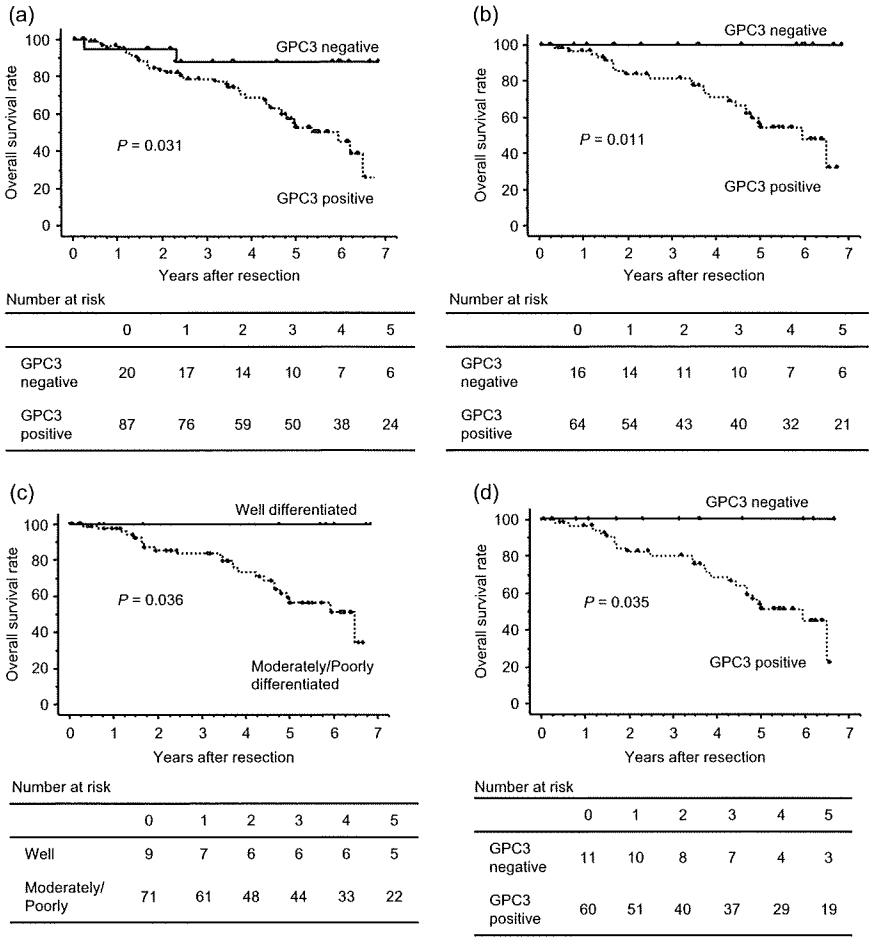

Fig. 2. Overall survival curves for the 107 hepatocellular carcinoma (HCC) patients stratified into those with glypican (GPC)-3-positive and GPC3-negative HCC. (a) Overall survival of patients with GPC3-positive HCC was shorter than those with GPC3-negative HCC ($P = 0.031$). (b) Overall survival curves in 80 of 107 HCC patients with initial treatment who underwent hepatectomy with positive and negative GPC3 expression. Patients with GPC3-positive HCC had a lower 5-year survival than those with GPC3-negative HCC ($P = 0.011$). (c) Overall survival curves in the 71 HCC patients with initial hepatectomy who exhibited well- and moderately and poorly differentiated HCC on histopathological examination. The 5-year survival rate was lower in the moderately and poorly differentiated GPC3-positive HCC than in the corresponding GPC3-negative HCC ($P = 0.036$). (d) Overall survival curves in the 71 initial treatment patients who underwent hepatectomy and exhibited moderately and poorly differentiated HCC on pathological examination with positive and negative GPC3 expression. The 5-year survival rate was lower in the GPC3-positive HCC patients than in the GPC3-negative HCC patients ($P = 0.035$).

Table 2. Prognostic factors for overall survival by univariate and multivariate analyses

Variable	No. patients	Univariate analysis		Multivariate analysis		
		5-year survival rate (%)	*P*-value	RR	95% CI	*P*-value
Age (years) (≥65/<65)	51/56	65.8/53.4	0.531			
Sex (male vs female)	85/22	56.1/72.7	0.403			
HBsAg (positive vs negative)	29/78	51.0/62.3	0.011	1.14	0.31–4.16	0.844
HCV (positive vs negative)	62/45	66.7/46.4	0.004	2.41	0.75–7.69	0.138
ICG R15 (%) (≥15 vs <15)	50/57	70.3/46.8	0.047	0.69	0.31–1.54	0.362
AFP (ng/mL) (≥50 vs <50)	45/62	49.1/65.1	0.132			
PIVKA-II (mAU/mL) (≥700 vs <700)	30/77	35.0/65.6	0.016	1.91	0.730–5.02	0.188
Tumor occurring (first vs recurrence)	80/27	62.8/50.2	0.019	1.83	0.78–4.31	0.167
No. tumors (solitary vs multiple)	75/32	65.7/42.7	0.009	3.53	1.41–8.00	0.006
Resection (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	29/78	36.5/67.1	0.005	1.71	0.52–5.60	0.374
Operation time (min) (>300 vs ≤300)	49/58	43.9/72.3	0.053			
Intraoperative blood loss (mL) (≥1300 vs <1300)	42/65	42.3/68.8	0.097			
Perioperative transfusion (present vs absent)	54/53	49.6/66.5	0.599			
Tumor size (mm) (>50 vs ≤50)	38/69	51.5/62.5	0.154			
Histological differentiation (well vs moderately and poorly)	12/95	77.8/56.4	0.102			
pStage (I vs II/III)	41/66	64.2/56.5	0.071			
Portal vein involvement (present vs absent)	47/60	64.9/58.5	0.369			
Hepatic vein involvement (present vs absent)	10/97	44.4/60.5	0.060			
Bile duct involvement (present vs absent)	12/95	20.0/62.7	0.004	0.94	0.31–2.85	0.912
Intrahepatic metastasis (present vs absent)	24/83	29.0/66.6	0.001	3.57	1.13–10.50	0.027
Non-cancerous lesion (cirrhosis vs non-cirrhosis)	40/67	53.6/61.9	0.232			
GPC3 staining (positive vs negative)	87/20	54.5/87.7	0.025	5.26	1.13–24.39	0.034

AFP, alpha-fetoprotein; CI, confidence interval; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; RR, relative risk; UICC, International Union against Cancer.

doi: 10.1111/j.1349-7006.2009.01206.x

In this study, the patients who were HCV positive, had higher ICG-R15 values, or portal vein involvement showed longer survival times, especially the patients who were HCV-positive or had higher ICG-R15 values, showed statistical significance in the univariate analysis. However, there was no statistical significance in these variables in the multivariate analysis. The reasons for these contradictive results in the univariate analysis are unclear.

In contrast, subgroup analysis did not reveal any significant difference in the disease-free survival rate between the GPC3-positive and GPC3-negative HCC patients (data not shown). The rate of recurrence in patients after surgery was 63.8% within the first 2 years after surgery among the previously treated patients in this study. Tumor recurrence in the GPC3-positive HCC patients occurred earlier than that in the GPC3-negative HCC patients until 9.7 months after the surgery among the patients who had received previous treatment. Two mechanisms of postoperative recurrence of HCC have been suggested: one is intrahepatic metastasis in the residual liver in a metachronous manner, and the other is multicentric hepatocarcinogenesis based on chronic hepatitis.[20–23] Some authors have suggested that early recurrence arises most often from intrahepatic metastases, whereas late recurrence is more likely to be multicentric in origin. Poon *et al.* and Portolani *et al.* reported that tumor factors like neoplastic vascular infiltration, but not host factors, were linked to early recurrence, whereas the risk of late recurrence was dependent on the underlying liver status.[21,22] These results indicate that GPC3 expression may indicate a high risk of intrahepatic recurrence.

Most of the GPC3 expression patterns in HCC cells showed the cytoplasmic pattern. There was no case that showed only the membrane pattern. Almost half of the HCC cases showed the mixed pattern (cytoplasm and membrane) and the other half showed only the cytoplasmic pattern.

There was no statistical significance between the mixed pattern (cytoplasm and membrane) and cytoplasmic pattern ($P = 0.297$) in Kaplan–Meier survival analysis. The functional difference between cytoplasmic GPC3 and membrane GPC3 is unknown, so further investigations are needed to clarify whether the different localization of staining has a different significance.

In addition to the investigation of its role as a prognostic indicator, a phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC is now underway; GPC3 is an ideal target for this therapy because it is more effective in patients with increased expression of GPC3, which is frequently observed in the later stages of HCC, as shown in the present study. The poor prognosis of patients with GPC3-positive HCC also prompted us to develop a strategy of anticancer immunotherapy,[24,25] that is, we may expect the effect of hepatocarcinogenesis prevention after surgery in patients with GPC3-positive HCC.

In summary, our study evaluated the prognostic significance of GPC3 expression at the protein level in clinical tissue specimens of HCC. The overall survival rate was significantly poorer in patients with elevated GPC3 expression in the tumor than in those with lower levels of GPC3 expression. Further functional characterization of GPC3 may be expected to lead to a better understanding of the molecular mechanisms underlying the development and progression of HCC.

Acknowledgments

This work was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan, a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare, Japan, and awardee of research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive 10-Year Strategy for Cancer Control (H.S.).

References

1 Thomas MB, Zhu AX. Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 2005; **23**: 2892–9.
2 Ibrahim S, Roychowdhury A, Hean TK. Risk factors for intrahepatic recurrence after hepatectomy for hepatocellular carcinoma. *Am J Surg* 2007; **194**: 17–22.
3 Mann CD, Neal CP, Garcea G, Manson MM, Dennison AR, Berry DP. Prognostic molecular markers in hepatocellular carcinoma: a systematic review. *Eur J Cancer* 2007; **43**: 979–92.
4 Filmus J. The contribution of *in vivo* manipulation of gene expression to the understanding of the function of glypicans. *Glycoconj J* 2002; **19**: 319–23.
5 Filmus J, Capurro M, Rast J. Glypicans. *Genome Biol* 2008; **9**: 224.
6 Capurro MI, Xiang YY, Lobe C, Filmus J. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. *Cancer Res* 2005; **65**: 6245–54.
7 Song HH, Shi W, Xiang YY, Filmus J. The loss of glypican-3 induces alterations in Wnt signaling. *J Biol Chem* 2005; **280**: 2116–25.
8 Stigliano I, Puricelli L, Filmus J, Sogayar MC, Bal de Kier Joffe E, Peters MG. Glypican-3 regulates migration, adhesion and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation. *Breast Cancer Res Treat* 2009; **114**: 251–62.
9 Torisu Y, Watanabe A, Nonaka A *et al.* Human homolog of NOTUM, overexpressed in hepatocellular carcinoma, is regulated transcriptionally by beta-catenin/TCF. *Cancer Sci* 2008; **99**: 1139–46.
10 Hippo Y, Watanabe K, Watanabe A *et al.* Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. *Cancer Res* 2004; **64**: 2418–23.
11 Capurro M, Wanless IR, Sherman M *et al.* Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology* 2003; **125**: 89–97.
12 Nakatsura T, Yoshitake Y, Senju S *et al.* Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. *Biochem Biophys Res Commun* 2003; **306**: 16–25.
13 Nakatsura T, Nishimura Y. Usefulness of the novel oncofetal antigen glypican-3 for diagnosis of hepatocellular carcinoma and melanoma. *Biodrugs* 2005; **19**: 71–7.
14 Ikuta Y, Nakatsura T, Kageshita T *et al.* Highly sensitive detection of melanoma at an early stage based on the increased serum secreted protein acidic and rich in cysteine and glypican-3 levels. *Clin Cancer Res* 2005; **11**: 8079–88.
15 Nakatsura T, Kageshita T, Ito S *et al.* Identification of glypican-3 as a novel tumor marker for melanoma. *Clin Cancer Res* 2004; **10**: 6612–21.
16 Wang XY, Degos F, Dubois S *et al.* Glypican-3 expression in hepatocellular tumors: diagnostic value for preneoplastic lesions and hepatocellular carcinomas. *Hum Pathol* 2006; **37**: 1435–41.
17 Libbrecht L, Severi T, Cassiman D *et al.* Glypican-3 expression distinguishes small hepatocellular carcinomas from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules. *Am J Surg Pathol* 2006; **30**: 1405–11.
18 Di Tommaso L, Franchi G, Park YN *et al.* Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007; **45**: 725–34.
19 Yamauchi N, Watanabe A, Hishinuma M *et al.* The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. *Mod Pathol* 2005; **18**: 1591–8.
20 Yamamoto J, Kosuge T, Takayama T *et al.* Recurrence of hepatocellular carcinoma after surgery. *Br J Surg* 1996; **83**: 1219–22.
21 Portolani N, Coniglio A, Ghidoni S *et al.* Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. *Ann Surg* 2006; **243**: 229–35.
22 Poon RT, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. *Cancer* 2000; **89**: 500–7.
23 Sakon M, Umeshita K, Nagano H *et al.* Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves. *Arch Surg* 2000; **135**: 1456–9.
24 Motomura Y, Ikuta Y, Kuronuma T *et al.* HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. *Int J Oncol* 2008; **32**: 985–90.
25 Komori H, Nakatsura T, Senju S *et al.* Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. *Clin Cancer Res* 2006; **12**: 2689–97.

