat model of ischemia/reperfusion injury. Dig Dis Sci. 2007;52:3029–37.
20. Jang JH, Kang KJ, Kang Y, Lee IS, Graf R, Clavien PA. Ischemic preconditioning and intermittent clamping confer protection against ischemic injury in the cirrhotic mouse liver. Liver Transpl. 2008;14:980–8.
21. Horiuchi T, Muraoka R, Tabo T, Uchinami M, Kimura N, Tanigawa N. Optimal cycles of hepatic ischemia and reperfusion for intermittent pedicle clamping during liver surgery. Arch Surg. 1995;130:754–8.
22. Miyagawa Y, Imamura H, Soeda J, Matsunaga K, Mochida S, Fujiwara K, et al. Fate of hepatocyte and sinusoidal lining cell function and kinetics after extended cold preservation and transplantation of the rat liver. Liver Transpl. 2002;8:370–81.
23. Peralta C, Closa D, Xaus C, Gelpi E, Rosello-Catafau J, Hotter G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. Hepatology. 1998;28:768–73.
24. Bowers BA, Branum GD, Rotolo FS, Watters CR, Meyers WC. Bile flow—an index of ischemic injury. J Surg Res. 1987;42:565–9.
25. Koeppel TA, Trauner M, Mennone A, Arrese M, Rios-Velez L, Boyer JL. Role of glutathione in hepatic bile formation during reperfusion after cold ischemia of the rat liver. J Hepatol. 1998;28:812–9.
26. Jaeschke H. Molecular mechanisms of hepatic ischemia–reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol. 2003;284:G15–26.
27. Liu P, Fisher MA, Farhood A, Smith CW, Jaeschke H. Beneficial effects of extracellular glutathione against endotoxin-induced liver injury during ischemia and reperfusion. Circ Shock. 1994;43:64–70.
28. Dunne JB, Davenport M, Williams R, Tredger JM. Evidence that *S*-adenosylmethionine and *N*-acetylcysteine reduce injury from sequential cold and warm ischemia in the isolated perfused rat liver. Transplantation. 1994;57:1161–7.
29. Kobayashi H, Kurokawa T, Kitahara S, Nonami T, Harada A, Nakao A, et al. The effects of gamma-glutamylcysteine ethyl ester, a prodrug of glutathione, on ischemia–reperfusion-induced liver injury in rats. Transplantation. 1992;54:414–8.
30. Serafin A, Rosello-Catafau J, Prats N, Xaus C, Gelpi E, Peralta C. Ischemic preconditioning increase the tolerance of fatty liver to hepatic ischemia–reperfusion injury in the rat. Am J Pathol. 2002;161:587–601.
31. Amersi F, Nelson SK, Shen XD, Kato H, Melinek J, Kupiec-Weglinski JW, et al. Bucillamine, a thiol antioxidant, prevents transplantation-associated reperfusion injury. Proc Natl Acad Sci USA. 2002;99:8915–20.
32. Schauer RJ, Gerbes AL, Vonier D, op den Winkel M, Fraunberger P, Bilzer M. Induction of cellular resistance against Kupffer cell-derived oxidant stress: a novel concept of hepatoprotection by ischemic preconditioning. Hepatology. 2003;37:286–295.
33. Accatino L, Pizarro M, Solís N, Arrese M, Koenig CS. Bile secretory function after warm hepatic ischemia–reperfusion injury in the rat. Liver Transpl. 2003;9:1199–210.
34. Lawson JA, Fisher MA, Simmons CA, Farhood A, Jaeschke H. Parenchymal cell apoptosis as a signal for sinusoidal sequestration and transendothelial migration of neutrophils in murine models endotoxin- and Fas-antibody-mediated liver injury. Hepatology. 1998;28:761–7.
35. Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of caspase-3 (CPP32)-like proteases is essential for TNF-a-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J Immunol. 1998;160:3480–6.
36. Gujral JS, Bucci TJ, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia–reperfusion in rats: apoptosis or necrosis? Hepatology. 2001;33:397–405.
37. Cursio R, Gugenheim J, Ricci JE, Crenesse D, Rostagno P, Maulon L, et al. A caspase inhibitor fully protects rats against lethal normothermic liver ischemia by inhibition of liver apoptosis. FASEB J. 1999;13:253–61.
38. Sindram D, Porte RJ, Hoffman MR, Bentley RC, Clavien PA. Platelets induce sinusoidal endothelial cell apoptosis upon reperfusion of the cold ischemic rat liver. Gastroenterology. 2000;118:183–91.
39. Kang KJ, Jang JH, Lim TJ, Kang Y, Park KK, Lee IS, et al. Optimal cycle of intermittent portal triad clamping during liver resection in the murine liver. Liver Transpl. 2004;10:794–801.
40. Kobayashi A, Imamura H, Isobe M, Matsuyama Y, Soeda J, Matsunaga K, et al. Mac-1(CD11b/CD18) and intercellular adhesion molecule-1 in ischemia–reperfusion injury of rat liver. Am J Physiol Gastrointest Liver Physiol. 2001;281:G577–85.
41. Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology. 2003;125:1246–57.
42. Schmidt-Nielsen K. Scaling: why is animal size so important? Cambridge: Cambridge University Press; 1984. p. 241.

