Table 1.
Patient profile

	Complete resection	Reductive resection	Radiotherapy	TACE/TAI	Chemotherapy	Palliative therapy
No. of patients (%)	16 (10)	16 (10)	18 (11)	73 (45)	8 (5)	30 (19)
Age (years)	57 ± 2.0	53 ± 2.8	59 ± 2.6	61 ± 0.9	62 ± 3.1	62 ± 1.7
Gender (M/F)	14/2	14/2	16/2	58/15	8/0	27/3
Virus type: B / C / non B non C	7/5/5	8/5/4	4/12/3	28/42/11	3/5/2	10/24/1
Aspartate aminotransferase (IU/dl)	54 ± 4	100 ± 21	89 ± 15	125 ± 15	73 ± 15	239 ± 90
Total bilirubin (mg/dl)	0.9 ± 0.04	0.8 ± 0.06	1.1 ± 0.2	1.3 ± 0.1	1.3 ± 0.3	4.8 ± 1.3
Albumin (g/dl)	3.9 ± 0.1	3.5 ± 0.1	3.3 ± 0.1	3.5 ± 0.1	3.6 ± 0.2	3.1 ± 0.1
ICG R15 (%)	14 ± 2.2	15 ± 1.7	27 ± 3.5	29 ± 1.9	12 ± 4.9	35 ± 4.3
Prothrombin activity (%)	79 ± 3.7	77 ± 3.3	71 ± 45	83 ± 10	68 ± 5.1	66 ± 3.6
Platelet ($\times 10^4/\mu l$)	19 ± 1.8	20 ± 1.9	16 ± 2.3	15 ± 1.0	14 ± 2.6	14 ± 1.3
AFP (pg/ml)	43 ± 27	83 ± 62	5.1 ± 1.9	62 ± 21	93 ± 93	82 ± 38
Child-Pugh score						
A	13	12	4	32	3	4
B	3	4	10	32	4	13
C	0	0	4	9	1	13

TACE / TAI: transcatheter arterial chemoembolization/transcatheter arterial infusion chemotherapy; ICG R15: Indocyanine green retention value at 15 minutes; AFP: α-fetoprotein.
Values represent (mean ± SE).

only TAI with farmorubicine is performed. After treatments with RR and RT, TACE/TAI are combined, repeated until the remnant tumor completely disappears on computed tomography angiography (CTA). management with respect to detection and staging of HCC is discussed with hepatobiliary-pancreatic surgeons, physicians, and diagnostic radiologists, and determined.

Statistical analysis was performed by the chi-square test and Mann–Whitney *U*-test. Survival rates were calculated using the Kaplan Meier method. Survival curves were compared using log-rank test. The Cox proportional hazard model was used to assess clinical factors influencing survival benefit in the RR group. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Tables 1 and 2 show patient characteristics in the main treatment groups. Among the 161 advanced HCC patients, 32 (20%) underwent surgical resection (16 CR, 16: RR). Eighteen patients (11%) received RT, 73 (45%) underwent TACE or TAI, 8 (5%) with distant metastases received systemic chemotherapy (CT), and 30 (19%) received palliative therapy (PT). Morbidity and mortality of 32 patients with surgical treatments (CR and RR) are demonstrated in Table 3. There was no statistical difference between the CR group and the RR group in the postoperative hospital stay. Table 4 and Fig. 2 show the median, 3-month, 1-year, and 5-year survival rates in each treatment group. There were 5-year survivors in the CR group only and the survival rate was 37%. Excluding the CR group, patients in the RR group had a higher 1-year survival rate than the other treatment groups. However, there was no significant difference in overall survival rates of the RR, RT, and TACE/TAI groups. Table 5 shows the results of univariate analysis used to identify significant preoperative factors for overall survival in patients treated with RR. Among the 13 preoperative factors, 4 significant factors were identified by univariate analysis: age, aspartate aminotransferase (AST), prothrombin activity (PA), and tumor size. Table 6 shows the results of multivariate analysis using Cox's proportional hazards model. Multivariate analysis was performed on the 4 preoperative factors (age, AST, PA, and tumor size) for overall survival that reached statistical significance in univariate analysis ($P < 0.05$), as shown in Table 5. Among them, serum PA was identified as a significant independent preoperative factor for overall survival. The distribution of serum PA for each patient treated with RR is shown in Fig. 3. The value of serum PA that gave the maximum sensitivity and specificity for the survival period was 78%. Figure 4 shows a comparison of overall survival rates after RR between serum PA of >78% and PA of ≤78%. Survival rate in patients with PA of ≤ 78% was significantly lower than that of patients with PA of >78% ($P = 0.0004$). Median survival time of patients with serum

Table 2.
Tumor factors of 161 patients with advanced hepatocellular carcinoma (HCC)

	Complete resection	Reductive resection	Radiotherapy	TACE/TAI	Chemotherapy	Palliative therapy
No. of patients (%)	16 (10)	16 (10)	18 (11)	73 (45)	8 (5)	30 (19)
Tumor size (cm)						
<5	1 (6)	0	3 (17)	11 (15)	2 (25)	2 (7)
5–10	7 (44)	6 (38)	9 (50)	42 (58)	2 (25)	19 (63)
>10	8 (50)	10 (63)	6 (33)	20 (27)	4 (50)	9 (30)
No. of tumor nodules						
1	9 (56)	0	8 (44)	12 (16)	4 (50)	4 (13)
2–5	3 (19)	5 (31)	1 (6)	8 (11)	3 (38)	2 (7)
>5	4 (25)	11 (69)	9 (50)	53 (72)	1 (13)	24 (80)
Bilobar tumors	2 (13)	14 (88)	13 (72)	54 (74)	5 (63)	27 (90)
Presence of major PVT	13 (81)	14 (88)	18 (100)	69 (95)	7 (88)	29 (97)
Presence of major HVT	8 (50)	4 (25)	2 (11)	15 (21)	3 (38)	9 (30)

TACE/TAI: transcatheter arterial chemoembolization/transcatheter arterial infusion chemotherapy; PVT: portal vein thrombosis; HVT: hepatic vein thrombosis.

Table 3.
Morbidity and mortality of 32 patients with surgical treatment

	Complete resection	Reductive resection	*P* value*
No. of patients	16	16	
Morbidity	10/16	13/16	NS
Wound infection	4	6	
Pleural effusion	3	2	
Ascites	2	0	
Bile leakage	4	7	
Mortality	1/16[a]	1/16[b]	NS
Postoperative hospital stay (days) (mean)	27	27	NS

NS: nonsignificant values.
[a]Death due to liver failure on postoperative 15th day.
[b]Death due to massive bleeding intraoperatively.
*Statistical significance between both groups was analyzed by chi-square test and Mann–Whitney *U*-test.

Table 4.
Survival rate of 161 patients with advanced hepatocellular carcinoma (HCC) in each treatment group

			Survivors (%)		
Treatment	Number	Median survival (months)	3-month survival	1-year survival	5-year survival
Complete resection	16	9.1	93	74	37
Reductive resection	16	6.2	93	39	0
Radiotherapy	18	6.6	72	28	0
TACE/TAI	73	3.8	67	25	0
Chemotherapy	8	3.0	50	0	0
Palliative therapy	30	0.9	10	0	0

TACE/TAI: transcatheter arterial chemoembolization/transcatheter arterial infusion chemotherapy.

PA of >78% who underwent RR was 13.9 months, and that of patients who underwent CR was 9.1 months, with no survival difference between CR patients and RR patients with PA of greater 78% (Fig. 5). Median survival time of patients with serum PA of ≤78% who underwent RR was 4.9 months, that of patients who received RT was 6.6 months, and that of patients received TACE/TAI was 3.8 months, with no survival difference among RT patients, TACE/TAI patients, and RR patients with PA less than 78% (Fig. 5).

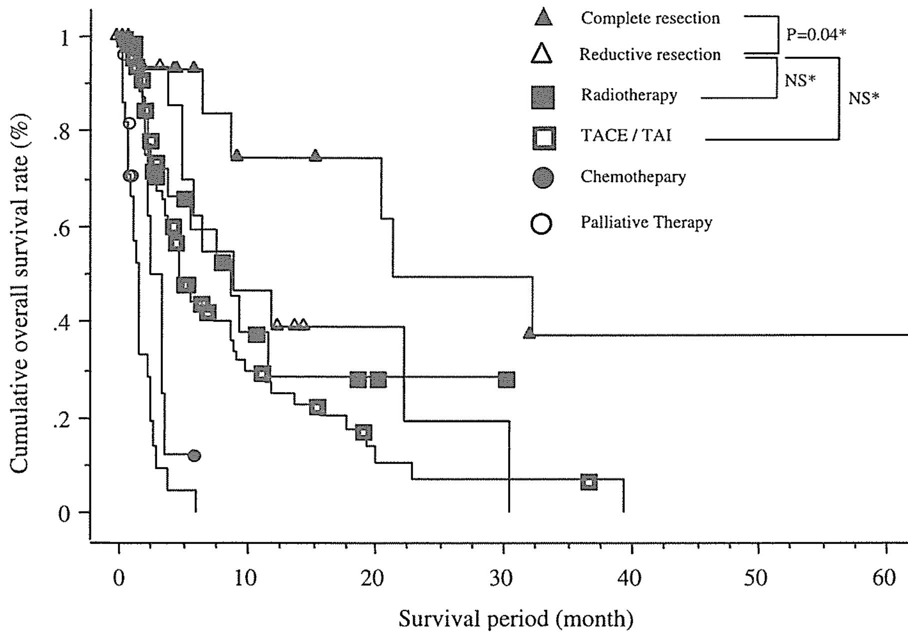

Figure 2. Cumulative survival curves obtained by the Kaplan–Meier method for each treatment (statistical significance between groups was analyzed by log-rank test).

Table 5.
Clinical factors influencing cumulative survival rate of patients in reductive resection group analyzed by univariate analysis

Clinical factors	*P* value
Age	0.0148*
Gender	0.8293
Aspartate aminotransferase	0.0136*
Total bilirubin	0.1719
Albumin	0.6645
ICG R15	0.0580
Prothrombin activity	0.0092*
Platelet	0.8851
AFP	0.5337
Tumor size	0.0072*
No. of tumor nodules	0.1192
Presence of major PVT	0.9641
Presence of major HVT	0.6589

ICG R15: Indocyanine green retention value at 15 minutes; PVT: portal vein thrombosis; HVT: hepatic vein thrombosis; AFP: α-fetoprotein.
*Statistically significant.

