PHASE I TRIAL OF A GLYPICAN-3-DERIVED PEPTIDE VACCINE FOR ADVANCED HEPATOCELLULAR CARCINOMA: IMMUNOLOGICAL EVIDENCE AND POTENTIAL FOR IMPROVING OVERALL SURVIVAL

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Running title

Phase I trial of GPC3 peptide vaccine in HCC patients.

Key words

Peptide vaccine, glypican-3, CTL, phase I, HCC.

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List of Abbreviations

GPC3, glypican-3; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; CTL, cytotoxic T lymphocyte; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin.
TRANSLATIONAL RELEVANCE

A cancer vaccine that induces cytotoxic T lymphocytes (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 demonstrated a correlation between immunological responses and antitumor efficacy in clinical trials in advanced HCC patients. 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 immunological 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 HCC patients receiving peptide vaccination. These observations suggest that GPC3-derived peptide vaccines could be a novel therapy for HCC patients.
ABSTRACT

Purpose: The carcinoembryonic antigen glypican-3 (GPC3) is an ideal target of anticancer immunotherapy against hepatocellular carcinoma (HCC). In this non-randomized, 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 one, 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 enzyme-linked immunospot assay, and the clinical outcomes tumor response, time to tumor progression, and overall survival.

Results: GPC3 vaccination was well-tolerated. One patient showed a partial response, and 19 patients showed stable disease two 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 overall survival. Overall survival 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 overall survival in HCC patients receiving peptide vaccination.
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 far, immunotherapy using tumor antigen-derived peptides has not demonstrated 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 GPC3298–306 (EYILSLEEL) and HLA-A*02:01-restricted GPC3144–152 (FVGEFFTDV) as peptides that can induce GPC3-reactive cytotoxic T lymphocytes (CTLs) without inducing autoimmunity (15, 16). Moreover, by performing a binding assay, we confirmed that HLA-A*02:01-restricted GPC3144–152 (FVGEFFTDV)
peptide can bind to HLA-A*02:06 and HLA-A*02:07 (unpublished data). 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 (PBMCs) 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 immunological 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 employed: diagnosis of HCC on the basis of imaging modalities or histological 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 four weeks; life expectancy ≥ 3 months; HLA-A24- or HLA-A2-positive status, as determined using
commercially-available genomic DNA typing tests (Mitsubishi Chemical Medience, Tokyo, Japan); Child-Pugh liver function class A–B; and adequate organ function (white blood cell count $\geq 3000/\mu$L, hemoglobin $\geq 8.0$ g/dL, platelets $\geq 50000/\mu$L, 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 non-randomized, open-label, phase I clinical trial with dose-escalation of the GPC3 peptides in patients with advanced HCC. HLA-A*24:02-restricted GPC3\textsubscript{298-306} peptide (EYILSLEEL) (American Peptide Company, Sunnyvale, CA) was used in HLA-A24-positive patients and HLA-A*02:01-restricted GPC3\textsubscript{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, Paris, France), by intradermal injection on days one, 15, and 29. The peptides and IFA were synthesized according to Good Manufacturing Practice guidelines. Administration of five incremental doses of peptide (0.3, 1.0, 3.0, 10, and 30 mg/body) was planned. We planned administer each dose to six patients, including at least each two patients given HLA-A2 or A24-restricted peptide. The primary endpoint was the safety of peptide vaccination. The secondary endpoints were immunological 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).

**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). Hematological examinations were conducted before each vaccination. The tumor size was evaluated by computed tomography or magnetic resonance imaging before vaccination, and then one month after the third vaccination. Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines and the modified RECIST (mRECIST) assessment (22).

**Measurement of Immunological Response**

*Ex vivo* interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay

An *ex vivo* IFN-γ ELISPOT assay was performed 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 two weeks after each vaccination and centrifuged with a Ficoll-Paque gradient. PBMCs were frozen prior to immunological 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. Non-cultured PBMCs (5 × 10^5 /well) were added to plates in the presence of peptide antigens (10 µg/mL) and incubated for 20 h at 37°C in 5% CO₂. 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, Oxford, UK) or HLA-A*24:02-restricted HIV_{583-591} (RYLKDQQLL) (ProImmune) were used as negative controls. The assays were performed in duplicate.

**Dextramer Staining and Flow Cytometry Analysis**

The PBMCs were stained with HLA-A*02:01 Dextramer-RPE (GPC3_{144-152}[FVGEFFTDV], HIV_{19-27} [TLNAWVKVV]; Immudex, Copenhagen, Denmark) and HLA-A*24:02 Dextramer-RPE (GPC3_{298-306}[EYILSLEEL], HIV_{583-591} [RYLKDQQLL]; Immudex) for 10 min at room temperature and with anti-CD8-FITC (ProImmune) for 20 min 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, Burlington, VT), CD8 (clone 1A5; dilution 1:80; Novocastra, Newcastle-upon-Tyne, UK), HLA class I (clone EMR8/5; dilution 1:2500; Hokudo, Sapporo, Japan), according to the manufacturers’
GPC3 Double-determinant (Sandwich) Enzyme-linked Immunosorbent Assay (ELISA)

Double-determinant (sandwich) ELISA of GPC3 was performed 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, Minneapolis, MN), 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 performed using the PASW Statistics software, version 18.0 (SPSS Inc, Chicago, IL). Statistical significance was defined by a value of $P < 0.05$.

