Table I. Patient characteristics and glypican-3-specific cytotoxic T lymphocytes.

Patient	HLA	Age (yrs.)	Gender	Etiology	Child-Pugh	No. of tumor	Tumor size (mm)	T[1]	N[1]	M[1]	AFP (<9.5 ng/ml)	PIVKA-II (<40 mAU/ml)	GPC3 expression[2]	GPC3-specific CTLs[3]			
														Pre	Post	Change	Δspot[4]
RFA1	A24	73	F	HBV	A	2	26	2	0	0	4.0	228	-	4	0	-	-4
RFA2	A24	68	M	HCV	B	1	20	1	0	0	5.0	300	+	10	24	+	+14
RFA3	A2	50	M	HCV	A	1	15	1	0	0	63.3	25	+	0	88	+	+88
RFA4	A24	79	F	HCV	A	1	10	1	0	0	484.2	30	+	0	10	+	+10
RFA5	A24	69	M	HCV	A	1	15	1	0	0	2.3	57	-	0	0	+/-	0
RFA6	A24	60	M	HCV	A	1	17	1	0	0	15.1	23	-	0	0	+/-	0
RFA7	A2	73	M	HCV	A	1	20	1	0	0	97.3	51	+	3	88	+	+85
RFA8	A2/A24	64	M	HBV/HCV	B	1	15	1	0	0	39.9	17	+	0	31	+	+31
RFA9	A2	60	M	HCV	B	1	10	1	0	0	92.0	19	-	19	15	-	-4
RES1	A24	48	M	HBV	A	1	20	1	0	0	19.7	38	+	32	15	-	-17
RES2	A24	66	F	HCV	A	1	26	2	0	0	63.4	77	+	20	3	-	-17
RES3	A24	64	M	HCV	A	2	30	2	0	0	10.1	276	+	12	0	-	-12
RES4	A2	72	M	-	A	1	60	2	0	0	9.2	1500	+	3	1	-	-2
RES5	A24	70	M	HCV	A	1	20	1	0	0	4.2	25	+	0	0	+/-	0
RES6	A24	42	M	HBV/HCV	A	2	98	3	0	0	15115.0	22477	+	50	30	-	-20
RES7	A2	75	M	-	A	1	75	2	0	0	22.8	10341	-	0	3	+	+3
RES8	A24	52	M	HCV	A	1	30	1	0	0	16.0	234	+	0	0	+/-	0
RES9	A24	60	M	HBV	A	1	30	1	0	0	15.6	23	-	0	0	+/-	0
TAE1	A2	64	M	-	A	3	30	2	0	0	10.7	98	+	0	330	+	+330
TAE2	A24	78	F	HCV	B	1	60	1	0	0	2483.0	3932	ND	34	0	-	-34
TAE3	A24	77	F	-	A	>5	35	3	0	0	180.2	11538	ND	0	3	+	+3
TAE4	A24	77	M	HCV	A	2	80	4	0	0	20014.0	241	ND	0	0	+/-	0
TAE5	A24	55	M	HBV	A	2	30	2	0	0	3.7	24	+	0	23	+	+23
TAE6	A24	77	M	-	A	>5	42	2	0	0	1407.0	1661	ND	0	20	+	+20
TAE7	A24	63	F	HCV	A	>5	32	2	0	0	640.3	270	ND	0	0	+/-	0
TAE8	A24	74	M	-	A	1	18	1	0	0	3.8	12	-	0	0	+/-	0
TAE9	A24	62	M	HCV	A	3	70	3	0	0	46.8	1907	ND	10	0	-	-10

[1]Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC). [2]GPC3 expression was evaluated by immunohistochemical staining; +, positive; -, negative. [3]Peripheral blood was taken from each patient before and after treatment, and GPC3-specific CTLs were measured by *ex vivo* interferon-γ enzyme-linked immunospot assay; +, increase; -, decrease; +/-, no change. [4]The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment. F, female; M, male; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; GPC3, glypican-3; ND, not determined.

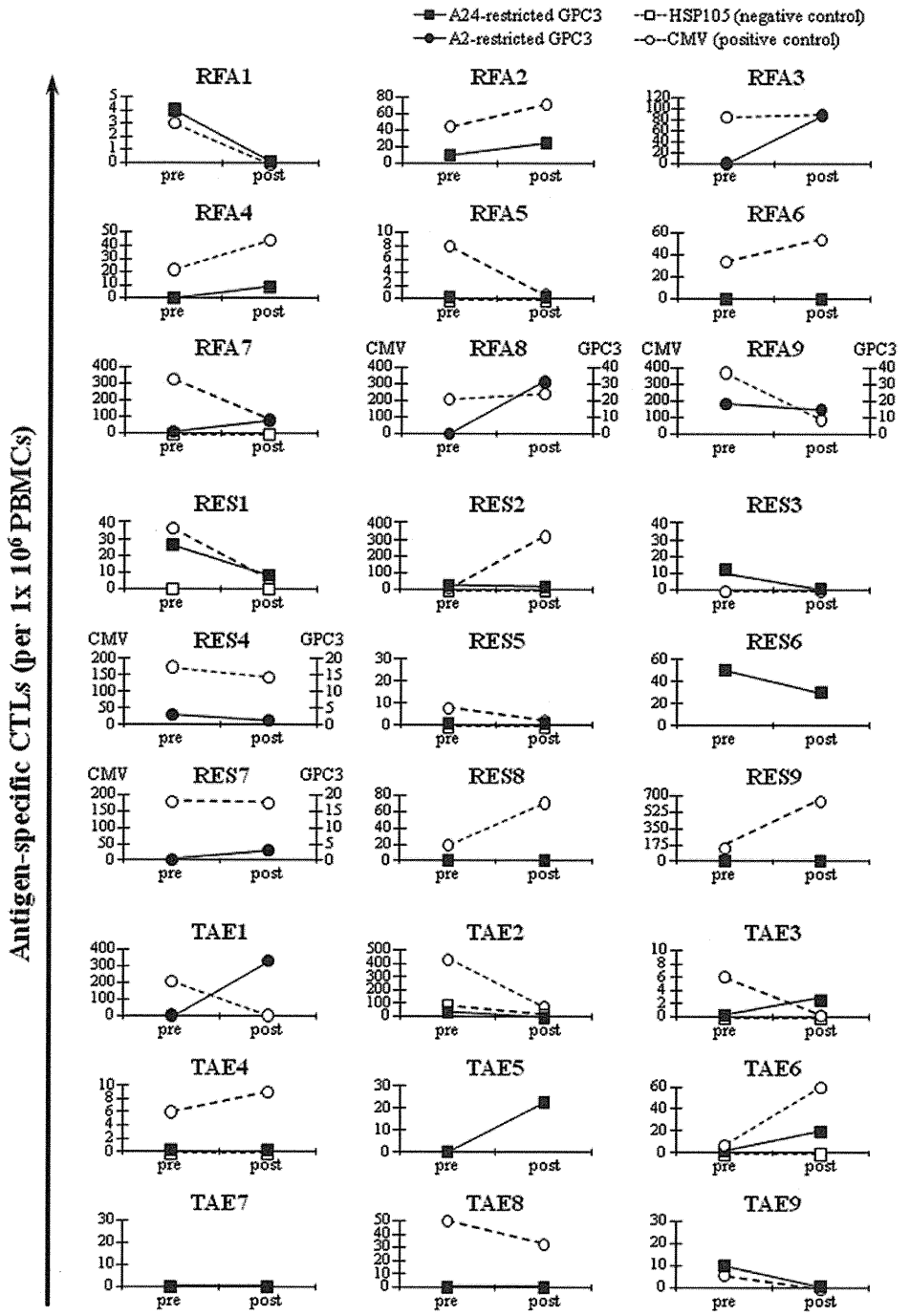

Figure 1. Kinetics of glypican-3 (GPC3)-specific CTLs between before and after treatment in each patient. A direct *ex vivo* interferon-γ enzyme-linked immunospot assay of PBMCs was performed before treatment and one month after treatment. The data are expressed as the number of interferon-γ producing cells, which indicate the CTLs specific with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) (■) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (●). Heat shock protein 105 (HSP105) peptide (□) and cytomegalovirus (CMV) peptide (○) were used as the negative and positive control, respectively.

kinetics of tumor markers indicated that their treatment was effective. The frequency of GPC3-specific CTLs increased after RFA (RFA3) and TACE (TAE5), whereas it decreased after surgical resection (RES6).

RFA has the potential to strongly induce T-cell-mediated immune response: A case report. A 70-year-old woman was admitted because of recurrent HCCs. Thirteen months earlier, the patient had undergone RFA for primary HCC located in the S5/8 region of the liver. CT detected two recurrent HCCs: one was contiguous to the previously ablated S5/8 region and the other was a distant tumor located in the S6 region. We performed surgical resection for these recurrent HCCs. Immunohistochemical examination of CD8 in the resected tumors revealed that a marked number of $CD8^+$ T cells had infiltrated not only into the surrounding recurrent tumor but also into the distant recurrent tumor after RFA (Fig. 3). On the other hand, few $CD4^+$ T cells were observed in these tumors (data not shown). Immunohistochemical analyses showed the expression of GPC3 and HLA class I in these tumors (data not

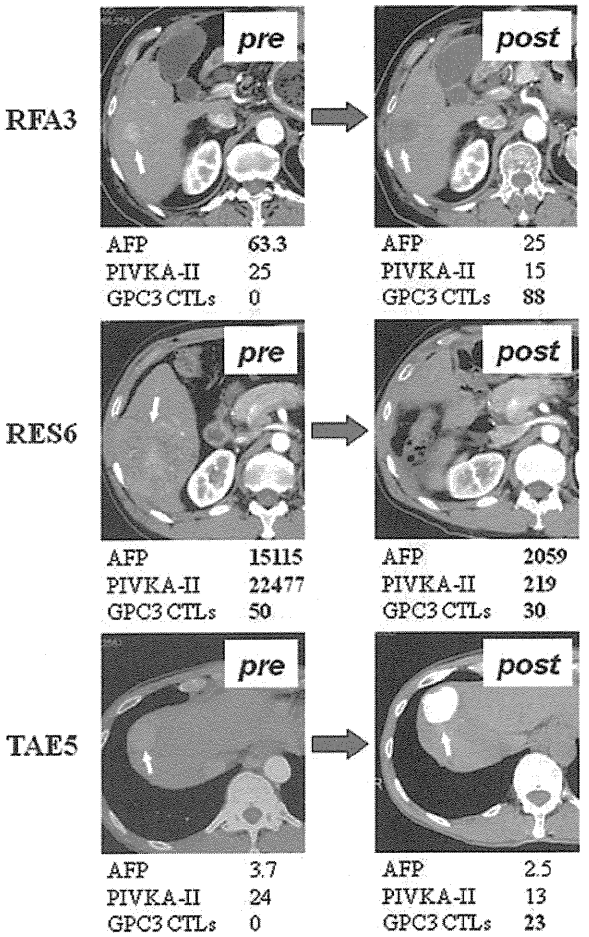

Figure 2. Changes in computed tomography images, serum levels of tumor markers, including α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II), and glypican-3 (GPC3)-specific CTLs in PBMCs between before and after treatment in patients RFA3, RES6, and TAE5. White arrows indicate nodules of hepatocellular carcinoma at pre- and post-treatment. The bold letters show the abnormal levels of tumor markers or the positive response of GPC3 specific CTLs.