Detection of glypican-3-specific CTLs in chronic hepatitis and liver cirrhosis

EMIKO HAYASHI[1], YUTAKA MOTOMURA[1], HIROFUMI SHIRAKAWA[1], TOSHIAKI YOSHIKAWA[1], NOBUYUKI OBA[2], SHUTA NISHINAKAGAWA[2], YASUHIRO MIZUGUCHI[2], TATSUYA KOJIMA[2], KAZUHIRO NOMURA[2] and TETSUYA NAKATSURA[1]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577; [2]Tokyo Rosai Hospital, Department of Internal Gastroenterology, 4-13-21 Omoriminami, Ota-ku, Tokyo 143-0013, Japan

Received February 10, 2009; Accepted March 26, 2009

DOI: 10.3892/or_00000418

Abstract. Glypican-3 (GPC3) is one of carcinoembryonic antigens known to be overexpressed in hepatocellular carcinoma (HCC). It has been suggested that GPC3 may be related to the development of HCC in a background of chronic hepatitis (CH) and liver cirrhosis (LC). Therefore, in an attempt to establish an early diagnostic marker of HCC, we quantified the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients. We selected CH and LC patients who were HCV-RNA (+) or HBs antigen (+) within 6 months prior to the study and had no HCC nodules as detected by imaging. A total of 56 patients with CH and LC, and 45 patients with HLA-A24$^+$ or HLA-A2$^+$ were enrolled for this investigation. After isolation of mononuclear cells from each patient's peripheral blood specimens, we performed ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides. In the ELISPOT assay, GPC3-specific CTLs were detected in 10 of the 45 CH and LC cases (22%). In addition, the plasma titers of anti-GPC3 IgG were increased in the CH and LC patients as compared with those in healthy donors. GPC3-specific CTLs were found to be present not only in patients with HCC, but also in patients with CH and LC. This suggests the possibility of GPC3-specific CTLs serving as a marker for the early diagnosis of imaging-invisible HCC.

Correspondence to: Dr Emiko Hayashi or Dr Tetsuya Nakatsura, Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
E-mail: ehayashi@east.ncc.go.jp
tnakatsu@east.ncc.go.jp

Abbreviations: GPC3, glypican-3; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

Key words: glypican-3, CTL, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma

Introduction

The prevalence of hepatocellular carcinoma (HCC) is increasing rapidly in both Asian and Western countries. It is clear that patients with hepatitis B- or C-associated liver cirrhosis are at a higher risk of developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at a higher risk of recurrence (2). Furthermore, the liver function of these patients is often very poor, which restricts further treatment options for recurrence. As a result, the prognosis of HCC remains poor, and the development of new therapies for the prevention of cancer development and recurrence, that is, adjuvant therapy, is urgently needed.

Glypican-3 (GPC3) has been reported to be overexpressed in most types of HCC (3-10) and melanoma in humans (6,8,9). GPC3 belongs to the six-member family of glypicans in mammals (11). GPC3 is a heparan sulfate proteoglycan that is bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor. GPC3 has been shown to regulate the signaling mediated by Wnts (12,13), Hedgehogs (14), fibroblast growth factors (15,16) and bone morphogenetic proteins (15,17). These signaling pathways are only partially dependent on the heparan sulfate chains (11,16,18). However, whether GPC3 plays an oncogenic role in HCC is still controversial.

We recently identified both HLA-A24 (A*2402) and H-2K^d-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL) and HLA-A2 (A*0201)-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We previously reported a preclinical study conducted in a mouse model with a view to designing an optimal schedule for clinical trials of a GPC3-derived peptide vaccine (20). We predicted that overexpression of GPC3 in HCC is related to the development of HCC in a background of chronic hepatitis (CH) and/or liver cirrhosis (LC). Towards establishing the possibility of early diagnosis of imaging-invisible HCC and vaccine therapy, we determined the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients.

Materials and methods

Patients, blood samples and cell lines. Blood samples from patients with CH and LC were collected during routine diagnostic procedures after obtaining their written consent at the Tokyo Rosai Hospital between October 2006 and October 2007. CH and LC patients who were confirmed to be HCV-RNA(+) or HBs antigen(+) within six months prior to registration were eligible for the study. The diagnosis of CH or LC was made clinically by imaging and laboratory data. The patients had no medical history of HCC, and no evidence of HCC on ultrasonography, CT (computed tomography) or MRI (magnetic resonance imaging) conducted prior to the registration.

Human liver cancer cell lines SK-Hep-1/GPC3, HepG2 and K562 were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS. SK-Hep-1/GPC3 has been described previously (19). HepG2 endogenously expressing GPC3 was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). HLA-class I deficient K562 was obtained from Kumamoto University. The origins and HLA genotypes of these cell lines have been described in previous reports (21,22).

Ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay. We isolated peripheral blood mononuclear cells (PBMCs) from the heparinized blood of HLA-A2$^+$ and/or HLA-A24$^+$ Japanese CH, LC or HCC patients and healthy donors by means of Ficoll-Conray density gradient centrifugation. IFN-γ production by the CTLs present in the PBMCs in the presence or absence of the GPC3 peptide was assessed by the ELISPOT assay (BD™ Bioscience, San Diego, CA), as described previously. Briefly, defrosted PBMCs (1x10^6/well) were cultured in 96-well flat-bottomed plates for the ELISPOT assay (BD Bioscience) with HLA-A2-restricted $GPC3_{44-52}$ (A2-1) (RLQPGLKWV), $GPC3_{144-152}$ (A2-3) (FVGEFFTDV), $GPC3_{155-163}$ (A2-4) (YILGSDINV) and HLA-A24-restricted $GPC3_{298-306}$ (A24-8) (EYILSLEEL) (10 μM) with 100 units/ml recombinant human IL-2 overnight *in vitro*. The negative control consisted of medium alone and the positive control included HLA-A24- or -A2-restricted cytomegalovirus. The number and area of the spots were automatically determined and subsequently analyzed with the ELISPOT system (Minerva Tech, Tokyo, Japan).

Induction of GPC3-reactive human CTLs and cytotoxic assay. We evaluated the cytotoxic activity of the CTLs that were induced with the GPC3 A2-3 peptide in the PBMCs isolated from the CH4 patient. PBMCs were isolated from HLA-A2$^+$ CH4 patient, distributed into 4 wells (3x10^5 cells/24-well), and cultured with the GPC3 A2-3 peptide. After culture for 7 and 14 days, the PBMCs cocultured with irradiated autologous monocyte-derived DCs obtained by positive selection with human CD14 Micro Beads (Miltenyi, Bergisch Gladbach, Germany) were pulsed with the GPC3 A2-3 peptide. The CD14$^+$ cells were cultured in the presence of 100 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Inc.) and 100 ng/ml of IL-4 (R&D Systems, Inc.) in RPMI-1640 (Sigma-Aldrich Corp., St. Louis, MO) containing 2% heat-inactivated autologous serum and 1% penicillin-streptomycin-glutamine (Gibco, Invitrogen, Ltd.; Paisley, Scotland, UK). After 5 days, TNFα (PEPRPTECH EC., London, UK) was added at the concentration of 20 ng/ml to induce maturation of the DCs. After 7 days, mature DCs were harvested and pulsed with 10 μM of the candidate peptides for 4 h at room temperature in RPMI. The peptide-pulsed DCs were then irradiated (3500 rads) and mixed at a ratio of 1:20 with autologous PBMCs.

These DCs were set up in 48-well culture plates; each well contained 1.5x10^4 peptide-pulsed DCs, 3x10^5 PBMCs and 5 ng/ml IL-7 (PEPRPTECH EC.) in 0.5 ml of RPMI containing 10% autologous serum. Three days after the start of the incubation, IL-2 (R&D Systems, Inc.) was added to these cultures at a final concentration of 10 U/ml. On days 7 and 14, the T cells were restimulated with the autologous DCs pulsed with the peptide.

After 21 days, the cells were recovered and analyzed for their cytotoxic activity against the target cells with the TERASCAN VPC system (Minerva Tech), as previously described (23). Briefly, SK-Hep-1/GPC3 (GPC3$^+$, A2$^+$, A24$^+$), HepG2 (GPC3$^+$, A2$^+$, A24$^+$) and K562 (HLA-class I$^-$) cells were used as the target cells and labeled with calcein-AM solution for 30 min at 37°C. The labeled cells were washed three times and distributed into a 96-well culture plate (1x10^4 per well) and then incubated with the effector cells for 5 h. The fluorescence intensity was measured before and after 5-h culture, and the Ag-specific cytotoxic activity was calculated using the following formula: cytotoxicity (%) = [(sample release) - (spontaneous release)]/[(maximum release) - (spontaneous release)] x 100.

ELISA for the detection of anti-GPC3 IgG antibodies. Recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) was diluted in 10 x Block Ace (Dainippon Pharmaceutical, Osaka) to a final concentration of 1 μg/ml, dispensed into 96-well plates (100 μl/well) and incubated overnight at 4°C. Then, the plates were blocked with Block Ace for 1 h at room temperature. Plasma samples from CH and LC patients and healthy controls (100 μl, 1:100 dilution) were added to each well, followed by incubation for 2 h at room temperature. After washing three times with PBS containing 0.05% Tween-20 (PBST), Peroxidase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratories, Inc., W. Baltimore, USA) was reacted for 30 min. The plates were washed with PBST and developed with Stable Peroxide Substrate Buffer (Pierce, Rockford, IL) for 20 min. After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 490 nm. All plasma samples were measured in duplicate and were randomly dispensed into the plates.

Statistical analysis. The two-tailed Student's t-test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay. Unpaired Mann-Whitney U tests were used for the evaluation of the significance of differences in the data obtained by ELISA. $P<0.05$ was considered to denote significant difference.