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 PS of 1; all others had a PS of 0. Staging was performed according to the 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 two of the 33 patients had undergone conventional chemotherapy, surgery, and trans-catheter arterial embolization prior to 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 five months immediately prior to vaccination (case 33). The gemcitabine therapy was discontinued due to nausea and lightheadedness. Other patients had undergone prior therapy, but all of them showed progression of the disease prior to enrollment in this study.

We evaluated the expression of GPC3 and HLA class I in the primary tumors that could be obtained (Supplementary Figure 1). 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 3 hematological adverse events (impaired liver function) were observed in four patients (cases 4, 6, 7, and 23). These four patients had progressively massive liver tumors. The effect and safety evaluation committee, including the external members, judged that these events were not related to the treatment, but rather to disease progression. All patients experienced grades 1 or 2 local skin reactions at the injection site. Transient immune-related events, including drug fever, rash, and flushing, were observed in most patients. Crotamiton, a scabicidal and antipruritic agent, was prescribed to the five patients who had mild itching, but no antipyretic analgesics were prescribed. These results suggest that GPC3 peptide vaccine therapy was well-tolerated.

GPC3 Peptide Vaccination Could Induce Peptide-specific CTLs in Most Patients

In order to determine whether the GPC3 peptide vaccine could induce a specific immune response, PBMCs, obtained from all patients before and after vaccination, were examined by ex vivo IFN-γ ELISPOT assay. After the third vaccination, the number of GPC3 peptide-specific CTLs in 5 × 10⁵ PBMCs was increased from 0 to 441 in case 32 (Figure 1A). As shown in Table 1, we found that the GPC3 peptide vaccine induced a GPC3-specific CTL response in 30 of the 33 patients (91%). GPC3-specific CTL frequency increased in a peptide dose-dependent manner (Figure 1B). Generally, CTLs for some tumor antigens cannot be directly detected ex vivo; they can only be detected after expansion by repeated in vitro stimulation with the antigenic peptide on appropriate antigen-presenting cells. This finding can be attributed to the sensitivity of the assay and the low frequency of tumor antigen-specific CTLs (23). Surprisingly,
GPC3-specific CTLs were directly detected *ex vivo* without *in vitro* peptide stimulation in almost all patients after GPC3 peptide vaccination.

We also analyzed the GPC3-specific CTL frequency by flow cytometry using the GPC3 peptide, Dextramer. The GPC3-specific CTL frequency is indicated as the percentage of both Dextramer-positive and CD8-positive cells before and after vaccination, as shown in Figure 1C. After the second vaccination, the frequency of GPC3-specific CTLs increased from 0 to 0.12% in case 32.

In many patients who were vaccinated only three times, the GPC3-specific CTL frequency decreased within two months after the third vaccination. We could vaccinate four or more times in 12 cases. In nine of these, the GPC3-specific CTL frequency increased after the fourth vaccination (data not shown).

**CTLs Infiltrated the Tumor after GPC3 Peptide Vaccination**

Tumor biopsy was performed (with informed consent) in seven patients to evaluate the therapeutic effect after vaccination. We evaluated infiltration of CD8-positive T cells by immunohistochemical staining. In case 8, liver biopsy was performed before and after vaccination. In case 11, neck lymph node metastasis was resected after vaccination. The specimen was compared with an abdominal lymph node metastasis sample obtained during a surgical procedure that this patient underwent prior to vaccination. While CD8-positive T cells did not infiltrate the tumor before vaccination, marked infiltration of CD8-positive T cells into the tumor was observed after vaccination in both cases (Figure 1D). In five of seven cases,
Clinical Responses

Patient characteristics and clinical responses in relation to GPC3-specific CTLs are shown in Table 1. Among the 33 patients, one (case 24) was judged to have a partial response (PR) and 19 patients stable disease (SD) for two months, according to RECIST. The assessment of tumor response according to mRECIST was the same as that according to RECIST in all 33 patients. The disease control rate (PR+SD) was 60.6% after two months. The median time to tumor progression (TTP) was 3.4 months (95% confidence interval (CI), 2.1 to 4.6). The median OS was 9.0 months (95% CI, 8.0 to 10.0).