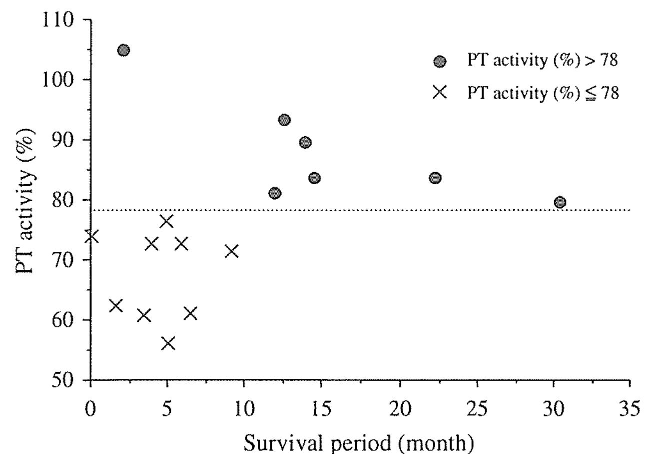

Figure 3. Patient distribution for each prothrombin (PT) activity in reductive resection group.

Table 6.
Clinical factors influencing cumulative survival rate of patients in reductive resection group analyzed by multivariate analysis

Clinical factors	*P* value
Age	0.5691
Aspartate aminotransferase	0.0894
Prothrombin activity	0.0203*
Tumor size	0.0927

*Statistically significant.

DISCUSSION

Development of TT in the first branch of the PV, the main PV, or the IVC via the HV is a feature of advanced HCC. The prognosis for such patients is extremely poor, and survival is generally limited to a few months after diagnosis.[3–15,19] Cases of advanced HCC with TT are usually not controllable by alternative therapies such as TACE/TAI, CT, and RT. As shown in Fig. 2, only patients who underwent CR of tumors with direct removal of TT had long-term survival. Our results demonstrated no significant difference between the RR group and other treatment groups (RT and TACE/TAI). However, if advanced HCC patients with TT cannot receive CR, RR with

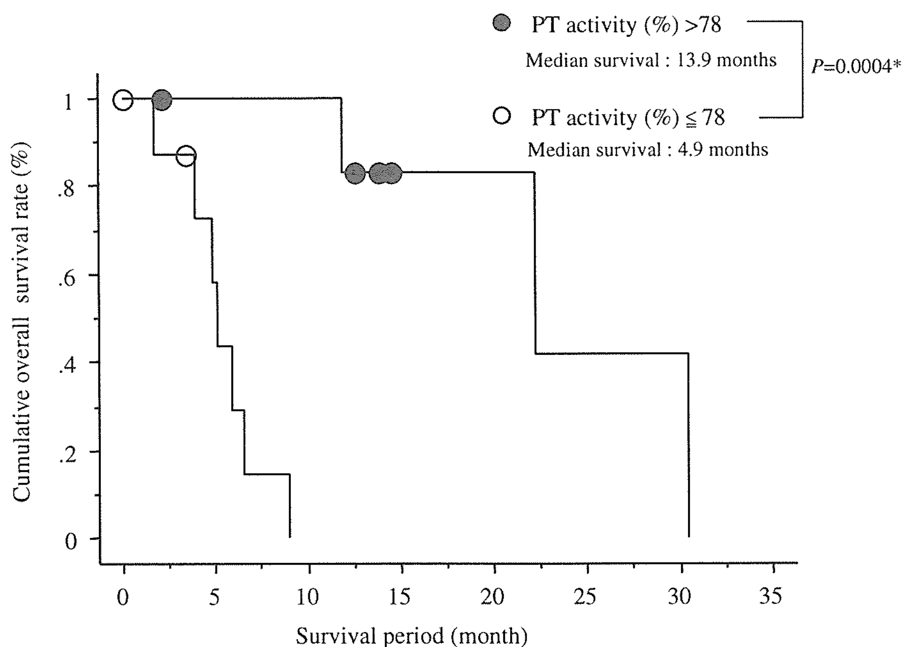

Figure 4. Cumulative survival curves obtained by the Kaplan-Meier method for each prothrombin (PT) activity in reductive resection group (statistical significance between groups was analyzed by log-rank test).

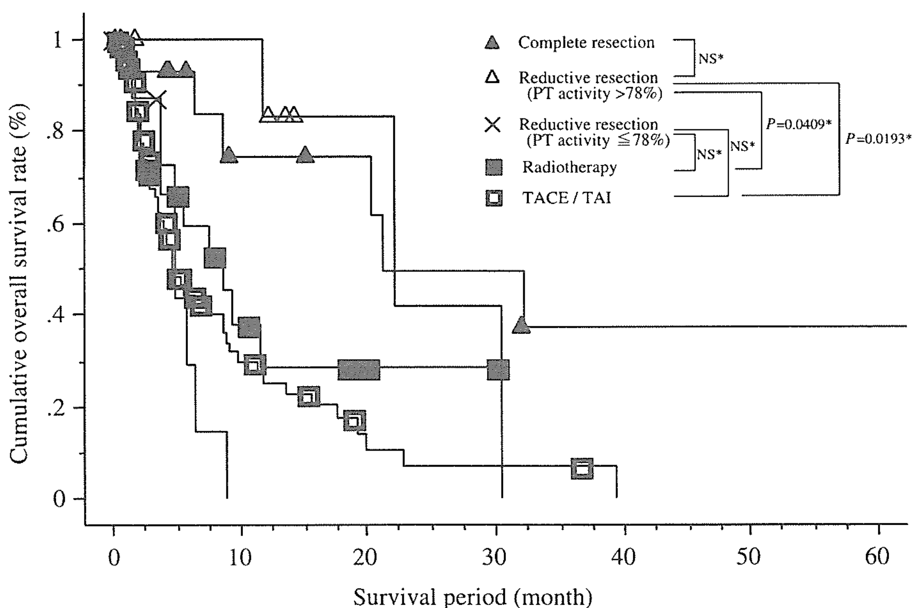

Figure 5. Comparison of cumulative survival curves obtained by the Kaplan–Meier method for each treatment group (statistical significance between groups was analyzed by log-rank test).

direct removal of TT might be the only treatment that offers hope of prolonged survival. We consider that RR holds the promise of prolonged survival in advanced HCC patients. There are several reports concerning the efficacy of RR for patients with advanced HCC.[15,19,34,35,37,38] Moreover, there are some reports that hepatectomy for large HCC (tumors greater than 10 cm) is a safe procedure when performed in high-volume centers.[22,36,39–42] We have also demonstrated that postoperative hospital stay in the RR for multiple large HCC was similar to the CR group and that RR could be performed safely. Although morbidity was high and one patient died within 30 days after operation in our surgical treatment groups (CR and RR groups: CR patient died in 1996 and RR patient died in 1995), morbidity was much lower recently because surgical technique and perioperative management were improving.

When we judged that the liver function would tolerate resection of the main tumor and direct removal of TT even if intrahepatic metastases remained in the remnant liver, we performed RR. In the present study, we evaluated the efficacy of RR for advanced HCC with major

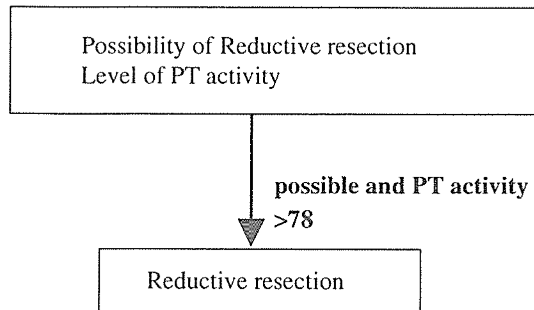

Figure 6. New indication of reductive resection for advanced hepatocellular carcinoma (HCC) with major vascular involvement.

vascular involvement. Yamamoto *et al.*[34] suggested remnant tumor index (RTI) as a significant prognostic factor in advanced HCC patients who underwent RR. RTI was calculated on the basis of the size and number of residual HCC nodules after RR. However, Asahara *et al.*[43] reported that long-term survivors did not always meet the criteria for RTI and that the indication for RR in HCC patients with multiple intrahepatic metastases was relatively small primary tumors that could be removed by single segmentectomy. These reports about the efficacy of RR included cases of advanced HCC without TT. In our study, we evaluated the characteristics of advanced HCC with major vascular involvement; primary tumors were larger than 5 cm in diameter, and the number of HCC nodules was greater than 5 in many cases. Our indication for RR for advanced HCC with TT in major vessels (first branch of the PV, the main PV, or the IVC) was serum PA of >78% (Fig. 6). Prolongation of survival in advanced HCC patients with serum PA of >78% can be expected with RR with direct removal of TT. The survival of these patients under our new indication for RR was similar to the survival of advanced HCC patients who underwent CR. On the other hand, the survival of advanced HCC patients with serum PA of ≤78% was similar to that of patients who received RT or TACE/TAI; therefore, it is not recommenced that patients with serum PA of ≤78% undergo RR with direct removal of TT. Unnecessary surgical stress can be avoided in patients who cannot be expected to achieve prolonged survival by RR.

In conclusion, if complete resection does not seem feasible for patients with advanced HCC with major vascular involvement on preoperative evaluation, the surgeon should still proceed with debulking surgery in those patients with serum PA of >78% because a reductive resection portends to survival benefit even if there is not complete clearance.

