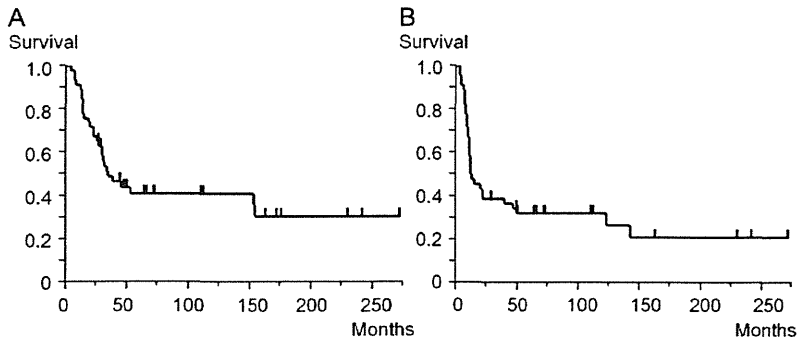

Figure 2. Disease-specific (A) and recurrence-free (B) survival for the patients overall analyzed by the Kaplan–Meier method.

Table 2. Univariate analyses of variables predicting disease-specific and recurrence-free survival

	P value	
	DSS	RFS
Extent of LN dissection		
Below vs. above iliac bifurcation	0.2042	0.1590
Histological type		
Mixed vs. pure UC	0.6140	0.7633
pT		
pT3–4 vs. pT2	0.1097	0.2830
pN		
pN2–3 vs. pN1	0.0224	0.0732
Total number of involved LNs (continuous)	0.0008	0.0018
Number of nodes resected (continuous)	0.9555	0.6407
LN density (continuous)	0.0112	0.0128
Maximum diameter of metastatic lesion in LN (continuous)	0.8079	0.8015
ENI of LN metastases		
Positive vs. negative	0.0338	0.0767
Adjuvant chemotherapy		
Yes vs. no	0.5230	0.0828

DSS, disease-specific survival; RFS, recurrence-free survival; UC, urothelial carcinoma.

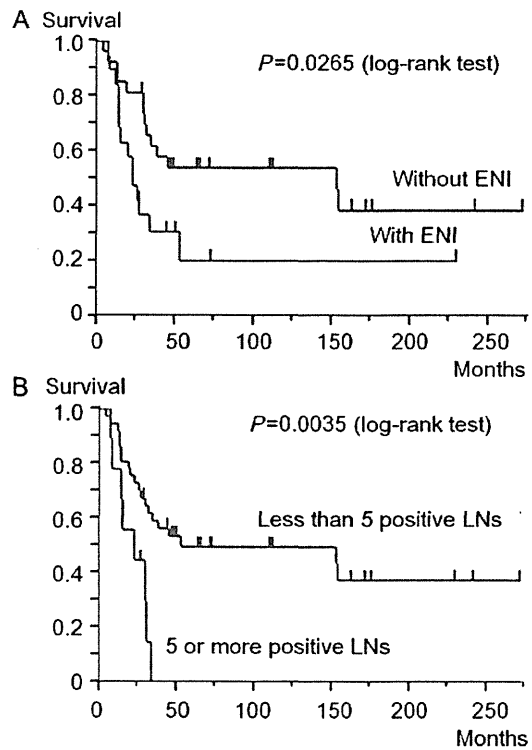

Figure 3. Disease-specific survival of patients with and without extranodal invasion (ENI) (A) and that of patients with fewer than five involved LNs or with 5 or more (B), analyzed by the Kaplan–Meier method.

were statistically significant variables predictive of DSS (Table 3). For RFS, the total number of involved LNs was the only variable showing a statistically significant correlation (Table 3).

Thus, ENI, the total number of positive nodes and the diameter of metastatic lesions were independently associated with DSS. Interestingly, the diameter of metastatic lesions was inversely correlated with poorer survival: some patients had a good outcome despite having sizable metastatic nodes. In fact, those with ENI-negative, large (>10 mm) metastatic nodes (seven patients) showed favorable clinical outcome. They showed better DSS and RFS than those with ENI-negative, multiple small metastatic lesions (<10 mm) (eight patients) ($P = 0.0156$ for DSS and $P = 0.0483$ for RFS). They also showed better DSS and marginally better RFS than those with ENI-positive nodes (19 patients) ($P = 0.0181$ for DSS and $P = 0.0527$ for RFS) (Figs 4 and 5).

DISCUSSION

The presence of histologically proven LN metastases is an adverse prognostic factor in patients who undergo radical cystectomy for bladder cancer with curative intent. However,

Table 3. Multivariate analyses of variables predicting disease-specific and recurrence-free survival

	Disease-specific survival		Recurrence-free survival	
	Risk ratio (95% CI)	*P* value	Risk ratio (95% CI)	*P*-value
Total number of involved LNs (continuous)	1.51 (1.21–1.89)	0.0003	1.31 (1.11–1.52)	0.0018
Maximum diameter of metastatic lesion in LN (continuous, unit: mm)	0.91 (0.84–0.99)	0.0214		
ENI				
Positive vs. negative	2.50 (1.02–6.09)	0.0445		

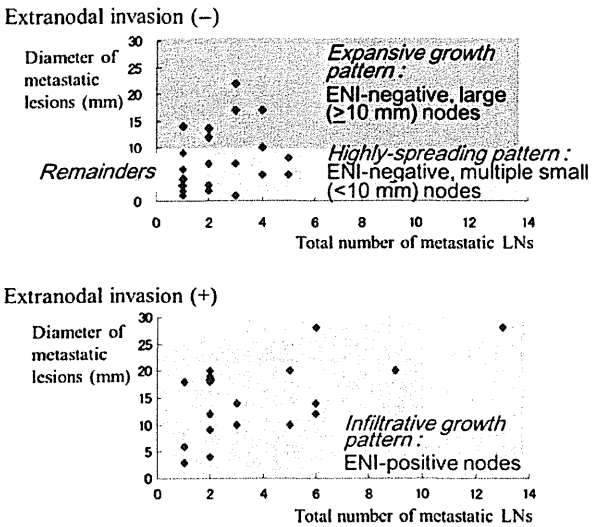

Figure 4. Distribution of the diameter of metastatic lesions and the number of involved LNs in the 27 patients with LN metastases without ENI and in the 19 patients with ENI.

the clinical outcome of patients with LN metastases is variable, and several clinicopathological factors have been reported to further stratify the risk of recurrence and death in such patients.

In our present study, the number of metastasis-positive nodes was the most significant variable predictive of poor survival. Patients with five or more positive nodes had a significantly worse outcome than those with fewer than five positive nodes ($P = 0.0035$ for DSS and $P = 0.0006$ for RFS). Previous studies have also reported similar data using cut-off points of 5, 6 and 8 for the number of LNs in order to discriminate groups with good or poor prognosis (3–6).

ENI is reported to represent an aggressive feature of metastatic cancers and to be a significant predictor of poorer outcome in cancers of the kidney, breast, lung, colon, head and neck, penis and ampulla of Vater (18–24). Reports examining the significance of ENI in bladder cancer are limited, and their results have been inconsistent. Three papers from a single center (the University of Bern, Switzerland) have suggested that ENI is the strongest independent prognostic factor for DSS (12–14). Two other studies reported that ENI was a significant prognostic factor for DSS in univariate analysis, but did not retain its significance in multivariate analysis (6,25). A paper from the M.D. Anderson Cancer Center reported that ENI was not associated with DSS ($P = 0.43$) or RFS ($P = 0.82$) (15). Thus, the conclusions have been inconsistent, and the prognostic significance of ENI in bladder cancers is still not fully understood. In the present study, ENI was detected in 19 (41.3%) of 46 node-positive patients, consistent with the reported incidence of ENI of 33–68% (6,12–15), and was associated with poorer prognosis of node-positive patients in both univariate and multivariate analyses.

It was somehow surprising that the diameter of metastatic lesions was inversely associated with poorer DSS. We did experience several patients with sizable nodal metastases who were cured with surgery alone or showed an indolent clinical course after tumor recurrence. LN diameter was significantly larger in ENI-positive cases than in ENI-negative ($P = 0.0002$, Mann–Whitney's test), and we focused attention on the outliers of this association; seven cases with ENI-negative, large (>10 mm) metastatic nodes. In fact, these seven cases showed favorable DSS, irrespective of the number of metastasis-positive nodes (Fig. 5). This type of tumors may have limited invasive potential and do not extend beyond the nodal capsule, thus being ENI-negative. In addition, they may have low metastasis potential, and consequently show local proliferation, resulting in a larger metastasis diameter (expansive growth pattern, Fig. 4). In contrast, patients with ENI-positive nodes (19 patients) had dismal clinical outcome (Fig. 5). ENI-positive nodes may represent aggressive invasive potential, resulting in the destruction of the LN capsule and invasion into surrounding tissues (infiltrative growth pattern, Fig. 4). There was a significant ($P < 0.0001$) positive correlation between diameter and total number of positive nodes with a Spearman correlation coefficient of 0.5617. Again, we focused attention on the outliers of this association. As shown in the upper panel of Fig. 4, eight cases had multiple small (<10 mm) LN metastases without ENI. These eight cases also showed poor outcome (Fig. 5). Multiple small LNs may represent spreading ability of cancer cells via lymphatic vessels before they form large metastatic lesions in LNs (highly spreading pattern, Fig. 4). Remainders (single small LN metastasis

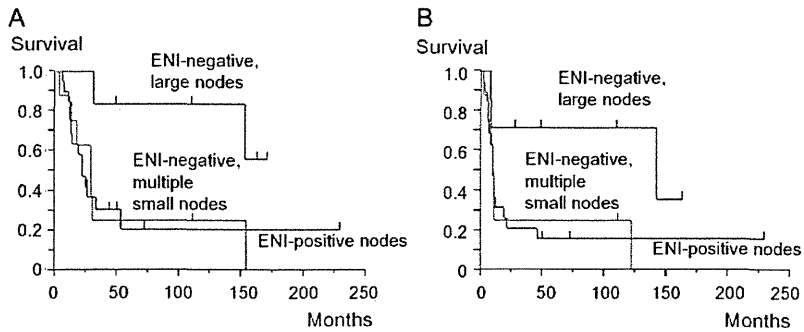

Figure 5. Disease-specific (A) and recurrence-free (B) survival stratified according to LN metastasis patterns, analyzed by the Kaplan–Meier method.

without ENI) were in the early phase of either of the three categories (12 patients).