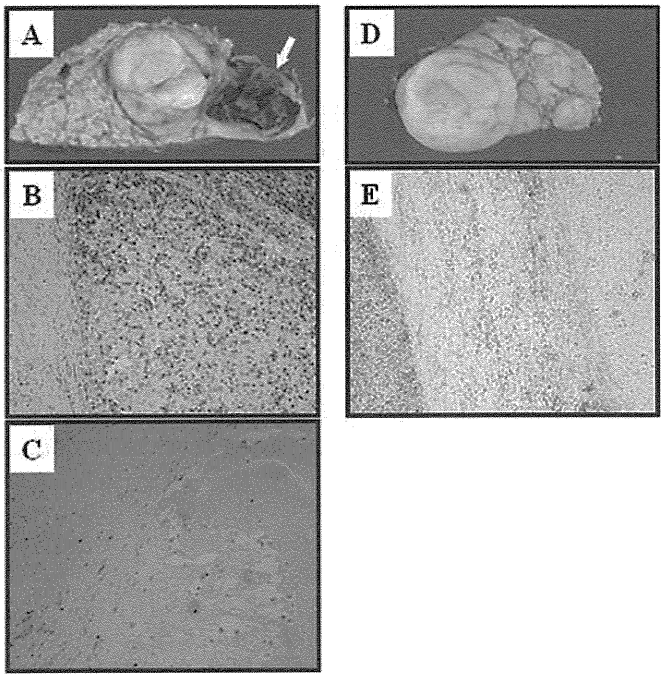

Figure 3. Macroscopic features and immunohistochemical examination of $CD8^+$ T cells in the resected tumors that had recurred after radiofrequency ablation. (A and D) show the cut surface of the resected specimens. (A) The white arrow indicates the post-ablated lesion to which a recurrent tumor was contiguous. The other recurrent tumor was distant from the post-ablated lesion (D). A marked number of $CD8^+$ T cells had infiltrated into the contiguous recurrent tumor (B) and the distant recurrent tumor (E), whereas few $CD8^+$ T cells had infiltrated into the post-ablated necrotic lesion (C). Magnification x100 (B and C) and x40 (E).

shown). These findings suggest that RFA not only activates the immune response systemically but also induces local infiltration of CTLs into the tumors.

Analysis of immune response induced by RFA in a mouse model. The experimental schedule is shown in Fig. 4A. The IFN-γ ELISPOT assay with $CD8^+$ T cells from the lymph nodes of mice demonstrated that the number of spots against both Colon 26 (P=0.049) and Colon 26/GPC3 (P=0.049) was larger after RFA compared to without treatment. On the other hand, the number of spots did not increase after surgical resection. These results suggest that RFA induced a significantly larger number of both Colon 26- and Colon 26/GPC3-reactive CTLs compared to no treatment or surgical resection (Fig. 4B).

The difference in number of spots between Colon 26 and Colon 26/GPC3 in each mouse, which represents GPC3-specific CTLs, is shown in Fig. 4C. As an effect of prior peptide vaccination, GPC3-specific CTLs were detected in the no treatment group. The frequency of GPC3-specific CTLs increased after RFA and decreased after surgical resection. As a result, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection (P=0.049). These results suggest that RFA induced a significantly larger number of GPC3-specific CTLs compared to surgical resection (Fig. 4C).

Discussion

We previously reported that 39% of HCC patients had detectable GPC3-specific CTLs by a direct *ex vivo* IFN-γ ELISPOT assay (25). In this study, GPC3-specific CTLs were detectable before treatment in 11 of 27 patients (41%). Additionally, when we analyzed the patients with a prior treatment for HCCs using the same methods, 11 of 21 (52%) patients had detectable GPC3-specific CTLs (data not shown). These results are favorable for anticancer immunotherapy because the antigen-specific T-cell-mediated immune response could be detected without *in vitro* stimulation. As for frequency, GPC3-specific CTLs were detectable in ~40% of HCC patients, whereas AFP-, human telomerase reverse transcriptase (hTERT)-, and multidrug resistance-associated protein 3 (MRP3)-specific CTLs have been detected in 5-20, 6-12, and 14-21% of HCC patients with a single epitope peptide, respectively (26-28). As for tumor stages, a GPC3-specific immune response is frequently detected even in the early stages (24), whereas AFP-specific CTLs are more frequently detected in patients with advanced HCC (26). These results suggest that GPC3 has strong immunogenicity and GPC3-specific T-cell-mediated immunotherapy is suitable for adjuvant therapy against HCC because the induction of tumor-specific immune response in

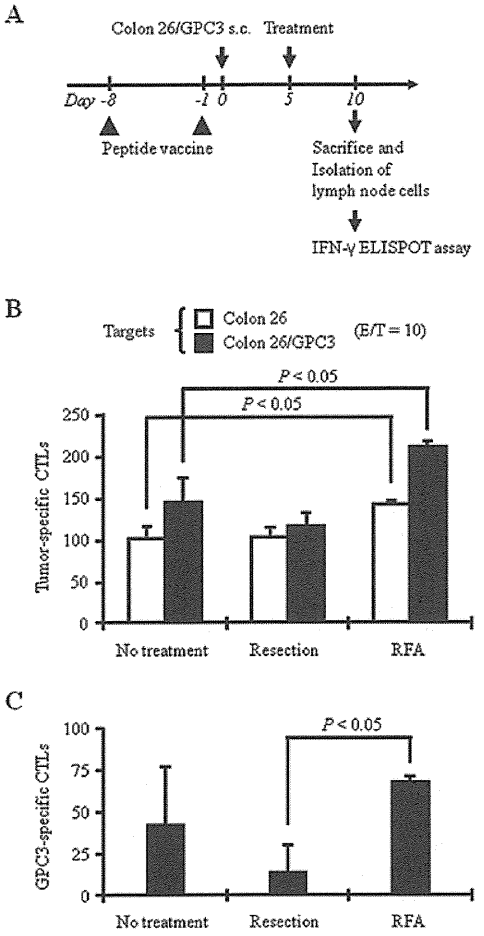

Figure 4. Investigation of the glypican-3 (GPC3)-specific immune response in a mouse model. (A) Experiment schedule. (B) An *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay of $CD8^+$ lymph node cells (effector, $3x10^5$ cells/well) against Colon 26 and Colon 26/GPC3 (target, $3x10^4$ cells/well). No treatment column indicates the group of mice that received only the peptide vaccination and no therapy for the established tumor. The data are expressed as the mean + SD. Three mice were used for each group. Effector/target ratio=10. (C) The frequency of GPC3-specific CTLs, which is calculated from the difference in the number of spots between Colon 26 and Colon 26/GPC3 in each mouse.

the early stages would be more effective for suppression of tumor growth.

The association between the induction of an antigen-specific immune response and the antigen expression in tumor tissue remains unclear. In this study, we obtained the result that the presence of GPC3-specific CTLs in PBMCs potentially had a positive correlation with GPC3 expression in tumor tissue, but the correlation was not statistically significant. On the other hand, Mizukoshi *et al* showed a negative correlation between the frequency of MRP3-specific CTLs and MRP3 expression level (28). Moreover, Benavides *et al* showed that even antigen-naïve patients had pre-existing immunity (29). First, this may be because of tumor heterogeneity of cancer tissue. In most cases, the whole tumor cannot be evaluated and, in the case of truly antigen-naïve patients, antigen-specific CTLs cannot exist in theory. Second, antigen expression may be negative if antigen-specific CTLs have killed all of the antigen-expressing tumor cells as described by Jäger *et al* (30). As for the changes in an antigen-specific immune response between before and after treatment, in this study, we showed impressive data that all patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas no patient with GPC3-expressing HCCs did after surgical resection.

This is the first study to compare locoregional therapies, including RFA, surgical resection, and TACE, in terms of antigen-specific T-cell response in HCC patients and tumor-bearing mice. Half the patients after RFA or TACE showed an increase in GPC3-specific CTLs, which might have been induced by the treatment, whereas only 1 of 9 patients after resection showed an increase and more than half the patients after resection showed a decrease. Similarly, the frequency of GPC3-specific CTLs increased after RFA and decreased after resection in a mouse model. These results suggest that RFA induced a stronger GPC3-specific immune response compared to surgical resection. RFA destroys tumor tissue and causes local necrosis followed by the release of tumor-associated antigens (12), whereas all of the tumor-associated antigens must be completely removed after resection. With regard to TACE, whereas the results of an IFN-γ ELISPOT assay after TACE were as encouraging as that after RFA, we have no other favorable data on the immune response after TACE. Although further investigation is required, TACE, which is also a necrosis-inducing treatment, might induce an antigen-specific immune response.

A limitation of this study is the patient selection in the three kinds of locoregional therapy. Current treatment guidelines for HCC including the Japanese ones, which we followed in this study, recommend RFA to earlier HCCs and TACE to more advanced HCCs than those which receive surgical resection (2,31-33). Therefore, selection bias is unavoidable under the circumstances. To overcome this problem, we added a murine study. The advantage of RFA over surgical resection in the induction of GPC3-specific CTLs was demonstrated also in a mouse model.

The correlation between antitumor immune response and clinical response is controversial. In this study, a significant contribution of GPC3-specific CTLs toward an optimal prognosis was not demonstrated. Mizukoshi *et al* reported that enhancement of T-cell response did not last for long and did not contribute to the prevention of HCC recurrence (34). In view of the highly complex nature of the human immune system, patient prognoses might not be determined only by the CTL response. Previous studies have demonstrated that the release of tumor-derived antigens by necrosis-inducing treatment causes sufficient signaling to activate not only antigen-specific CTL response but also antigen-specific helper T-cell response (35,36), antigen-specific antibody response (36), and non-antigen-specific natural killer cell response (37). However, the mechanisms for cancer escape from immunosurveillance would suppress the efficiency of these immune responses (38). In the literature, tumor-infiltrating lymphocytes in HCC are associated with better prognosis (39), but, in our case, tumor-infiltrating CTLs were actually insufficient for suppression of cancer recurrence despite the massive infiltration. For successful anticancer immunotherapy, the development of an innovative strategy to link antitumor immune response with clinical response and to provide a survival benefit for cancer patients is necessary, and so we have just started the clinical trial of a GPC3-derived peptide vaccine for adjuvant therapy after RFA.