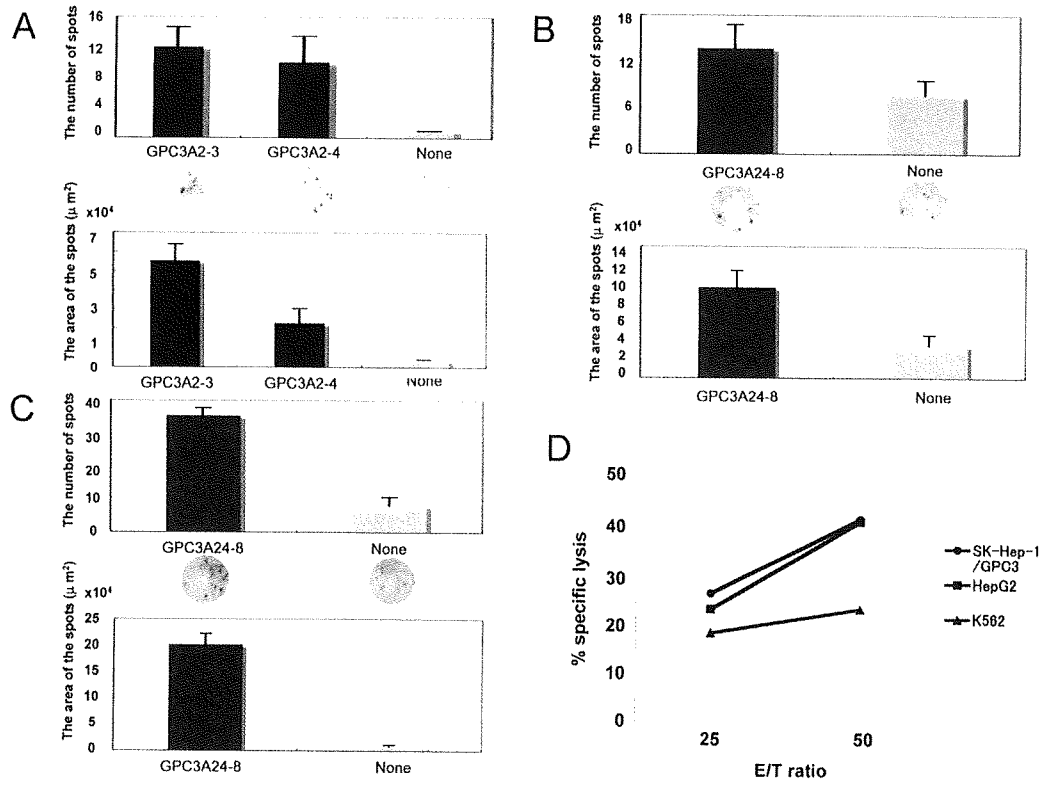

Figure 1. Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2⁺ or HLA-A24⁺ CH and LC patients and the cytotoxicity of the CTLs induced by stimulation with the GPC3 (A2-3) peptide. GPC3-specific CD8⁺ T cells were detected in the chronic hepatitis [(A), HLA-A2⁺ CH4 patient; (B), HLA-A24⁺ CH5 patient] and liver cirrhosis [(C), HLA-A24⁺ LC5 patient]. IFN-γ produced by the peptide-specific T cells was measured by the IFN-γ-ELISPOT assay (middle column). The number and area of spots are shown in the upper and lower panels, respectively. Lysis of human hepatoma cell lines SK-Hep-1/GPC3 (circles) and HepG2 (squares) expressing GPC3 and HLA-A2 by GPC3-specific CTLs was observed following stimulation with the GPC3 A2-3 peptide (FVGEFFTDV) [(D), HLA-A2⁺ CH4 patient]. An HLA-classI⁻ K562 human erythromyeloblastoid leukemia cell line was used as the negative control (triangles).

Results

Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2⁺ or HLA-A24⁺ CH, LC and HCC patients. We evaluated the frequency of CTLs that recognized the GPC3 A2-1, A2-3, A2-4 or A24-8 peptide in the PBMCs of CH, LC and HCC patients. The CH and LC patients enrolled in this study were 34 male and 22 female patients. The average age of the patients was 64 years. HCV and HBV infection was found in 54 and 2 patients, respectively. The 56 patients were 33 CH and 23 LC cases. Mean serum α-fetoprotein (AFP) was 13.3±21.1 ng/ml (normal <20 ng/ml). In regard to the HLA genotype, 10, 22 and 13 patients, respectively, were HLA-A2⁺, HLA-A24⁺ and HLA-A2⁺/24⁺. On the other hand, there were 11 patients who were HLA-A2⁻/A24⁻. In this investigation, we enrolled the 45 patients who were HLA-A2⁺ or HLA-A24⁺.

We determined the presence of CTLs in the PBMCs of the CH and LC patients by ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides (Fig. 1, Table I). The representative data of the ELISPOT assay are highlighted. Interestingly, in the CH4 patient, the spots and areas were highly developed in the GPC3 A2-3 and A2-4 peptide-stimulated PBMCs (Fig. 1A). However, few spots and areas were detected in the negative control (no peptide). In addition, GPC3 A24-8 peptide-restricted CTLs were also detected in the CH5 and LC5 patients (Fig. 1B and C). These results suggest that GPC3-specific CTLs are present in the PBMCs of some of CH and LC patients.

Cytotoxicity of CTLs induced by stimulation with the GPC3 (A2-3) peptide. To clarify the cytotoxic activity of GPC3-specific CTLs induced by stimulation with the GPC3 peptide, the HCC cell line, SK-Hep-1/GPC3, transfected with GPC3 and expressing HLA-A2 and HLA-A24 were used as the target cells (Fig. 1D). The CTLs induced from the PBMCs of CH4 (Table I) patient by stimulation with the GPC3 A2-3 peptide showed specific cytotoxicity against the SK-Hep-1/GPC3 and HepG2 cells. On the other hand, no GPC3-specific cytotoxicity was observed against the HLA-classI⁻ K562 cells. These results indicate that GPC3-peptide-specific CTLs induced from CH4 (Table I) patient are cytotoxic against the GPC3-expressing target HCC cells.

Frequency of HLA-A2⁺ or HLA-A24⁺ CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in PBMC The frequency of patients with GPC3-specific CTLs in their PBMCs is shown in Fig. 2, while the clinical backgrounds of the CH, LC and HCC patients are summarized in Table II. CTL positivity was observed in 5 of 26 CH patients (19%), 5 of 19 LC patients (26%), and 21 of 54 HCC patients (39%). In addition, the percentage of CTL-positive patients tended to

Table I. Detection of GPC3-specific CTLs in the PBMCs of chronic hepatitis/liver cirrhosis patients by ELISPOT assay.

	Peptide/Peptide sequence									
	GPC3 A2-1/RLQPGLKWV		GPC3 A2-3/FVGEFFTDV		GPC3 A2-4/YILGSDINV		GPC3 A24-8/RYILSLEEL		No peptide	
	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)
CH[b]1(A*0201)	**1.0±0.0**[c]	**25905.0±8487.8**	**2.0±1.0**	**2826.0±3079.5**	**1.6±1.1**	**13895.0±4486.8**	NT	NT	0.0±0.0	0.0±0.0
CH2 (A*0201)	**1.0±1.7**	**707.0±1223.6**	**1.6±1.1**	**6830.0±6934.2**	**2.6±1.1**	**3297.0±3263.1**	NT	NT	0.0±0.0	0.0±0.0
CH3 (A*0201)	NT[d]	NT	**18.3±5.5**	**85100.0±17050.1**	**15.6±2.5**	**20173.0±4728.4**	NT	NT	8.0±1.7	8045.0±1849.1
CH4 (A*0201)	NT	NT	**12.0±2.6**	**55187.0±8618.4**	**10.0±3.4**	**22832.0±7632.2**	NT	NT	1.0±0.0	3853.0±375.2
CH5 (A*2402)	NT	NT	NT	NT	NT	NT	**13.3±3.7**	**101736.0±54505.9**	7.0±1.0	36502.5±14892.4
LC[b]1 (A*0201)	**1.0±0.0**	**1060.0±815.7**	**2.1±0.2**	**2944.0±815.7**	**6.3±0.5**	**50162.0±4283.0**	NT	NT	0.5±0.0	354.0±0.0
LC2 (A*0201)	**24.0±3.0**	**55891.2±23304.1**	**8.0±2.0**	**45971.9±25440.5**	**8.0±1.0**	**103961.4±13618.6**	NT	NT	4.3±0.5	2098.3±2166.5
LC3 (A*0201)	**1.3±0.5**	**2355.0±2855.2**	**3.6±1.5**	**8007.0±6564.4**	**11.3±5.7**	**100323.0±70946.1**	NT	NT	2.0±3.4	2826.0±4894.7
LC4 (A*2402)	NT	NT	NT	NT	NT	NT	**14.0±8.0**	**41331.0±31472.6**	3.0±0.0	7065.0±3996.5
LC5 (A*2402)	NT	NT	NT	NT	NT	NT	**35.3±2.3**	**200882.0±21210.9**	8.3±2.3	8714.0±2855.5

[a]CH, chronic hepatitis. [b]LC, liver cirrhosis. [c]We show values higher than the value for 'No peptide' by a bold font. [d]NT, not tested.

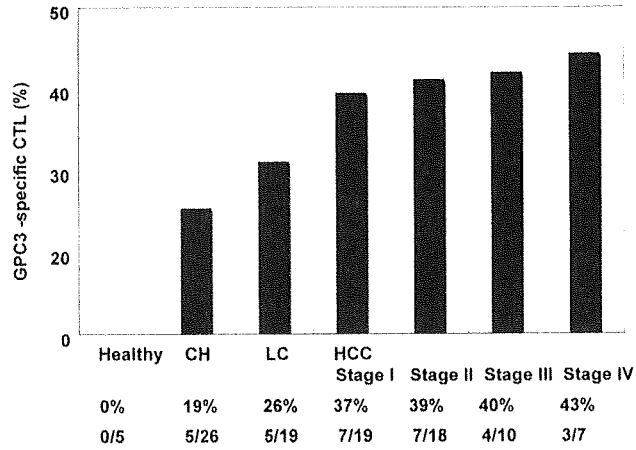

Figure 2. Frequency of HLA-A2[+] or HLA-A24[+] CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in the PBMCs. GPC3-peptide-specific CTLs were detected in 19 and 26% of the patients with CH and LC, respectively. In the HCC patients, the percentage of these CTLs tended to increase with increasing stage of progression of the disease: 37% (stage I), 39% (stage II), 40% (stage III) and 43% (stage IV).

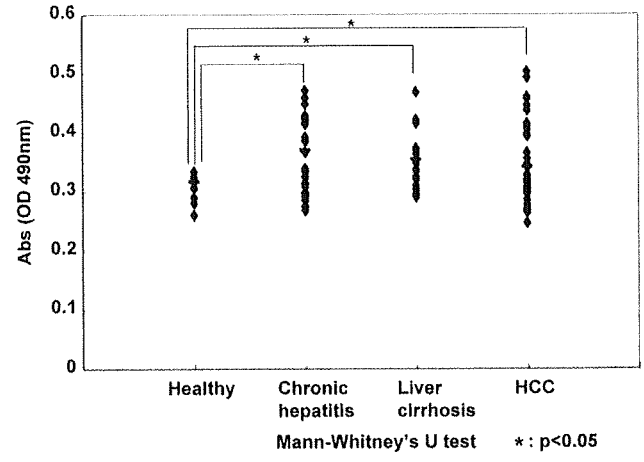

Figure 3. Plasma titers of anti-GPC3 IgG in the CH, LC and HCC patients. Anti-GPC3 IgG was detected by ELISA using recombinant GPC3 protein. A significantly higher titer of IgG to GPC3 was observed in the CH ($p<0.05$), LC ($p<0.05$) and HCC patients ($p<0.05$) as compared with that in healthy donors. *$p<0.05$ (Mann-Whitney U test).

increase with increasing clinical stage of HCC; stage I (7/19, 37%), stage II (7/18, 39%), stage III (4/10, 40%), and stage IV (3/7, 43%) (Table II). There were no CTL-positive cases (0/5, 0%) in healthy donors.

Anti-GPC3 IgG in the plasma in patients with CH, LC and HCC. To examine the quantitative titers of anti-GPC3 IgG in the plasma of patients with CH, LC and HCC, we carried out ELISA using the recombinant GPC3 protein (Fig. 3). The titers in the CH, LC and HCC patients were significantly higher as compared with the peak titer in healthy controls. These results indicate that the GPC3 antigen is expressed not only in HCC patients, but also in CH and LC patients.

Table II. Number of CTL-negative and -positive cases in chronic hepatitis, liver cirrhosis and HCC patients.