Our data show that the clinical outcome of node-positive patients is not simply stratified according to well-accepted variables, such as the number of positive nodes, that reflect the tumor burden. It is also affected by tumor biology including properties such as invasiveness and metastasis potential, which is reflected in pathological characteristics such as ENI and the diameter of nodal lesions.

In conclusion, the present study has shown that the total number of involved nodes, ENI and the diameter of metastatic lesions are significantly associated with DSS of patients with node-positive urothelial bladder carcinoma. Differences in the characteristics of metastatic nodes such as expansive growth/infiltrative growth/highly spreading pattern would result in the variable clinical outcome. Even the patients with large LN metastases may benefit from meticulous LN dissection if the tumor showed expansive growth pattern.

Acknowledgement

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Conflict of interest statement

None declared.

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Identification of an H2-K^b or H2-D^b restricted and glypican-3-derived cytotoxic T-lymphocyte epitope peptide

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Abstract. Glypican-3 (GPC3) is overexpressed in human hepatocellular carcinoma (HCC) but not expressed in normal tissues except for placenta and fetal liver and therefore is an ideal target for cancer immunotherapy. In this study, we identified an H2-K^b or H2-D^b restricted and murine GPC3 (mGPC3)-derived cytotoxic T-lymphocyte (CTL) epitope peptide in C57BL/6 (B6) mice, which can be used in the design of preclinical studies of various therapies with GPC3-target immunotherapy *in vivo*. First, 11 types of 9- to 10-mer peptides predicted to bind with H2-K^b or H2-D^b were selected from the mGPC3 amino acid sequence based on the binding score as calculated by the BIMAS software. We evaluated the peptide-binding affinity and confirmed that all peptides were able to bind to H2-K^b or H2-D^b by *in vitro* cellular binding assay. Subsequently, a mixed peptide vaccine and single peptide vaccine were given to B6 mice to evaluate immunogenic potential of the 11 selected peptides. Using the splenocytes from peptide-vaccinated mice, interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays showed that mGPC3-1$_{127\text{-}136}$ (AMFKNNYPSL) peptide was the most efficient for inducing CTLs among the 11 peptides. Next, we demonstrated that the mGPC3-1 peptide-specific CTL line could recognize mGPC3-expressing cancer cells, suggesting that mGPC3-1 peptide was an endogenously presented peptide. In conclusion, we identified mGPC3-1 as an H2-K^b or H2-D^b restricted, mGPC3-derived CTL epitope peptide.

Introduction

Liver cancer ranks fifth in frequency in the world and is the third most common cause of lethal cancer (1). Liver cancer consists of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), with HCC as the most common. Regarding HCC therapy, hepatectomy, percutaneous local therapy and transcatheter arterial embolization (TAE) are common, but the recurrence rate with conventional therapies for advanced HCC patients is still high (2). Therefore, developing a novel curative therapy or an effective adjuvant therapy for HCC is important.

Recently, immunotherapy, which consists of a peptide vaccine, protein vaccine, or DNA vaccine, has become a potentially promising option for HCC (3,4). Many tumor antigen-derived peptides recognized by cytotoxic T-lymphocyte (CTL) have been identified (5). However, to date, vaccine therapy using these peptides has not proven adequate antitumor efficacy in clinical trials for advanced HCC patients (6-8).

In HCC, glypican-3 (GPC3) is overexpressed and is not expressed in normal tissues except for the placenta and embryonic liver (9). Hence, GPC3 is a novel target molecule in HCC patients. GPC3 is a member of the heparan sulfate proteoglycan family and the glypican family regulates cell growth and division through Wnt signaling, Hedgehogs, fibroblast growth factors and bone morphogenetic proteins (10-12). We previously identified HLA-A*24:02-restricted GPC3$_{298\text{-}306}$ (EYILSLEEL) and HLA-A*02:01-restricted GPC3$_{144\text{-}152}$ (FVGEFFTDV) peptides and showed that both peptides can induce GPC3-specific CTLs without an autoimmune response (13,14). Clinical trials of a GPC3-derived peptide vaccine for HCC patients are currently in progress. The phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC showed safety as well as immunological evidence and potential for improving overall survival (15-17). The phase I clinical trial suggested that the GPC3-derived peptide vaccine could be an attractive approach for treatment of HCC, however, the effect of tumor reduction was limited. Therefore, further studies are needed to enhance the effect of GPC3-targeted immunotherapy and to establish a GPC3-specific CTL-inducible mouse model. We previously conducted a preclinical study of the GPC3-derived peptide vaccine using HLA-A2.1 transgenic mice (18). The treatment model experiment using HLA transgenic mice is limited.

Mice with the C57BL/6 (B6) background have been reported to spontaneously develop liver cancer (19,20). Recently, the NASH mouse model (named STAM mice C57BL/6N-NASH), which had a B6 background and spontaneously developed liver

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Key words: glypican-3, cytotoxic T-lymphocyte epitope, hepatocellular carcinoma, mouse

cancer, was exploited by Stelic Institute & Co. In this mouse model, the cancer incidence rate is high and cancer incident time is short, thus, STAM mice C57BL/6N-NASH is an attractive model for studying GPC3-targeted therapy for HCC. Therefore, identification of a mouse major histocompatibility complex (MHC) class I epitope peptide to induce GPC3-specific CTL was needed for establishment of the appropriate mouse model.

Strategies to identify epitope peptides have previously been reported (21-24). A summary of our strategy follows. First, peptides binding MHC class I epitope were predicted from antigen amino acid sequences *in silico* by prediction software and the ability of the predicted peptides to bind MHC class I was confirmed *in vitro* by a binding assay. Then, the immunogenic potential of the predicted peptides was examined by *in vivo* immunization or *in vitro* stimulation. Lastly, whether peptides that have immunogenic potential are presented by cells endogenously expressing the antigen was confirmed. In summary, we identified peptides with immunogenic potential that were presented by cells endogenously expressing the antigen. We attempted to identify H2-K^b or H2-D^b restricted, GPC3-derived CTL epitope peptides in C57BL/6 mice based on the above strategy.

Materials and methods

Mice. C57BL/6 (B6) mice were purchased from Charles River Laboratories Japan, Inc. and STAM mice C57BL/6N-NASH were a gift from this company. Mice were maintained under the institutional guidelines set by the Animal Research Committee of the National Cancer Center Hospital East. Mice were housed in specific pathogen-free (SPF) conditions with a 12-h light cycle and food and water at *ad libitum*. Six to eight-week-old female B6 mice were used in all experiments and STAM mice C57BL/6N-NASH were provided with a very high-fat rodent diet (rodent diet with 60% kcal% fat, Research Diet Inc.). All animal procedures were performed according to the guidelines for Animal Research Committee of the National Cancer Center, Japan.

Cell lines and transfection. B6 thymoma RMA and RMA-S cell lines, which have H2-K^b and -D^b as MHC class I epitopes, were maintained in our laboratory. RMA-S is an antigen processing-defective cell line and the cells cannot present endogenous antigens with MHC class I epitopes (25). To obtain RMA transiently expressing murine GPC3 (RMA-GPC3-puro), RMA (GPC3-negative) was transfected with pCAGGS-mGPC3-internal ribosomal entry site (IRES)-puromycin-resistant (puro-R) using Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocols. As negative control, RMA, which was transfected with pCAGGS-IRES-puro-R in a similar way, was named RMA-puro. Expression of murine GPC3 (mGPC3) in RMA-GPC3-puro or RMA-puro was confirmed by reverse transcription polymerase chain reaction (RT-PCR). All cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco).

RT-PCR. Total ribonucleic acid was isolated from RMA-GPC3-puro or RMA-puro homogenized with the TRIzol Reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's protocols. The first-strand complementary deoxyribonucleic acid (cDNA) was synthesized with a PrimeScript® II 1st strand cDNA Synthesis kit (Takara Bio Inc., Japan), then mGPC3 was amplified using a Takara PCR Amplification kit (Takara Bio Inc.). The amplification protocol was as follows: 150 sec at 94°C for initial denaturation, 35 amplification cycles at 58°C for 40 sec and 72°C for 40 sec, followed by a final extension at 72°C for 5 min. The primer sequences for mGPC3 were as follows: sense, 5'-ACGGGATGGTGAAA GTGAAGA-3' and antisense, 5'-GAAAGAGAAAAGAGGGA AACA-3'. The primer sequences for β-actin were as follows: sense, 5'-GAGCAATGATCTTGATCTTCAT-3' and antisense, 5'-TCCATCATGAACTGTGACGT-3'. PCR products were visualized by ethidium bromide staining after separation on a 1% agarose gel. After normalization using β-actin messenger ribonucleic acid (mRNA) as a control, we compared the expression of mGPC3 mRNA.