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Selection Criteria for Reduction Hepatectomy in Multiple Advanced Hepatocellular Carcinoma

NAOTO GOTOHDA[1], HIROSHI ISHII[2], MASARU KONISHI[1], TOSHIO NAKAGOHRI[1], SHINICHIRO TAKAHASHI[1], JUNJI FURUSE[2], MASAHIRO YOSHINO[2] and TAIRA KINOSHITA[1]

[1]*Department of Hepatobiliary Pancreatic Surgery and* [2]*Department of Hepatobiliary Pancreatic Medical Oncology, National Cancer Center Hospital East, Chiba, 277-8577, Japan*

Abstract. *Background: Few studies have compared the prognostic impact of reduction hepatectomy (RH) for advanced hepatocellular carcinoma (HCC) with that of non-surgical treatment or curative hepatectomy. Patients and Methods: The treatment outcome of 30 RH patients was compared to that of two control groups: control group A, including 71 patients who underwent curative hepatectomy; control group B, including 106 patients who did not receive definitive local therapy or best supportive care. Results: In patients with tumor extension in >50% of the liver, 1-year survival rates for patients according to treatment (RH, control A and B) were 50%, 90% and 11% and 3-year survival rates were 42%, 60% and 0%, respectively. There was no significant difference between RH and control A, but there was a significant difference between RH and control B. Conclusion: RH could be recommended to patients with multiple advanced HCC extending to >50% of the whole liver.*

Hepatic resection is the only treatment that offers a hope of cure with long-term survival in patients with HCC. However, the indications for radical hepatectomy for hepatocellular carcinoma (HCC) remain limited for patients with multiple intrahepatic metastases (1). If hepatectomy with curative intent for multiple advanced HCCs cannot be performed, the possibility of volume reduction hepatectomy (RH) for those HCCs is often considered, with the aim of decreasing target tumor cells so that effective post-operative treatment can be carried out. The efficacy of RH for advanced HCC has been reported by several studies (2-5), and we have also performed RH for multiple advanced HCC.

The aim of this study was to evaluate the long-term results of RH for patients with multiple HCC, and to validate the indications for RH compared to other treatments and optimize patient selection.

Correspondence to: Naoto Gotohda, MD, Department of Hepatobiliary Pancreatic Surgery, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba, 277-8577, Japan. Tel: +81 4 7133 1111, Fax: +81 4 7131 4724, e-mail: ngotohda@east.ncc.go.jp

Key Words: Reduction hepatectomy, advanced hepatocellular carcinoma, selection criteria.

Patients and Methods

From July 1992 to May 2005, 1,001 patients with a diagnosis of HCC with no distant (extrahepatic) metastases were examined consecutively at the National Cancer Center Hospital East, Japan. Of these HCC patients, 30 underwent RH. Their treatment outcome was compared retrospectively with that of two control groups selected among the remaining 971 patients with a similar background to the RH patients. The selection criteria (background factors) were: total bilirubin <2.4 mg/dl, albumin >2.7 g/dl, no ascites, Child-Pugh score <9 points, multiple tumors, main tumor >44 mm in diameter, Okuda stage I or II (6), and BCLC (Barcelona Clinic Liver Cancer) stage B or C (7). Control A included 71 patients who underwent hepatectomy with curative intent. Control B included 106 patients who did not receive definitive local therapy (hepatectomy, percutaneous ablation therapy or proton beam radiotherapy) or best supportive care.

The indications for hepatectomy were determined according to criteria based on tumor extension and hepatic functional reserve (8, 9). As our treatment strategy for advanced HCC, whether or not hepatectomy with curative intent could be performed was considered first. When hepatectomy with curative intent could not be performed, the possibility of RH was considered. RH was defined at our institute as resection of the main tumor or main tumor plus satellite tumors around the main tumor, with satellite tumors in the remnant liver classified as unresectable. If this surgical treatment for advanced HCC was not feasible, radiotherapy (including proton beam radiotherapy) or transcatheter arterial chemoembolization/infusion chemotherapy (TACE/TAI) tolerance was considered. If liver function and portal vein flow were poor due to tumor thrombi, only TAI with farmorubicin was administered. If TAI was not feasible, best supportive care was considered. After RH, TACE or TAI treatment was repeated until the residual tumor had completely disappeared on CT.

Table I. *Patient characteristics.*

	RH	Control A	*p*-value	Control B	*p*-value
No. of patients	30	71		106	
Patient factors					
Age (yr)	60 (27-76)	65 (28-84)	**0.013***	63 (39-86)	**0.010***
Gender (M/F)	29/1	62/9	0.274§	90/16	0.119§
HBsAg positive	7	18	1.000§	14	0.250§
Anti-HCVAb positive	15	40	1.000§	74	0.052§
Total bilirubin (mg/dl)	0.9 (0.4-2.3)	0.8 (0.4-1.8)	0.390*	1.0 (0.4-2.2)	0.100*
Albumin (g/dl)	3.6 (2.8-4.3)	3.7 (2.8-4.7)	0.088*	3.5 (2.8-4.3)	0.351*
AFP (pg/ml)	540 (1.8-970,000)	71 (1.7-380,000)	0.126*	400 (1.7-350,000)	0.937*
Child-Pugh stage (A/B)	26/4	63/8	0.746§	83/23	0.438§
Okuda classification (I/II)	18/12	60/11	**0.010§**	84/22	0.054§
BCLC staging (B/C)	14/16	56/15	0.002§	74/32	0.029§
Tumor factors					
Size of main tumor (mm)	120 (45-200)	80 (45-220)	**0.001***	70 (45-150)	**<0.001***
Vascular invasion positive	16	15	**0.002§**	32	**0.029§**
Tumor extension in >50%	12	10	**0.007§**	11	**<0.001***

Values represent median (range). RH: reduction hepatectomy, HBsAg: hepatitis B surface antigen, Anti-HCVAb: anti-hepatitis C virus antibody, AFP: α-fetoprotein, BCLC staging: Barcelona Clinic Liver Cancer staging. Statistical significance was analyzed by Chi-square test§ and Mann-Whitney's *U*-test*.

Results

The patient characteristics are listed in Table I. As patients with similar background factors to those in RH patients were selected, no significant differences were found between the RH, control A and B groups. Cumulative 1-year survival rates for patients in RH, control A and B groups were 29%, 85% and 61%, and cumulative 3-year survival rates were 17%, 63% and 16%, respectively. Significant differences were found in survival between control group A and RH, and between control group A and control group B.

In cases where the main tumor was greater than 100 mm in diameter, cumulative 1- and 3-year survival rates for RH, control A and B groups were 32 and 26%, 82 and 45% and 31 and 0%, respectively. Significant differences were found between control A and RH, and between control A and control B group. In patients with vascular invasion, 1-year survival rates for RH, control A and B groups were 13%, 73% and 29%, while 3-year survival rates were 13%, 38% and 3%, respectively. In patients with tumor extension in >50% of the liver, 1-year survival rates for RH, control A and B groups were 50%, 90% and 11%, while 3-year survival rates were 42%, 60% and 0%, respectively. There was no significant difference between RH and control group A, but there was a significant difference between RH and control B (Figure 1). The characteristics of patients with tumor extension in >50% of the liver are shown in Table II. Total bilirubin was higher in control group B compared to RH group, while the other patient factors were similar between the three groups. Although larger HCCs were included in the RH group compared to control B group, the vascular invasion factor was similar.

Discussion

The background factors of patients treated with RH in our hospital were investigated first, using various prognostic scoring systems (Okuda stage, CLIP (10), JIS (11) , Tokyo score (12) and BCLC). There were no RH patients with Okuda stage III or BCLC stage A or D. As the RH patient distribution by the Okuda and BCLC staging systems was characteristic and not biased, they were used to unify the background factors of HCC patients. Several reports, after validation of the various prognostic staging systems for HCC, have indicated that the BCLC staging system provided the best independent prediction of survival (13, 14).

Patient factors were unified as much as possible, and two comparison groups were used (curative hepatectomy group and other local therapy group). After overall survival rates in each group had been compared, RH was definitively inferior to curative hepatectomy, and similar to other local therapies (including best supportive care).

However, although all patients in this study had multiple HCCs, three tumor factors (tumor size, vascular invasion and tumor extension) were further advanced in RH group compared to control A and B groups. These tumor factors were then estimated separately, and survival rates in each group with the same tumor condition were compared.

Table II. *Characteristics of patients with tumor extension in >50% of liver.*

	RH	Control A	*p*-value	Control B	*p*-value
No. of patients	12	10		11	
Patient factors					
Age (yr)	60 (27-74)	66 (45-70)	0.147*	61 (50-70)	0.389*
Gender (M/F)	12/0	9/1	0.455§	8/3	0.093§
HBsAg positive	3	2	1.000§	2	1.000§
Anti-HCVAb positive	4	4	1.000§	8	0.100§
Total bilirubin (mg/dl)	0.9 (0.5-1.2)	0.8 (0.5-1.0)	0.230*	1.4 (0.8-2.2)	**0.003***
Albumin (g/dl)	3.5 (2.8-4.0)	3.7 (3.0-4.3)	0.186*	3.6 (2.8-4.0)	0.687*
AFP (pg/ml)	6,400 (2.5-240,000)	290 (1.7-380,000)	0.210*	19,000 (3.5-92,000)	0.854*
Child-Pugh stage (A/B)	10/2	8/2	1.000§	6/5	0.193§
Okuda classification (I/II)	0/12	0/10		0/11	
BCLC staging (B/C)	5/7	7/3	0.231§	6/5	0.684§
Tumor factors					
Size of main tumor (mm)	160 (130-200)	130 (53-220)	0.079*	130 (90-150)	**0.006***
Vascular invasion positive	7	3	0.231§	5	0.684§

Values represent median (range). RH: reduction hepatectomy, HBsAg: hepatitis B surface antigen, Anti-HCVAb: anti-hepatitis C virus antibody, AFP: α-fetoprotein, BCLC staging: Barcelona Clinic Liver Cancer staging. Statistical significance was analyzed by Chi-square test§ and Mann-Whitney's *U*-test*.

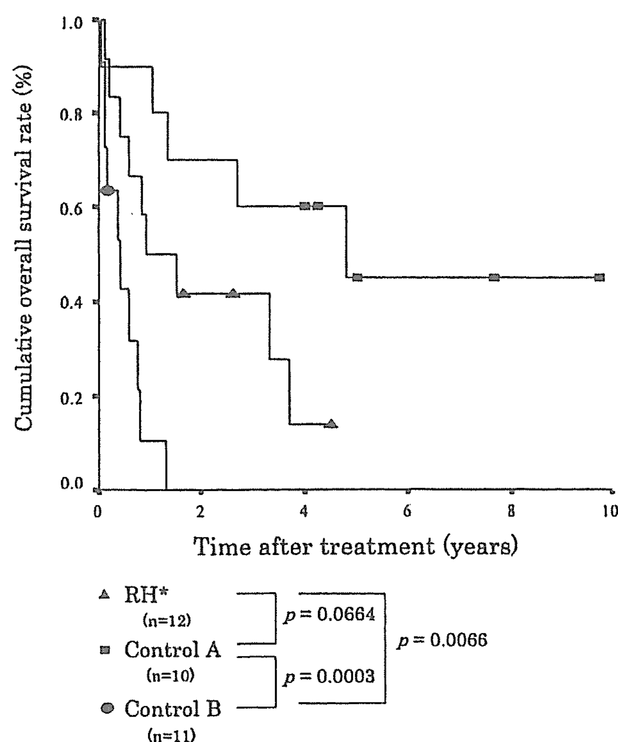

Figure 1. *Kaplan-Meier estimated survival curves according to treatment in patients with tumor extension in >50% of liver. *RH: reduction hepatectomy. Statistical significance was analyzed by log-rank test.*

After those comparisons, the survival benefit of RH for advanced multiple HCCs with tumor extension in >50% of the liver was found to be equivalent to that of curative hepatectomy and superior to that of other local therapy (including best supportive care). Since RH is intended to decrease the tumor volume in the liver, this result could suggest that RH is more effective for prolonged survival benefit if tumor extension in the liver is larger.