	Healthy (n=5)		Chronic hepatitis (n=33)		Liver cirrhosis (n=23)		HCC (n=54)	
Group	Negative (n=5) mean (±SD)	Positive (n=0) mean (±SD)	Negative (n=28) mean (±SD)	Positive (n=5) mean (±SD)	Negative (n=19) mean (±SD)	Positive (n=5) mean (±SD)	Negative (n=33) mean (±SD)	Positive (n=21) mean (±SD)
Age	31.2±7.1	-	61.6±11.2	60.6±12.9	67.3±10.1	71.0±2.7	65.8±7.9	64.0±10.5
Male	4	0	16	3	12	3	28	15
Female	1	0	12	2	6	2	5	6
HCV/HBV								
+/-	ND	ND	5	26	18	5	18	14
-/+	ND	ND	2	0	0	0	4	2
+/+	ND	ND	0	0	0	0	2	2
-/-	ND	ND	0	0	0	0	9	3
AFP (ng/ml)	ND	ND	9.5±18.9	9.6±7.3	21.2±25.4	8.8±7.7	26335.1±143782.5	1431.5±3574.9
HLA-								
$A02^+$	3	0	3	3	2	2	13	8
$A24^+$	2	0	12	1	7	2	18	11
$A02^+/24^+$	0	0	6	1	5	1	2	2
$A02^-/24^-$	0	0	7	0	4	0	0	0

Discussion

The oncofetal antigen GPC3 is known to be overexpressed in HCCs (3-10) and melanomas (6,8,9). We recently identified GPC3-specific peptides restricted to HLA-A24 (A^*2402) and H-$2K^d$, or HLA-A2 (A^*0201), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We are currently conducting a phase I clinical trial of peptide vaccine prepared using these peptides against advanced HCC. In addition, in the near future, we propose to carry out a phase II clinical trial of the vaccine in HCC patients as well as CH and LC patients to evaluate its efficacy in preventing the onset of HCC. We report the finding of GPC3-specific CTLs in CH and LC patients for the first time in this study. Furthermore, the plasma titers of anti-GPC3 IgG in the CH and LC patients were also found to be significantly increased as compared with those in healthy donors.

It has been suggested that GPC3-specific CTLs may be derived from clinically invisible pre-neoplastic or neoplastic nodular lesions. In previous studies, expression of GPC3 was reported in 2/23 (8%) cirrhotic low-grade dysplastic nodules, and 2/9 (22%) (24), 2/22 (9%) (25) or 6/31 (19%) high-grade dysplastic nodules (26). In one study, among 5 adenomas with malignant characteristics, 3 (60%) showed immunoreactivity for GPC3 in the malignant regions (24). Other studies reported positive staining for GPC3 in 12/20 (60%) (24) and 22/32 (69%) cases (25) of early HCC. Meanwhile, the serum titers of the elevated GPC3 antigen in HCC cases were reported to be correlated with the clinical stage of HCC (19). In our study, we noted an increase of the plasma titers of anti-GPC3 IgG antibody in CH, LC and HCC patients. In addition, the frequency of patients with GPC3-specific CTLs appeared to increase with the stage of progression of the liver disease. These results suggest that GPC3 expression and the appearance of GPC3-specific CTLs may be prediagnostic markers of HCC.

On the other hand, the increase in the frequency of GPC3-specific CTLs and titers of anti-GPC3 IgG in the peripheral blood might be related to the expression of GPC3 in CH with high grade inflammation and LC. In this study, we did not perform immunohistochemical examination for GPC3, because needle biopsy of the liver in our patients was not conducted in our collaborative clinic. Previous studies have demonstrated GPC3 expression by immunohistochemistry in 25/30 (83%) cases of CH with high grade inflammation (27) and 11/95 (12%) cases of LC (26), indicating that GPC3 might be expressed in CH with high-grade inflammation and some LC patients, resulting in the appearance of GPC3-specific CTLs in the PBMCs of these patients.

During the 1-year follow-up of this study, onset of HCC was not observed in any of the 10 CH and LC patients who were positive for GPC3-specific CTLs in the peripheral blood; on the other hand, 2 (1CH and 1LC) patients who were negative for GPC3-specific CTLs showed development of HCC. It would, therefore, seem that the GPC3-specific CTLs might prevent the development of HCC or be predictive of a favorable prognosis of non-neoplastic liver lesions. However, our examination was limited to only HLA-A24- and A2-positive patients, and moreover, we followed up the patients for only one year. Therefore, careful long-term observation of a larger number of CH and LC cases is necessary to determine the role of GPC3-specific CTLs in patients with CH and LC.

In this study, we demonstrated an increase of GPC3-specific CTLs and high titers of anti-GPC3 IgG in CH and LC patients. Thus, GPC3-specific CTLs and anti-GPC3 IgG

may possibly be markers of early imaging-invisible HCC. In addition, active immunotherapy using GPC3 peptides may prevent the development of both non-neoplastic and neoplastic lesions of the liver.

Acknowledgements

We thank Ms. Chinatsu Kojima for her help with the preparation of this manuscript. This study was supported in part by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan, the Foundation for Promotion of Cancer Research in Japan, the Japan Research Foundation for Clinical Pharmacology, and a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan (E.H.).

References

1. Schafer DF and Sorrell MF: Hepatocellular carcinoma. Lancet 353: 1253-1257, 1999.
2. Tung-Ping Poon R, Fan ST and Wong J: Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg 232: 10-24, 2000.
3. Hippo Y, Watanabe K, Watanabe A, *et al*: Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 64: 2418-2423, 2004.
4. Capurro M, Wanless IR, Sherman M, *et al*: Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 125: 89-97, 2003.
5. Nakatsura T, Yoshitake Y, Senju S, *et al*: Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 306: 16-25, 2003.
6. Nakatsura T, Kageshita T, Ito S, *et al*: Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 10: 6612-6621, 2004.
7. Zhu ZW, Friess H, Wang L, *et al*: Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut 48: 558-564, 2001.
8. Ikuta Y, Nakatsura T, Kageshita T, *et al*: Highly sensitive detection of melanoma at an early stage based on the increased serum secreted protein acidic and rich in cysteine and glypican-3 levels. Clin Cancer Res 11: 8079-8088, 2005.
9. Nakatsura T and Nishimura Y: Usefulness of the novel oncofetal antigen glypican-3 for diagnosis of hepatocellular carcinoma and melanoma. BioDrugs 19: 71-77, 2005.
10. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
11. Filmus J, Capurro M and Rast J: Glypicans. Genome Biol 9: 224, 2008.
12. Song HH, Shi W, Xiang YY, *et al*: The loss of glypican-3 induces alterations in Wnt signaling. J Biol Chem 280: 2116-2125, 2005.
13. De Cat B, Muyldermans SY, Coomans C, *et al*: Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. J Cell Biol 163: 625-635, 2003.
14. Capurro MI, Xu P, Shi W, *et al*: Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. Dev Cell 14: 700-711, 2008.
15. Grisaru S, Cano-Gauci D, Tee J, *et al*: Glypican-3 modulates BMP- and FGF-mediated effects during renal branching morphogenesis. Dev Biol 231: 31-46, 2001.
16. Lai JP, Sandhu DS, Yu C, *et al*: Sulfatase 2 up-regulates glypican 3, promotes fibroblast growth factor signaling, and decreases survival in hepatocellular carcinoma. Hepatology 47: 1211-1222, 2008.
17. Midorikawa Y, Ishikawa S, Iwanari H, *et al*: Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. Int J Cancer 103: 455-465, 2003.
18. Kwack MH, Choi BY and Sung YK: Cellular changes resulting from forced expression of glypican-3 in hepatocellular carcinoma cells. Mol Cells 21: 224-228, 2006.
19. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
20. Motomura Y, Ikuta Y, Kuronuma T, *et al*: HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol 32: 985-990, 2008.
21. Wadee AA, Paterson A, Coplan KA, *et al*: HLA expression in hepatocellular carcinoma cell lines. Clin Exp Immunol 97: 328-333, 1994.
22. Matsui M, Machida S, Itani-Yohda T, *et al*: Downregulation of the proteasome subunits, transporter, and antigen presentation in hepatocellular carcinoma, and their restoration by interferon-gamma. J Gastroenterol Hepatol 17: 897-907, 2002.
23. Muneta Y, Nagaya H, Minagawa Y, *et al*: Expression and one-step purification of bovine interleukin-21 (IL-21) in silkworms using a hybrid baculovirus expression system. Biotechnol Lett 26: 1453-1458, 2004.
24. Wang XY, Degos F, Dubois S, *et al*: Glypican-3 expression in hepatocellular tumors: diagnostic value for preneoplastic lesions and hepatocellular carcinomas. Hum Pathol 37: 1435-1441, 2006.
25. Di Tommaso L, Franchi G, Park YN, *et al*: Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. Hepatology 45: 725-734, 2007.
26. Baumhoer D, Tornillo L, Stadlmann S, *et al*: Glypican 3 expression in human non-neoplastic, preneoplastic, and neoplastic tissues: a tissue microarray analysis of 4,387 tissue samples. Am J Clin Pathol 129: 899-906, 2008.
27. Abdul-Al HM, Makhlouf HR, Wang G, *et al*: Glypican-3 expression in benign liver tissue with active hepatitis C: implications for the diagnosis of hepatocellular carcinoma. Hum Pathol 39: 209-212, 2008.

World J Surg (2009) 33:312–317
DOI 10.1007/s00268-008-9831-2

World Journal of Surgery

Risk Factors of Surgical Site Infection After Hepatectomy for Liver Cancers

Shin Kobayashi · Naoto Gotohda · Toshio Nakagohri · Shinichiro Takahashi · Masaru Konishi · Taira Kinoshita

Published online: 21 November 2008

Abstract
Background Risk factors of surgical site infection (SSI) after hepatectomy under the guideline of Centers for Disease Control and Prevention (CDC) are not well examined.
Methods Hospital records of consecutive patients who underwent hepatectomy without biliary reconstruction for liver cancers were reviewed retrospectively. Prophylactic antibiotics were given to patients just before skin incision and every 3 hours during the operations. Clinicopathological factors were compared between patients who developed SSI and those without it.
Results There were 405 patients identified, and the incidence of SSI was 23 cases (5.8%). In multivariate analysis, intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were significant risk factors of SSI after hepatectomy.
Conclusions Prophylactic antibiotics were necessary only during the operation for most patients who underwent hepatectomy without biliary reconstruction. However, patients with intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years are at risk to develop SSI and might need additional administration of prophylactic antibiotics after surgery.

Introduction

Use of antibiotics is one of the main techniques to prevent surgical site infection (SSI) after surgery. There has been tremendous accumulation of evidence during the last three decades with regard to the optimal methods of its administration [1]. The Centers for Disease Control and Prevention (CDC) recommended in its 1999 guideline to maintain therapeutic levels of prophylactic antibiotic during the operation and, at most, a few hours after closure of incisions [2]. However, it is well known that incidence of SSI is greatly influenced by patients' underlying general status and perioperative factors [3]. Disease and procedure-specific risks and use of prophylactic antibiotics are not well examined, except for colorectal surgery [4, 5], open heart surgery [6], cholecystectomy [7, 8], etc.

It is suggested that hepatectomy suppresses Kupffer cell and T-cell function significantly, which renders patients immunosuppressive [9]. Postoperative infection, including SSI, deteriorates hepatic failure in cases with limited hepatic functional reserve. There is a wide variety in operation time, blood loss, transfusion requirement, etc., depending on the extent of parenchymal resection. Underlying cirrhosis and hypoalbuminemia inhibits normal wound healing [10]. However, perioperative factors that should be considered a significant risk to develop SSI after hepatectomy have not been clear. The purpose of this study was to analyze the risk factors of SSI after hepatectomy with prophylactic antibiotics under CDC guideline and to clarify who might benefit from additional administration of prophylactic antibiotics after operation.

Materials and methods

Patients who underwent hepatectomy for liver cancers from November 2002 to December 2006 at National Cancer Center East Hospital, Kashiwa, Japan, were identified and reviewed retrospectively. Patients who

S. Kobayashi (✉) · N. Gotohda · T. Nakagohri · S. Takahashi · M. Konishi · T. Kinoshita
National Cancer Center East Hospital, 6-5-1, Chiba, Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
e-mail: shkobaya@east.ncc.go.jp

underwent hepatectomy without biliary reconstruction regardless of diagnosis were included in the study. Patients who underwent cholecystectomy along with hepatectomy were included in the study, but those who underwent simultaneous procedures, such as colorectal resection or stoma closure, were excluded from the study.