Generation of bone marrow-derived dendritic cells (BM-DCs) from BM cells. BM cells (4x10^6) from B6 mice were cultured in RPMI-1640 containing FBS (10%), 2-mercaptoethanol (2-ME, 50 μM) and murine granulocyte macrophage colony-stimulating factor (mGM-CSF, 20 ng/ml) for 1 week.

Peptides. Eleven types of 9- to 10-mer peptides predicted to bind with H2-K^b or H2-D^b were selected from mGPC3 amino acid sequences (accession code AAH36126) based on the binding score as calculated by BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, USA) and 11 synthetic peptides (custom ordered) were purchased from Scrum Inc. (Tables I and II). The 11 amino acid sequences were as follows: mGPC3-1, AMFKNNYPSL; mGPC3-2, SLFPVIYTQM; mGPC3-3, LFPVIYTQM; mGPC3-4, KSFINFYSAL; mGPC3-5, LTARLNMEQL; mGPC3-6, LGSDINVDDM; mGPC3-7, QYVQKNGGKL; mGPC3-8, YVQKNGGKL; mGPC3-9, DTLCWNGQEL; mGPC3-10, RNGMKNQFNL; mGPC3-11, MKNQFNLHEL. Each peptide was dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Japan) and each peptide's density was 10 mg/ml.

H2-K^b or H2-D^b binding assay. To evaluate the binding affinity of the predicted peptides to H2-K^b or H2-D^b molecules, an *in vitro* cellular binding assay was performed as previously reported (23,26). Briefly, after incubation of RMA-S cells in culture medium at 26°C overnight, cells (1x10^6) were washed with PBS and suspended in 100 μl Opti-MEM® (Invitrogen) with or without 10 μg peptide, followed by incubation at 26°C for 3 h and then at 37°C for 3 h. After washing with PBS, H2-K^b or H2-D^b expression was measured with a BD FACSCanto™ II flow cytometer (BD) using FITC-conjugated H2-K^b (BioLegend Inc., AF6-88.5) or H2-D^b (BioLegend Inc., KH95) specific monoclonal antibody and mean fluorescence intensity (MFI) was recorded. Percent MFI increase was calculated as follows: percent MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

Vaccination. The mixed peptide vaccine per mouse consisted of 5 μl mGPC3-1 to mGPC3-11 solution, 55 μl sodium bicar-

Table I. Synthetic peptides predicted to bind with H2-K^b.

	Peptide sequence (position)	Binding score[a]
mGPC3-1	AMFKNNYPSL (127-136)	52.8
mGPC3-2	SLFPVIYTQM (172-181)	44
mGPC3-3	LFPVIYTQM (173-181)	66
mGPC3-4	KSFINFYSAL (395-404)	40

[a]Binding scores were estimated by using BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

Table II. Synthetic peptides predicted to bind with H2-D^b.

	Peptide sequence (position)	Binding score[a]
mGPC3-5	LTARLNMEQL (82-91)	200
mGPC3-1	AMFKNNYPSL (127-136)	343.2
mGPC3-6	LGSDINVDDM (156-165)	260
mGPC3-7	QYVQKNGGKL (331-340)	720
mGPC3-8	YVQKNGGKL (332-340)	240
mGPC3-9	DTLCWNGQEL (418-127)	600
mGPC3-10	RNGMKNQFNL (437-446)	200
mGPC3-11	MKNQFNLHEL (440-449)	288

[a]Binding scores were estimated by using BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

bonate solution and 110 μl incomplete Freund's adjuvant (IFA). Single peptide vaccine per mouse consisted of 5 μl peptide, 45 μl sodium bicarbonate solution and 50 μl IFA. Each vaccine solution was emulsified. The mice were immunized by intradermal injection at the base of the tail every 7 days for a total of two vaccinations. Similarly, STAM mice C57BL/6N-NASH were immunized seven times with the mGPC3-1 peptide vaccine.

Restimulation of splenocytes obtained from immunized mice. Seven days after the last immunization, splenocytes were collected and cluster of differentiation 8 (CD8) positive splenocytes were isolated by positive selection with anti-CD8 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. CD8-positive splenocytes were cocultured with BM-DCs pulsed with each peptide as previously described (13). Seven days after coculture, the detection of antigen-specific T cells producing interferon (IFN)-γ was performed using the BD ELISPOT kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocols.

Establishment of GPC3-1-specific CTL line. The GPC3-1-specific CTL line was established as previously described (27). Splenocytes (1x10^4) derived from B6 mice immunized with the GPC3-1 peptide vaccine were cocultured with B6-derived and irradiated (35 Gy) splenocytes (5x10^4) in RPMI-1640 contained with FBS (10%), sodium pyruvate (1 mM, Gibco), MEM non-essential amino acid solution (1X, Gibco) and 2-ME (50 μM). Seven days later, recombinant interleukin-2 (rIL-2, 50 U/ml, Nipro, Osaka, Japan) was added to the culture medium.

IFN-γ enzyme-linked immunospot (ELISPOT) analysis. IFN-γ ELISPOT assay was performed according to the manufacturer's protocols. Briefly, restimulated CD8-positive splenocytes (5x10^4) as target cells were added to the plate and then BM-DCs (5x10^4) pulsed with each peptide (10 μg/ml) as effector cells or non-pulsed BM-DCs (5x10^4) as control and target cells were added to the plate, which was then incubated for 20 h at 37°C, 5% CO_2. Using the GPC3-1-reactive CTL line (1x10^5) as effector cells, RMA-S (5x10^4) pulsed with each peptide (10 μg/ml) as target cells and non-pulsed RMA-S as control and target cells (5x10^4), the plate was incubated for 20 h at 37°C, 5% CO_2. Using the mGPC3-1-reactive CTL line (1x10^5) as effector cells, RMA-GPC3-puro as target cells (5x10^5) and RMA-puro (5x10^5) as control and target cells, the plate was incubated for 48 h at 37°C, 5% CO_2. The number of spots was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay. Cytotoxic activity against target cells was analyzed using the Terascan VPC system (Minerva Tech) as previously described (28). Target cells were incubated with calcein AM (Dojindo, Kumamoto, Japan) solution for 30 min at 37°C and labeled. Then the labeled cells were incubated with effector cells for 4 h. Fluorescence intensity was measured before and after the culture and specific cytotoxic activity was evaluated using the following formula: % cytotoxicity = {1- [(average fluorescence of the sample wells - average fluorescence of the maximal release control wells) - (average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]} x 100%.

Statistical analysis. Statistical analyses were performed with a Mann-Whitney U test (n=3). Significant differences were defined as $^*p<0.05$ or $R^2>0.5$.

Results

Evaluation of selected peptide-binding affinity to H2-K^b or H2-D^b. The selected 11 peptides derived from mGPC3 by the BIMAS software were evaluated by an *in vitro* binding assay to determine each peptide's binding affinity to H2-K^b or H2-D^b. The peptide with the highest binding affinity for H2-K^b was mGPC3-2 (percent MFI, 376.6%), followed by the mGPC3-3 peptide (128.0%) and the mGPC3-1 peptide (72.7%) (Fig. 1A). That for H2-D^b was mGPC3-10 peptide (539.1%) followed by the mGPC3-1 peptide (298.2%) and the mGPC3-8 peptide (191.1%) (Fig. 1B). These results show that all 11 peptides could bind H2-K^b or H2-D^b, although the binding score calculated by the BIMAS software did not correlate with the actual binding affinity (Fig. 1C and D).

Induction of CTL response against mGPC3-derived peptides in B6 mice. The vaccine schedule was performed as follows (Fig. 2A): At days 0 and 7, peptide vaccine was given. At day 14, primed mice were sacrificed and CD8-positive

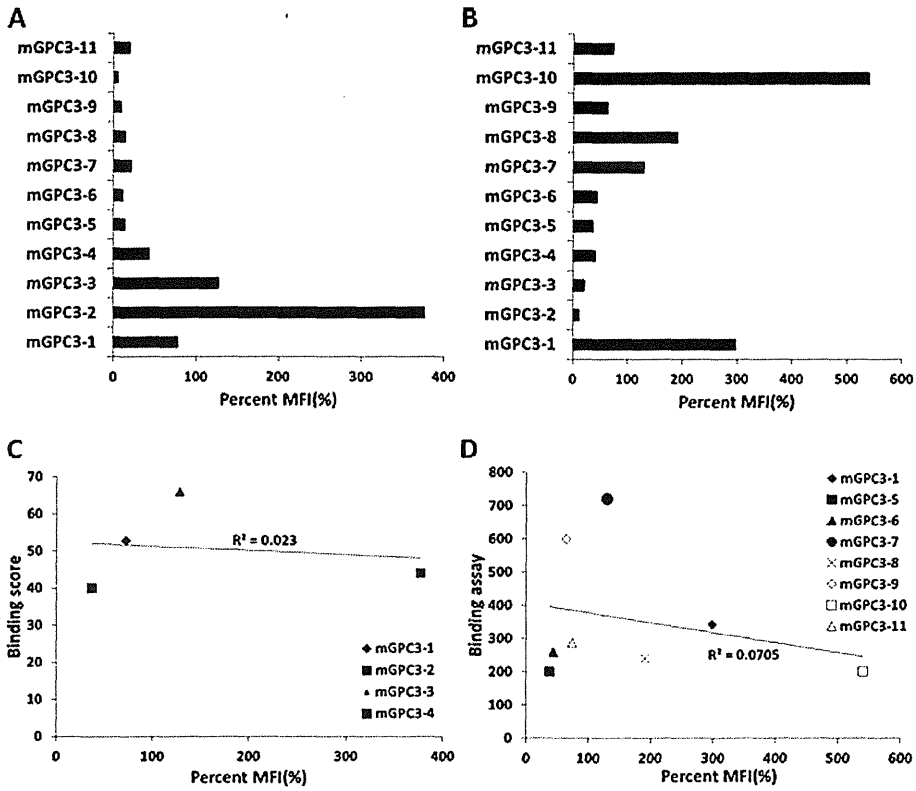

Figure 1. *In vitro* cellular peptide binding assays to H2-K^b (A) or H2-D^b (B) were performed using a FACS system. Comparison of BIMAS binding score with percent MFI for H2-K^b (C) or H2-D^b (D). Percent MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

splenocytes were collected. CD8-positive splenocytes were restimulated with BM-DCs pulsed with each peptide. At day 21, the peptide's immunogenic potential was evaluated by IFN-γ ELISPOT assay.