With regard to the effectiveness of RH for advanced HCC, Yamamoto *et al.* (4) reported that RH was effective in patients with fewer and smaller tumors left in the residual liver and no extrahepatic metastasis. However, only surgically treated patients were compared in the report. Wakabayashi *et al.* (5) compared two groups of patients (RH group and non-surgical group), and showed that RH had survival benefit. However, their background factors differed from ours, and the authors commented that unintended patient selection bias may have affected the results of the study. Although our evaluated patient factors were selected retrospectively, as shown in Table II, our treatment outcome was compared in groups selected to have equivalent factors, and, thus, our results were considered to be unbiased.

In conclusion, the present study suggested that RH for multiple advanced HCC with tumor extension in >50% of the liver might hold promise of survival benefit.

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Randomized, double-blind, placebo-controlled trial of bovine lactoferrin in patients with chronic hepatitis C

Hideki Ueno,[1,16] Tosiya Sato,[2] Seiichiro Yamamoto,[3] Katsuaki Tanaka,[4] Shinichi Ohkawa,[5] Hitoshi Takagi,[6] Osamu Yokosuka,[7] Junji Furuse,[8] Hidetsugu Saito,[9] Akira Sawaki,[10] Hiroshi Kasugai,[11] Yukio Osaki,[12] Shigetoshi Fujiyama,[13] Keiko Sato,[14] Keiji Wakabayashi[15] and Takuji Okusaka[1]

[1]Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital, Tokyo 104-0045; [2]Department of Biostatistics, Kyoto University School of Public Health, Kyoto 606-8501; [3]Biometric Research Section, Statistics and Cancer Control Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo 104-0045; [4]Gastroenterological Center, Yokohama City University Medical Center, Kanagawa 232-0024; [5]Division of Hepatobiliary and Pancreatic Oncology, Kanagawa Cancer Center Hospital, Kanagawa 241-0815; [6]Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Gunma 371-8511; [7]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba 260-0856; [8]Hepatobiliary and Pancreatic Oncology Division, National Cancer Center Hospital East, Chiba 277-0882; [9]Department of Internal Medicine, Keio University Hospital, Tokyo 160-8582; [10]Department of Gastroenterology, Aichi Cancer Center Hospital, Aichi 464-8681; [11]Department of Gastrointestinal Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka 537-8511; [12]Department of Gastroenterology and Hepatology, Osaka Red Cross Hospital, Osaka 543-8555; [13]Department of Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556; [14]Genetic Counselling and Clinical Research Unit, Kyoto University School of Public Health, Kyoto 606-8501; [15]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan

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Several studies have suggested that lactoferrin administration may decrease the serum level of hepatitis C virus (HCV) RNA in patients with chronic hepatitis C. The aim of the present study was to confirm the efficacy of orally administered bovine lactoferrin (bLF) in patients with chronic hepatitis C. The patients with chronic hepatitis C randomly received either oral bLF at a dose of 1.8 g daily for 12 weeks, or an oral placebo. The primary endpoint was the virologic response, defined as a 50% or greater decrease in serum HCV RNA level at 12 weeks compared with the baseline. The secondary endpoint was the biochemical response, which was defined as a 50% or greater decrease in the serum alanine aminotransferase (ALT) level at 12 weeks compared with the baseline. One hundred and ninety-eight of 199 patients were evaluable for efficacy and safety. bLF treatment was well tolerated and no serious toxicities were observed. A virologic response was achieved in 14 of 97 patients (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group. There was no significant difference in virologic response rates between the two groups (–4.4%, 95% confidence interval –14.8, 6.1). In addition, bLF intake did not have any favorable effect on the serum ALT level. The virologic responses were not different between two groups in any subgroup analysis. In conclusion, orally administered bLF does not demonstrate any significant efficacy in patients with chronic hepatitis C. (*Cancer Sci* 2006; 97: 1105–1110)

Hepatitis C virus is a leading cause of chronic liver disease in Japan, and nearly two million people are estimated to be infected.[1] It is well known that HCV infection frequently causes chronic hepatitis, and that chronic hepatitis eventually progresses to liver cirrhosis and HCC approximately 30 years after HCV infection.[2] In Japan, more than 30 000 people die of HCC annually, and approximately 80% of HCC patients are infected with HCV.[3] Therefore, effective anti-HCV therapy is necessary to reduce the number of patients suffering from cirrhosis or HCC. To date, interferon-based therapy is the only effective treatment used clinically for chronic hepatitis C. A sustained complete virologic response (loss of detectable serum HCV RNA) occurs in 15–20% of patients with chronic hepatitis C after interferon therapy.[4] Moreover, recent studies have demonstrated that interferon with ribavirin or peginterferon with ribavirin improves the sustained complete virologic response rate by up to 40–50%.[5,6] However, because more than half of patients do not respond to interferon therapy, and because interferon therapy sometimes induces strong adverse effects, further developments in the treatment of chronic hepatitis C are required.

Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is present mainly in breast milk and other exocrine secretions. Several biological activities of lactoferrin have been demonstrated, including regulation of iron absorption in the intestine and modulation of immunoreactions.[7] Lactoferrin also plays an important role in human innate defense mechanisms against bacteria, fungi and viruses.[8] *In vitro* studies to date have shown that lactoferrin has antiviral effects against human immunodeficiency virus-1 and human cytomegalovirus.[9] Recent experimental studies have suggested that lactoferrin has antiviral effect against HCV.[10–12] Yi *et al.* have reported that lactoferrin binds to HCV envelope proteins *in vitro*.[10] Ikeda *et al.* have reported that lactoferrin prevents HCV infection in cultured human hepatocytes, and suggested that the anti-HCV activity of lactoferrin might be related to its direct binding to viral surfaces.[11,12] In addition, recent clinical studies have demonstrated the potential efficacy of lactoferrin against chronic hepatitis C.[13,14] Tanaka *et al.* reported that 8-week oral administration of bLF at a dose of 1.8 or 3.6 g/day decreased the serum level of HCV RNA markedly in three of four patients with a low pre-treatment HCV RNA level (<100 Kcopy/mL).[13] Iwasa *et al.* administered bLF (3.6 g/day) orally to 15 patients with high viral loads (≥100 KIU/mL), and reported that the mean serum HCV RNA level decreased significantly from 1106 KIU/mL at entry to 612 KIU/mL after 6 months of treatment ($P < 0.01$).[14] Based on these promising findings, we planned to investigate the efficacy of orally administered bLF in patients with chronic hepatitis C. First, we conducted a dose-finding study in 45 patients with chronic hepatitis C.[15] In that study, three dose levels of bLF (1.8, 3.6 and 7.2 g/day) were scheduled, and 15 patients at each dose level received the determined dose of bLF for 8 weeks. bLF treatment was well tolerated up to 7.2 g/day, and no serious adverse events were observed. Although no relationship between bLF dose and efficacy was recognized, a 50% or greater decrease in the serum HCV RNA level was seen in four of 45 patients (8.9%). Furthermore, the HCV RNA level was decreased by 50% or more in eight patients (17.8%) at week 8 after the end of treatment. These results encouraged us to conduct further investigations, and the present randomized

[16]To whom correspondence should be addressed. E-mail: hiueno@ncc.go.jp
Abbreviations: ALT, alanine aminotransferase; bLF, bovine lactoferrin; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL, interleukin; NK, natural killer.

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trial was designed to clarify the anti-HCV activity of bLF in patients with chronic hepatitis C.

Patients and Methods

Patients. Each patient was required to meet the following eligibility criteria: 20–74 years of age; positivity for anti-HCV antibody; an HCV RNA level of 0.5–850 KIU/mL evaluated within 1 month before entry; a sustained elevation of serum ALT level for at least 6 months; a serum ALT level of at least twice the upper normal limit evaluated within 1 month before entry; no evidence of HCC on the basis of ultrasonography or computed tomography carried out within 3 months before entry; and adequate bone marrow function (white blood cell count ≥ 4000/mm^3, platelet count ≥ 100 000/mm^3, and hemoglobin level ≥ 11 g/dL), liver function (total bilirubin level ≥ 2.0 mg/dL, serum albumin level ≥ 3.5 g/dL, and serum aspartate aminotransferase and ALT level ≥ 200 IU/L) and renal function (normal serum creatinine and blood urea nitrogen levels).

The exclusion criteria were: positivity for hepatitis B surface antigen; interferon therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin therapy within 1 month before entry; past or present history of bLF tablet intake; pregnant or lactating females; severe hepatic disease (e.g. autoimmune hepatitis and primary biliary cirrhosis); other serious medical conditions (e.g. gastrointestinal bleeding, active infection, severe pulmonary disease and psychiatric disorders).

Methods. This double-blind, placebo-controlled phase III trial was conducted at 11 centers in Japan. The study was approved by the institutional review board at each center, and all the participants provided written informed consent. Eligible participants were assigned randomly to one of two treatment groups in equal proportions using permutation blocks stratified by centers. A randomization list was drawn up using the SAS random number generator at the data center (Quintiles Transnational Japan K. K. Tokyo, Japan). The treatments consisted of bLF at a dose of 1.8 g/day or a placebo, administered orally twice daily for 12 weeks. In the current study, bLF at 1.8 g/day was selected on the basis of the previous dose-finding study, which indicated that there was no significant relationship between bLF dose (range, 1.8–7.2 g/day) and anti-HCV activity.[15] After the treatment allocation, the data center sent a numbered container of bLF or placebo tablets to a participant. During treatment, combined use of interferon, immunomodulatory therapy, corticosteroid and intravenous glycyrrhizin was prohibited. bLF (450 mg/tablet) and placebo tablets were provided by Morinaga Milk Industries (Tokyo, Japan).