The extent of hepatectomy was evaluated according to the disease progression, liver function, and general condition of patients [11]. Tumor progression and resectability was assessed by imaging studies, such as contrast enhanced computed tomography (CT) scans, magnetic resonance imaging (MRI), hepatic arterial angiography, ultrasound, and chest x-ray. Liver function was assessed by liver biochemistry test, Child-Pugh grade [12], and the indocyanine green retention rate at 15 minutes [13]. All patients were reviewed before surgery at weekly conferences by hepatic surgeons, medical oncologists, and interventional radiologists to discuss whether the planned procedures were appropriate. Hepatic resection was performed under intraoperative ultrasonographic guidance by the pean fracture method with or without inflow occlusion (Pringle's maneuver). Anatomic hepatectomy was performed whenever possible, whereas partial resection was performed in consideration of limited liver functional reserve or anatomic location of the tumor. During parenchymal resection, all blood vessels and bile ducts were ligated whenever possible with 2-0 or 3-0 braided silk or vessel clip. One or two closed drains were inserted at the end of operation in the right subphrenic space or wherever close to the resected liver parenchyma. Drains were removed when no rebleeding or bile leakage was observed on postoperative day (POD) 3 or 4.

SSI was defined as a condition in which purulent discharge was observed from any incision or space that was manipulated during an operation within 30 days after the operation with or without microbiological evidence as in the guideline issued by CDC [2], and it was identified retrospectively by reviewing clinical records of patients who underwent hepatectomy. Remote site infection was defined as a condition in which fever and leukocytosis were present with bacteria in sputum, urine, catheter-tip, blood, or other body fluid/space, or according to the physician's judgment regardless of microbiological evidence.

Patients were usually given two doses of cefazolin as prophylactic antibiotics. One gram of cefazolin was administered to patients within 30 minutes before skin incision and another dose 3 hours later. When the operation lasted more than 3 hours, additional doses were given every 3 hours thereafter during the operation. No antibiotics were given after incisions were closed if patients had already received two doses of cefazolin.

All data were compiled in a database for analysis (Microsoft Excel and SPSS 11.0 J for Windows). Differences between numerical variables were tested with Mann-Whitney U test and those between categorical variables were tested with χ^2 statistics. Multivariate analysis was performed with logistic regression test. $p < 0.05$ was deemed significant.

Results

During the period of study, 405 patients underwent hepatectomy without biliary reconstruction for primary or secondary liver cancers at National Cancer Center East Hospital, Kashiwa, Japan. Of these 405 patients, 23 patients (5.8%) developed SSI (incisional, 20; organ/space, 3). Incisional SSIs were treated by opening incisions and organ/space SSIs were treated by drainage under ultrasound guidance. The patient characteristics and demographic variables are listed in Table 1. No differences in these basic characteristics, except age, were observed between patients with SSI and those without it. Mean age of patients with SSI was 68.2 years and was statistically older than those without SSI. A cutoff value of aged 65 years had the highest statistical power ($p = 0.016$). Patients' ASA score, comorbidities, and underlying liver pathology were statistically similar between the two groups.

Culture results of infecting organisms included *Bacteroides faragilis* (n = 3), *Staphylococcus aureus* (n = 2), *Klebsiera oxytoca* (n = 1), *Serratia marcescens* (n = 1), *Escherichia coli* (n = 1), *Streptoccoccus anginosus* (n = 1), *Streptococcus constellatus* (n = 1), *Enterobacter cloacae* (n = 1), *Citrobacter braakii* (n = 1), *Citrobacter freundii* (n = 1), Corynebacterium species (n = 1), and Candida species (n = 1).

The perioperative variables are listed in Table 2. Operation time, red blood cell (RBC) transfusion requirement, RBC transfusion volume, and intraoperative bowel injury were statistically different between the two groups. Blood loss did not reach statistical significance, but cutoff value of 2000 ml had the significant power to predict SSI ($p = 0.003$). Multivariate analysis of those variables found that intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were the significant risk factors to develop SSI after hepatectomy without biliary reconstruction (Table 3). Rates of SSI increased dramatically with the number of risk factors present (Fig. 1). Patients with two or more risk factors were statistically more likely to develop SSI than those with none or only one risk factor.

During the same period, three patients died within 30 days from the operations. One patient died from pulmonary embolism on POD 3, another died from brain stroke on POD 3, and the other died from esophageal varix rupture on POD 9. Incidence of remote site infection was

Table 1 Patient characteristics and demographic variables for patients with SSI compared with those without it

	SSI (−) (N = 382)	SSI (+) (N = 23)	*P* value
Age (yr)[a]	63.7 ± 0.5	68.2 ± 2	*0.034*
≥65[b]	194 (50.9)	18 (78.3)	*0.016*
<65	188 (49.1)	5 (21.7)	
Gender[b]			0.809
Male	285 (74.6)	18 (78.3)	
Female	97 (25.4)	5 (21.7)	
Body mass index (kg/m^2)[a]	23.8 ± 0.6	23.6 ± 0.7	0.583
Diabetes mellitus[b]	75 (19.6)	1 (4.5)	0.095
ASA score[b]			0.488
1	111 (29.5)	7 (30.4)	
2	243 (64.6)	16 (69.6)	
3	22 (5.9)		
Diagnosis[b]			0.566
HCC	239 (62.6)	13 (56.5)	
Metastases	126 (33)	8 (34.8)	
Others	16 (4.5)	2 (8.7)	
Viral hepatitis serology[b]			0.858
HBV	51 (14)	3 (13)	
HCV	141 (38.7)	8 (34.8)	
HBV and HCV	7 (1.9)		
Liver parenchyma[b]			0.758
Chronic hepatitis	105 (29.6)	9 (39.1)	
Liver cirrhosis	93 (26.2)	5 (21.7)	
Child class[b]			0.634
A	355 (94.4)	21 (91.3)	
B	21 (5.6)	2 (8.7)	
ICG15R[a]	14.6 ± 0.4	15.5 ± 1.6	0.571

[a] Mann-Whitney *U* test

[b] χ^2 test

Data are numbers with percentages in parentheses or means ± standard error of the mean

ASA American society of anesthesiology, *HCC* hepatocellular carcinoma, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG15R* indocyanin green 15 min retention rate

11 (2.5%) (pneumonia (n = 6), urinary tract infection (n = 1), catheter infection (n = 1), epididymitis (n = 1), unknown origin (n = 2)). Other morbidities included bile leak (n = 9), retractable ascites (n = 6), ileus (n = 4), transient renal insufficiency (n = 4), rebleeding (n = 3), pleural effusion (n = 3), skin rash (n = 2), poor oral intake (n = 2), delirium (n = 1), transient heart failure (n = 1), pulmonary embolism (n = 1), upper gastrointestinal bleeding (n = 1), wound dehiscence (n = 1). There were four reoperations for three rebleedings and one wound dehiscence.

Discussion

Our study clearly demonstrated the risk factors of SSI after hepatectomy with prophylactic antibiotics under the CDC guideline. Intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were the significant risk factors. Although both alimentary tract surgery and hepatobiliary surgery are classified as clean-contaminated [14], biliary tract without calculus is normally sterile contrary to the alimentary tract, which has high bacterial densities [15, 16]. Intraoperative bowel injury is suspected to contaminate surgical field of hepatectomy without biliary reconstruction and to increase the risk of SSI. Blood loss reduces the concentration of antibiotics and is found to be a risk factor of SSI [17, 18]; 1500 ml to 2000 ml of blood loss is the suggested threshold to administer additional doses of cefazolin to maintain a concentration higher than the minimum inhibitory concentration for the common infecting organisms [19, 20]. Our threshold of 2000 ml of blood loss is compatible with previous findings. Elderly patients also are reported to be susceptible to SSI [18, 21]. Because aging involves complex physiologic changes, it is difficult to clarify a definitive mechanism of the vulnerability of elderly patients. Reduction in immune function is one suggested mechanism [10].

Rates of SSI increased dramatically with the number of the three risk factors present (Fig. 1). According to the National Nosocomical Infections Surveillance (NNIS) report, rates of SSI after hepatopancreaticobiliary complex

Table 2 Perioperative variables for patients with SSI compared with those without it

	SSI (−) (N = 382)	SSI (+) (N = 23)	*P* value
Operation time (min)[a]	210 ± 19	269 ± 23	*0.021*
≥300[b]	68 (17.8)	9 (39.1)	*0.017*
<300	313 (82.2)	14 (60.9)	
Pringle time (min)[a]	63.3 ± 2.1	75.9 ± 9.7	0.259
None[b]	26 (7.3)	0 (0)	0.23
>0	331 (92.7)	20 (100)	
Repeat resection[b]	110 (28.8)	4 (17.4)	0.338
Blood loss (ml)[a]	1070 ± 69	1928 ± 470	0.068
≥2000[b]	50 (13.2)	9 (39.1)	*0.003*
<2000	332 (86.8)	14 (60.9)	
RBC transfusion (ml)[a]	177 ± 29	537 ± 192	*0.003*
None[b]	297 (78.2)	12 (52.2)	*0.009*
>0	83 (21.8)	11 (47.8)	
Intraoperative bowel injury[b]	3 (0.8)	4 (17.4)	*<0.001*
Bile leak[b]	7 (1.8)	2 (22.2)	0.087
Resected segments (Couinaud)[b]			0.96
<2	285 (74.8)	16 (69.6)	
2–3	42 (11)	3 (13)	
≥4	54 (14.2)	4 (17.4)	
Resected weight (g)[a]	221 ± 19	269 ± 77	0.281
Largest tumor size (cm)[a]	3.8 ± 0.2	3.7 ± 0.4	0.253
NNIS index[b]			0.184
0	293 (76.9)	14 (60.9)	
1	86 (22.6)	9 (39.1)	
2	2 (0.5)		
Postoperative length of stay[a]	10.2 ± 0.2	23.7 ± 5.7	*<0.001*

[a] Mann-Whitney *U* test

[b] χ^2 test

Data are numbers with percentages in parentheses or means ± standard error of the mean

RBC red blood cell, *NNIS* national nosocomical infection surveillance

Table 3 Multivariate analysis of SSI risk factors

	P value	Odds ratio (95% confidence intervals)
Age ≥65 yr	*0.027*	3.4 (1.15–10.05)
Blood loss ≥2000 ml	*0.004*	4.4 (1.63–11.91)
Intraoperative bowel injury	*<0.001*	20.08 (4–100.8)
RBC transfusion	0.62	1.51 (0.31–7.42)
Operation time >300 min	0.67	1.35 (0.34–5.32)

SSI risk factors identified by univariate analysis were compared by multivariate analysis (logistic regression test)

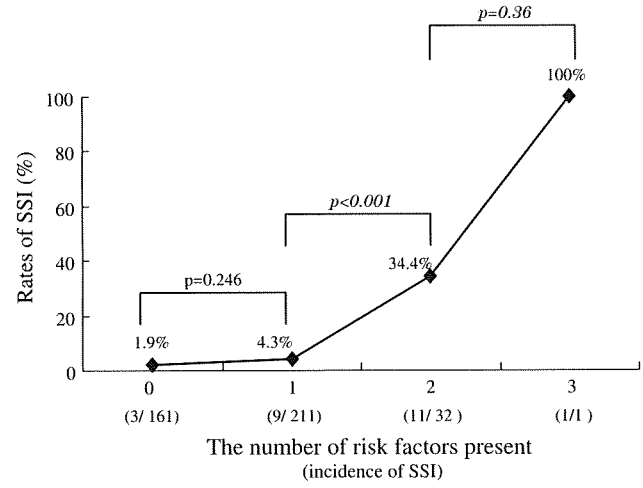

Fig. 1 Rates of SSI increased with the number of risk factors present. Rates of SSI were not statistically different between patients with one risk factor and those without any factors. However, patients with two or more risk factors developed SSI at a significantly higher rate than those with none or only one risk factor

surgery range from 3.24–7.04% [22]. Other reported rates of SSI after hepatectomy range from 4.6–25.2% [23, 24]. Compared with those previously reported rates, the rates of SSI for patients with none or only one risk factor, 1.9% and 4.3% respectively, are considered allowable. Prophylactic antibiotics for hepatectomy without biliary reconstruction are necessary only during operations for patients with none or only one risk factor. However, patients with two or more risk factors developed SSI at statistically higher rates. Fujita et al. [4] reported that two additional doses of postoperative antibiotics reduced the incidence of incisional SSI from 14.2% to 4.3% compared with single-dose preoperative administration in elective colorectal surgery

[4]. Additional administration of postoperative antibiotics maintains therapeutic levels for longer hours and reduces the incidence of SSI more effectively for patients at higher risk. Although there have been no published data concerning the effectiveness of postoperative administration of antibiotics in hepatectomy, Fig. 1 illustrates that patients with two or more risk factors may receive some additional doses of postoperative antibiotics as in colorectal surgery. Appropriate doses of additional antibiotics are matters to be discussed.