The mixed peptide vaccination was performed to evaluate immunogenic potential of the 11 peptides and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with each peptide and non-pulsed BM-DCs as target cells. The CD8-positive splenocytes from mice primed with the mixed vaccine released more IFN-γ to BM-DCs pulsed with mGPC3-1 peptide (average number of spots, 44.3±15.3) and mGPC3-4 peptide (average number of spots, 7.6±3.2) than to non-pulsed BM-DCs (average number of spots, 0.3±0.5). These results suggest that the mGPC3-1 and mGPC3-4 peptides had immunogenic potential and were able to induce peptide-specific CTLs in B6 mice primed by the mixed vaccine system (Fig. 2B and C).

Next, to confirm whether the peptides are CTL-inducible peptides, a single peptide vaccine was given and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with either peptide and non-pulsed BM-DCs as target cells. The CD8-positive cells from mice immunized with mGPC3-1 peptide released more IFN-γ to BM-DCs pulsed with mGPC3-1 peptide (average number of spots, 101.0±33.2) than to non-pulsed BM-DCs (average number of spots, 2.1±3.7) (Fig. 2D and E). The CD8-positive cells from mice immunized with mGPC3-4 peptide released more IFN-γ to BM-DCs pulsed with mGPC3-4 peptide (average number of spots, 5.3±4.0) than to non-pulsed BM-DCs (average number of spots, 1.8±0.7), but no significant differences were observed (Fig. 2F and G). These results suggest that mGPC3-1 peptide is more efficient for inducing CTLs than the mGPC3-4 peptide in a single peptide vaccine system.

Taken together, the above results suggest that mGPC3-1 peptide is the most efficient peptide for inducing CTLs among the 11 peptides.

mGPC3-1 peptide-specific CTL line recognition of target cells endogenously expressing mGPC3. To further investigate the ability of mGPC3-1 peptide-specific CTLs induced by peptide vaccination, we established a CTL line from immunized mice according to the above described protocol. IFN-γ ELISPOT assays were performed using RMA-S pulsed with mGPC3-1 peptide and non-pulsed RMA-S to confirm whether the CTL line had mGPC3-1 peptide specificity. The CTL line clearly released more IFN-γ to RMA-S pulsed with mGPC3-1 peptide than to non-pulsed RMA-S, which suggests that the CTL line is the mGPC3-1 peptide-specific CTL (Fig. 3A).

Subsequently, a cytotoxicity assay was performed to confirm whether the mGPC3-1-specific CTLs could kill RMA-S pulsed with mGPC3-1 peptide. The CTLs killed RMA-S pulsed with the mGPC3-1 peptide (16.4%) better than non-pulsed RMA-S (2.2%), suggesting that the mGPC3-1-specific CTL line could specifically recognize and kill RMA-S pulsed with the mGPC3-1 peptide (Fig. 3B).

Finally, we examined whether the CTL line could recognize RMA GPC3-puro endogenously expressing mGPC3. Expression of mGPC3 in RMA-GPC3-puro and RMA-puro

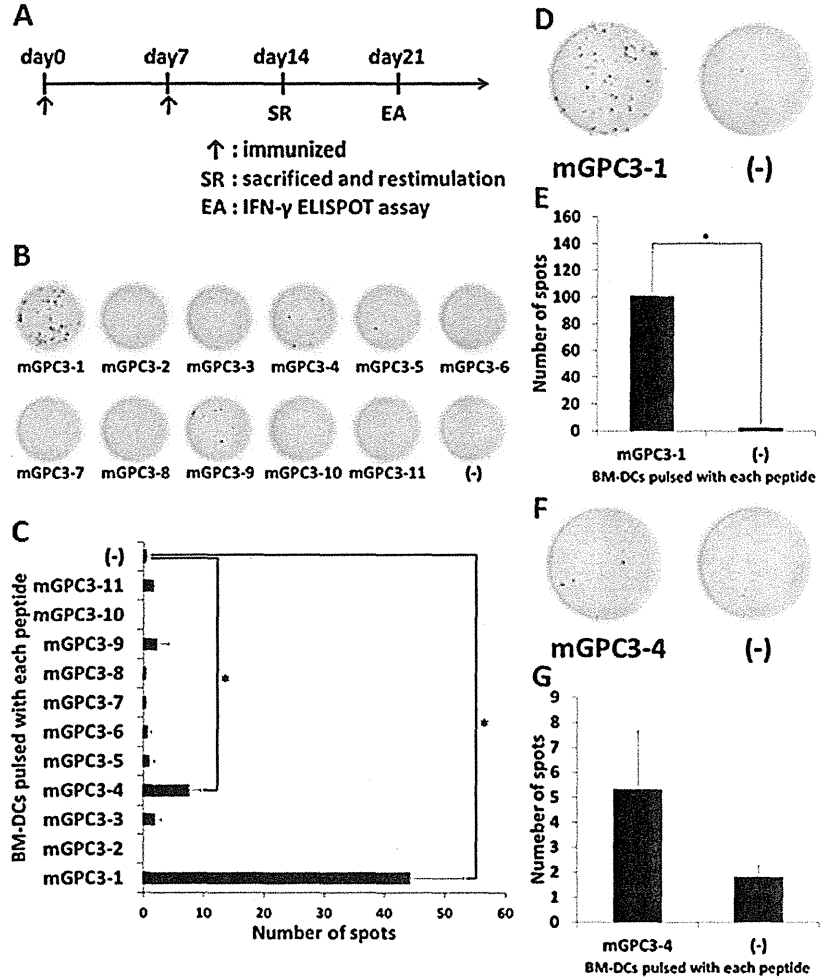

Figure 2. *In vivo* mixed peptide vaccine and single peptide vaccine. Analysis was performed for each vaccine. (A) Schedule of mixed peptide vaccine and single peptide vaccine. (B and C) The mixed peptide vaccine was given to mice and the responses of CD8-positive cells to the 11 peptides were examined. IFN-γ ELISPOT assays were performed using BM-DCs pulsed with each peptide and non-pulsed BM-DCs as target cells (n=3, $^*p<0.05$). Representative data are shown (B). To confirm whether mGPC3-1 or mGPC3-4 was a CTL-inducible peptide, the single peptide vaccine was given. (D and E) mGPC3-1 peptide vaccine was given and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with mGPC3-1 and non-pulsed BM-DCs as target cells (n=3, $^*p<0.05$). Representative data are shown D). (F and G) mGPC3-4 peptide vaccine was given and IFN-γ ELISPOT assays were performed using BM-DCs pulsed with mGPC3-4 and non-pulsed BM-DCs as target cells (n=3). Representative data are shown (F).

was confirmed by RT-PCR. The results showed that RMA-GPC3-puro expressed mGPC3 and RMA-puro did not express mGPC3 (Fig. 3C). IFN-γ ELISPOT assays were performed using RMA-GPC3-puro and RMA-puro as target cells to investigate whether the CTL line could recognize RMA-GPC3-puro expressing endogenous mGPC3. The CTL line released more IFN-γ to RMA-GPC3-puro (average number of spots, 32.2±5.0) than to RMA-puro (average number of spots, 18.2±6.2). This result suggests that the mGPC3-1 peptide is an endogenously presented peptide (Fig. 3D).

CTL response against the mGPC3-derived peptides induced in STAM mice. Previously, the NASH mouse model (named STAM mice C57BL/6N-NASH) was exploited by Stelic Institute & Co. and STAM mice with a B6 background spontaneously developed liver cancer. We observed that liver cancer developed in 18-week-old STAM mice (Fig. 4A and B). Furthermore, to verify whether mGPC3-1 peptide-specific CTLs were induced in STAM mice C57BL/6N-NASH, a mGPC3-1 peptide vaccine was given and an IFN-γ ELISPOT assay was performed using RMA-S pulsed with mGPC3-1 peptide or non-pulsed RMA-S. The CD8-positive cells derived from immunized mice released IFN-γ only to pulsed RMA-S (average number of spots, 100±74.3), not to non-pulsed RMA-S (average number of spots, 0.0±0.0) (Fig. 4C and E). However, the CD8-positive cells derived from unimmunized mice did not release IFN-γ to either pulsed (average number of spots, 0±0.0 or non-pulsed (average number of spots, 0.0±0.0) RMA-S (Fig. 4D and E). These results suggest that peptide-specific mGPC3-1 could be induced in STAM mice C57BL/6N-NASH immunized with the mGPC3-1 peptide vaccine but could not be induced in un-immunized STAM mice C57BL/6N-NASH.