In conclusion, our results demonstrate that RFA has a stronger effect on the immune system compared with surgical resection. Although further investigation is necessary, the data on immune response support the rationale for combined immunotherapy for HCC patients.

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CLINICAL INVESTIGATION **Liver**

DOSE–VOLUME HISTOGRAM ANALYSIS OF THE SAFETY OF PROTON BEAM THERAPY FOR UNRESECTABLE HEPATOCELLULAR CARCINOMA

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Purpose: To evaluate the safety and efficacy of radiotherapy using proton beam (PRT) for unresectable hepatocellular carcinoma.

Methods and Materials: Sixty consecutive patients who underwent PRT between May 1999 and July 2007 were analyzed. There were 42 males and 18 females, with a median age of 70 years (48–92 years). All but 1 patient had a single lesion with a median diameter of 45 mm (20–100 mm). Total PRT dose/fractionation was 76–cobalt Gray equivalent (CGE)/20 fractions in 46 patients, 65 CGE/26 fractions in 11 patients, and 60 CGE/10 fractions in 3 patients. The risk of developing proton-induced hepatic insufficiency (PHI) was estimated using dose-volume histograms and an indocyanine-green retention rate at 15 minutes (ICG R15).

Results: None of the 20 patients with ICG R15 of less than 20% developed PHI, whereas 6 of 8 patients with ICG R15 values of 50% or higher developed PHI. Among 32 patients whose ICG R15 ranged from 20% to 49.9%, PHI was observed only in patients who had received 30 CGE (V30) to more than 25% of the noncancerous parts of the liver (n = 5) Local progression-free and overall survival rates at 3 years were 90% (95% confidence interval [CI], 80–99%) and 56% (95% CI, 43–69%), respectively. A gastrointestinal toxicity of Grade ≥2 was observed in 3 patients.

Conclusions: ICG R15 and V30 are recommended as useful predictors for the risk of developing PHI, which should be incorporated into multidisciplinary treatment plans for patients with this disease. © **2011 Elsevier Inc.**

Hepatocellular carcinoma, Proton beam radiotherapy, Dose–volume histogram, Radiation tolerance of the liver.

INTRODUCTION

Recent improvements in diagnostic imaging and radiotherapy (RT) techniques have made high-dose radiotherapy a safe and effective treatment for selected patients with unresectable hepatocellular carcinoma (HCC) (1). Charged-particle radiotherapy can potentially deliver considerably larger doses of RT to liver tumors, with greater sparing of normal tissues, and proton beam radiotherapy (PRT) for HCC using aggressively high total and fractional RT doses has been investigated during the last 2 decades. The results have shown local control rates ranging from 75% to 96% and overall survival (OAS) rates exceeding 50% at 2 years in groups of patients that include those who had HCC tumors of ≥5 cm in diameter (2–4). HCC has a high propensity for venous invasion, which is frequently associated with multiple tumors within resected specimens (5–9). In this context, the extent of resection was determined while considering potential tumor spread via portal blood flow and the necessity of preserving a functional liver reserve (5, 7, 10). Even in preselected patients who underwent hepatectomy, more than 50% of tumors with diameters greater than 4 cm demonstrated microscopic vascular invasion (8, 11). Consequently, it will become more crucial to consider the influence of vascular invasion on undetectable tumor dissemination at the periphery of the gross tumor in RT for unresectable HCC.

Given the high probability of obtaining local control by using PRT, an appropriate definition of the clinical target volume (CTV) according to patterns of tumor spread and patients' functional liver reserves is extremely important in order to maximize the therapeutic ratio. Ideally, the entire portal segment that contains HCC nodules should be covered within the CTV when the tumor shows macro- or microscopic vascular invasion. This requires a considerably larger

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irradiated volume even with PRT, partly because of unavoidable uncertainty in treatment planning without using intraoperative ultrasonography (7). Another possible way to eradicate satellite HCC nodules, which are disseminated via portal blood flow, is transarterial chemoembolization (TACE). Currently, the standard treatment for patients with unresectable HCC that is not amenable to local ablation therapy is TACE instead of best supportive care (12). The OAS rate at 3 years after TACE ranges from 32% to 47% in patients with stage III cancer and with liver damage A to B, according to the staging system used in a nationwide cohort study conducted by the Liver Cancer Study Group of Japan (13). Considering that the tumoricidal effect of TACE in HCC with vascular invasion is frequently incomplete (13), a significant benefit of adding PRT to TACE would be expected. However, presently, there has been no robust evidence supporting this concept. Before we examine the validity of targeting the entire anatomical portal segment containing HCC in a multidisciplinary approach that includes PRT, practical methods to estimate the safety of PRT according to the dose–volume histogram (DVH) should be established in patients who have various levels of severity of liver dysfunction. Findings from our previous study consisting of 30 patients suggested that the risk of proton-induced hepatic insufficiency (PHI) could be predicted by the indocyanine green clearance test and the retention rate at 15 minutes (ICG R15) in combination with DVH parameters (14) such as percentages of hepatic noncancerous portions receiving doses of >30 cobalt-Gray-equivalent (CGE) (3). We have subsequently accumulated data from additional patients in clinical practice. The clinical results were evaluated, and we have again used the DVH analysis to examine the relationship between probability of PHI and dose–volume parameters.

METHODS AND MATERIALS

Patients

Patient eligibility was reported previously (3); in brief, they were required to have uni- or bidimensional measurable HCC nodules of ≤10 cm in maximum diameter on computed tomography (CT) and/or magnetic resonance imaging (MRI) without evidence of extrahepatic tumor spread. All patients had a white blood cell count of ≥2,000/mm^3; a hemoglobin level of ≥7.5 g/dl; a platelet count of ≥25,000/ mm^3; and adequate hepatic function (total bilirubin, ≤3.0 mg/dl; alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase of <5.0× normal; no ascites). Patients who had multicentric HCC nodules were not considered as candidates for PRT, except for those who fulfilled the following two conditions: (*1*) multiple nodules could be encompassed within a single clinical target volume; and (*2*) lesions other than those of the targeted tumor were judged to be controlled with prior surgery and/or local ablation therapy. This retrospective study was approved by the institutional ethics committee, and written informed consent was obtained from all patients.

Treatment Planning

ICG R15 was measured in all patients to quantitatively assess the hepatic functional reserve. Serological testing for hepatitis B surface antigen and anti-hepatitis C antibody was done. All patients were judged to be unresectable by expert hepatobiliary surgeons at our institution, based on the patient's serum bilirubin level, ICG R15, and expected volume of resected liver (10). Percutaneous fine-needle biopsies were performed for all patients unless they had radiologically compatible, postsurgical recurrent HCC (3).

Treatment methods were published previously (3). In brief, gross tumor volume (GTV) was defined using a treatment-planning CT scan, and CTV and planning target volume (PTV) were defined as follows in all but 2 patients: CTV = GTV + 5 mm, and PTV = CTV + 3 mm of lateral, craniocaudal, and anteroposterior margins. CTV encompassed the entire volume of the right lobe in 1 patient who had a tumor of 4 cm in diameter that broadly attached to the bifurcation of the right anterior and posterior portal veins. In this patient, right portal vein embolization was done to facilitate compensatory hypertrophy of the left lobe for expected surgery. However, the patient was finally judged to be unresectable, and PRT was selected. Another patient was treated with a CTV encompassing the entire right anterior portal segment because a tumor of 2 cm in diameter had invaded the bifurcation of the right anterosuperior and anteroinferior portal vein associating with daughter HCC at the right anterosuperior portal segment. The beam energy and spread-out Bragg peak (15) were fine-tuned so that a 90% isodose volume of the prescribed dose encompassed the PTV.

Forty-six patients received PRT to a total dose of 76 CGE in 3.8 CGE once-daily fractions, four to five fractions in a week. Another 3 patients underwent 60 CGE /10 fractions/2 weeks, depending on availability of the proton beam. Eleven patients whose PTV encompassed the gastrointestinal wall received 65 CGE in 2.5 CGE /fraction, five fractions per week. All patients were treated using a 150- to 190-MV proton beam. The relative biological effectiveness of our proton beam was defined as 1.1 (16). No concomitant treatment such as TACE, local ablation, or systemic therapy was allowed during or after the PRT, unless a treatment failure was detected. Both scanning of CT images for treatment planning and irradiation by the proton beam were done during the exhalation phase using the respiration-gated irradiation system and intrahepatic fiducial markers as previously reported (3).

Outcomes

Death from any cause was defined as an event in calculation of OAS, whereas tumor recurrences at any site or patient deaths were defined as events in disease-free survival (DFS). An increase of the tumor diameter within the PTV was defined as local progression, and patients who died without evidence of local progression were censored at the time of last radiographic examination. Adverse events were reviewed weekly during the PRT regimen by means of physical examination, complete blood count, liver function tests, and other biochemical profiles as indicated. The severity of adverse events was assessed using the National Cancer Institute common terminology criteria for adverse events, version 3.0. After completion of PRT, reviews that monitored disease status, including CT and/or MRI examinations and long-term toxicity, were done at a minimum frequency of every 3 months in all 60 patients. The percentages of hepatic noncancerous portions (entire liver volume minus gross tumor volume) receiving CGE doses of >0 (V0), ≥10 (V10), ≥20 (V20), ≥30 (V30), ≥40 (V40), and ≥50 (V50) were calculated using PRT planning software (PT-PLAN/NDOSE System, Sumitomo Heavy Industries Ltd., Tokyo, Japan), and their influence on the outcomes were analyzed (3). Time-to-event analyses were done using Kaplan-Meier estimates from the start of PRT. The differences between time-to-event curves were evaluated with the log-rank test. Multivariate analyses were performed with Cox's proportional hazards model.

RESULTS

Patients

A total of 60 patients with HCC underwent PRT in our institution between May 1999 and July 2007. Approximately 1400 patients with HCC were newly presented to our institution during this study period and about 35%, 30%, 25%, and the remainder primarily treated with hepatectomy, TACE, percutaneous local ablation, and other treatments, respectively. Therefore 60 patients in this study corresponded to approximately 4% of overall, or 7% of patients with unresectable HCC. Patient characteristics at the start of PRT are listed in Table 1. All patients had underlying chronic liver disease. One patient had a history of schistosomiasis, and another patient had autoimmune hepatitis as the cause of liver cirrhosis. Five additional patients were diagnosed with liver cirrhosis caused by non-B, non-C hepatitis. A total of 24 patients received PRT as the first treatment for their HCC. Ten patients had postsurgical recurrences, 22 patients received unsuccessful local ablation and/or TACE to the targeted tumor, and 4 patients underwent successful local ablation to a tumor other than the target prior to PRT. Histological confirmation was not obtained in 1 patient who had a tumor with typical radiographic features compatible with HCC (3). Six patients had HCC nodules of ≤3 cm in diameter; however, they were not considered candidates for local ablation therapy because of the tumor locations, which were in close proximity to the great vessels or the lung.