There were five infecting organisms that were resistant to cefazolin: *Bacteroides fragilis*, *Enterobacter, cloacae*, *Serratia marcescens*, Corynebacterium species, and Citrobacter species. Because some patients lack microbiologic data, a definitive conclusion about the optimum choice of prophylactic antibiotics was not possible. However, it is evident that cefazolin alone was effective for most patients who underwent hepatectomy without biliary reconstruction. Two of the seven patients with intraoperative bowel injury developed SSI with *Bacteroides fragilis*. Because likely pathogens in alimentary tract surgery are gram-negative bacilli and anaerobes [2], postoperative antibiotics with anaerobic coverage might be more effective for patients with intraoperative bowel injury.

Postoperative infections, especially organ/space SSI, sometimes deteriorate hepatic function and may cause mortalities. We experienced 23 SSIs and 11 remote site infections, but none of the patients died from those infections. We speculate that our strict evaluation of extent of hepatectomy using CT volumetry and liver function test precluded some excessive hepatic resection and saved postoperative hepatic function. Postoperative infection is more likely to occur in patients with hepatic dysfunction [25]. Our relatively low rate of major hepatectomy in consideration of hepatic functional reserve might be related to the fewer incidence of SSI.

RBC transfusion requirement and operation time were significant risk factors of SSI in univariate analysis, but not in multivariate analysis. Transfusion has immunosuppressive effects on postoperative patients via reductions in natural killer cell number and cytotoxic T-cell function [26, 27] and is reported to be a risk factor of SSI in colorectal surgery [28, 29]. However, controversy exists concerning the causal relationship between transfusion and SSI [30], and a recent meta-analysis denies the association between transfusion and postoperative infection [31]. Our result is consistent with the meta-analysis. Operation time is another reported risk factor of SSI [18]. Cefazolin exhibits time-dependent decrease in serum and tissue concentration, and additional administrations are recommended every 3 or 4 hours during operation to maintain therapeutic levels of cefazolin [2]. Because all of our patients received a second dose of cefazolin at 3 hours from incision, serum and tissue concentration of cefazolin was expected to exceed therapeutic levels during the whole time of operations for most patients. Influence of operation time on the incidence of SSI was suspected to be minimized with additional dose of cefazolin at 3 hours from incision.

Abdominal drainage after elective hepatectomy is controversial. Some randomized, controlled trials (RCTs) reported increased incidence of SSI and other morbidities associated with abdominal drainage and denied the routine placement of drainage catheters [32, 33]. However, the routine drainage group in those RCTs had drainage catheters placed for at least 5 to 9 days, which was unnecessarily long. We almost routinely placed drainage catheters but removed them on POD 3/4 or earlier if postoperative bleeding and bile leakage were denied. Early removal of prophylactic drains prevents intra-abdominal infections [34]. We do not consider that abdominal drainage causes more infections if drains are removed on POD 3/4 or earlier.

Our study has several limitations. First, SSI was detected indirectly by retrospectively reviewing patient records and laboratory data. It has been suggested to be a less accurate method than prospective direct observation of surgical sites [2]. Some SSI might be possibly undetected because of inappropriate patient records. However, indirect case-finding by reviewing daily records and laboratory data is the most widespread method of surveillance in the medical literature. Its reported sensitivity is as high as 83.8–92.3% compared with prospective direct finding of SSI [35]. Since then, we do not consider that our surveillance method precludes the importance of our findings. Second, it is a single-center study. Our department is one of the highest volume centers in Japan and performs 250 hepatopancreaticobiliary cancer surgeries in a year. Also, we do not perform operations on patients with end-stage renal disease on dialysis due to inadequacies of dialysis facilities. Our relatively low rate of SSI incidence may be attributable to the high volume of cases and to the patient selection.

Conclusions

Our study demonstrated that prophylactic antibiotics were necessary only during operations and, at most, a few hours after closure of incisions in most of the patients who underwent hepatectomy without biliary reconstruction. However, patients with intraoperative bowel injury, blood loss >2000 ml, and age older than 65 years were at risk for developing SSI. Patients with two or more risk factors may receive additional doses of postoperative antibiotics to prevent SSI more effectively.

References

1. Nichols RL (1996) Surgical infections: prevention and treatment-1965 to 1995. Am J Surg 172:68–74
2. Mangram AJ, Horan TC, Pearson ML et al (1999) Guideline for prevention of surgical site infection, 1999. Infect Control Hosp Epidemiol 20:247–278
3. Culver DH, Horan TC, Gaynes RP et al (1991) Surgical wound infection rates by wound class, operative procedure, and patient risk index. Am J Med 91(3B):152S–157S
4. Fujita S, Saito N, Yamada T et al (2007) Randomized, multicenter trial of antibiotic prophylaxis in elective colorectal surgery. Arch Surg 142:657–661
5. Tang R, Chen HH, Wang YL et al (2001) Risk factors for surgical site infection after elective resection of the colon and rectum: a single-center prospective study of 2, 809 consecutive patients. Ann Surg 234:181–189
6. Harbarth S, Samore MH, Lichtenberg D et al (2000) Prolonged antibiotic prophylaxis after cardiovascular surgery and its effect on surgical site infections and antimicrobial resistance. Circulation 101:2916–2921
7. Dobay KJ, Freier DT, Albear P (1999) The absent role of prophylactic antibiotics in low-risk patients undergoing laparoscopic cholecystectomy. Am Surg 65:226–228
8. Higgins A, London J, Charland S et al (1999) Prophylactic antibiotics for elective laparoscopic cholecystectomy: are they necessary? Arch Surg 134:611–613
9. Karpoff HM, Tung C, Ng B et al (1996) Interferon gamma protects against hepatic tumor growth in rats by increasing Kupffer cell tumoricidal activity. Hepatology 24:374–379
10. Silverstein JH (2002) Physiologic changes associated with aging. In: O'Leary JP (ed) The physiologic basis of surgery, 2nd edn. Lippincott Williams & Wilkins, Philadelphia, pp 705–711
11. Makuuchi M, Kosuge T, Takayama T et al (1993) Surgery for small liver cancers. Semin Surg Oncol 9:298–304
12. Pugh RN, Murray-Lyon IM, Dawson JL et al (1973) Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 60:646–649
13. Lau H, Man K, Fan ST et al (1997) Evaluation of preoperative hepatic function in patients with hepatocellular carcinoma undergoing hepatectomy. Br J Surg 84:1255–1259
14. Talbot TR, Kaiser AB (2005) Postoperative infections and antimicrobial prophylaxis. In: Mandell GL (ed) Principles and practice of infectious diseases, 6th edn. Elsevier, Philadelphia
15. Fry DE (2002) Surgical infection. In: O'Leary JP (ed) The physiologic basis of surgery, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, pp 212–258
16. Ahrendt SA, Pitt HA (2001) Biliary tract. In: Townsend CMJ (ed) Textbook of surgery. The biological basis of modern surgical practice, 16th edn. Saunders, Philadelphia, pp 1076–1111
17. Griffiths J, Demianczuk N, Cordoviz M et al (2005) Surgical site infection following elective caesarian section: a case-control study of postdischarge surveillance. J Obstet Gynaecol Can 27:340–344
18. Yamamoto S, Kanamaru S, Kunishima Y et al (2005) Perioperative antimicrobial prophylaxis in urology: a multi-center prospective study. J Chemother 17:189–197
19. Swoboda SM, Merz C, Kostuik J et al (1996) Does intraoperative blood loss affect antibiotic serum and tissue concentrations? Arch Surg 131:1165–1171
20. Meter JJ, Polly DWJ, Brueckner RP et al (1996) Effect of intraoperative blood loss on the serum level of cefazolin in patients managed with total hip arthroplasty. A prospective, controlled study. J Bone Joint Surg Am 78:1201–1205
21. Yoshida J, Shinohara M, Ishikawa M et al (2006) Surgical site infection in general and thoracic surgery: surveillance of 2663 cases in a Japanese Teaching Hospital. Surg Today 36:114–118
22. Gaynes RP, Culver DH, Horan TC et al (2001) Surgical site infection (SSI) rates in the United States, 1992–1998: The National Nosocomial Infections Surveillance System Basic SSI Risk Index. Clin Infect Dis 33(2):S69–S77
23. Togo S, Matsuo K, Tanaka K et al (2007) Perioperative infection control and its effectiveness in hepatectomy patients. J Gastroenterol Hepatol 22:1942–1948
24. Wu CC, Yeh DC, Lin MC et al (1998) Prospective randomized trial of systemic antibiotics in patients undergoing liver resection. Br J Surg 85:489–493
25. Schindl MJ, Redhead DN, Fearon KC et al (2005) The value of residual liver volume as a predictor of hepatic dysfunction and infection after major liver resection. Gut 54:289–296
26. Gascon P, Zoumbos NC, Young NS (1984) Immunological abnormalities in patients receiving multiple blood transfusions. Ann Intern Med 100:173–177
27. Kaplan J, Samaik S, Gitlin J et al (1984) Diminished helper/suppressor lymphocyte ratios and natural killer activity in recipients of repeated blood transfusions. Blood 64:308–310
28. Vamvakas EC, Carven JH, Hibberd PL (1996) Blood transfusion and infection after colorectal cancer surgery. Transfusion 36:1000–1008
29. Mynster T, Christensen IF, Moesgaard F et al (2000) Effects of the combination of blood transfusion and postoperative infectious complications on prognosis after surgery for colorectal cancer. Br J Surg 87:1553–1562
30. Vamvakas EC, Carven JH (1998) Transfusion of white-cell-containing allogeneic blood components and postoperative wound infection: effect of confounding factors. Transfus Med 8:29–36
31. Vamvakas EC (2007) White-blood-cell-containing allogeneic blood transfusion and postoperative infection or mortality: an updated meta-analysis. Vox Sang 92:224–232
32. Fong Y, Brennan M, Brown K et al (1996) Drainage is unnecessary after elective liver resection. Am J Surg 171:158–162
33. Liu CL, Fan ST, Lo CM et al (2004) Abdominal drainage after hepatic resection is contraindicated in patients with chronic liver disease. Ann Surg 239:194–201
34. Kawai M, Tani M, Terasawa H et al (2006) Early removal of prophylactic drains reduces the risk of intra-abdominal infections in patients with pancreatic head resection. Ann Surg 244:1–7
35. Cardo DM, Falk PS, Mayhall CG (1993) Validation of surgical wound surveillance. Infect Control Hop Epidemiol 14:211–215

J Gastroenterol (2010) 45:60–67
DOI 10.1007/s00535-009-0109-8

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Transcatheter arterial infusion chemotherapy with cisplatin–lipiodol suspension in patients with hepatocellular carcinoma

Masafumi Ikeda · Seishi Maeda · Hiroshi Ashihara · Hiroyasu Nagahama · Motohiko Tanaka · Yutaka Sasaki

Received: 25 October 2008 / Accepted: 12 July 2009 / Published online: 5 August 2009

Abstract
Purpose The aim of this study was to investigate the antitumor efficacy of treatment, identify prognostic factors, and construct a prognostic index in patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy (TAI) using cisplatin suspended in lipiodol.
Methods We analyzed the outcomes in a total of 94 consecutive patients with previously untreated hepatocellular carcinoma who were treated by TAI using cisplatin suspended in lipiodol.
Results Twenty-seven patients (29%) showed complete response and 21 patients (22%) showed partial response, with an overall response rate of 51% (95% confidence interval, 41–61%). The median survival time was 2.5 years and the proportions of survivors at 1, 2, and 5 years were 81.6, 65.2, and 18.3%, respectively. The results of multivariate analysis indicated a significant association of serum albumin ≥3.0 g/dL, maximum tumor size ≤3.0 cm, absence of ascites, and unilateral distribution of the tumors with a favorable survival. For clinical application, we also propose a prognostic index based on a combination of these prognostic factors. Based on this index, the patients were classified into three groups: those with good, intermediate, and poor prognosis. The median survival times in these three groups were 4.3, 2.7, and 1.1 years, respectively ($p < 0.01$).
Conclusions TAI with cisplatin suspended in lipiodol exhibited favorable tumor efficacy and survival in patients with hepatocellular carcinoma. The prognostic factors identified and the index proposed based on these factors may be useful for predicting life expectancy, determining treatment strategies, and designing future clinical trials.