Discussion

HCC is the most common liver cancer and the recurrence rate for treated HCC patients is high, thus establishment of an effective preventative method, such as a vaccination to prevent

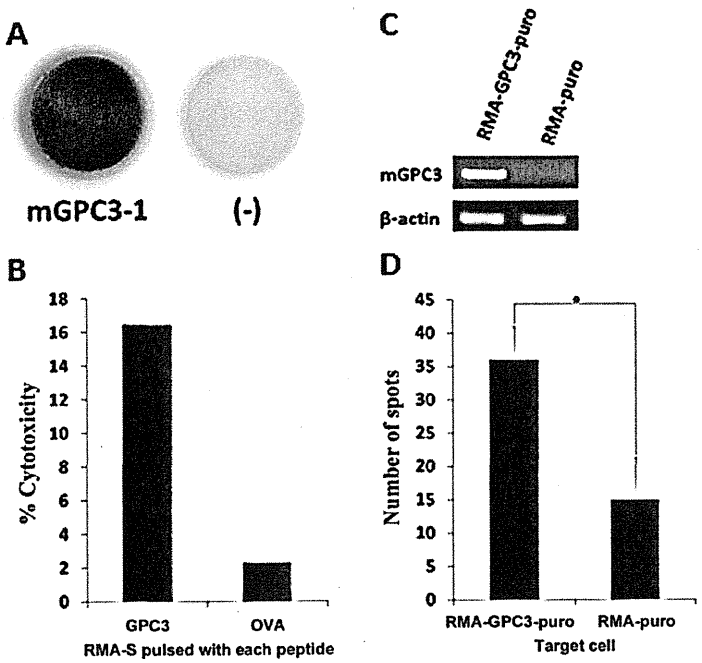

Figure 3. Analysis of established CTL line. (A) IFN-γ ELISPOT assays were performed using GPC3-1 pulsed or non-pulsed RMA-S as target cells. (B) Cytotoxicity assays were performed using GPC3-1 pulsed or unpulsed RMA-S as target cells. Percent cytotoxicity = {1- [(average fluorescence of the sample wells - average fluorescence of the maximal release control wells) - (average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]} x 100%. (C) mGPC3 expression of RMA-GPC3-puro and RMA-GPC3 by RT-PCR. (D) IFN-γ ELISPOT assays were performed using RMA-GPC3-puro and RMA-puro as target cells (n=3, $^*p>0.05$).

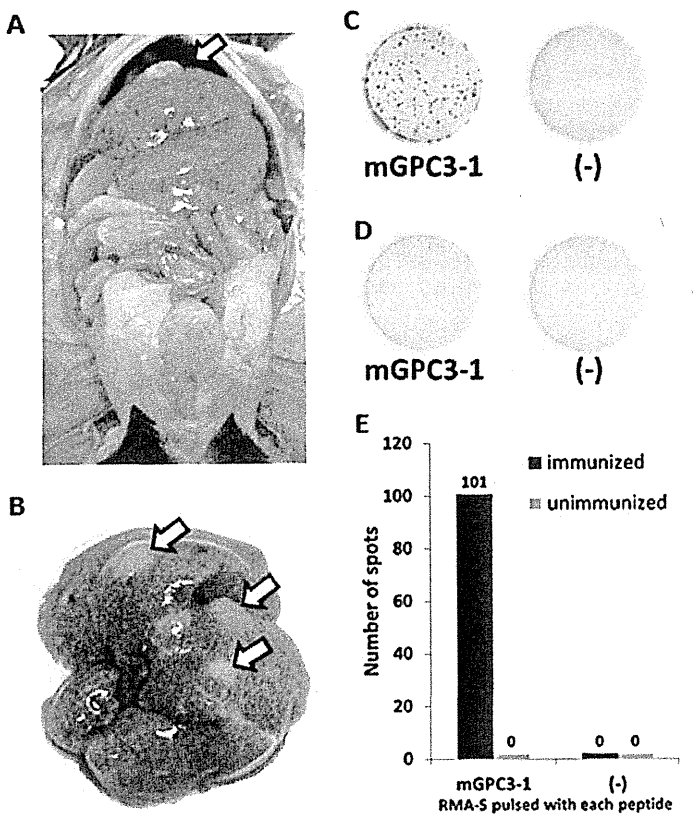

Figure 4. Analysis of STAM mice C57BL/6N-NASH. (A and B) Liver cancer was observed in 18-week-old STAM mice C57BL/6N-NASH. (C-E) To confirm whether mGPC3-1 peptide-specific CTL was induced, the mGPC3-1 peptide vaccine was given to STAM mice C57BL/6N-NASH and the IFN-γ ELISPOT assay was performed. Arrow indicates the area of developing cancer (C and E). The CD8-positive cells derived from immunized mice released IFN-γ to RMA-S pulsed with mGPC3-1 peptide (n=3). Representative data are shown (C). (D and E) As a control, IFN-γ ELISPOT assays were performed using the CD8-positive cells derived from unprimed mice. Representative data are shown (D).

the occurrence and recurrence of HCC, is needed. GPC3 is overexpressed in HCC and is not expressed in normal tissue except for the placenta and embryonic liver. Clinical trials of a GPC3-derived peptide vaccine for HCC have been performed and a phase I clinical trial has shown the safety and immunological and clinical potential of the vaccine (15,16). Moreover, to study the preventive effect as a potential of the GPC3-derived peptide vaccine, we attempted to establish a mouse model to induce GPC3-specific CTLs by the peptide vaccine.

First, mGPC3-derived peptides binding to H2-K^b or H2-D^b were determined *in silico* using BIMAS software. Moreover, a binding assay was performed *in vitro* and showed that all peptides predicted by the BIMAS software could bind H2-K^b and H2-D^b. However, the BIMAS score did not correlate with the actual binding affinity.

Peptides that can bind to MHC class I are not always able to induce peptide-specific CTLs (21,29). Therefore, to investigate actual CTL-inducible peptides among the 11 selected peptides, a mixed peptide vaccine and single peptide vaccine were given to mice. These results (Fig. 2) suggested that mGPC3-1 could induce peptide-specific CTLs. In addition, antigen-derived and CTL-inducible peptides are not necessarily presented by cancer cells endogenously expressing the antigen (23,30). Hence, we confirmed whether the mGPC3-1 peptide-specific CTL line could recognize RMA-GPC3-puro endogenously expressing mGPC3 (Fig. 3D). Furthermore, confirming whether the mGPC3-1 peptide-specific CTL line killed cancer cells presenting the mGPC3-1 peptide is important, thus a cytotoxicity assay was performed (Fig. 3B).

Mice with a B6 background that spontaneously develop liver cancer have been reported (19,20). These mice enable investigations as to whether a peptide vaccine for GPC3 has a preventive capability. Recently, the STAM mice C57BL/6N-NASH was established as a non-alcoholic-steatohepatitis (NASH) mouse model by Stelic Institute & Co. STAM mice C57BL/6N-NASH are drug-treated B6 mice and liver cancer occurs spontaneously and early in NASH mice. Therefore, this mouse is an attractive model for studying the preventive effects of a cancer vaccine. We showed that mGPC3-1 peptide-specific CTL could be induced in STAM mice C57BL/6N-NASH (Fig. 4E). Simultaneously, we established a liver cancer cell line derived from STAM mice C57BL/6N-NASH and observed the cancer cell line expressed mGPC3 (data not shown).

However, the GPC3 peptide vaccine did not prevent the occurrence of liver cancer in STAM mice C57BL/6N-NASH (data not shown). Therefore, further research to develop strong GPC3-specific immunotherapies or combinational approaches in an appropriate mouse model is needed. Identification of an H2-K^b or H2-D^b restricted, GPC3-derived peptide is the first step. The established cell line from STAM mice C57BL/6N-NASH, which show GPC3 expression, may help us to develop a new mouse model system for a GPC3-targeted therapy.

In conclusion, mGPC3-$1_{127-136}$ AMFKNNYPSL was identified as an H2-K^b or H2-D^b restricted, GPC3-derived CTL most-inducible epitope peptide and mGPC3-1 peptide-specific CTL can kill RMA-S pulsed with the mGPC3-1 peptide. Furthermore, we established an mGPC3-1-specific CTL-inducible model in B6 mice using an mGPC3-1 peptide vaccine.

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A glypican-3-derived peptide vaccine against hepatocellular carcinoma

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Keywords: clinical trial, cytotoxic T lymphocyte, glypican-3, hepatocellular carcinoma, peptide vaccine

The results of a Phase I clinical trial in which a glypican-3 (GPC3)-derived peptide was tested in advanced hepatocellular carcinoma patients point to a strong correlation between immunological and clinical responses. This commentary reviews our fundamental studies and clinical trials on the GPC3-derived peptide vaccine.