Table 1. Characteristics of patients

Characteristics	No. of patients (%)
Age (years)	
Median	70
Range	48–92
Gender	
Male	42 (70)
Female	18 (30)
ECOG performance status	
0–1	57 (95)
2	3 (5)
Viral markers	
Hepatitis B surface antigen-positive	3 (5)
Hepatitis C antibody-positive	49 (82)
Both positive	1 (2)
Both negative	7 (12)
Child-Pugh classification	
A	47 (78)
B	13 (22)
C	0
% patients with pretreatment ICG R15 values	
<20	20 (20)
20–40	25 (55)
40–50	7 (12)
≥50	8 (13)
Tumor size (mm)	
Median	45
Range	20–90
20–50	42 (70)
>50	18 (30)
Macroscopic vascular invasion	
Yes	42 (70)
No	18 (30)
Morphology of primary tumor	
Single nodular	45 (75)
Multinodular, aggregating	9 (15)
Diffuse	5 (8)
Portal vein tumor thrombosis	1 (2)
Serum alpha-fetoprotein level (IU/mL)	
<300	41 (68)
≥300	19 (32)
Histology	
Well-differentiated	15 (25)
Moderately-differentiated	28 (47)
Poorly-differentiated	7 (12)
Differentiation not specified	9 (15)
Negative (radiological diagnosis only)	1 (2)
Prior treatment	
None	24 (40)
Surgery	10 (17)
Local ablation/TACE	26 (43)

Adverse events during PRT

All patients completed the treatment plan. Prolongation of the overall treatment time for more than 1 week occurred in 4 patients: treatment of 3 patients was extended due to availability of the proton beam machine, and 1 patient's treatment was extended because of fever associated with grade 3 elevation of total bilirubin that spontaneously resolved within a week. A total of 14 patients experienced transient grade 3 leukopenia and/or thrombocytopenia without infection or bleeding that necessitated treatment. In addition, 8 patients experiencing grade 3 elevation of transaminases without clinical manifestation of hepatic insufficiency maintained good performance status. PRT was not discontinued for these patients; nevertheless, these events spontaneously resolved within 1 to 2 weeks.

Estimation of the risk of PHI by DVH analysis

Development of hepatic insufficiency presented with anicteric ascites and/or asterixis within 6 months after completion of PRT in the absence of disease progression was defined as PHI. Eleven patients, all of whom received a total PRT dose of 76 CGE, developed PHI at 1 to 6 months (median, 2 months) after completion of PRT without elevation of serum bilirubin and transaminases of more than threefold above normal levels. DVHs for hepatic noncancerous portions were drawn according to pretreatment ICG R15 values (Fig. 1A–C). Results showed that all 20 patients with ICG R15 of <20% were free of PHI, regardless of the DVH, for 2 to 94 months (median, 44 months). On the other hand, 6 of 8 patients with pretreatment ICG R15 values of ≥50% died of PHI with (n = 3) or without (n = 3) evidence of HCC recurrence at 2 to 15 months (median, 8 months). There was no obvious relationship between DVH and development of PHI in these 8 patients, as shown in Fig. 1C.

Among 32 patients whose ICG R15 values ranged from 20% to 49.9%, 5 patients developed PHI. The V0 to V50 in these 32 patients are shown in Fig. 2. Differences in distributions of these DVH parameters between patients who did

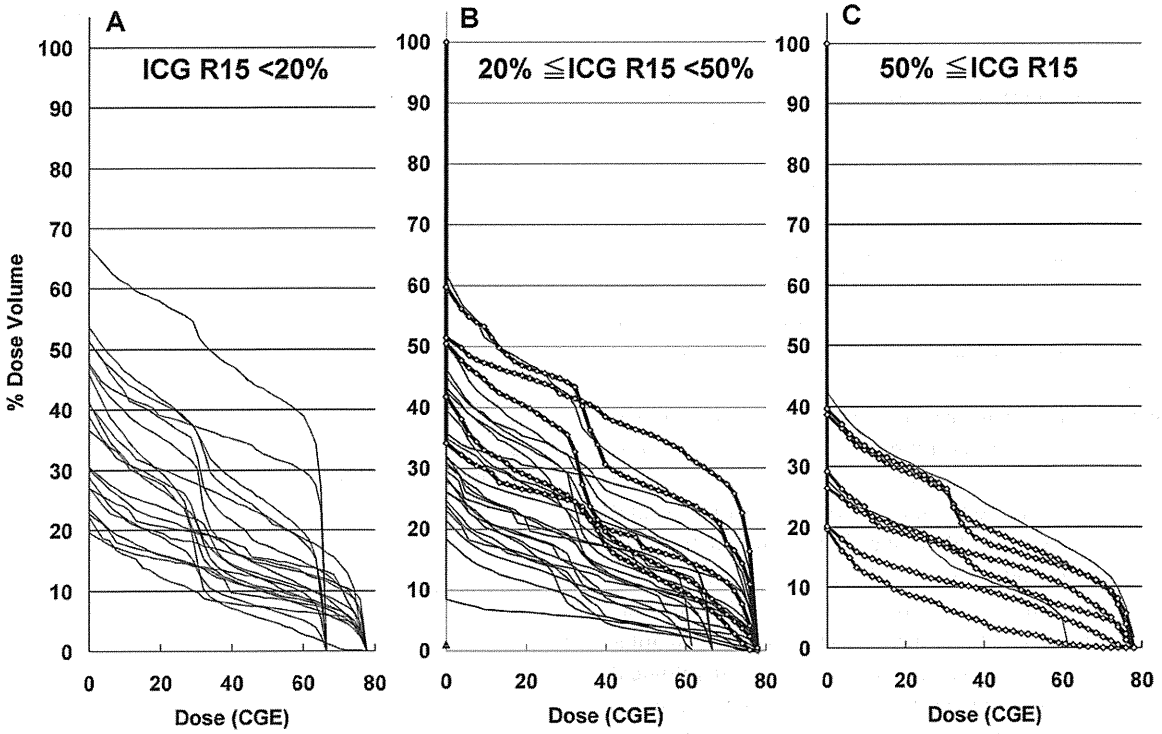

Fig. 1. DVH are shown for all patients according to their pretreatment ICG R15 values, as noted in each panel. Thick lines with rhomboid symbols represent DVHs for patients suffering from hepatic insufficiency within 6 months after completion of PRT.

and did not develop PHI were statistically significant, with p values of 0.012 in V0, 0.009 in V10, 0.012 in V20, 0.006 in V30, 0.016 in V40, and 0.024 in V50 (Mann-Whitney U test). The lowest p value was observed in the difference at V30. Among 32 patients whose ICG R15 values ranged from 20% to 49.9%, none of the 21 patients whose V30 were <25% experienced PHI, whereas 5 of 11 patients (45%) whose V30 was ≥25% developed PHI (p = 0.037, Mann-Whitney U test). The incidence of PHI was 2/25 (8%) in Child-Pugh class A patients, whereas PHI incidence was 3/7 (43%) in class B patients in this group of 32 patients (p = 0.218, Mann-Whitney U test). Of 5 patients who experienced PHI, 1 died at 8 months without evidence of HCC recurrence. PHI spontaneously resolved in 4 patients; 2 patients died of intrahepatic recurrence at 22 and 71 months, respectively; 1 patient died of brain metastasis at 8 months; and 1 patient was alive and disease free at 50 months. In both of the patients who survived for more than 4 years despite development of PHI, the pretreatment functional liver reserve was Child-Pugh class A and ICG R15 was less than 40%. On the other hand, all 3 patients who experienced PHI and died within 2 years had Child-Pugh class B liver functions. Relationships between ICG R15 and V30 according to occurrence of PHI in Child-Pugh class A and B patients are shown in Fig. 3a and b, respectively.

Other serious adverse events

Three patients experienced a gastrointestinal toxicity grade of ≥2. One patient developed hemorrhagic duodenitis associated with anemia at 2 months after completion of 76 CGE/20 fractions/30 days of PRT. The dose administered to the duodenum was estimated to be 50 to 80% of the prescribed dose. Bypass surgery was attempted to alleviate the symptoms; however, this patient died of postoperative hepatic failure at 6 months. Two patients received 65 CGE/26 fractions of PRT, with the entire circumference of the gastrointestinal walls covered within the PTV. One of these 2 patients experienced grade 3 hemorrhagic ulcer at the ascending colon, within the PTV. The patient was managed successfully with right hemicolectomy at 10 months; however, the patient

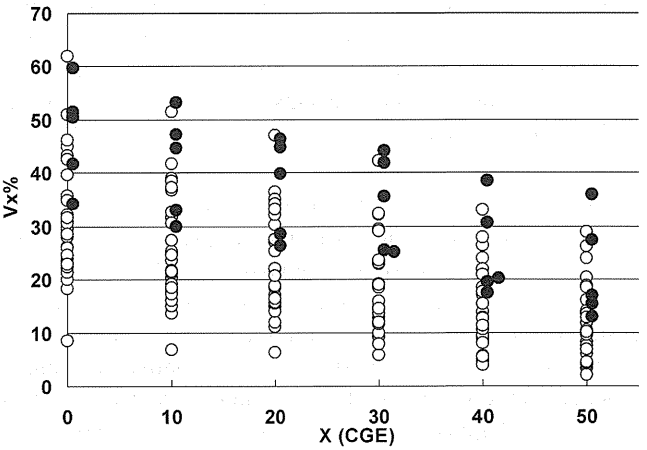

Fig. 2. Distribution of V0 to V50 in DVHs for 32 patients whose pretreatment ICG R15 values ranged from 20% to 49.9%. Open circles represent values for patients who did not experience PHI, whereas closed circles represent those who developed PHI.