In the current study, we tested the hypothesis that oral administration of bLF would: (1) reduce the serum HCV RNA level; and (2) reduce the serum ALT level in patients with chronic hepatitis C. In addition, we investigated the influence of orally administered bLF on systemic immune response in a small group of participants. The participants were evaluated every 4 weeks as outpatients until 4 weeks after completion of treatment. Serum HCV RNA level and serum ALT level were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after treatment. Serum HCV RNA level was determined by reverse transcription–polymerase chain reaction using the Amplicor-HCV monitor V 2.0 kit with a sensitivity of 0.5 KIU/mL (Roche Diagnostics, Tokyo, Japan). Anti-HCV antibody was determined by chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Tokyo, Japan). HCV serotyping was carried out as described previously.[16] HCV serotype 1 corresponds to genotypes 1a and 1b of the Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.[17] Serum concentration of IL-18 was measured in participants at two institutions (National Cancer Center Hospital and Osaka Red Cross Hospital), and the percentage of CD4$^+$, CD8$^+$, CD16$^+$ and CD56$^+$ peripheral blood lymphocytes was measured in participants at the National Cancer Center Hospital. IL-18 and all lymphocytes were measured before treatment, during treatment at weeks 4, 8 and 12, and at 4 weeks after completion of treatment. Serum concentration of IL-18 was assayed with a human IL-18 enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan). Lymphocyte surface phenotypes of CD4, CD8, CD16 and CD56 were determined by flow cytometry.

Adverse events were graded for severity according to the Japan Society for Cancer Therapy criteria,[18] which are similar to the National Cancer Institute Common Toxicity criteria. During treatment, participants were asked to record in a daily journal both compliance and any adverse events they experienced.

Assessment of efficacy and statistical analysis. Analyses were carried out on an intention to treat basis. The primary endpoint was a virologic response. In the current study, we defined a virologic response as a 50% or greater decrease in the serum HCV RNA level at 12 weeks compared with the baseline. Secondary endpoints were a biochemical response, as were changes in serum HCV RNA level and serum ALT level. If the serum ALT level at 12 weeks showed both a ≥50% decrease compared with the baseline and was ≤ twice the upper normal limit, we considered it a biochemical response. Response rate was calculated as the number of responders divided by the total number in each group. Participants whose HCV RNA (or ALT) data at 12 weeks were missing were included only in the denominator. Change in HCV RNA level (or ALT level) was calculated as the logarithm of the HCV RNA level (or ALT level) at 12 weeks minus the logarithm of these at the baseline. Differences in the virologic or biochemical response rates between two groups were analyzed using a test for the difference between two proportions. Differences in the change in HCV RNA level or ALT level between two groups were analyzed using a test for the difference between two means. In addition to the above planned analyses, subgroup analyses for virologic response were carried out based on pretreatment variables including age, serum HCV RNA level and HCV serotype. In a small group of participants, change in the serum concentration of IL-18 and changes in the percentage of CD4$^+$, CD8$^+$, CD16$^+$ and CD56$^+$ peripheral blood lymphocytes during the study period were investigated. Analyses were carried out using JMP4.0 and PC SAS Release v.8.02 (SAS Institute Japan Ltd, Tokyo, Japan). All P-values are two-tailed, and differences at $P < 0.05$ were regarded as statistically significant.

We estimated that a total of 250 participants would be the maximum to enroll for a 2-year enrollment period. Subsequent power analysis revealed that 125 participants per group would have 75% power to detect a 10% difference in the virologic response rate (15 vs 5%) at the 5% level of significance. An interim analysis by the independent data monitoring committee was planned after the first 125 participants had been enrolled. All trial personnel and participants were blinded to treatment assignment for the duration of the trial. Only the trial statistician and the independent data monitoring committee saw unblinded data. In the interim analysis of the primary endpoint, the O'Brien–Fleming method was used.[19]

Results

Patients. Enrollment began at seven institutions in April 2001. Because 250 participants were not enrolled for the 2 years planned originally, we extended the registration period for one more year and increased the number of participating institutions from seven to 11. An interim analysis was carried out in March 2004 with the data from the first 125 participants. Because the results of the interim analysis indicated that it was highly unlikely that a significant difference in treatment efficacy between

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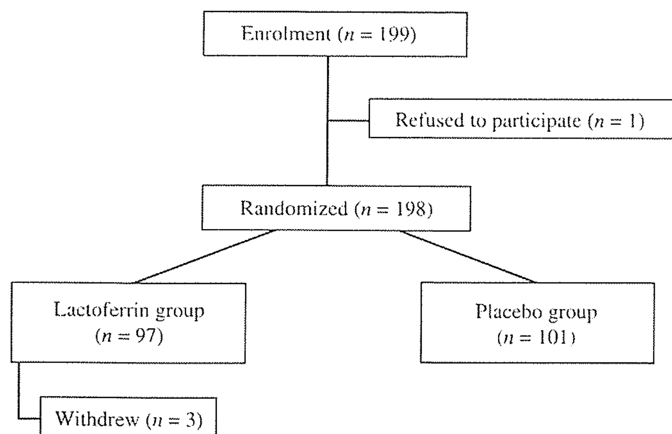

Fig. 1. Flow diagram of participant enrolment.

Table 1. Baseline characteristics of the patients

Characteristic	Bovine lactoferrin	Placebo
No. patients	97	101
Age (years)†	61 (29–74)	58 (31–74)
Sex (male/female)	53/44	55/46
History of interferon therapy	25	29
ALT level (IU/L)†	91 (41–340)	98 (27–250)
HCV RNA level (KIU/mL)†	378 (8.8–960)	452 (8.0–1560)
HCV serotype (1/2/ND)	78/17/1	76/22/3

†Median (range). ALT, alanine aminotransferase; HCV, hepatitis C virus; ND, not determined.

the two groups would be observed with the planned full enrollment of 250 participants, the data monitoring committee recommended discontinuation of further enrollment. Therefore, enrollment was stopped on 31 March 2004, at which point 199 participants had been enrolled. Because one patient refused to participate in the study before randomization, efficacy and safety were analyzed in the remaining 198 participants (97 bLF and 101 placebo) (Fig. 1). Although three participants in the bLF group discontinued treatment for reasons other than an adverse event, the remaining 195 participants completed the scheduled 12 weeks of treatment. The baseline characteristics of the 198 participants are shown in Table 1. There was no significant difference between the bLF and placebo groups regarding the pretreatment characteristics including age, sex, serum ALT level and serum HCV RNA level.

Virologic efficacy. Virologic response, the primary endpoint, was assessed in all 198 participants who received at least one dose of treatment. Virologic response was observed in 14 of 97 participants (14.4%) in the bLF group, and in 19 of 101 (18.8%) in the placebo group (Table 2). No complete virologic response (loss of detectable serum HCV RNA) was seen in either of the groups. There was no significant difference in the virologic response rate with bLF treatment in comparison with the placebo (–4.4%, 95% CI –14.8, 6.1). Change in the HCV RNA level at 12 weeks compared with the baseline was assessed in 190 participants (93 bLF group, 97 placebo group), excluding eight participants for whom HCV RNA data at 12 weeks were lacking. The change in the mean logarithm of the HCV RNA level was –0.09 in the bLF group and –0.09 in the placebo group, indicating no significant difference between the groups (P = 1.00).

Biochemical efficacy. Biochemical response was assessed in 198 participants. Biochemical response was seen in six of 97 participants (6.2%) in the bLF group, and in four of 101 participants (4.0%) in the placebo group (Table 2). No significant difference in the biochemical response rate was seen between the groups (2.2%, 95% CI –3.9, 8.3). Change in the serum AST level was assessed in 192 participants (93 bLF group, 99 placebo group), excluding six participants for whom ALT data at 12 weeks were lacking. The change in the mean logarithm of the ALT level was –0.085 in the bLF group and –0.080 in the placebo group, indicating no significant difference (P = 0.93).

Subgroup analysis. The rates of virologic response with respect to pretreatment variables are presented in Table 3. Among participants with a low HCV RNA level (<100 KIU/mL), the virologic response rate was 29.4% in the bLF group and 15.4% in the placebo group, indicating no significant difference between the groups (14.0%, 95% CI –15.2, 43.2). The virologic responses were also not different between two groups in other subgroup analyses such as age, sex and HCV serotype.

Analysis of IL-18 and lymphocytes. The serum concentration of IL-18 was measured in 73 participants enrolled at the National Cancer Center Hospital and Osaka Red Cross Hospital (36 bLF, 37 placebo). Figure 2 shows the changes in the mean IL-18 levels in the bLF group and placebo group. The mean IL-18 levels in the bLF and placebo groups were 293.9 pg/mL and 309.9 pg/dL at the baseline and 280.7 pg/mL and 291.5 pg/mL at 12 weeks, respectively. The corresponding changes in the mean IL-18 level at 12 weeks were –14.5 pg/mL and –15.9 pg/mL, respectively, indicating no significant difference between the groups (P = 0.91). Similarly, there were no significant differences between the groups at any other points during the study period. The percentage of lymphocyte was measured in 46 participants at the National Cancer Center Hospital (bLF 23, placebo 23), and the results are shown in Fig. 3. The percentage of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ peripheral blood lymphocytes remained almost unchanged throughout the study in both groups, and the differences between them were not significant.

Safety. Safety was assessed in 198 participants who received at least one dose of bLF or placebo during the study. The bLF treatment was well tolerated, and no serious complications occurred during the treatment. Although minor adverse events including neutropenia, γ-GTP elevation and hyperglycemia were observed in participants treated with bLF, their frequency and intensity did not differ from those in the placebo group. HCC was detected in one participant in the bLF group and in one participant in the placebo group during the study period.

Discussion

The present study was carried out to confirm the anti-HCV activity of orally administered bLF in patients with chronic hepatitis C. A virologic response (a 50% or greater decrease in the serum level of HCV RNA at 12 weeks compared with the baseline) was observed in 14 of 97 participants (14.4%) in the bLF group, and 19 of 101 (18.8%) in the placebo group, the difference between the groups being non-significant. The virologic responses were not different between two groups in any subgroup analysis. Furthermore, bLF intake did not have any favorable effect on the serum ALT level. On the basis of these results, we concluded that orally administered bLF did not have any efficacy, including anti-HCV activity, in patients with chronic hepatitis C.