The induction of tumor-specific responses in the absence autoimmunity is the ideal goal of immunotherapy. Since the identification of tumor-associated antigens in hepatocellular carcinoma (HCC), immunotherapeutic approaches have been based on the generation of tumor-specific $CD8^+$ T cells that recognize peptides of 8–11 residues derived from intracellular proteins and presented in association with MHC Class I molecules.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans, which are attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. We identified GPC3 as a carcinoembryonic antigen and suggested that it would consitute an ideal target for HCC immunotherapy, due to its specific overexpression in HCC (in 81% of paitents) and its correlation with poor prognosis.[1–4] Furthermore, we identified both HLA-A24*(A*2402)*-restricted and H-$2K^d$-restricted $GPC3_{298-306}$ (EYILSLEEL), as well as HLA-A2*(A*0201)*-restricted $GPC3_{144-152}$ (FVGEFFTDV), as peptides that can induce GPC3-reactive cytotoxic T lymphocytes (CTLs) but not autoimmunity.[2,5] HLA-A24 and A2 are the most common MHC Class I alleles in the Japanese population. By performing a binding assay, we confirmed that the HLA-A*02:01-restricted $GPC3_{144-152}$ peptide can also bind to HLA-A*02:06 and HLA-A*02:07. We then conducted a preclinical study in mice to design an optimal schedule for a clinical trial with the GPC3-derived peptide vaccine (**Fig. 1**). This study showed that incomplete Freund's adjuvant (IFA) is indispensable for GPC3 peptide-based immunotherapy, and that the immunological effects of the peptide vaccine are dose-dependent.[6]

Based on these results, we conducted a Phase I clinical trial using this GPC3-derived peptide vaccine in patients with advanced HCC, which has recently been concluded.[7] In this study, 33 patients with advanced HCC received GPC3 peptide vaccination with dose-escalation. Peptides were emulsified with IFA and administered in liquid form by intradermal injection on days 1, 15 and 29. The $GPC3_{298-306}$ peptide was used in HLA-A24-positive patients and the $GPC3_{144-152}$ peptide in HLA-A2-positive patients.

In this trial, we collected evidence of immune responses, demonstrated antitumor effects, and demonstrated the safety of our GPC3-derived peptide vaccine. One patient manifested a partial response (PR) and 4 out of 19 patients with stable disease (SD) exhibit tumor necrosis or regression that did not meet the criteria for PRs. Two months after initiation of treatment, the disease control rate (PR+SD) was 60.6%. When we analyzed the frequency of GPC3-specific CTLs ex vivo by interferon γ (IFNγ) enzyme-linked immunospot (ELISPOT) assays, we could detect GPC3 peptide-specific CTLs in the peripheral blood of most patients. Alongside, we established several $GPC3_{144-152}$ peptide-specific CTL clones from peripheral blood mononuclear cells (PBMCs) of patients vaccinated in this trial.[8] Tumor biopsies were performed in seven patients to evaluate the infiltration of $CD8^+$ T cells by immunohistochemistry. In five cases, we observed a marked intratumoral infiltration of $CD8^+$ T cells upon vaccination.

A correlation between immunological and clinical responses is nowadays a required as proof for the clinical efficacy of immunotherapy. The frequency of GPC3 peptide-specific CTLs in the peripheral blood correlated with overall survival in HCC patients who received the peptide vaccination. In multivariate analysis, the frequency of GPC3-peptide-specific CTLs constitute the only predictive factor for overall survival in this trial. Analysis of all 33 patients showed a median overall survival of 12.2 mo (95% CI, 6.5–18.0) in patients with a high frequency of GPC3-specific CTLs, compared with 8.5 mo (95% CI, 3.7–13.1) in individuals with a low GPC3-specific CTL frequency ($p = 0.033$). These observations suggest that GPC3-derived peptide vaccines represent a novel immunotherapeutic strategy for patients with HCC, with a potential to improve overall survival.

We subsequently conducted a Phase II study of the GPC3-derived peptide vaccine

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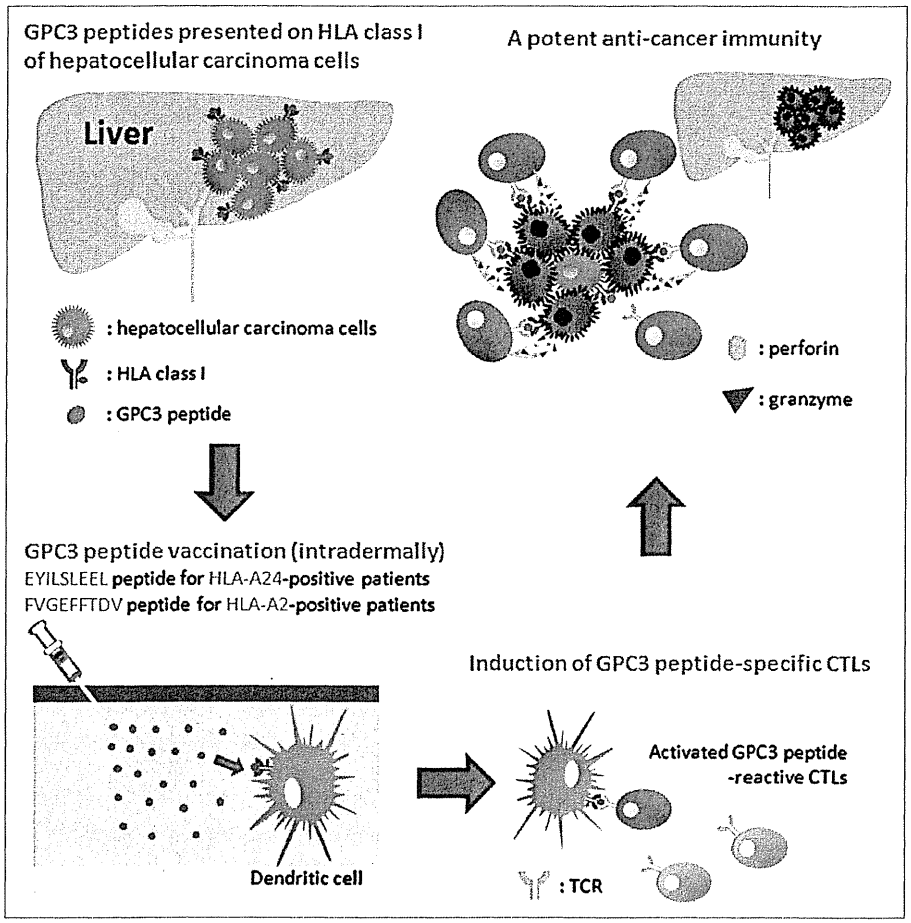

Figure 1. Mechanism of action of the GPC3-derived peptide vaccination. Most patients with hepatocellular carcinoma (HCC) exhibit an HLA-restricted glypican-3 (GPC3)-derived peptide presented in association with MHC Class I molecules. In clinical trials based on GPC3-derived peptide vaccines in HCC patients, the $GPC3_{298-306}$ (EYILSLEEL) peptide was used in HLA-A24-positive patients and the $GPC3_{144-152}$ (FVGEFFTDV) peptide in HLA-A2-positive patients. The peptides were administered with incomplete Freund's adjuvant by intradermal injection, leading to engulfment and cross-presentation by dendritic cells. Dendritic cells are capable of inducing GPC3 peptide-specific cytotocxic T lymphocytes (CTLs), which mediate anticancer immune responses.

as an adjuvant therapy for patients with HCC (UMIN-CTR: 000002614). Forty patients with HCC who had undergone surgery or radiofrequency ablation were enrolled in this Phase II, open-label, single-arm trial. Ten vaccinations were performed over 1 year after curative treatment. Primary endpoints were the 1- and 2-year recurrence rates, while secondary endpoints were immunological responses, as measured by IFNγ ELISPOT. The correlation between the time of recurrence and immunological responses is currently being analyzed.

In the Phase I trial, we did not confirm whether the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific. To address this issue, we are initiating a pilot study of liver biopsies performed before and after GPC3 peptide vaccination for advanced HCC (UMIN-CTR: 000005093).

GPC3 is overexpressed in several malignant tumors, including ovarian clear cell carcinoma (CCC), which is normally characterized by a poor prognosis due to low sensitivity to conventional chemotherapy. We confirmed that a $GPC3_{144-152}$ peptide-specific CTL clone can recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines using an IFNγ ELISPOT assay, and that is can kill ovarian CCC cell lines.[9] We are currently conducting a Phase II study with a GPC3-derived peptide vaccine in ovarian CCC patients (UMIN-CTR: 000003696).

We expect that the results of these trials will provide a rationale for larger randomized clinical trials that determine the efficacy of GPC3-derived peptide vaccines. In addition, as the antitumor effect of the peptide vaccine alone is not dramatic in advanced cancer patients, we aim to develop combinational approaches[9] or strong antigen-specific immunotherapies, including adoptive cell transfer approaches following lymphodepletion.[10] Finally, clinical trials of the adoptive cell transfer of GPC3-specific CTLs in patients with HCC in Japan are planned. Well-designed clinical trials using innovative immunotherapeutic approaches will lead to the development of efficient new therapies for the treatment of GPC3-expressing tumors.

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Cancer Therapy: Clinical

Clinical Cancer Research

Phase I Trial of a Glypican-3–Derived Peptide Vaccine for Advanced Hepatocellular Carcinoma: Immunologic Evidence and Potential for Improving Overall Survival

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Abstract

Purpose: The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target of anticancer immunotherapy against hepatocellular carcinoma (HCC). In this nonrandomized, open-label, phase I clinical trial, we analyzed the safety and efficacy of GPC3 peptide vaccination in patients with advanced HCC.

Experimental Design: Thirty-three patients with advanced HCC underwent GPC3 peptide vaccination (intradermal injections on days 1, 15, and 29 with dose escalation). The primary endpoint was the safety of GPC3 peptide vaccination. The secondary endpoints were immune response, as measured by IFN-γ ELISPOT assay, and the clinical outcomes tumor response, time to tumor progression, and overall survival (OS).

Results: GPC3 vaccination was well-tolerated. One patient showed a partial response, and 19 patients showed stable disease 2 months after initiation of treatment. Four of the 19 patients with stable disease had tumor necrosis or regression that did not meet the criteria for a partial response. Levels of the tumor markers α-fetoprotein and/or des-γ-carboxy prothrombin temporarily decreased in nine patients. The GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 patients. Furthermore, GPC3-specific CTL frequency after vaccination correlated with OS. OS was significantly longer in patients with high GPC3-specific CTL frequencies ($N = 15$) than in those with low frequencies ($N = 18$; $P = 0.033$).