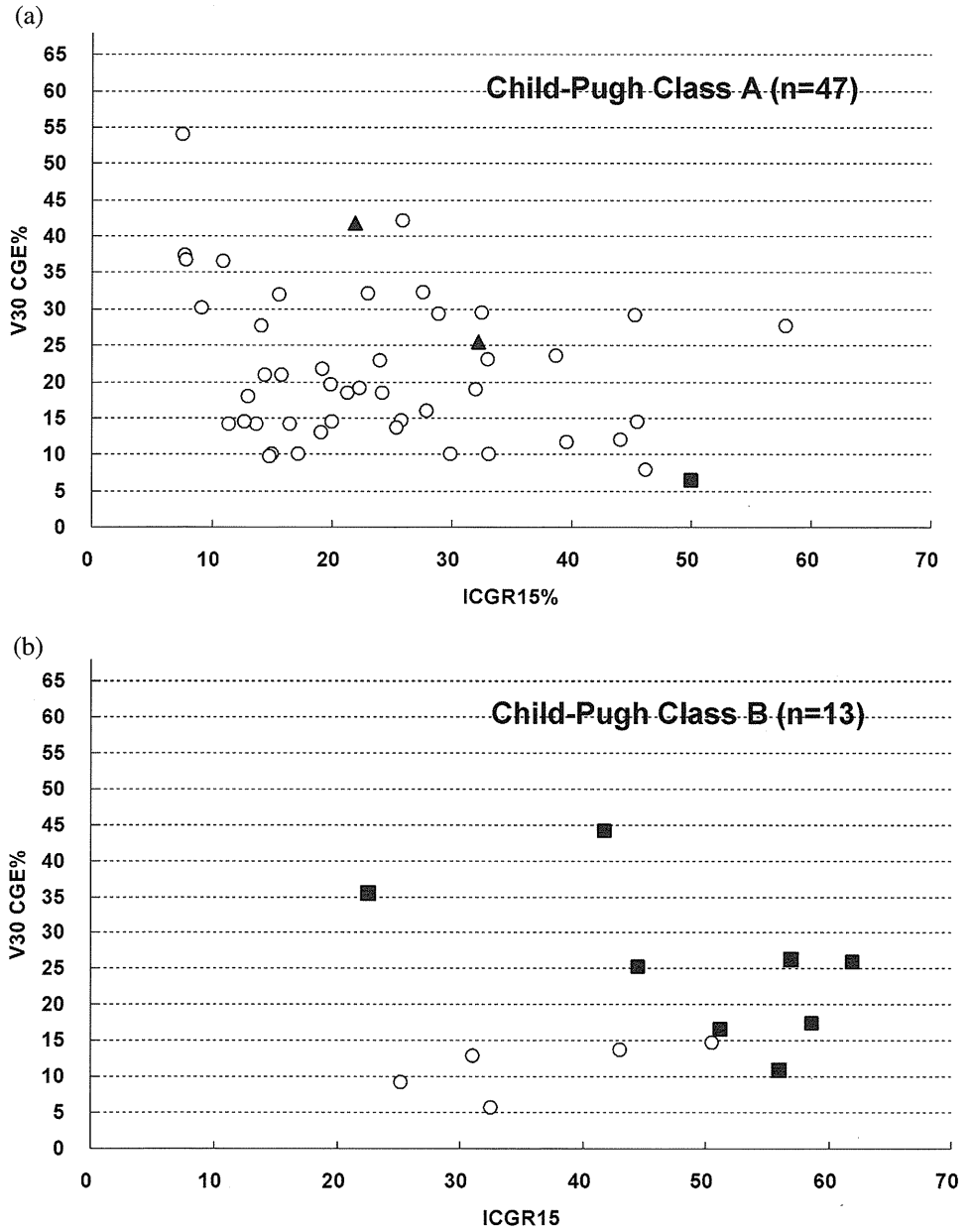

Fig. 3. Scattergram of V30 in each patient who had pretreatment liver functions classified as Child-Pugh class A (a) and class B (b), as shown in each panel, according to the ICG R15 value. Open circles represent values in patients who did not experience PHI. Closed squares represent those who developed PHI and died within 2 years with ($n = 5$) or without ($n = 4$) disease recurrence. Closed triangles represent those who experienced transient PHI and survived for more than 4 years after commencement of PRT.

died of local recurrence and subsequent hepatic failure at 23 months. The other patient developed grade 2 esophagitis within the PTV at 7 months. Repetitive balloon dilatations were required to alleviate the patient's dysphagia; however, the patient was alive without disease and taking a normal diet at 30 months. There were no other observations made of adverse events of Grade ≥3 in any of the patients.

Tumor control and survival

At the time of analysis in August 2009, 42 patients had already died because of intrahepatic recurrence in 27, nodal recurrence in 1, distant metastasis in 3, hepatic insufficiency without recurrence in 9, comorbidity in 1, and senility in 1. Forty of these 42 patients had been free from local progression until death; the durations ranged from 2 to 77 months (median, 20 months). Two patients who experienced local progression died subsequently. A total of 15 patients were alive at 25 to 92 months (median, 43 months) without local progression. Three patients were alive at 49, 53, and 94 months, respectively, after salvage treatment for local progression, using local ablation in 2 and TACE in 1 A total of 37 patients achieved complete disappearance of the primary tumor at 1 to 50 months (median, 10 months) post-PRT. Eighteen patients had residual tumor masses on CT

and/or MRI for 2 to 44 months (median, 21 months) until the time of death or last follow-up visit without local progression. The local progression-free (LPF) rates at 3 and 5 years were 90% (95% confidence interval [CI], 80%–99%) and 86% (95% CI, 74%–98%), respectively.

Of 5 patients who experienced local progression, 3 patients underwent 65 CGE/26 fractions, and 2 patients received 76 CGE/20 fractions of PRT. All 3 patients who received 60 CGE/10 fractions were free from local progression at 6, 30, and 51 months, respectively. LPF rates at 3 and 5 years for 46 patients who received 76 CGE/20 fractions were 97% (95% CI, 92%–100%) and 93% (95% CI, 83%–100%), respectively. LPF rates at 3 years for 11 patients who underwent 65 CGE/26 fractions of PRT were 56% (95% CI, 16%–95%) and was worse than that in patients who received 76 CGE/20 fractions with statistical significance ($p = 0.005$).

A total of 32 patients developed intrahepatic tumor recurrences that were outside of the PTV at 1 to 62 months (median, 20 months). Nine of these tumors occurred within the same segment of the primary tumor. Nodal recurrence at the hepatoduodenal ligament and distant metastasis were observed as the first sites of failure in 2 and 3 patients, respectively. In addition to the above-mentioned five deaths from PHI or postsurgical mortality, 4 patients died of hepatic failure because of underlying liver disease at 17 to 23 months, and 2 patients died from other reasons (comorbidity or senility) without evidence of HCC recurrence. Seven patients remained alive and disease free at 27 to 51 months (median, 30 months). The median survival time for all 60 patients was 41 months, and actuarial OAS rates at 3 and 5 years were 56% (95% CI, 43%–69%) and 25% (12%–39%), respectively. DFS rates at 3 and 5 years were 18% (95% CI, 7%–29%) and 4% (95% CI, 0%–12%), respectively, as shown in Fig. 4. Two Child-Pugh class A patients who underwent PRT with the CTV covering the entire right lobe or right anterior portal segment were alive and disease free at 50 and 26 months, respectively. The former patient had a pre-PRT ICG R15 of 22% and received a V30 of 42% and experienced transient PHI that resolved spontaneously; the latter patient, whose corresponding parameters were 8% and 37%, respectively, did not experience PHI.

Factor analysis

Univariate analyses revealed that factors related to functional liver reserve and occurrence of PHI had significant influence on OAS ($p < 0.05$). Liver function (Child-Pugh class A or B) and prior treatment (none or recurrent) were independent and significant prognostic factors ($p < 0.002$), and occurrence of PHI had marginal significance ($p = 0.011$) by multivariate analysis, as shown in Table 2. The DFS rate at 3 years for 24 patients who had no prior treatment for HCC was 35% (95% CI, 14%–56%), whereas DFS for the remaining 36 patients was 7% (95% CI, 0%–17%) ($p = 0.011$). In Child-Pugh class A patients, OAS at 3 and 5 years for those who had no prior treatment ($n = 17$) was 76% (95% CI, 56%–97%) and 59% (95% CI, 33%–86%), respectively, and 63% (95% CI, 45%–80%) and 25% (95% CI, 7%–42%), respectively, for 30 patients with recurrent tumor ($p = 0.060$). In Child-Pugh class B patients, the 2-year OAS for patients without PHI ($n = 5$) was 80% (95% CI, 45%–100%), while 8 patients who developed PHI died within 2 years with ($n = 5$) or without ($n = 3$) HCC recurrence ($p = 0.009$).

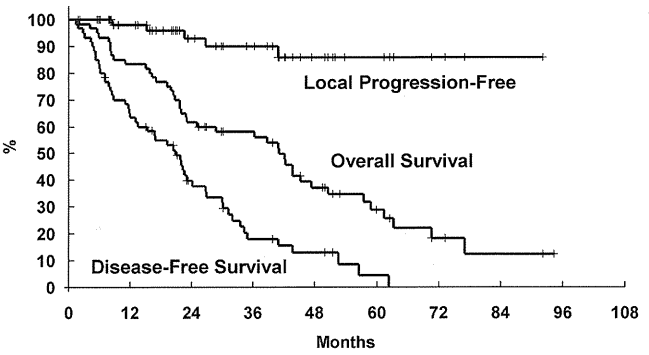

Fig. 4. Kaplan-Meier estimation of local progression-free survival, OAS, and disease-free survival rates for all 60 patients.

DISCUSSION

The promising tumoricidal effect of PRT using aggressive escalation of total and fractional doses, which has been repeatedly reported previously, was reproduced in this study (3, 4). The estimated actuarial local progression-free rate within the PTV in patients receiving 76 CGE/20 fractions exceeded 90% at 3 years. DFS at 3 years for patients who underwent PRT as an initial treatment ($n = 24$) was 35%, and, among them, OAS at 3 years was 76% in Child-Pugh class A patients ($n = 17$). These results are comparable to those observed after surgical treatment (17). Although the number of patients was small, these data indicate that appropriate local control with PRT may provide survival benefit in adequately selected patients with unresectable HCC. The fact that 9 of the 32 intrahepatic HCC recurrences occurred within the same anatomical portal segments showed that it should still be possible to improve the progression-free rate by defining the CTV so it covers undetectable tumor spread via the portal blood flow.