The virologic response rate of 14.4% observed in the bLF group was somewhat higher than that reported in the previous dose-finding study,[15] in which four of 45 patients (8.9%) showed a virologic response at the end of bLF treatment. Nevertheless, the current study failed to demonstrate any anti-HCV activity of bLF, because a similar virologic response rate to that in the bLF group was seen in the placebo group. Having designed this randomized study, we assumed that a virologic

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Table 2. **Virologic and biochemical efficacy**

Characteristic	Bovine Lactoferrin	Placebo	Difference (95% CI)	*P*-value
Virologic efficacy				
Response rate (%)	14.4	18.8	−4.4 (−14.8, 6.1)	
Change in HCV RNA level†	−0.09	−0.09		1.00
Biochemical efficacy				
Response rate (%)	6.2	4.0	2.2 (−3.9, 8.3)	
Change in ALT level†	−0.085	−0.080		0.93

†Mean logarithm. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

Table 3. Virologic response rate as a function of baseline variables

Variable	Bovine lactoferrin (*n* = 97)		Placebo (*n* = 101)		Difference	
	Response/total	%	Response/total	%	%	95% CI
Age						
<65 years	12/62	19.4	14/77	18.2	1.2	−11.9, 14.2
≥65 years	2/35	5.7	5/24	20.8	−15.1	−33.1, 2.9
Sex						
Male	10/53	18.9	10/55	18.2	0.7	−14.0, 15.3
Female	4/44	9.1	9/46	19.6	−10.5	−24.7, 3.8
ALT level						
<100 IU/L	6/57	10.5	7/51	13.7	−3.2	−15.6, 9.2
≥100 IU/L	8/40	20.0	12/50	24.0	−4.0	−21.1, 13.1
HCV RNA level						
<100 KIU/mL	5/17	29.4	2/13	15.4	14.0	−15.2, 43.2
≥100 KIU/mL	9/80	11.3	17/88	19.3	−8.0	−18.8, 2.7
HCV serotype†						
1	11/78	14.1	16/76	21.1	−7.0	−18.9, 5.0
2	3/18	16.7	2/22	9.1	7.6	−31.4, 28.6

†Hepatitis C virus serotype was not measured in four patients. ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus.

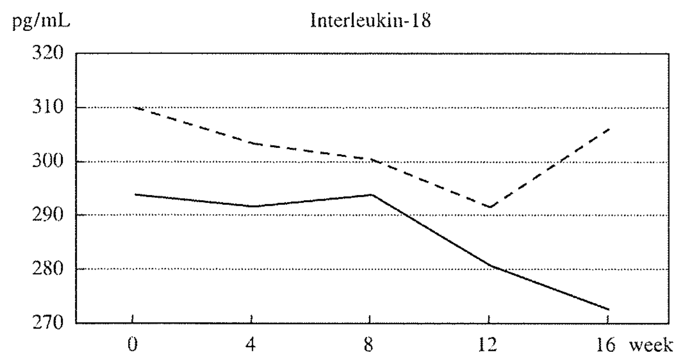

Fig. 2. Changes in the mean serum concentration of interleukin-18 in the bovine lactoferrin group (straight line, *n* = 36) and the placebo group (dotted line, *n* = 37).

response rate of around 5% would be seen in the placebo group due to spontaneous remission of viral activity. However, contrary to our expectation, 19 of 101 participants (18.8%) in the placebo group showed a ≥50% decrease in the HCV RNA level at 12 weeks, indicating that our assumption was inappropriate. Our results suggested that in order to assess the reduction of the HCV RNA level, periodic evaluation would be necessary to exclude the influence of spontaneous fluctuation of HCV RNA.

Several experimental studies have suggested that lactoferrin has some activity against HCV. Yi *et al.*[10] reported that lactoferrin binds to the HCV E1 and E2 envelope proteins *in vitro*, and Ikeda *et al.*[11,12] reported that lactoferrin prevents HCV infection in cultured human hepatocytes. They suggested that the anti-HCV activity of lactoferrin might be due to a neutralizing efficacy, in which the administered lactoferrin became bound directly to the HCV virion, thus inhibiting adsorption of the HCV-lactoferrin complex into human hepatocytes. Therefore, intravenous administration of lactoferrin might improve the viremic state in patients with chronic hepatitis C. However, for practical application, administration of lactoferrin directly into blood does not seem to be a suitable approach because lactoferrin is a large glycoprotein molecule (80 kDa) that may cause allergic reactions. Therefore, oral administration of bLF was selected for the present study, even though the metabolism and mechanism of ingested lactoferrin are yet to be clarified. As to absorption, it has been reported that intact lactoferrin and its fragments are present in the urine of human milk-fed preterm infants.[20] However, in adult rats, lactoferrin and its fragments are not detectable in portal blood after bLF ingestion,[21] and in adult humans, the serum lactoferrin level does not increase after oral administration of recombinant human lactoferrin.[22] However, several studies have suggested that orally administered lactoferrin might enhance immune responses via cytokine production.[23,24] It has been reported that oral administration of bLF to mice enhances the production of IL-18 and interferon-γ in the mucosa of the small intestine, and increases the number of $CD4^+$, $CD8^+$ and NK cells in the small-intestinal epithelium.[25,26] Varadhachary *et al.* reported that oral administration of recombinant human lactoferrin to mice stimulates IL-18 production from gut enterocytes, and augments the NK activity of spleen cells and production of blood $CD8^+$ cells.[27] Furthermore, a recent clinical study has demonstrated that oral administration of bLF (0.6 g/day) for 3 months in 36 patients with chronic hepatitis C increased the serum IL-18 level significantly compared with the

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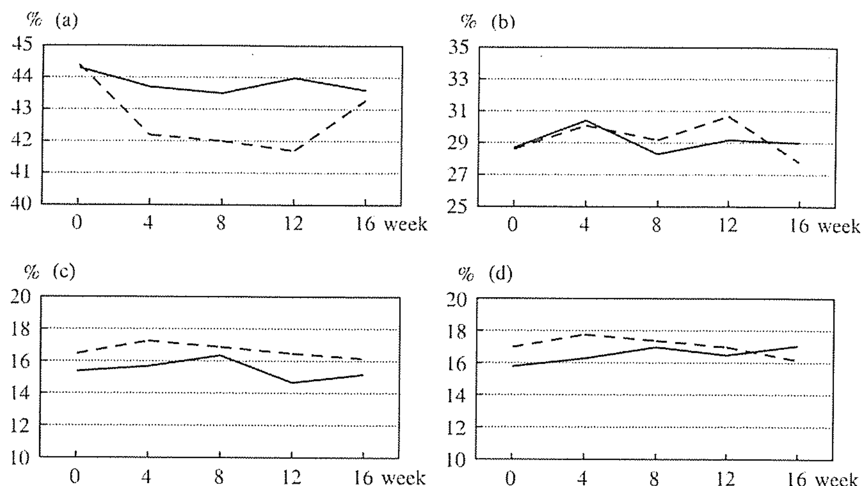

Fig. 3. Changes in the mean percentages of (a) CD4+, (b) CD8+, (c) CD16+ and (d) CD56+ peripheral blood lymphocytes in the bovine lactoferrin group (straight line, $n = 23$) and the placebo group (dotted line, $n = 23$).

baseline.[28] However, our study found no evidence that oral administration of bLF influences the serum concentration of IL-18 or the percentage of CD4+, CD8+, CD16+ and CD56+ lymphocytes. Further investigations are required to clarify the peripheral and systemic effects of orally administered lactoferrin. In addition, as many *in vitro* studies have suggested that lactoferrin has direct binding neutralizing efficacy against HCV,[29–31] further investigations are needed to devise a means of delivering lactoferrin or its fragment into the bloodstream safely and effectively.

Recently, several studies have investigated the value of adding lactoferrin to interferon therapy for chronic hepatitis C. Hirashima *et al.* randomly assigned 21 patients with chronic hepatitis C to either a consensus interferon plus oral lactoferrin (3.0 g/day) group or a consensus interferon monotherapy group.[32] Three of 10 patients in the consensus interferon plus lactoferrin group showed a sustained complete virologic response, as did four of 11 patients in the consensus interferon group, indicating no statistically significant difference between the groups. Ishibashi *et al.* conducted a randomized controlled trial to investigate the efficacy of interferon α-2b and ribavirin plus oral lactoferrin (0.6 g/day) compared with interferon α-2b and ribavirin plus placebo in 36 patients with chronic hepatitis C.[33] A sustained complete virologic response was seen in six of 18 patients in the lactoferrin group and in five of 18 patients in the placebo group, there being no statistically significant difference between the groups ($P = 0.7$). Although the numbers of patients recruited in the two randomized trials were small, these results suggested that the additional value of oral lactoferrin combined with interferon therapy would be negative for the treatment of chronic hepatitis C.

In summary, oral administration of bLF at a dose of 1.8 g/day for 12 weeks showed an acceptable safety profile in patients with chronic hepatitis C. However, there was no significant difference in the virologic responses between patients who received oral bLF and those receiving placebo. In addition, bLF intake did not have any favorable effect on the serum ALT level. These findings do not support the practical use of oral bLF in patients with chronic hepatitis C.

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Identification of HLA-A2- or HLA-A24-Restricted CTL Epitopes Possibly Useful for Glypican-3-Specific Immunotherapy of Hepatocellular Carcinoma

Hiroyuki Komori,[1,2] Tetsuya Nakatsura,[1] Satoru Senju,[1] Yoshihiro Yoshitake,[1] Yutaka Motomura,[1,2] Yoshiaki Ikuta,[1,2] Daiki Fukuma,[1] Kazunori Yokomine,[1] Michiko Harao,[1,2] Toru Beppu,[2] Masanori Matsui,[3] Toshihiko Torigoe,[4] Noriyuki Sato,[4] Hideo Baba,[2] and Yasuharu Nishimura[1]

Abstract **Purpose and Experimental Design:** We previously reported that glypican-3 (GPC3) was overexpressed, specifically in hepatocellular carcinoma (HCC) and melanoma in humans, and it was useful as a novel tumor marker. We also reported that the preimmunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse GPC3$_{298-306}$ (EYILSLEEL) peptide prevented the growth of tumor-expressing mouse GPC3. Because of similarities in the peptide binding motifs between H-2K^d and HLA-A24 (*A*2402*), the GPC3$_{298-306}$ peptide therefore seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. In this report, we investigated whether the GPC3$_{298-306}$ peptide could induce GPC3-reactive CTLs from the peripheral blood mononuclear cells (PBMC) of HLA-A24 (*A*2402*)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice to identify the HLA-A2 (*A*0201*)–restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2$^+$ HCC patients.