Conclusions: GPC3-derived peptide vaccination was well-tolerated, and measurable immune responses and antitumor efficacy were noted. This is the first study to show that peptide-specific CTL frequency can be a predictive marker of OS in patients with HCC receiving peptide vaccination. *Clin Cancer Res; 18(13); 3686–96. ©2012 AACR.*

Introduction

While primary liver cancer, which predominantly consists of hepatocellular carcinoma (HCC), is the sixth most common cancer worldwide, it has a very poor prognosis, which makes it the third leading cause of cancer mortality (1). One of the major reasons for the poor prognosis of HCC is the limited availability of treatment options for advanced disease. The molecular-targeted agent sorafenib was recently proven to prolong overall survival (OS) in patients with advanced HCC and has become the standard drug for first-line systemic treatment (2, 3). However, according to Response Evaluation Criteria in Solid Tumors (RECIST), the response rate for sorafenib is quite low, and the incidence of adverse drug reactions is high, especially in elderly patients (4). Moreover, no second-line treatment has been established for patients when sorafenib treatment has failed. Therefore, new treatment modalities are urgently required to prolong survival in patients with advanced HCC while minimizing the risk of adverse reactions.

Immunotherapy is a potentially attractive option for HCC. Many tumor antigens identified in HCC are potential antigens for peptide vaccines (5, 6). However, thus

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Translational Relevance

A cancer vaccine that induces CTLs to tumor-associated antigens is a potentially attractive option for hepatocellular carcinoma (HCC). However, thus far, immunotherapy using tumor antigen–derived peptides has not showed a correlation between immunologic responses and antitumor efficacy in clinical trials in patients with advanced HCC. Glypican-3 (GPC3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC and correlates with poor prognosis.

In a phase I clinical study, we investigated the safety and antitumor effects of, and immunologic response to, a GPC3-derived peptide vaccine. Our results show that GPC3 peptide–specific CTLs appeared in peripheral blood and that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination.

This is the first study to show that peptide-specific CTL frequency was correlated with overall survival in patients with HCC receiving peptide vaccination. These observations suggest that GPC3-derived peptide vaccines could be a novel therapy for patients with HCC.

far, immunotherapy using tumor antigen–derived peptides has not showed adequate antitumor efficacy in clinical trials in patients with advanced HCC (7–9). The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target for anticancer immunotherapy against HCC because it is specifically overexpressed in HCC (72%–81%) and correlates with a poor prognosis (10–14). We identified HLA-A*24:02–restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01–restricted $GPC3_{144-152}$ (FVGEFFTDV) as peptides that can induce GPC3-reactive CTLs without inducing autoimmunity (15, 16). Moreover, by conducting a binding assay, we confirmed that HLA-A*02:01–restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide can bind to HLA-A*02:06 and HLA-A*02:07. HLA-A24 is the most common HLA class I allele in the Japanese population, and 60% of Japanese individuals (95% of whom have an A*24:02 genotype), 20% of Caucasians, and 12% of Africans are positive for HLA-A24 (17, 18). HLA-A2 is also expressed in Japanese (40%) and other ethnic populations, with an estimated frequency of 50% in Caucasians (17, 19). In a preclinical study using a mouse model, we developed an optimal schedule for human clinical trials of a GPC3-derived peptide vaccine (20). On the basis of these results, we conducted a phase I clinical trial of this GPC3-derived peptide vaccine in patients with advanced HCC. We previously reported that several $GPC3_{144-152}$ peptide-specific CTL clones were established from peripheral blood mononuclear cells (PBMC) of patients vaccinated with HLA-A2–restricted $GPC3_{144-152}$ peptide in this trial (21). We recently completed this phase I clinical trial of the GPC3-derived peptide vaccine. We evaluated the vaccine's safety, tolerability, recommended phase II dose, and immunologic and clinical responses in this trial.

Materials and Methods

Patient eligibility

This phase I trial was approved by the Ethics Committee of the National Cancer Center and was carried out from February, 2007, to November, 2009. Patients with advanced or metastatic HCC were enrolled after providing written, informed consent. The following eligibility criteria were used: diagnosis of HCC on the basis of imaging modalities or histologic examinations; no expectation of response to other therapies; an Eastern Cooperative Oncology Group performance status of 0–1; age between 20 and 80 years; no prior therapy within 4 weeks; life expectancy $\geq$3 months; HLA-A24- or HLA-A2–positive status, as determined using commercially available genomic DNA typing tests (Mitsubishi Chemical Medience); Child-Pugh liver function class A and B; and adequate organ function (white blood cell count $\geq$3,000/μL, hemoglobin $\geq$8.0 g/dL, platelets $\geq$50,000/μL, total bilirubin $\leq$3.0 mg/dL, aspartate aminotransferase $\leq$200 IU/L, alanine aminotransferase $\leq$200 IU/L, and serum creatinine $\leq$1.5 mg/dL). The following exclusion criteria were applied: massive ascites; known brain metastasis; pregnancy or lactation; known history of HIV infection; clinically serious infection; severe cardiac insufficiency; other active malignancy; history of organ allograft; immunodeficiency or history of splenectomy; concurrent treatment with steroids or immunosuppressive agents; and unsuitability for the trial, based on clinical judgment.

Study design and endpoints

This study was a nonrandomized, open-label, phase I clinical trial with dose escalation of the GPC3 peptides in patients with advanced HCC. HLA-A*24:02–restricted $GPC3_{298-306}$ peptide (EYILSLEEL; American Peptide Company) was used in HLA-A24–positive patients and HLA-A*02:01–restricted $GPC3_{144-152}$ peptide (FVGEFFTDV; American Peptide Company) in HLA-A2–positive patients. Peptides were administered in liquid form, emulsified with incomplete Freund's adjuvant (IFA; Montanide ISA-51VG, SEPPIC), by intradermal injection on days 1, 15, and 29. The peptides and IFA were synthesized according to Good Manufacturing Practice guidelines. Administration of 5 incremental doses of peptide (0.3, 1.0, 3.0, 10, and 30 mg/body) was planned. We planned administer each dose to 6 patients, including at least each 2 patients given HLA-A2 or A24-restricted peptide. The primary endpoint was the safety of peptide vaccination. The secondary endpoints were immunologic responses, clinical outcomes, and determination of the optimal dose of peptide for further clinical trials. This study was approved by the Ethics Committee of the National Cancer Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000001395).

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Table 1. Patient characteristics, clinical response, and GPC3-specific CTL response

Dose of peptide, mg	No.	Age/sex	Stage[a] (UICC/ LCSGJ)		PS	Child–Pugh	Hepatic virus infection[b]	Prior therapy[c]	Tumor response[d]	PFS, mo	Os, mo	HLA-A	The spot number of GPC3-specific CTL[e]			Expression in the primary tumor[f]	
													Prevaccine	Postvaccine	Increased CTL	GPC3	HLA class I
0.3	1	75/M	II	III	0	B	C	TAE, PEI, RFA, S-1	PD	2	9	2402	1	8	+	1+	1+
	2	77/M	IV	IVB	0	A	C	PEI, Proton, TAE, TAI	SD	3	11	2402	0	5	+	1+	1+
	3	67/M	IV	IVB	0	A	–	Ope	SD	3	8	0206/0207	22	20	–	2+	1+
	4	51/M	IIIA	IVA	0	B	B	Ope, TAE, TAI	PD	1	2	0201	0	7	+	NA	NA
	5	62/M	IIIA	III	0	A	–	TAI	PD	2	5	0201	0	9	+	1+	1+
	6	69/M	IV	IVB	0	A	–		PD	0	1	0201	10	9	–	NA	NA
	7	59/M	IIIA	IVA	0	A	B	Ope, TAE, TAI	PD	2	3	2402	0	3	+	1+	1+
	8	55/M	IIIA	III	0	A	C	MCT, PEI, TAE, RT, Sor, S-1	SD	3	17	0201	1	5	+	1+	1+
1.0	9	68/F	IIIC	IVA	0	A	C	PEI, TAE, RFA	SD	4	13	0201	8	8	–	NA	NA
	10	72/M	IIIA	IVA	0	B	C	Ope, MCT, RFA, PEI, Sor, TAE, RT, S-1	SD	4	9	0201	8	51	+	1+	1+
	11	60/M	IIIC	IVA	0	A	C	TAE, RFA	SD	4	9	2402	0	11	+	–	1+
	12	62/M	II	III	0	A	–	RFA, PEI, TAE	PD	2	5	0201/0206	0	12	+	–	1+
	13	44/M	IV	IVB	0	A	B	TAE, RFA, PEI, RT	PD	2	24	2402	6	73	+	1+	2+
	14	42/F	IV	IVB	0	A	–		SD	4	14	2402	1	132	+	2+	1+
3.0	15	67/F	IV	IVB	0	A	–	Ope, PEI, TAE, Proton	SD	5	9	0201	0	23	+	1+	1+
	16	58/M	IIIA	III	0	A	–	Ope, TAE, S-1, TAE	SD	5	7	0201	0	101	+	1+	1+
	17	75/M	IIIC	IVA	0	A	C	RFA, TAE	PD	2	7	2402	0	69	+	–	1+
	18	70/M	IV	IVB	1	A	C	Ope, RT	SD	4	14	2402	0	72	+	1+	1+
	19	76/M	IIIA	III	0	B	C	Ope, TAE, TAI	SD	2	3	2402	31	68	+	1+	1+
	20	73/M	II	II	1	A	–	Ope, TAE	SD	8	>34	2402	0	124	+	1+	1+
10	21	52/M	IV	IVB	0	A	B	Ope, TAE, S-1	SD	4	8	0201	1	100	+	2+	1+
	22	71/M	IIIC	IVA	0	A	–	Ope	SD	4	>32	2402	0	171	+	–	1+
	23	70/M	IV	IVB	0	A	B	Ope, TAI, TAE, PEI	PD	2	6	0201	0	5	+	1+	–
	27	56/M	IV	IVB	0	A	C	TAE, UFT	SD	6	>23	2402	64	69	+	NA	NA
	28	57/M	IIIA	IVA	1	B	C	TAE, RFA, TAI	PD	1	1	2402	0	4	+	NA	NA
	29	68/M	IIIA	IVA	0	A	C	Ope, TAE, TAI	PD	2	4	0201	1	125	+	1+	2+
	33	76/M	IV	IVB	0	A	C	Ope, TAE, MCT, RFA, GEM	SD	4	>16	2402	0	5	+	2+	1+
30	24	75/F	IV	IVB	1	A	C	Ope, RFA, RT	PR	5	12	0207	11	196	+	1+	1+
	25	52/M	IV	IVB	0	A	B	Ope, RFA, TAE, RT, UFT	PD	2	12	0206	2	151	+	2+	2+