As shown in Fig. 3, no patient who had ICG R15 of less than 20% experienced PHI. In addition, only Child-Pugh class A patients with pre-PRT ICG R15 of less than 40% survived for longer than 4 years despite development of PHI. One of them underwent systematic portal segmental irradiation with the CTV covering the entire right lobe, and the details for this patient will be reported separately. On the other hand, all patients who had pre-PRT liver functions classified as Child-Pugh class B and/or ICG R15 of 40% or higher died within 2 years when they developed PHI. This suggests that the role of systematic portal irradiation requiring a large irradiated volume should be pursued further in Child-Pugh class A patients with favorable ICG R15 values; otherwise, the CTV should be confined to the GTV with adequate margins. Furthermore, in patients who have ICG R15 of 50% or

Table 2. Factors related to overall survival

Factor	No. of patients	% of OAS at 3 years (MST, months)	Univariate *p* value	Multivariate *p* value, hazard ratio (95% CI)
Age				
<70	29	55 (41)	0.660	0.087
≥70	31	61 (42)		0.52 (0.24–1.10)
Gender				
Male	42	62 (41)	0.332	0.194
Female	18	44 (42)		0.62 (0.29–1.30)
Tumor size (mm)				
<50	36	66 (44)	0.178	0.070
≥50	24	46 (23)		0.54 (0.28–1.05)
Pretreatment ICG R15				
<40%	45	67 (44)	0.002	
≥40%	15	33 (15)		
Child-Pugh classification				
A	47	68 (45)	<0.001	<0.001
B	13	23 (15)		0.19 (0.07–0.50)
Serum alfa-fetoprotein level (IU/mL)				
<300	41	61 (42)	0.617	0.618
≥300	19	53 (39)		0.83 (0.39–1.74)
PHI				
No	49	65 (44)	0.001	0.011
Yes	11	18 (9)		0.29 (0.11–0.76)
% of patients receiving V30				
<25%	40	57	0.724	
≥25%	20	60		
Total dose = 65 Gy				
Yes	11	44 (29)	0.646	0.185
No	49	61 (42)		1.88 (0.73–4.76)
Prior treatment				
None	24	67 (47)	0.112	0.002
Recurrence	36	53 (36)		0.32 (0.15–0.66)

Abbreviations: OAS = overall survival; MST = median survival time; CI = confidence interval; PHI = proton-induced hepatic insufficiency.

higher, the indication for PRT should be considered with extreme caution to prevent life-threatening PHI, as shown in Fig. 3.

Results of this retrospective study showed 56% OAS at 3 years in all patients and 68% in 47 Child-Pugh class A patients. All of them were judged strictly as unresectable and not amenable to local ablation. Therefore, a survival benefit of adding PRT to TACE could be expected, which should be tested in randomized trials. Suitable candidates for such a study may be patients who have unresectable HCC of >4 cm in diameter (*i.e.*, a high probability of microscopic vascular invasion) or who show macroscopic vascular invasion, which is amenable to selective segmental TACE as a curative treatment. Nevertheless, before developing that kind of randomized study, data should still be compiled regarding the safety and patterns of failure after PRT combined with TACE while ICG R15 and V30 are taken into account. Preliminary results of hypofractionated stereotactic body radiotherapy for patients with relatively small primary or metastatic liver tumors showed 70% to >90% of objective response rates and 20 or more months of median survival time (1, 18–20). Mature data regarding the relationship between oncological outcomes and tumor characteristics, as well as functional reserve of the liver, are needed to optimize cost-effectiveness of localized, high-dose RT using X-ray or charged particles for treatment of this disease. Nonetheless, RT should have no role in preventing multifocal tumorigenesis, which will be continuously encountered by multidisciplinary approaches (21).

The risk of developing serious gastrointestinal sequela after PRT is another important issue to consider in patients who have HCC located adjacent to the digestive tract. We attempted once-daily fractionation of PRT with 65 CGE/26 fractions. However, 2 of 11 patients who received this treatment developed gastrointestinal toxicity grade of ≥2. Moreover, these 11 patients showed significantly worse LPF rates than those who received 76 CGE/20 fractions of PRT. Three patients who received 60 CGE/10 fractions of PRT were controlled locally. Although our current data are based on a limited number of patients, precluding definitive conclusions, they suggest a low α/β ratio (22) of HCC, and this assumption should be examined further in clinical trials. Based on currently available data, efforts to exclude the gastrointestinal loop from the PTV by using, for example, surgical manipulations, seem to be positively considered in order to expand the role of PRT for HCC.

CONCLUSIONS

In conclusion, PRT achieved excellent local progression-free rates when aggressive, high-dose/fractionation was administered. Child-Pugh class A patients with ICG R15 of less than 40% tolerated PRT of a large irradiated volume well, despite development of transient PHI. However, in Child-Pugh class B patients, it seems reasonable to minimize the irradiated volume to prevent detrimental liver damage induced by PRT and underlying liver diseases. A V30 of less than 25% in the noncancerous portion of the liver is considered an indicator of the safety of PRT in patients who have pre-PRT ICG R15 of 20% to 50%. We believe that there are extremely few indications for PRT in patients who have ICG R15 of 50% or higher. Gastrointestinal toxicity is a major drawback of PRT for tumors adjacent to the gastrointestinal tract, and surgical manipulation to exclude the intestinal loop from the PTV should be positively considered as indicated. If these issues are carefully considered, with special attention to the patterns of tumor spread, when determining the

CTV, aggressive high-dose PRT could become a legitimate treatment for a certain population of patients with unresectable HCC for whom there is no standard treatment available other than TACE or liver transplantation.

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第7回 DIA 日本年会
グローバル開発における日本の役割・日本の貢献
大学・研究機関・企業での臨床試験の実際
国立がん研究センター東病院での
がんペプチドワクチン療法臨床試験の取り組み

独立行政法人国立がん研究センター東病院　がん治療開発部機能再生室長　中面　哲也

我々は，国立がん研究センターで医師主導の臨床研究として「がんペプチドワクチン（peptide vaccine）療法」を行っていますが，このような免疫療法はまだエビデンス（evidence）が確立されておらず，我々が臨床研究をどんどんやっていかないといけない分野だと思っています。その中から良いものがあれば製薬会社にぜひ拾っていただいて，開発していただきたいと思っております。全部最初から治験でやるというのは無理がありますので，我々のような者が医師主導の臨床研究を行って，いろいろな抗原をどんどんみつけて良いものを製薬会社の人に拾っていただき，治験として開発していただくというのが良いと思っております。なお，本日（2010.10.29）は，この後，癌治療学会ともブッキングしておりまして，パネルディスカッションは残念ながら出られないのですが，ご容赦ください。

先日の朝日新聞の記事にありましたように，がんワクチンがちょっと叩かれています。明日（2010.10.30）もがん免疫学会主催で緊急のシンポジウムが東京大学医科学研究所で開催されます。私はそちらでも話しますけれども，今日は20分の持ち時間なので，いろいろ話したいことはあるのですが，限られた時間内で，できるだけのことを話したいと思います。詳細は「臨床医薬」にも掲載されますし，西條長宏先生が監修された「Mebio」という雑誌の2010年12月号に特集が組まれますので，そちらもご覧いただければと思います。

私はもともと外科医で肝胆膵外科のレジデント（resident）をやっていたということもあり，国立がん研究センターに呼ばれて丸5年になります。江角先生に呼んでいただいたのですが，最初はこのGlypican-3（GPC3）のペプチドワクチンのフェーズⅠ（phase Ⅰ）を立ち上げるということで呼ばれました。国立がんセンターというところは化学療法が専門の先生が多いため，免疫療法を信用していない先生が多くて，かなり立ち上げに苦労しました。この臨床試験も，倫理審査委員会に出してから承認までに1年間かかりました。最近では西條先生はじめ諸先生方もペプチドワクチンをある程度評価してくださっていることから，がんセンターの中でも免疫療法の地位は確立できたと考えております。ただ，まだまだエビデンスが足りませんので，我々は一生懸命頑張っていきたいと思っています。

今日は主にこのGPC3についての発表になります。

GPC3というのは，もともと当時東京大学医科学研究所の中村祐輔先生のラボ（labo）のcDNAマイクロアレイ（microarray）のデータからみつけたものです。肝細胞がんの8割で高発現している遺伝子で，正常の臓器にはほとんど発現していない遺伝子としてみつけました。胎盤と胎生期の肝臓で発現している遺伝子で，胎盤と胎生期の肝臓は免疫学的に守られていますので，これがもし有望な抗原であれば，副作用が起きない理想的な抗原になるということでみつけました。蛋白レベルで免疫染色をしても肝細胞がんでは染まりますが胆管細胞がんでは

全く染まらず，きれいな特異性があり，胎盤と胎生期の肝臓ではヒトでもマウスでも同じように発現が認められます。

GPC3は，肝細胞がんでは8割の人が陽性なのですが，陰性の2割の人は予後が良くて，陽性の8割の人は予後が悪いという結果が得られています。

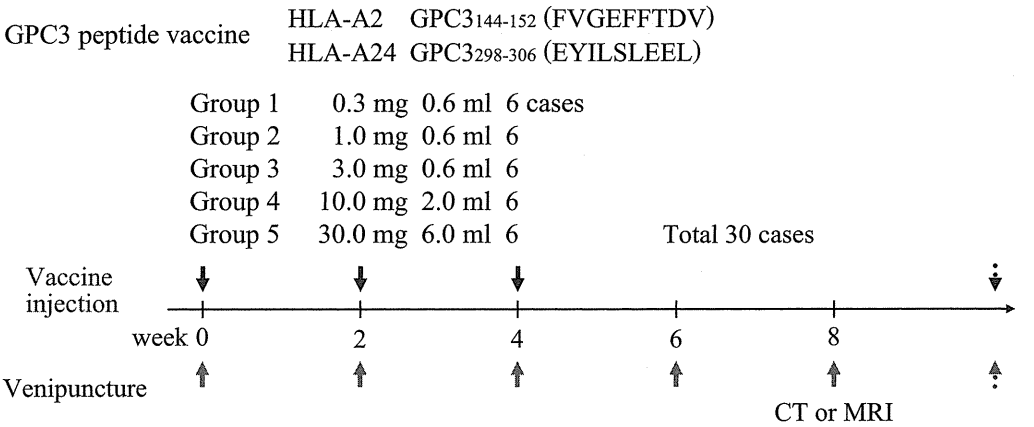
我々は熊本大学時代からこのGPC3におけるHLA-A24とHLA-A2，つまり，日本人に多いタイプのHLAに対するペプチドを同定して参りました。HLA-A24が日本人の6割で，HLA-A2は欧米白人においてメジャーなものですが，日本人の4割が陽性です。2つのペプチドを合わせると，日本人の85%に対応可能です。マウスの実験もいろいろとやり，ペプチドが有望で副作用を起こさない抗原であるということを証明してきました（**表1**）。

苦労した末に，2007年2月にスタートした臨床第1相試験は，進行肝細胞がんを対象としたスタディ（study）です。当初は再発予防のアジュバント・セッティング（aduvant setting）でやろうとしたのですが，「進行がんでやりなさい」ということで，進行がんでスタートしました。使っているのはこの2種類のペプチドで，あまり免疫療法には投与量は関係ないといわれていたのですが，抗がん剤に倣う形で，0.3mg，1 mg，3 mgとドース・エスカレーション（dose escalation）していく設定にしました。

3 mgが一番有望な結果が出ましたので，10mg，30mgを追加することになりましたが，**図1**の

表1　GPC3 is an ideal tumor antigen for immunotherapy in mouse models

We identified
HLA-A24 (*A*2402*)-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL),
HLA-A2 (*A*0201*)-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV),
Nakatsura T. *Clin. Cancer Res.* 10 : 8630-8640 2004.
Komori H. *Clin. Cancer Res.* 12 : 2689-2697 2006.
IFA is one of indispensable adjuvants for peptide-based immunotherapy, and the immunological effect of peptide vaccines depends on the dose of peptide injected.
Motomura Y. *Int. J. Oncol.* 32 : 985-990, 2008.