Results: We found that the GPC3$_{144-152}$ (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) transgenic mice without inducing autoimmunity. In five out of eight HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3$_{144-152}$ peptide-reactive CTLs were generated from PBMCs by *in vitro* stimulation with the peptide and the GPC3$_{298-306}$ peptide-reactive CTLs were also generated from PBMCs in four of six HLA-A24$^+$ GPC3$^+$ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusion: Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Hepatocellular carcinoma (HCC) is now spreading rapidly, especially in Asian and Western countries. It is clear that patients with hepatitis B or C–based liver cirrhosis are at high risk for developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at high risk for recurrence (2). Furthermore, the liver function of these patients is often very poor, so further treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor, and new therapies for the prevention of cancer development and recurrence, i.e., adjuvant therapy, is urgently needed. As for melanoma, the age-adjusted incidence rates have been increasing in most fair-skinned populations in recent decades (3). In 2005, it is estimated that 59,580 Americans will be diagnosed to have melanoma, and 7,770 will die from the disease (4).

We and others previously reported that glypican-3 (GPC3) was overexpressed in most types of HCC (5–9) and melanoma in humans (8), and we also previously reported that an H-2K^d-restricted antigenic peptide, the mouse GPC3$_{298-306}$ (EYILSLEEL) peptide, could be recognized by mouse CD8$^+$ CTLs. In addition, these CTLs rejected tumor expressing mouse GPC3 both *in vitro* and *in vivo* (10). Because the structural motifs of peptides bound to HLA-A24 (*A*2402*) and mouse H-2K^d are similar, we investigated whether the GPC3 peptide was also useful as a cancer immunotherapy modality for HLA-A24$^+$ HCC patients. The gene frequency of HLA-A24 (*A*2402*) is relatively high in Asian populations, especially in the Japanese, whereas it is low in Caucasians. On the other hand, The gene frequency of

Authors' Affiliations: Departments of [1]Immunogenetics and [2]Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University; [3]Department of Microbiology, Saitama Medical School, Saitama; and [4]Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan
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Note: T. Nakatsura is currently in the Immunotherapy Section, Investigative Treatment Division, Center for Innovative Medicine, National Cancer Center East, Kashiwa, Japan.
Requests for reprints: Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. Phone: 81-96-373-5310, Fax: 81-96-373-5314; E-mail: mxnishim@gpo.kumamoto-u.ac.jp.
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HLA-A2 (*A*0201*) is high among various ethic groups, including both Asians and Caucasians (11). Therefore, it is suggested that the HLA-A2-restricted and GPC3-derived CTL epitopes might be very useful for the immunotherapy of many patients with HCC and melanoma all over the world. In the present study, we identified human GPC3-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 or HLA-A24-restricted epitope peptides could induce GPC3-reactive CTLs from peripheral blood mononuclear cells (PBMC) of patients with HCC.

Materials and Methods

Mouse. HLA-A2.1 (HHD) Tgm; H-$2D^{b-/-}\beta 2m^{-/-}$ double knockout mice introduced with human β2m-HLA-A2.1 (α1 α2)-H-$2D^b$ (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were generated in the Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France (12, 13) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan).

Patients, blood samples, and cell lines. Blood samples from patients with HCC were obtained during routine diagnostic procedures after obtaining a formal agreement signed by the patients in Kumamoto University Hospital from April to September 2005. Human liver cancer cell lines, SK-Hep-1 and T2-A0201 (a TAP-deficient and HLA-A*0201-positive cell line; refs. 14, 15), were provided by Kyogo Ito of Kurume University. Human liver cancer cell lines HepG2 and HuH-7 endogenously expressing GPC3, and $GPC3^-$ colon cancer cell line SW620, were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). C1R-A*2402 (an *HLA-A*2402* transfectant of C1R cells expressing a trace amount of HLA class I molecule; ref. 15) were generous gifts from Dr. Masafumi Takiguchi. The expression of HLA-A2 and HLA-A24 in these cell lines were examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 and anti-HLA-A24 mAb (One Lambda, Inc., Canoga Park, CA), respectively, in order to select target cell lines for CTL assays. The origins and HLA genotypes of these cell lines have been described elsewhere (16, 17). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS.

Induction of GPC3-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay. Human GPC3-derived peptides (purity >90%) sharing the amino acid sequences with mouse GPC3 and carrying binding motifs for *HLA-A*0201*-encoded molecules, were identified using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and we purchased a total of nine peptides carrying HLA-A2 (*A*0201*) binding motifs (Table 1) from Biologica (Tokyo, Japan). The immunizations of mice with peptides were done as previously described (7). In brief, bone marrow (BM) cells (2×10^6) from HLA-A2.1 (HHD) Tgm were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) and 2ME (0.8 ng/mL) for 7 days in 10-cm plastic dishes, and these BM-dendritic cells (DC) were pulsed with the mixture of GPC3 peptides carrying HLA-A2 binding motifs (1 μmol/L for each peptide) at 37°C for 2 hours. We primed the HLA-A2.1 (HHD) Tgm with this syngeneic BM-DC vaccine (5×10^5/mouse) into the peritoneal cavity once a week for two weeks. Seven days after the last immunization, the spleens were collected and $CD4^-$ spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production by $CD4^+$ spleen cells cocultured with the BM-DC. The $CD4^-$ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DC (2×10^5/well) pulsed with each peptide *in vitro*. Then, 6 days later, the frequency of cells producing IFN-γ/2×10^4 $CD4^-$ spleen cells upon stimulation with syngeneic BM-DC (1×10^4/well), pulsed with or without each peptide, was assayed in an enzyme-linked immunospot (ELISPOT) assay as previously described (18).

Induction of GPC3-reactive human CTLs. We isolated PBMCs from the heparinized blood of HLA-$A24^+$ and/or HLA-$A2^+$ Japanese patients with HCC or healthy donors by means of Ficoll-Conray density gradient centrifugation, and peripheral monocyte-derived DCs were generated as described previously (19, 20). $CD8^+$ T cells were isolated using CD8 microbeads (Miltenyi Biotec) from the PBMC of the same donors, and thereafter, peptide-reactive $CD8^+$ CTLs were generated (19, 20). Five days after the last stimulation, the cytotoxic activities of the CTLs were measured by a ^{51}Cr release assay.

CTL responses against cancer cell lines. CTLs were cocultured with each cancer cell line as a target cell (5×10^3/well) at the indicated effector/target ratio and ^{51}Cr release assay was done as described (21). The blocking of HLA-class I or HLA-DR, was done as follows. Before the coculture of CTLs with a cancer cell line in a ^{51}Cr release assay or ELISPOT assay, target cancer cells were incubated for 1 hour with 10 μg/mL anti-class I mAb W6/32 or 10 μg/mL anti-HLA-DR mAb, H-DR-1, and then the effects of mAbs on either the cytotoxic activity or production of IFN-γ by CTLs were examined as reported previously (22).

Histologic and immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the $GPC3_{144-152}$ peptides and the staining of

Table 1. GPC3-derived peptides conserved between human and mouse GPC3 and predicted to be bound to HLA-A2 (*A*0201*)

A2-binding peptide	Position	Subsequence residue listing	HLA-A2 binding score*
GPC3A2-1	44-52	RLQPGLKWV	879
GPC3A2-2	102-110	FLIIQNAAV	319
GPC3A2-3	144-152	FVGEFFTDV	828
GPC3A2-4	155-163	YILGSDINV	162
GPC3A2-5	169-177	ELFDSLFPV	1055
GPC3A2-6	254-268	RMLTRMWYC	1259
GPC3A2-7	281-289	VMQGCMAGV	196
GPC3A2-8	326-334	TIHDSIQYV	496
GPC3A2-9	522-560	FLAELAYDL	402

*Binding scores were estimated by using BIMAS software (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform).

apoptotic cells with terminal deoxynucleotidyl transferase-mediated nick end labeling methods (ApopTag fluorescein *in situ* apoptosis detection kits; Serologicals Corporation, Norcross, GA) in tumor specimens of patients with HCC were done as described previously (23, 24). In addition, immunohistochemical staining of HLA-class I in HCC tumor tissue specimens were done by using anti-HLA-class I mAb, EMR 8-5.[5]

Detection by ELISA of the serum-soluble GPC3 protein. Detection of the serum-soluble GPC3 protein was done by an indirect ELISA using the rabbit anti-GPC3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (7). We used recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) as a standard, and the presence of >106 ng/mL of serum GPC3 protein was considered to be positive.

Transfer of CTLs to the NOD/SCID mice implanted with a human HCC cell line. The transfer of GPC3-reactive CTLs to the immunodeficient mice implanted with a human HCC cell line was done as described previously (7). Briefly, we s.c. inoculated SK-Hep1/GPC3 cells (1×10^7) positive for both HLA-A2 and HLA-A24 at the right flank of NOD/SCID mice. When the diameter of these tumors reached 5×5 mm on day 9 after tumor inoculation into mice, we intravenously injected the mixture of GPC3 peptide-reactive CTL lines or irrelevant HIV peptides; HLA-A2-restricted SLYNTYATL peptide and HLA-A24-restricted RYLRDQQLL peptide, stimulated $CD8^+$ T cells (3×10^6) established from four HLA-A24-positive or two HLA-A2-positive HCC patients, or saline alone. T cells were i.v. injected one more times on day 14. The $CD8^+$ T cells stimulated with HLA-A24-restricted $GPC3_{298-306}$ peptide or HIV (RYLRDQQLL) peptide and derived from two independent $HLA\text{-}A24^+$ HCC patients were mixed, and injected into three NOD/SCID mice on day 9, and the mixture of peptide-stimulated $CD8^+$ T cells from two other $HLA\text{-}A24^+$ HCC patients distinct from the T cell donors at the first injection, were injected into the mice on day 14. The HLA-A2-restricted peptide-stimulated $CD8^+$ T cells from one $HLA\text{-}A2^+$ HCC patient were also injected into a NOD/SCID mouse on day 9, followed by the injection on day 14 with the peptide-stimulated $CD8^+$ T cells derived from another $HLA\text{-}A2^+$ HCC patient.