(Continued on the following page)

Table 1. Patient characteristics, clinical response, and GPC3-specific CTL response (Cont'd)

Dose of peptide, mg	No.	Age/sex	Stage[a] (UICC/ LCSGJ)		PS	Child–Pugh	Hepatic virus infection[b]	Prior therapy[c]	Tumor response[d]	PFS, mo	Os, mo	HLA-A	The spot number of GPC3-specific CTL[e]			Expression in the primary tumor[f]	
													Prevaccine	Postvaccine	Increased CTL	GPC3	HLA class I
	26	75/F	II	II	0	B	C	MCT, RFA, TAE, TAI	SD	2	8	2402	0	16	+	NA	NA
	30	69/M	IV	IVB	1	A	–	Ope, TAI, UFT, GEM+CDDP, RT	SD	4	6	2402	2	34	+	1+	–
	31	53/M	IV	IVB	0	B	B	TAE, RFA	SD	4	14	2402	0	7	+	NA	NA
	32	67/M	IV	IVB	0	A	B	Ope, Sor, TAE	PD	2	>17	0201	0	441	+	–	–

Abbreviation: PD, progressive disease; PFS, progression-free survival; PS, performance status.

[a]Stage: staging was carried out according to the TNM classification for HCC (Union for International Cancer Control, UICC) and the Japanese integrated staging system (Liver Cancer Study Group of Japan, LCSGJ).

[b]Hepatic virus infection B. HBsAg was examined by radioimmunoassay. C: HCV was detected by RT-PCR.

[c]Prior therapy: Ope, surgery; TAE, transcatheter arterial embolization; PEI, percutaneous ethanol injection therapy; RFA, radiofrequency ablation; S-1, tegafur, gimeracil, oteracil potassium; proton, proton beam therapy; TAI, transcatheter arterial injection; RT, radiotherapy; Sor, sorafenib; MCT, microwave coagulation therapy; UFT, tegafur plus uracil; GEM, gemcitabine; CDDP, *cis*-diamminedichloroplatinum.

[d]Tumor responses were evaluated according to RECIST guidelines and modified RECIST (mRECIST) assessment. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients.

[e]Number of GPC3-specific CTL spots. The number of GPC3 peptide–specific CTL spots (postvaccination) was the maximum number of spots in an *ex vivo* IFN-γ ELISPOT assay for GPC3 peptide, carried out after vaccination and using 5×10^5 PBMCs.

[f]Expression of GPC3 and HLA class I was determined by immunohistochemistry. Degree of staining of tumor cells for GPC3: –, no reactivity; 1+, weak reactivity; 2+, strong reactivity; NA, not analyzed. Degree of staining of tumor cells for HLA class I: –, no membranous reactivity; 1+, weak membranous reactivity; 2+, strong membranous reactivity; NA, not analyzed.

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Evaluation of toxicity and clinical response

Patients were evaluated for signs of toxicity during and after vaccination. Adverse events were graded according to the Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Hematologic examinations were conducted before each vaccination. The tumor size was evaluated by computed tomography (CT) or MRI before vaccination, and then 1 month after the third vaccination. Tumor responses were evaluated according to the RECIST guidelines and the modified RECIST (mRECIST) assessment (22).

Measurement of immunologic response

Ex vivo *IFN-γ enzyme-linked immunospot assay.* An *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted to measure the antigen-specific CTL response, as described previously (21). Briefly, peripheral blood (30 mL) was obtained from each patient before the first vaccination and 2 weeks after each vaccination and centrifuged with a Ficoll-Paque gradient. PBMCs were frozen before immunologic analysis. All PBMCs obtained from an individual patient were incubated in the same plate and analyzed by *ex vivo* IFN-γ ELISPOT assay at the same time. Noncultured PBMCs (5×10^5 per well) were added to plates in the presence of peptide antigens (10 μg/mL) and incubated for 20 hours at 37°C in 5% CO_2. The GPC3 antigen was the HLA-A2–restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide or HLA-A*24:02–restricted $GPC3_{298-306}$ peptide (EYILSLEEL). PBMCs plus HLA-A2–restricted HIV_{19-27} (TLNAWVKVV) peptide (ProImmune) or HLA-A*24:02–restricted $HIV_{583-591}$ (RYLKDQQLL; ProImmune) were used as negative controls. The assays were conducted in duplicate.

Dextramer staining and flow cytometric analysis. The PBMCs were stained with HLA-A*02:01 Dextramer-RPE [$GPC3_{144-152}$ (FVGEFFTDV), HIV_{19-27} (TLNAWVKVV); Immudex] and HLA-A*24:02 Dextramer-RPE [$GPC3_{298-306}$ (EYILSLEEL), $HIV_{583-591}$ (RYLKDQQLL); Immudex] for 10 minutes at room temperature and with anti-CD8-FITC (ProImmune) for 20 minutes at 4°C. Flow cytometry was carried out using a FACSAria cell sorter (BD Biosciences), as described previously (21).

Immunohistochemical analysis. Biopsy specimens were taken from some of the vaccinated patients, each of whom provided informed consent. Specimens were stained with hematoxylin and eosin or monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8 (clone 1A5; dilution 1:80; Novocastra), HLA class I (clone EMR8/5; dilution 1:2,500; Hokudo), according to the manufacturers' directions.

GPC3 double-determinant (sandwich) ELISA. Double-determinant (sandwich) ELISA of GPC3 was carried out as described previously (10). The serum-soluble protein GPC3 was detected by indirect ELISA using an anti-human GPC3 monoclonal antibody (clone 1G12; BioMosaics Inc.), and anti-human GPC3 sheep polyclonal antibody (R&D Systems), and recombinant human GPC3 (#211-GP/CF; R&D Systems).

Statistical analysis

OS rates were analyzed by the Kaplan–Meier method. Prognostic factors were evaluated using the log-rank test and Cox proportional hazard models. All statistical analyses were conducted using the PASW Statistics software, version 18.0 (SPSS Inc.). Statistical significance was defined by a value of P less than 0.05.

Results

Patient characteristics

Thirty-three patients were enrolled in this study (Table 1). None of the patients dropped out because of adverse events caused by peptide vaccination. Two patients (cases 4 and 6) discontinued the regimen after the second vaccination because of liver function impairment resulting from tumor progression. One patient (case 28) could not undergo a CT scan after the third vaccination because of tumor progression. These patients were judged to have disease progression, but were not removed from the analyses at the advice of the effect and safety evaluation committee, including the external members. All patients received adequate follow-up to monitor toxicity. The median follow-up period was 9.0 months (range, 1.1–34.1 months). Of the 33 patients, 28 were male. Their average age was 64.3 years (range, 42–77 years). Five patients had a performance status (PS) of 1; all others had a PS of 0. Staging was conducted according to the tumor-node-metastasis (TNM) classification for HCC (Union for International Cancer Control). Sixteen patients were diagnosed with stage IV disease. Seven patients had Child-Pugh class B disease, and all others Child-Pugh class A disease. Twenty-three patients (70%) had a hepatic virus infection. All but 2 of the 33 patients had undergone conventional chemotherapy, surgery, and transcatheter arterial embolization before receiving GPC3 peptide vaccine therapy. At the time of the trial's initiation, sorafenib had not been approved by the drug administration in Japan. Only a few patients had received sorafenib as prior therapy in this phase I trial. One patient treated with gemcitabine had had stable disease for 5 months immediately before vaccination (case 33). The gemcitabine therapy was discontinued because of nausea and lightheadedness. Other patients had undergone prior therapy, but all of them showed progression of the disease before enrollment in this study.

We evaluated the expression of GPC3 and HLA class I in the primary tumors that could be obtained (Supplementary Fig. S1). GPC3 expression was detected in 21 of 26 patients (81%), consistent with previous reports (10–14). Cell membrane expression of HLA class I was evident in 23 of 26 patients (88%; Table 1).

GPC3 peptide vaccine was well-tolerated

The adverse events observed in this trial are listed in Table 2. Dose-limiting toxicity and dose-specific adverse events were not seen. Grade III hematologic adverse events (impaired liver function) were observed in 4 patients (cases 4, 6, 7, and 23). These 4 patients had progressively massive