- The principal endopoints : toxicity and immunological responses
- The secondary endopoint : clinical responses.
  Clinical responses at 2 months after 1st vaccination (RECIST criteria)
  Monitoring the level of serum tumor markers

図1　Phase I clinical study of GPC3 derived peptide vaccine

量をみると分かるように，3 mg まではペプチドが溶けるのですが 30mg となるとペプチドは溶けないので，混ぜる IFA（Incomplete Freund's adjuvant：不完全フロイントアジュバント）の量も 3 倍，10 倍になりました。6 cc の中には 3 cc の IFA が入っていますので，30mg の場合は IFA の副作用が 10 倍高いということになります。進行がんでフェーズⅠですので，限られた設定で 2 週間に 1 回，3 回ワクチンして，2 ヵ月間で評価するという設定をとらざるを得ませんでしたが，患者さんの QOL（Quality Of Life）がかなり良かったものですから，「もっと打ってくれ」という患者さんの要望が強くなりました。そこで，最初のころの前半の患者さんは継続投与ができなかったのですが，あとで倫理審査委員会に承認していただいて，後半の 12 人は継続投与が可能になっています。フェーズⅠのエンドポイント(end point)は，安全性と免疫学的な有効性，そして二次的にクリニカル（clinical）なレスポンス（response）をみるということです。

ペプチドワクチンは「何で効くのか」というメカニズム（mechanism）なのですが，まず肝細胞がんの HLA（Human Leucocyte Antigens：ヒトリンパ球抗原）class1 には――すべての細胞に HLA class1 が出ています――**図 2** で示す GPC3 のペプチドが出ています。通常はキラー T 細胞が GPC3 のペプチドを見分けて殺しているはずなのですけれども，その機構が破綻しているのが，がん患者さんにあらわれているがんの塊です。正常細胞にはこの GPC3 のペプチドは出ていません。このペプチドは 9 アミノ酸が繋がったものですから人工的につくることができ，GMP（Good Manufacturing Practice：優良医薬品製造基準）グレードのものをつくってもらい使っております。それを IFA と混ぜて白い液を患者さんの脇の下の皮膚，皮内に注射しています。皮内にはランゲルハンス（Langerhans）細胞というプロフェッショナル（professional）抗原提示細胞がたくさんおり，ふだんはこのランゲルハンス細胞は自分の細胞の中でできたペプチドを出しているのですが，大量に GPC3 のペプチドを打ちますと，これが置きかわって，GPC3 ペプチドを乗せたランゲルハンス細胞がたくさんでき上がります。それが脇の下のリンパ節に移動して，GPC3 のペプチドを認識する T-cell receptor（T 細胞抗原受容体）を持つ CD8 陽性のキラー T 細胞（Killer T cell）を活性化して増やすというメカニズムになっており，増えたキラー T 細胞は GPC3 のペプチドを出している肝臓がんの細胞だ

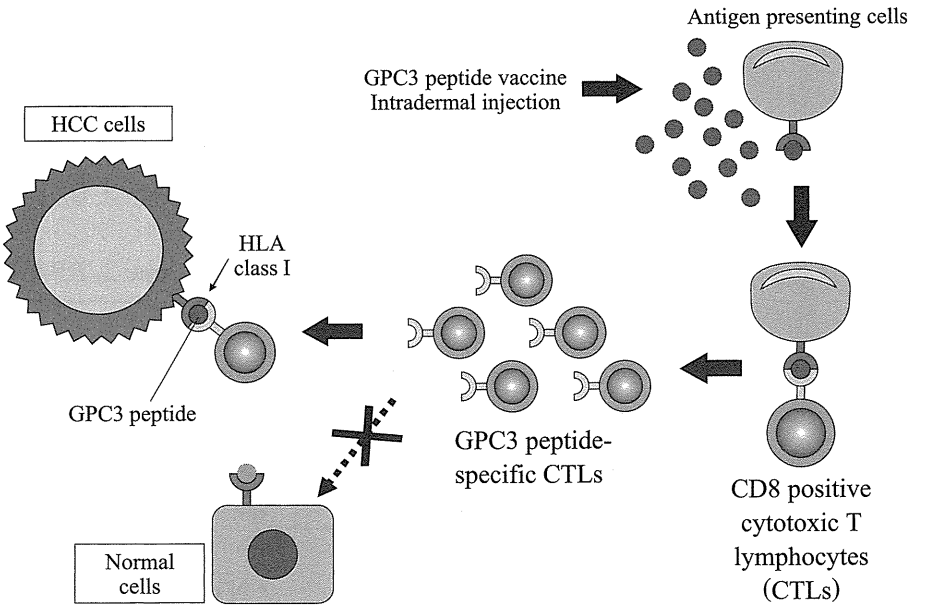


図 2　The mechanism of inducing tumorspecific CTLs by peptide vaccine

けを殺して，正常は殺さないという仕組みです。

今回のフェーズⅠは，33人がエンロール（enroll：登録）して，3人は3回ワクチンの2ヵ月が完遂できませんでしたので，今回は完遂できた30人での解析結果を示します（**図3**）。

対象は，比較的肝機能の良い人が入っているのですけれども，半分はステージ4（Stage 4）の遠隔転移のある患者さんで，半分は肝臓のがんが大きくて治療できない，有効な治療がないという患者さんが入っております。HLA-A24とA2は半分ぐらいが入っていまして，前治療は様々で，いろいろな治療が抵抗性になった患者さんが30人入っています（**表2**）。

GPC3の免疫染色とHLA class1の免疫染色における30人の内訳ですが，8割以上の人がGPC3も発現していて，HLA class1も発現しているという患者さんたちです（**表3**）。

まず安全性ですが，DLT（Dose-Limiting Toxicity：用量規制毒性）は一例も出現しませんでした。グレード2までの副作用でおさまっています。全員に起こるのが，投与局所の発赤，

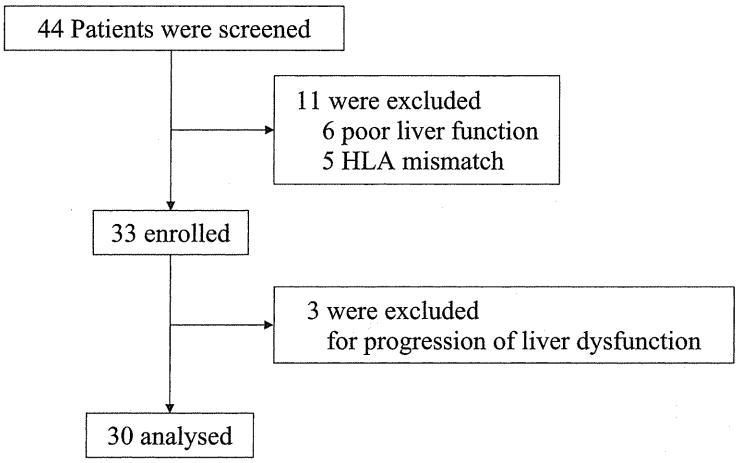


図3 Patient's Enrollment

表2 Patient characteristics

Age : 42-77 (mean 64.83) Sex M 25 F 5
PS 0 : 29 1 : 1 Child-Pugh A : 25 B : 5
Stage Ⅱ : 5, ⅢA : 6, ⅢC : 4, Ⅳ: 15
HCV : 14, HBV : 7, NBNC ; 9
HLA-A24 :16, HLA-A2 :14 (0201 : 11, 0206 : 3, 0207 : 2)
Prior therapy
TACE 8, TAI 6, Chemotherapy 7, RT 5, ope 2, RFA 1, none 1

表3 Immunohistochemical analysis of GPC3 and HLA class I

• Glypican-3 (GPC3)	• HLA class I
• ++ : 5 (19.2%)	• ++ : 3 (11.5%)
• + : 16 (61.5%)	• + : 20 (76.9%)
• − : 5 (19.2%)	• − : 3 (11.5%)
• NT : 4	• NT : 4
• Positive rate 80.7%	• Positive rate 88.4%

硬結で，これはグレード1で起こります。打ったときの一過性の発赤ですが，これは7割の人に認められ異所性の発疹が2人に認められました。37度5分以上の熱は一過性の熱なのですが，30人中6人に認められ38度以上は3人でした。いずれも解熱鎮痛剤を使用しないで，一過性に自然解熱しており，かゆみが5例あり，この5人には抗ヒスタミン剤の塗り薬を処方しています（**表4**）。

次に免疫学的な有効性について述べます。ペプチドを打つと本当にペプチド特異的キラーT細胞はできるのかというところがこの試験の大きなポイントで，エクスビボ（ex vivo：生体外）のIFN-γ（Interferon-gamma：インターフェロンガンマ）のエリスポット・アッセイ（ELISPOT assay：測定法）とデキストラマー（Dextramer）を使ったフローサイトメーター（flow cytometer）の解析を行いました。HIV（Human Immunodeficiency Virus）のエイズウイルスのペプチドに対するキラーT細胞は我々の体にはいませんので，これがネガティブコントロール（negative control）となります。打ったGPC3のペプチドに対するキラーT細胞はどれぐらいいるかというアッセイ（assay）ですが，この30mgを3回打った患者さんでは，投与前は血液中の50万個のリンパ球の中には1個もペプチド特異的キラーT細胞はいないのですが，2回ワクチンを打った後に50万個中441個，0.09%のペプチド特異的なキラーT細胞が出現しました。フローサイトメーターでも同じで，この0.1%というのはどのくらいインパクトを持つかといいますと，全身で1兆個のリンパ球がいますけれども，10億個のペプチド特異的なキラーT細胞が，わずか2回か3回のワクチンで誘導されたことになります。

全員でこの解析をやり，ほとんどの患者さんでワクチン前に比べてワクチン後にペプチド特異的なキラーT細胞の数が増えています。しかも，その投与量の依存性が確認されていて，投与量が増えれば増えるほど，最高の数が**図4**のように増えていっているということが分かります。

平均と中央値をとってみても，0.3mgのワクチンよりも3 mgのワクチンが良いということは明らかで，「30mgは意味があるか」というのは議論があるところでした（**図5**）。