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Superselective transarterial chemoembolization for hepatocellular carcinoma. Validation of treatment algorithm proposed by Japanese guidelines

Kenichi Takayasu[1,*,†], Shigeki Arii[2,†], Masatoshi Kudo[3,†], Takafumi Ichida[4,†], Osamu Matsui[5,†], Namiki Izumi[6,†], Yutaka Matsuyama[7,†], Michiie Sakamoto[8,†], Osamu Nakashima[9,†], Yonson Ku[10,†], Norihiro Kokudo[11,†], Masatoshi Makuuchi[12,†]

[1]*Diagnostic Radiology, National Cancer Center Hospital, Tokyo, Japan;* [2]*Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University Graduate School of Medicine, Tokyo, Japan;* [3]*Department of Gastroenterology and Hepatology, Kinki University School of Medicine, Sayama, Japan;* [4]*Department of Hepatology and Gastroenterology, Juntendo Shizuoka Hospital, Izunokuni, Japan;* [5]*Department of Radiology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan;* [6]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Japan;* [7]*Department of Biostatistics, School of Public Health, University of Tokyo, Tokyo, Japan;* [8]*Department of Pathology, Keio University School of Medicine, Tokyo, Japan;* [9]*Department of Clinical Laboratory Medicine, Kurume University Hospital, Kurume, Japan;* [10]*Department of Surgery, Kobe University Graduate School of Medicine, Kobe, Japan;* [11]*Hepato-Biliary-Pancreatic Surgery Division, Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;* [12]*Surgery, Japanese Red Cross Medical Center, Tokyo, Japan*

Background & Aims: Transcatheter arterial chemoembolization with lipiodol (TACE) is widely performed in patients with hepatocellular carcinoma (HCC) unsuitable for curative treatment. It has recently been recommended for patients with 2 or 3 tumors >3 cm or ⩾4 tumors in a treatment algorithm proposed by Japanese guidelines. However, the best indication and appropriateness of the algorithm for TACE are still unclear.

Methods: In 4966 HCC patients who underwent TACE, survival was evaluated based on tumor number, size and liver function; and the adequacy of the algorithm for TACE was validated. Exclusion criteria were: vascular invasion, extrahepatic metastasis, and prior treatment. The mean follow up period was 1.6 years.

Results: The overall median and 5-year survivals were 3.3 years and 34%, respectively. Multivariate analysis revealed that Child–Pugh class, tumor number, size, alpha-fetoprotein, and des-gamma carboxy-prothrombin were independent predictors. The survival rate decreased as the tumor number ($p = 0.0001$) and size increased ($p = 0.04$ to $p = 0.0001$) in all but one subgroup in both Child–Pugh-A and -B. The stratification of these patients to four treatments in the algorithm showed potential ability to discriminate survivals of the resection and ablation (non-TACE) groups from those of the TACE group in Child–Pugh-B and partially in A.

Conclusions: TACE showed higher survival rates in patients with fewer tumor numbers, smaller tumor size, and better liver function. The treatment algorithm proposed by the Japanese guidelines might be appropriate to discriminate the survival of patients with non-TACE from TACE therapy.

Cancer

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide with 626,000 new cases every year, and is the third most common cause of death from cancer [1]. The frequency of curative treatment, such as resection, local ablation, and/or liver transplantation is low (only 30%) due to advanced cancer stage and associated liver cirrhosis at the time of diagnosis [2]. Among several treatments, transcatheter arterial chemoembolization with lipiodol (TACE) is widely performed in patients with unresectable HCC at an initial and recurrent time, which accounts for 32% and 58% of all treatment modalities, respectively, in Japan [3]. Superselective TACE is indispensable to maximize the effect in targeted tumors and to minimize liver injury [4].

Recently, two treatment algorithms for HCC were proposed: the Barcelona Clinic Liver Cancer (BCLC) classification, in 2001 [5] and the Japanese guidelines, in 2005 [6]. The first one recommends TACE in patients with multi-nodular HCC in Child–Pugh A or B in the intermediate stage, while the second recommends TACE in patients with 2 or 3 tumors, >3 cm in diameter or ⩾4 tumors in liver damage A or B. In both guidelines, vascular invasion and/or extrahepatic spread are excluded. However, the survival rate of TACE-stratified to recommended treatment of the Japanese guidelines algorithm and its appropriateness have not

Keywords: Hepatocellular carcinoma (HCC); Transcatheter arterial chemoembolization (TACE); Prognostic factor; Validation of treatment algorithm; Japanese guidelines.

Received 6 August 2011; received in revised form 15 October 2011; accepted 26 October 2011; available online 13 December 2011

* Corresponding author. Address: Diagnostic Radiology, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan. Tel.: +81 3 3542 2511; fax: +81 3 3542 3815.

E-mail address: ktakayas@ncc.go.jp (K. Takayasu).

† For the Liver Cancer Study Group of Japan.