Keywords Hepatocellular carcinoma · Transcatheter arterial infusion chemotherapy · Cisplatin · Prognosis

Abbreviations

HCC	Hepatocellular carcinoma
TAE	Transcatheter arterial chemoembolization
TAI	Transcatheter arterial infusion chemotherapy
CT	Computed tomography
AFP	Serum alpha-fetoprotein
PIVKA II	Protein induced by vitamin K absence or antagonist-II
CR	Complete response
PR	Partial response

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and its incidence is continuing to increase worldwide. However, the prognosis of advanced HCC remains unsatisfactory [1]. Curative therapies such as resection, liver transplantation, and local ablative treatments may offer a chance of improved life

M. Ikeda · H. Ashihara · H. Nagahama · M. Tanaka · Y. Sasaki (✉)
Department of Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan
e-mail: sasakiy@kumamoto-u.ac.jp

M. Ikeda
Division of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan

S. Maeda
Department of Gastroenterology and Hepatology, Tamana Regional Health Medical Center, Kumamoto, Japan

expectancy, but these treatment modalities are applicable to only a small proportion of all HCC patients. Transcatheter arterial chemoembolization (TAE) has been recognized as an effective palliative treatment option for patients with advanced HCC, because two meta-analyses [2, 3] of seven randomized controlled trials [4–10] showed that TAE significantly improves the survival of unresectable HCC patients with preserved hepatic function [1]. Transcatheter arterial infusion chemotherapy (TAI) is also often used for the treatment of advanced HCC, but a consensus regarding the most effective chemotherapeutic regimen has not yet been reached [11, 12]. Lipiodol, a lipid lymphographic agent, is selectively retained by HCC tissues for prolonged periods in comparison with non-cancerous tissues, and is therefore commonly mixed with anticancer agents to allow these agents to be retained for prolonged periods of time in the target tumor [13–15]. In a randomized controlled trial of TAE and TAI with zinostatin stimalamer and lipiodol, TAE did not yield superior survival as compared to TAI in patients with advanced unresectable HCC [16]. Our previous analysis also revealed that TAE did not significantly improve the survival of patients with HCC in comparison with TAI using cisplatin suspended in lipiodol, even though TAE is known to have higher antitumor efficacy than TAI [17]. Thus, TAI may have a higher efficacy on survival compared to TAE. If the appropriate indications for TAI can be expanded, additional embolization may not be necessary in some patients, considering that TAE has more deleterious effects on the liver functions than TAI [17, 18]. However, proper patient selection for TAI with lipiodol has not yet been fully investigated, although those for TAI without lipiodol [19–21] and for TAE [22–24] have been frequently analyzed. Analysis of prognostic factors would suggest appropriate patient selection for TAI. The present study was conducted to investigate the antitumor efficacy of the treatment, and to evaluate a number of variables that may affect survival in patients with HCC treated by TAI using cisplatin suspended in lipiodol; we have proposed a prognostic index in patients treated with TAI based on the results of our analyses.

Materials and methods

Patients

Between October 1987 and May 1996, 94 consecutive patients with previously untreated HCC were treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol at Kumamoto University Hospital, Japan. The study subjects were patients who were judged to be suitable candidates for TAI (Table 1). HCC was diagnosed on the basis of histological examination or distinctive findings on computed tomography (CT) and/or angiography, associated with elevated serum levels of serum alpha-fetoprotein (AFP) or protein induced by vitamin K absence or antagonist-II (PIVKA II). Pretreatment evaluation included a complete medical history and careful physical examination. The laboratory procedures included complete

Table 1 Patient characteristics

	No of patients (%)
Host-related variables	
Age (years)	
Median [range]	64 [41–81]
Gender	
Male	62 (66%)
Blood transfusion	
Present	28 (30%)
Alcohol abuse[a]	
Present	11 (12%)
Smoking habit[b]	
Present	31 (33%)
Hepatitis B surface antigen	
Positive	14 (15%)
Hepatitis C antibody	
Positive	76 (81%)
Ascites	
Present	14 (15%)
Child-Pugh class	
A	45 (48%)
B	48 (51%)
C	1 (1%)
Tumor-related variables	
Number of tumors	
Multiple	53 (56%)
Tumor distribution	
Unilateral	70 (74%)
Maximum tumor size (cm)	
Median [range]	2.9 [1.5–12.0]
Portal vein invasion	
Present	7 (7%)
Alpha-fetoprotein (ng/mL)	
Median [range]	36.9 [1.9–17,100]
PIVKA II (mAU/mL)	
Median [range]	30 [0–6,000]
Other variables	
Modified Japan Integrated Stage	
Median [range]	2 [0–5]

PIVKA II protein induced by vitamin K absence or antagonist-II

[a] Ethanol intake ≥80 g/day for ≥5 years

[b] >20 cigarettes/day for >10 years

differential blood count, biochemistry tests, viral markers, including serum hepatitis B surface antigen and serum hepatitis C antibody, and tumor markers, including the serum levels of AFP and PIVKA II. Before treatment, a chest X-ray and ultrasonography and CT of the abdomen were obtained to evaluate the extent and size of the tumors and to exclude the presence of extrahepatic metastasis. The number, size, and distribution of the tumors were examined by CT and/or angiography. Written informed consent was obtained from all the patients prior to the start of the treatment.

Treatment procedure

Following conventional visceral angiography, TAI was performed by selectively introducing a catheter into the proper, right or left hepatic artery, or a branch of the artery feeding the tumor and injecting cisplatin suspended in lipiodol (iodized oil; Guerbet, Paris, France). The dose of the drug was determined based on the tumor size and liver function. The cisplatin suspension in lipiodol was prepared by the following procedure [25]: cisplatin powder, produced by evaporating water and sodium chloride from cisplatin solution, was sterilized by heating and subsequently suspended in lipiodol with a mortar and pestle under sterile conditions. The content of cisplatin in the lipiodol was adjusted to 20 mg/mL.

After the treatment, follow-up examinations, including CT, tumor marker measurement, and serum biochemistry, were performed, first at one month after the treatment completion and subsequently every 3–4 months. The transcatheter arterial treatments were repeated when relapse of the treated lesions and/or new hepatic lesions were seen.

Evaluation of the antitumor efficacy

The antitumor effect was assessed by contrast-enhanced CT or magnetic resonance imaging at one month after the treatment. Lipiodol accumulation in the tumor was regarded as representing necrotic tissue, because earlier studies have shown that areas on the CT showing lipiodol retention correspond to necrotic areas in the tumors [13–15]. We defined complete response (CR) as disappearance or 100% necrosis of all tumors, and partial response (PR) as >50% reduction and/or necrosis in the sum of all measurable tumors. Progressive disease was defined as more than 25% enlargement in the sum of all lesions and/or the appearance of any new lesions. Stable disease was considered as any disease that did not qualify for classification as CR, PR or progressive disease.

Factors analyzed

The relationships of pretreatment clinical variables to survival were investigated by univariate and multivariate analyses. The pretreatment variables were chosen based on their possible effects on the prognosis and tumor response indicated by previous investigations [1–12, 16–30] or suggested by our own clinical experience. Each of the variables, which were classified as host-related or tumor-related, was divided into two subgroups in accordance with clinically meaningful values for easy application in clinical practice, as shown in Table 2.

Overall survival was measured from the date of initial treatment to the date of death or last follow-up. Survival curves were calculated by the Kaplan–Meier method, and differences in survival were evaluated by the log rank test. The Cox proportional hazard model was used to determine the most significant variables related to survival. Forward and backward stepwise regression procedures based on the partial likelihood ratio were used to determine the major independent predictors of survival. A prognostic index based on the regression coefficients derived from all variables identified by the multivariate analysis was constructed. Stratification of the patients was conducted on the basis of this prognostic index. All p values presented in this report are of the two-tailed type. Differences at $p < 0.05$ were considered to be significant.

Results

Patient characteristics

The characteristics of all the 94 patients are shown in Table 1. There were 62 males (66%) and 32 females (34%), with a median age of 64 (range 41–81) years. There were 45 patients (48%), 48 patients (51%) and 1 patient (1%) with Child-Pugh stage A, B, and C [29], respectively. Fifty-three patients (56%) had multiple tumors, and the median maximum tumor size was 2.9 (range 1.5–12.0) cm. The median modified Japan Integrated Stage [30] was 2 (range 0–5). The median number of courses of TAI was two (range 1–9) during the follow-up period, and the median follow-up duration was 2.5 years (range 0.2–8.4 years). The median dose of cisplatin at first TAI was 50 (range 20–150) mg per treatment.

Treatment efficacy and survival

Twenty-seven patients (29%) showed CR and 21 patients (22%) showed PR, with an overall response rate of 51% (95% confidence interval, 41–61%). The median survival time was 2.5 years, and the proportions of survivors at 1, 2, 3, and 5 years were 81.6, 65.2, 39.8, and 18.3%, respectively (Fig. 1). The cause of death was tumor progression in 47 patients, hepatic failure in 25 patients, rupture of esophageal varices in 4 patients, and other causes in 6