もう1つ，今回のエビデンスとしては生検はしづらいのですが，7人の患者さんからワクチン後に腫瘍の生検をさせていただきました。そうしますと，7人中5人でワクチン前のがんの中には茶色に染まったキラーT細胞はほとんどいないのですが，ワクチン後にがんの中にキラーT細胞がたくさん入っている像が確認できました。

臨床効果ですけれども，ネクローシス（necrosis）が起こった患者さんの例では，この患者さんでは一番大きい腫瘍に真っ黒い壊死が起こり，こちらの患者さんの場合たくさんの肝細胞がんの中に壊死が起こりました。

次に縮小した症例ですけれども，肺門のリンパ節が縮小した症例と，胸部のリンパ節が縮小した症例があります。

表4 Toxicity

Adverse Event (CTCAE v3.0)	Grade 1	Grade 2
Allergic reaction (Flushing or Rash) 24 (80%)	transient flushing 22 (73.3%)	ectopic rash or flushing 2 (6.7%)
Allergic reaction (Drug fever) 6 (20%)	drug fever of ＜38°C 3 (10%)	drug fever of ≧38°C 3 (10%)
Injection site reaction 30 (100%)	erythema ; induration 30 (100%)	pain or swelling with inflamation 0
Itching 5 (16.7%)	mild or focal itcing 5 (16.7%)	severe or wide itcing 0

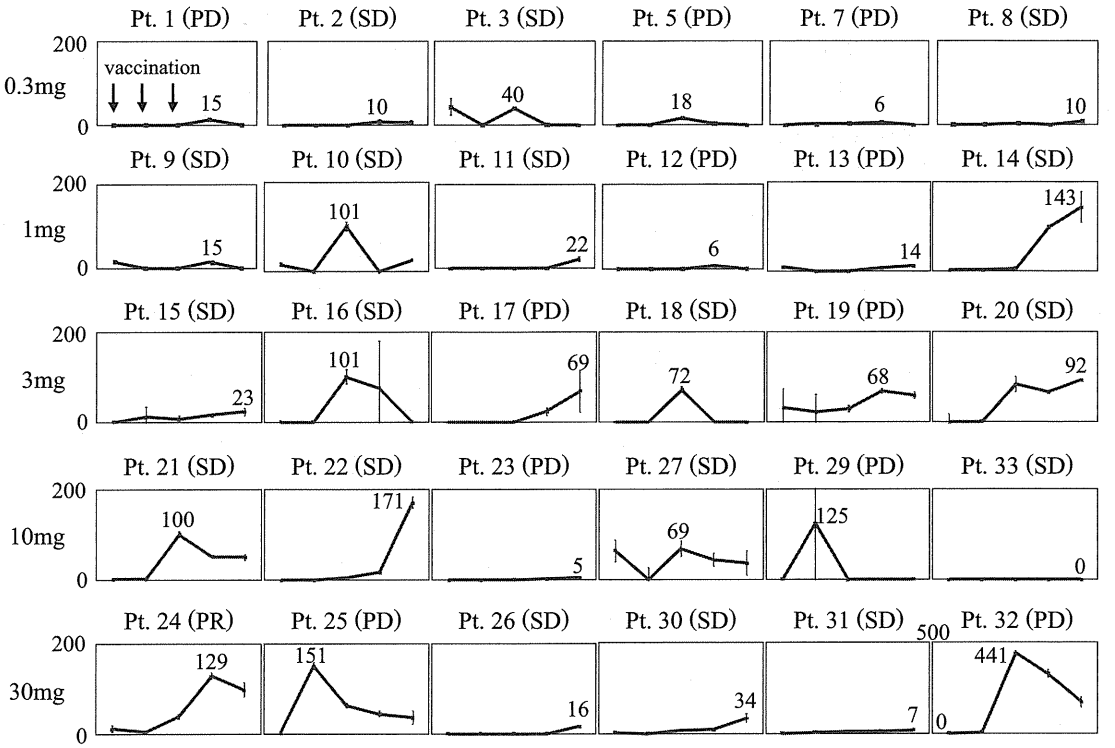


図 4　Immunological responses (1)

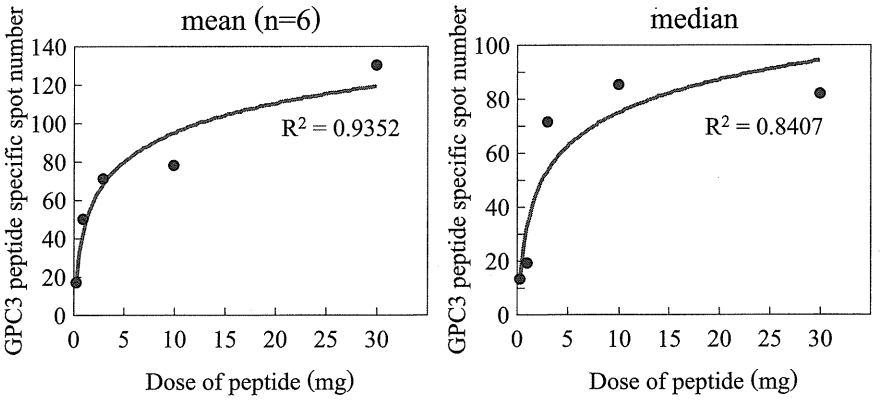


図 5　Immunological responses (2)

次に一番レスポンスがあった PR（Partial Response：部分寛解）の患者さんの結果を示します。ワクチン前の状態で，多発の肝細胞がん，骨転移，肺転移，リンパ節転移の患者さんが 30mg を 3 回打った結果，5.5cm の骨転移が大幅に縮小して，別の骨転移も真っ黒く壊死になってしまいました。1.4cm の肝臓内の腫瘍 2 個は完全に消えて，エコー（echo：超音波検査）でもみえなくなりました。1.4cm の腫瘍が，CT（Computed Tomography）上は同じ大きさで残っているようにみえるのですが，ここを生検させてもらうと，何とがんの中の半分はキラー T 細胞という状態で，がんの量としては半分になっていることが分かりました。

表 5 が臨床効果のまとめです。RECIST criteria（Response Evaluation Criteria In Solid Tumors によるガイドライン）で 1 例の PR と，2 ヵ月間で 12 例の SD（Stable Disease：不変）ということになっています。ネクローシスか，サイズの縮小がみられた患者さんが 30 例中 5

表5　Clinical responses at 2 months after 1st vaccination (RECIST criteria)

peptide dosage	Clinical response				necrosis or size reduction of tumors	decrease tumor markers in the sera
	PR	SD	PR+SD	PD		
0.3mg	0	3	3 (50.0%)	3	1	1/6 (16.7%)
1.0mg	0	4	4 (66.7%)	2	0	4/6 (66.7%)
3.0mg	0	4	4 (66.7%)	2	3	5/5 (100%)
10mg	0	4	4 (66.7%)	2	0	6/6 (100%)
30mg	1	3	4 (66.7%)	2	1	6/6 (100%)
total	1	18	19 (63.3%)	11	5 (16.7%)	22/29 (75.9%)

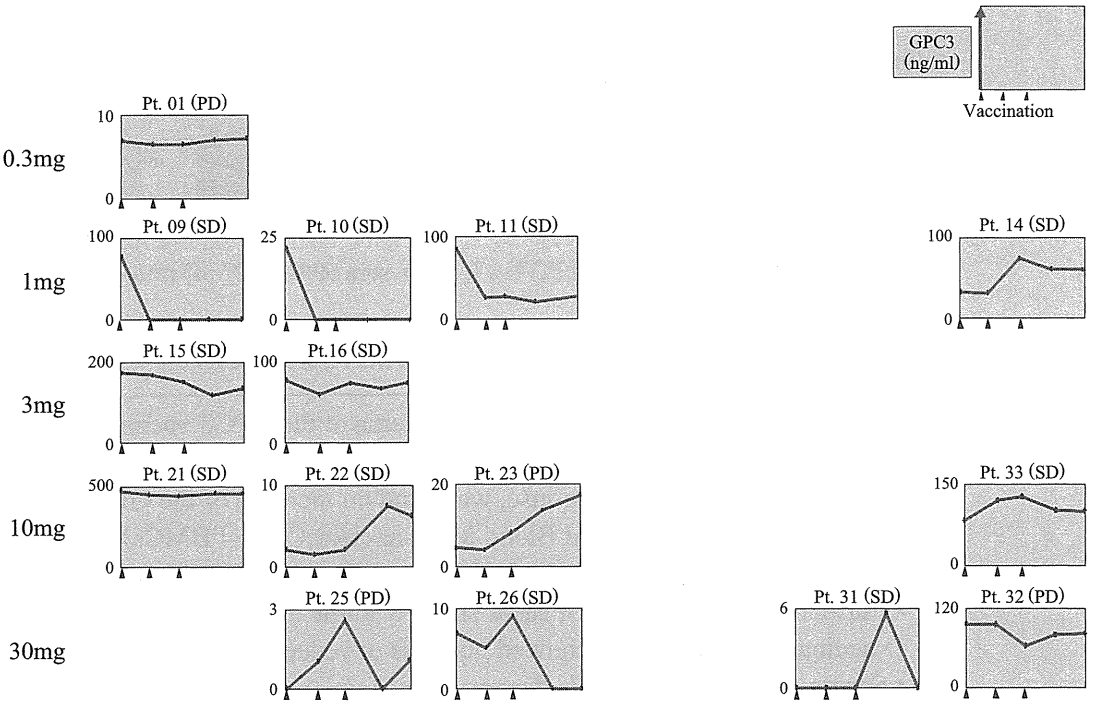


図6　Decrease of GPC3 in the sera 100% 15/15 SD rate：73% 11/15

例で，AFP（Alpha Fetoproteins：癌胎児性蛋白），PIVKA-Ⅱ（Protein Induced by Vitamin K absence or Antagonist-Ⅱ：ビタミンK依存性凝固因子前駆体Ⅱ），GPC3の腫瘍マーカー（tumor marker）が2ヵ月の間に1回でも下がった患者さんは76%という結果です。

GPC3は腫瘍マーカーにもなるのですが，投与前に陽性だった15例全員がワクチン期間中に1回はGPC3が下がり，その下がった人のSD率は73%ですので，全体のSD率63%より高いということで，バイオマーカー（Biomarker：生物指標化合物）にもなり得ると考えています（図6）。

PIVKA-Ⅱも同じで，6割の人がワクチン投与によって下がったのですが，下がった人は75%がSDになっています（図7）。

今回投与量は0.3mgから30mgまで，3回しか打っていない人が18人含まれていますので，全く意味がないのですが，overall survival（OS：全生存期間 9ヵ月）はソラフェニブ（sorafenib：複数のキナーゼ阻害薬）のフェーズⅡの結果と比べると，遜色ない結果が得られています。本当に進行がんの方の予後を延ばせるのかというのは，今後のフェーズⅡとしての