Statistical analysis. The two-tailed Student's t test was used to evaluate the statistical significance of differences in the data obtained by ELISPOT assay. The statistical significance of the differences in several factors between patients showing a successful CTL induction and other patients was assessed by a χ^2 test. $P < 0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Identification of HLA-A2-restricted CTL epitopes by using HLA-A2.1 (HHD) Tgm. To identify HLA-A2-restricted epitopes by using HLA-A2.1 (HHD) Tgm, we selected nine kinds of peptides having amino acid sequences conserved between human and mouse GPC3 and having high predicted binding scores to HLA-A2 (*A*0201*; Table 1). $CD4^-$ spleen cells from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of these nine peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide, and we found that $CD4^-$ spleen cells stimulated *in vitro* with the $GPC3_{144-152}$ peptide produced the largest amount of IFN-γ in a peptide-specific manner in ELISPOT assays. These $CD4^-$ spleen cells (2×10^4/well), showed 36 ± 2.85 spot counts/well, in response to the BM-DCs pulsed with the $GPC3_{144-152}$ peptide, whereas they showed 23 ± 1.84 spot counts/well in the presence of BM-DCs without peptide loading ($P < 0.005$) indicating that about $(36\text{-}23) / 2 \times 10^4 = 0.065\%$ of $CD4^-$ spleen cells were reactive to the GPC3 peptide. When we used syngeneic BM-DCs pulsed with a HLA-A2-binding HIV-derived peptide; SLYNTYATL as a control, no significant response (8.84 ± 1.73) was observed. The summation of the diameter of the IFN-γ ELISPOT observed in $CD4^-$ spleen cells stimulated with the $GPC3_{144-152}$ peptide pulsed BM-DCs was $1,878 \pm 131$ μm, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was 437 ± 77 μm, and that observed in the presence of BM-DC without peptide loading was 762 ± 131 μm ($P < 0.001$). These assays were done thrice with similar results. As shown in Fig. 1B, the differences in the spot counts (left) or spot diameters (right) between stimulations with peptide pulsed BM-DC and BM-DC without peptide loading clearly revealed the $GPC3_{144-152}$ peptide-specific response of $CD4^-$ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the $GPC3_{144-152}$ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this $GPC3_{144-152}$ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, $GPC3_{144-152}$, did not induce autoimmunity in HLA-A2.1 (HHD) Tgm. It is well known that melanocyte-differentiation antigens such as MART-1 or gp100 are very useful for immunotherapy of melanoma patients, but they sometimes cause autoimmunity, such as vitiligo or uveitis, following vaccination. We previously reported that the immunization of the $GPC3_{298-306}$ peptide did not cause autoimmunity in BALB/c mouse (9). To investigate whether the immunization of mice with HLA-A2-restricted GPC3-derived peptides causes autoimmunity, the immunohistochemical staining of several organs with anti-CD4 and anti-CD8 mAb was done in HLA-A2.1 (HHD) Tgm immunized with a mixture of nine GPC3 peptides 7 days before the analysis. As shown in Fig. 2, we could not find any pathologic changes, such as lymphocyte infiltration or tissue destruction and repair in skin, lung, brain, heart, liver, and kidney of HLA-A2.1 (HHD) Tgm. The same result was also observed when mice were vaccinated with the $GPC3_{144-152}$ peptide alone ($n = 3$; data not shown). These results indicate that the $GPC3_{144-152}$ peptide-reactive $CD8^+$ CTLs do not attack the normal tissue specimens that we investigated.

Induction of GPC3-reactive CTLs from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. We evaluated the cytotoxic activity of CTLs that were induced with the $GPC3_{298-306}$ or $GPC3_{144-152}$ peptide from PBMCs isolated from HCC patients. PBMCs were isolated from HCC patients positive for HLA-A24 and/or HLA-A2, and $CD8^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide as described in Materials and Methods. CTLs from PBMCs of $HLA\text{-}A2^+$ HCC patients stimulated with the $GPC3_{144-152}$ peptide or CTLs from PBMCs of $HLA\text{-}A24^+$ HCC patients stimulated with the $GPC3_{298-306}$ peptide exhibited cytotoxicity against peptide-pulsed target cells. The representative data of CTLs restricted by HLA-A2 or HLA-A24 were shown in Fig. 3A. The CTLs induced from PBMCs of patient A2-8 showed cytotoxic activity to T2-A0201 cells (HLA-A2+) pulsed with the $GPC3_{144-152}$ peptide, but not to T2-A0201 cells without peptide loading by ^{51}Cr release assay. The CTLs induced from PBMCs of patient A24-12 exhibited cytotoxic

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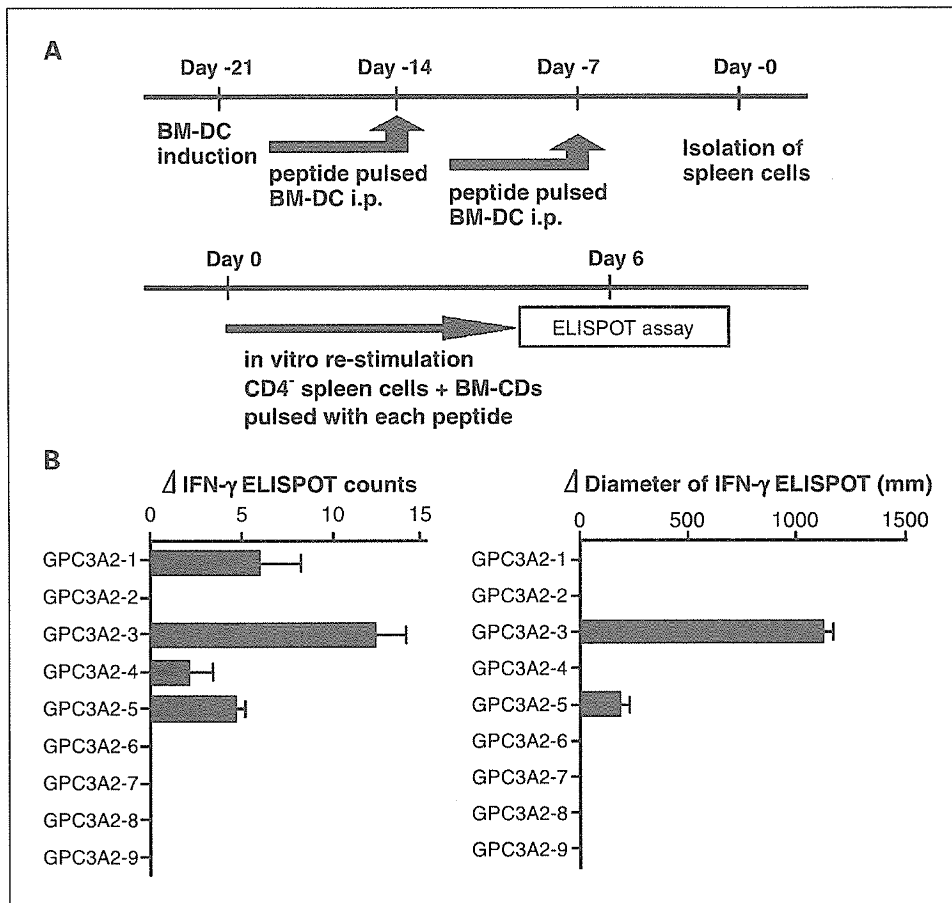

Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. *A,* protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (*A*0201*) binding motif into the peritoneal cavity once a week for two weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in ELISPOT assay to evaluate GPC3-specific response of CTLs. *B, bar graph,* IFN-γ ELISPOT counts/2×10^4 $CD4^-$ spleen cells cocultured with peptide pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*left*). *Bar graph,* summation of IFN-γ ELISPOT diameters/2×10^4 $CD4^-$ spleen cells cocultured with peptide-pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*right*). *Columns,* mean of triplicate assays; *bars,* SE. All assays were done thrice with similar results.

activity to the C1R-A*2402 cells (HLA-A24+) pulsed with the $GPC3_{298-306}$ peptide, but not to C1R-A*2402 cells without peptide loading. These results indicate that these CTLs had peptide-specific cytotoxicity. Other CTLs induced from the nine patients A2-1, A2-2, A2-3, A2-4, A24-1, A24-3, A24-4, A24-6, and A24-7 similarly exhibited peptide-specific cytotoxicity against peptide-pulsed target cells (data not shown).

Furthermore, we used GPC3 transfectants, SK-Hep1/GPC3 (GPC3+, HLA-A2+, HLA-A24+) or SW620/GPC3 (GPC3+, HLA-A2+, HLA-A24+) as target cells and examined whether we could find GPC3-specific cytotoxic activity of CTLs. As shown in Fig. 3B, the CTLs induced from PBMCs of patient A2-3 by stimulation with the $GPC3_{144-152}$ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the $GPC3_{298-306}$ peptide–induced CTLs showed specific cytotoxicity against SW620/GPC3 in patient A24-7 or against SK-Hep1/GPC3 in patient A24-12, but not against SK-Hep1 or SW620, respectively, which did not endogenously express GPC3. These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 or HLA-A24 can be expressed on the cell surface of cancer cells to be recognized by the CTLs.

When we think about the application of GPC3 to cancer immunotherapy, the most important point is that these GPC3-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing GPC3. We thus investigated whether these CTLs could kill human HCC cell lines expressing both endogenous GPC3 and the restriction HLA class I molecules. As shown in Fig. 3C, we could generate GPC3-reactive CTLs by stimulation with the $GPC3_{144-152}$ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

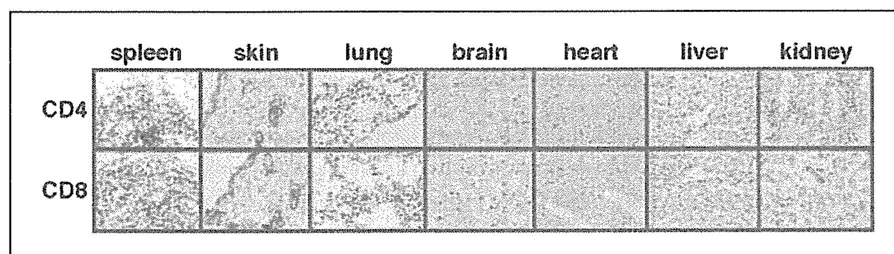

Fig. 2. Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the $GPC3_{144-152}$ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, ×200).