Table 2 Univariate analysis of prognostic factors in patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol

	n	Median survival (years)	2-year survival (%)	Hazard ratio	*p* value
Host-related variables					
Age (years)					
≥60	67	2.5	65		
<60	27	2.6	54	0.98 (0.60–1.59)	0.93
Gender					
Female	32	2.7	66		
Male	62	2.4	60	0.99 (0.61–1.56)	0.97
Blood transfusion					
Present	28	2.5	60		
Absent	66	2.7	63	0.77 (0.48–1.24)	0.28
Alcohol abuse[a]					
Present	11	2.0	55		
Absent	83	2.6	63	0.63 (0.33–1.20)	0.16
Smoking habit[b]					
Absent	63	2.5	59		
Present	31	3.4	69	0.79 (0.50–1.27)	0.33
HBs Ag					
Negative	80	2.5	64		
Positive	14	1.8	46	0.77 (0.40–1.49)	0.45
HCV Ab					
Negative	18	1.9	47		
Positive	76	2.5	65	0.93 (0.53–1.64)	0.81
Ascites					
Present	14	1.4	21		
Absent	80	2.8	69	0.29 (0.16–0.53)	**<0.01**
WBC ($\times 10^4/mm^3$)					
≤4.0	51	2.5	61		
>4.0	43	2.5	64	0.76 (0.49–1.19)	0.23
Hemoglobin (g/dL)					
<10	17	2.4	59		
≥10	77	2.6	63	0.69 (0.40–1.19)	0.18
Platelet ($\times 10^4/mm^3$)					
<7.5	36	2.5	67		
≥7.5	58	2.5	59	0.89 (0.57–1.37)	0.59
Total bilirubin (mg/dL)					
≥2.0	13	1.8	46		
<2.0	81	2.7	65	0.59 (0.32–1.09)	0.09
Albumin (g/dL)					
<3.0	33	1.6	35		
≥3.0	61	4.0	76	0.29 (0.18–0.47)	**<0.01**
AST (U/L)					
≥85	24	2.4	58		
<85	70	2.8	63	0.63 (0.38–1.04)	0.07
ALT (U/L)					
≥92	21	2.4	57		
<92	73	2.7	63	0.74 (0.44–1.24)	0.25
LDH (U/L)					
≥500	9	1.8	44		
<500	85	2.5	64	0.76 (0.36–1.58)	0.46
Prothrombin time (%)					
<70	41	2.4	58		
≥70	53	2.7	65	0.93 (0.60–1.45)	0.76
ICG R15 (%)					
≥30	46	2.2	52		
<30	43	3.4	71	0.68 (0.43–1.07)	0.09
Tumor-related variables					
Number of tumors					
Multiple	53	2.0	51		
Single	41	2.8	76	0.63 (0.41–0.98)	**<0.05**
Tumor distribution					
Bilateral	24	1.1	27		
Unilateral	70	2.8	73	0.39 (0.24–0.65)	**<0.01**
Maximum tumor size (cm)					
>3.0	40	1.6	42		
≤3.0	54	3.2	76	0.41 (0.26–0.66)	**<0.01**
Portal vein invasion					
Present	7	1.0	17		
Absent	87	2.6	65	0.36 (0.15–0.84)	**<0.05**
Alpha-fetoprotein (ng/mL)					
≥100	46	2.4	57		
<100	48	2.6	67	0.66 (0.42–1.02)	0.06
PIVKA II (mAU/mL)					
≥100	14	1.1	34		
<100	80	2.7	67	0.53 (0.29–0.97)	**<0.05**

p values lesser than 0.05 are given in bold

HBs Ag hepatitis B surface antigen, *HCV Ab* hepatitis C antibody, *WBC* white blood cell count, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactic dehydrogenase, *ICG* indocyanine green test, *PIVKA II* protein induced by vitamin K absence or antagonist-II

[a] Ethanol intake ≥80 g/day for ≥5 years

[b] >20 cigarettes/day for >10 years

patients. Neither severe toxicity including renal dysfunction or thrombocytopenia, nor complication or treatment related death were seen in the present study.

Univariate and multivariate analysis

The median survival times, two-year survival, hazard ratios and *p* values of the survival time for univariate analysis are shown in Table 2. Among the host-related factors, absence of ascites and a serum albumin level of >3.0 g/dL were

significantly associated with a longer survival time. Among the tumor-related factors, single nodule, unilateral distribution of tumors, maximum tumor size <3.0 cm, absence of portal vein invasion, and PIVKA II level <100 mAU/mL were significantly associated with a longer survival time. The results of multivariate analysis using the Cox proportional hazard model are shown in Table 3. In the multivariate analyses, only those variables identified as significant by the univariate analysis were entered. Serum albumin ≥3.0 g/dL, maximum tumor size <3.0 cm, absence of ascites, and unilateral distribution of the tumors were significantly associated with favorable survival.

Risk groups based on the regression model

For the clinical application of these findings, a prognostic index was calculated based on the regression coefficients derived from the four variables identified by multivariate analysis (Table 3), as follows: prognostic index = score for albumin (0 for ≥3.0, 1 for <3.0 g/dL) + score for ascites (0 for absence, 1 for presence) + score for maximum tumor size (0 for ≤3.0, 1 for >3.0 cm) + score for tumor distribution (0 for unilateral, 1 for bilateral). The index values ranged from 0 to 4. The patients were then classified into three groups according to the prognostic index, as follows: good prognosis group (Group A: prognostic index = 0, $n = 31$ patients) (equivalent to patients with none of the four prognostic factors); intermediate prognosis group (Group B: prognostic index = 1, $n = 28$ patients) (equivalent to patients with one of the four prognostic factors); poor prognosis group (Group C: prognostic index ≥ 2, $n = 35$ patients) (equivalent to patients with two or more of the four prognostic factors). The survival curves for the three groups are shown in Fig. 2. The median survival times in the good, intermediate, and poor prognosis groups were 4.3, 2.7, and 1.1 years, respectively. There were significant differences in the survival time among the three groups ($p < 0.01$).

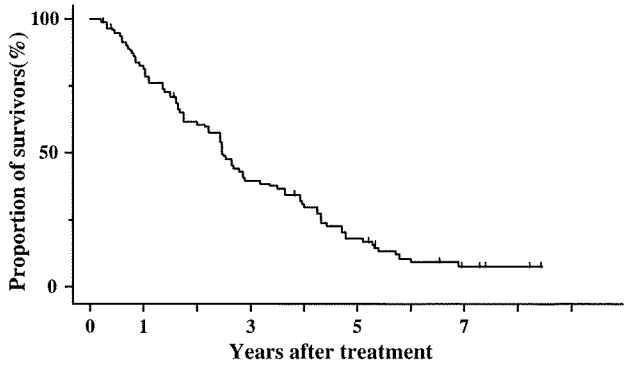

Fig. 1 Overall survival curve for all patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol. *Tick marks* indicate censored cases

Discussion

TAE has been widely used for cases with unresectable HCC and is currently the mainstay of non-surgical treatment for HCC, because it has been shown to exert a marked antitumor effect against HCC and can be administered for any type of HCC, regardless of the size, location or number of tumors [1]. In addition, the survival benefit of this treatment modality has been verified by two meta-analyses [2, 3] of seven randomized controlled trials [4–10]. However, TAE has deleterious effects on liver functions, thereby impairing the baseline prognosis. On the other hand, TAI has milder hepatotoxicity, but also shows a lower antitumor efficacy against advanced HCC than TAE. However, in a randomized controlled trial of TAE versus TAI with zinostatin-stimalamer and lipiodol, TAI and TAE were reported to yield comparable survival [16]. Moreover, the result of our retrospective analysis of TAE versus TAI using cisplatin–lipiodol suspension indicated similar outcomes for the two modalities [17]. From the results of these two studies, we could not conclude that additional embolization is not necessary for the treatment of advanced HCC, but there may be a subset of patients of advanced HCC in which TAI alone may yield sufficient treatment efficacy and survival. Therefore, this analysis of prognostic factors was carried out to enable identification of appropriate candidates for TAI using cisplatin–lipiodol suspension among HCC patients with no prior treatment. This single-institution study was undertaken using a unified method for tumor staging and identical procedures for treatment, follow-up, and supportive care throughout the duration of the study, to enable us to obtain reliable results for confirming important

Table 3 Significant prognostic factors determined by multivariate analysis with the Cox proportional hazard model

Variable	Coefficient	Hazard ratio (95% confidence intervals)	*p* value
Albumin ≥3.0 g/dL	0.94	0.39 (0.23–0.66)	<0.001
Maximum tumor size ≤3.0 cm	1.01	0.37 (0.19–0.69)	0.001
Absence of ascites	0.81	0.45 (0.11–0.40)	0.002
Unilateral tumor distribution	0.77	0.46 (0.27–0.79)	0.004

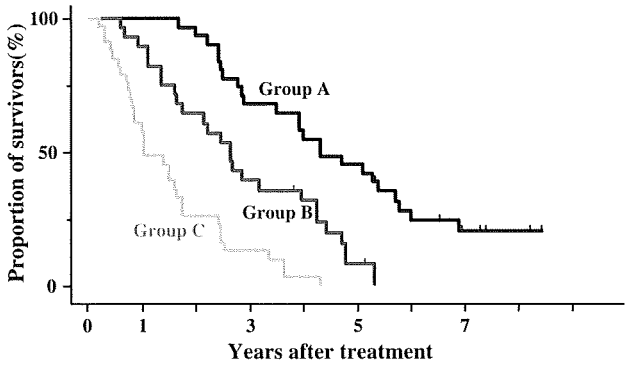

Fig. 2 Survival curves for the three groups determined by a prognostic index. *Group A* good prognosis (31 patients), *Group B* intermediate prognosis (28 patients), *Group C* poor prognosis (35 patients). *Tick marks* indicate censored cases

prognostic factors, predicting life expectancy and designing future clinical trials of TAI for HCC.

In this study, cisplatin was administered as the anticancer agent for TAI. Cisplatin has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. It is one of the key drugs for advanced HCC, that constituted a component of the combined chemotherapeutic regimen used in three of the seven randomized controlled trials of TAE reported until date [6, 7, 9]. In Japan, a favorable tumor response (33.8%) was reported in a clinical study of intra-arterial administration of cisplatin for advanced HCC [21], and the treatment has been approved for the treatment of HCC by the Ministry of Health, Labour and Welfare of Japan. Lipiodol has been used as a carrier for anticancer agents in targeting chemotherapy [13–15], and a suspension of cisplatin powder in lipiodol was used in this study. It has been reported that stronger antitumor effect is obtained by hepatic arterial administration of a combination of lipiodol and an anticancer agent than by that of an anticancer agent alone [26]. Recently, a lipophilic cisplatin derivative that can be suspended in lipiodol, SM-11355, was reported to show promising tumor efficacy (CR rate: 56%) in a phase II trial, and further trial is ongoing [27]. Therefore, combined therapy with cisplatin and lipiodol has been expected to become established as a valid option for the treatment of HCC. The response rate (51%: 95% confidence interval, 41–61%) at one month obtained in this study was more favorable than that in a clinical study of cisplatin alone, because TAI with an emulsion of an anticancer agent and lipiodol could be expected to exert more potent effects than an anticancer agent alone. However, follow-up at one month might be insufficient for evaluation of the rate/pattern of recurrence of HCC.

The median survival time and survival rates at two years in the current study were 2.5 years and 65.2%, respectively. These results were comparable or superior to those of TAE reported from the aforementioned seven randomized controlled trials [4–10]. Although the study was based on a retrospective cohort design, the treatment efficacy of TAI with cisplatin–lipiodol suspension was promising and comparable to that of TAE for HCC.

In regard to the host-related factors, absence of ascites and a serum albumin level >3.0 g/dL were found to be favorable prognostic factors by multivariate analysis. Ascites and albumin are the most important factors to consider when evaluating the hepatic reserve, being included in both the Okuda staging system [28] and Child-Pugh classification [29], and have been shown to be prognostic factor in previous studies of patients with advanced HCC [19, 20, 22–24]. In regard to the tumor-related factors, a maximum tumor size $\leq$3.0 cm and unilateral distribution of the tumors were identified as being significantly associated with a longer survival time by multivariate analysis. Increased tumor size and bilateral distribution of tumors are the well-known unfavorable prognostic factors in HCC patients, and have been shown to be correlated with increased tumor volume and poorer differentiation of HCC, which reflect a more advanced stage and higher malignant potential of the tumors [22]. However, these prognostic factors for TAI with lipiodol in this study were similar to those identified for TAI without lipiodol [19–21] or TAE in previous reports [22–24], and no specific prognostic factors for TAI could be identified in this study.

For clinical application of these findings, we propose a prognostic index based on the independent prognostic factors identified in this study. Patients could be classified into three groups: those with good, intermediate, and poor prognosis ($p < 0.0001$) (Fig. 2). This index consists of both hepatic reserve and tumor stage, like the modified JIS score [30], and it differs from the Child-Pugh stage or TNM stage which are, respectively, based on either only the hepatic reserve or tumor stage. An index based on both the hepatic reserve and tumor stage might enable a more accurate prediction of life expectancy and stratification of the group into more distinct prognoses. This index can be easily calculated, because it is based on variables obtained during routine examinations before TAI. It can, therefore, be used to stratify patients with HCC before TAI according to the predicted survival. Accordingly, patients with good prognosis may obtain sufficient treatment efficacy and survival with TAI alone. In contrast, patients with a poor prognosis may be treated with supportive care only because of the extremely short median survival (1.1 years) expected, or may be treated other more aggressive treatments, such as more intensive chemotherapy. Recently, systemic chemotherapy for advanced HCC has become an important treatment modality, because sorafenib has been proven to confer a survival benefit and to show promise as a standard