In case 24, supraclavicular lymph node metastases markedly regressed, two liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (Figure 2A, B). We performed a biopsy of the remaining liver tumor and the thoracic bone metastasis after obtaining informed consent. Immunohistochemical staining showed expression of GPC3 and HLA class I on cells in the remaining liver tumor (Figure 2C). Surprisingly, we detected massive infiltration of CD8-positive T cells into the remaining liver tumor by immunohistochemical staining. No viable tumor cells were found in the biopsy specimens of the thoracic bone metastasis.

Four other patients (cases 1, 15, 16, and 17) had tumor necrosis or partial tumor reduction that did not meet the PR criteria.

Serum levels of AFP and DCP are useful tumor markers of HCC (24). The levels of AFP or DCP decreased temporarily at least once in nine of the 33 patients during the two-month period.
(Supplementary Table 1). In seven of these nine patients, the levels of DCP fell to < 30% of baseline values. In 15 of 32 patients, GPC3 protein was detectable in serum before vaccination. The serum levels of GPC3 temporarily decreased at least once in 12 of these 15 patients (data not shown).

These results suggest that there is not the duration of the responses in regards to CTL induction and tumor responses in this phase I trial.

**Overall Survival was Correlated with GPC3-specific CTL Frequency**

We also examined prognostic factors (Table 3). Fifty GPC3 peptide-specific CTL spots were detected in an *ex vivo* IFN-γ ELISPOT assay performed using 5 × 10^5 PBMCs, which means that the GPC3 peptide-specific CTL frequency in peripheral lymphocytes was is 1 × 10^{-4}%. We focused on these 50 spots in order to elucidate prognostic factors. Univariate analysis indicated that distant metastasis (-) (*p* = 0.032), invasion of the IVC or portal vein (*p* = 0.040), AFP ≥ 100 ng/ml (*p* = 0.003), tumor size ≥ 10 cm (*p* = 0.003), and GPC3-specific CTL frequency < 50 were prognostic factors for OS. Furthermore, AFP ≥ 100 ng/ml (*p* = 0.004; hazard ratio (HR) = 4.66; 95% CI, 1.61 to 13.19), tumor size ≥ 10 cm (*p* = 0.003; HR = 4.36; 95% CI, 1.58 to 12.05) and GPC3-specific CTL frequency < 50 (*p* = 0.032; HR = 2.71; 95% CI, 1.09 to 6.72) were prognostic factors for OS in a multivariate analysis. We showed that GPC3-specific CTL frequency could be a predictive marker of the effects of GPC3 peptide vaccination. We compared patients with GPC3-specific CTL frequencies ≥ 50 (N = 15) with those with GPC3-specific CTL frequencies < 50 (N = 18) and found that there was no significant
difference in clinical background. We only found a significant difference ($p = 0.004$) for vaccine consumption ($\geq 1.0$ vs. $<1.0$ mg) (Supplementary Table 2). Analysis of all 33 patients showed that the median OS was 12.2 months (95% CI, 6.5 to 18.0) in patients with GPC3-specific CTL frequencies $\geq 50$, compared with 8.5 months (95% CI, 3.7 to 13.1) in those with GPC3-specific CTL frequencies $<50$ ($p = 0.033$) (Figure 3).

DISCUSSION

We did not observe dose-limiting toxicity in this study. It was difficult to determine the maximum tolerated dose of peptide. A peptide dose of greater than 1.0 mg was required for adequate induction of GPC3-specific CTLs. However, it was complicated to inject more than 10 mg of peptide intradermally because injection mixtures contained both peptide and IFA, and doses of peptide vaccine $> 10$ mg emulsified with IFA (consisting of 2 mL of fluid, including 1 mL of IFA), increased local skin reactions (induration, blushing) at the injection site (Supplementary Figure 2). Therefore, a dose of peptide of 3.0 mg is recommended for future clinical trials.

We evaluated the expression of GPC3 in the primary tumors of 26 patients by immunohistochemistry. Among the 21 patients with low GPC3 expression (degree of staining - or 1+), one patient was judged to have a partial response, and three patients have shown long-term survival. We do not suggest that only patients with high GPC3 expression (degree of staining 2+) should be enrolled in further clinical trials.

We studied immunological responses using an $ex$ $vivo$ IFN-$\gamma$ ELISPOT assay. The GPC3 peptide vaccine induced GPC3-specific CTL responses in 30 of the 33 patients. In contrast,
clear immune responses were not observed in HCC patients in another vaccination trial (9). Differences in tumor antigen may account for the differences in immune response between the two vaccination trials. Previous studies have shown that GPC3 is also overexpressed in other malignant tumors, including melanomas, Wilms’ tumor, hepatoblastoma, ovarian clear cell carcinoma, and lung squamous cell carcinoma (12, 25-28). GPC3 might also be an effective target for immunotherapy against these tumors (29, 30).

In our study, none of the patients in the 0.1 mg dose group showed more than 50 GPC3 peptide-specific CTL spots. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. Previously, Salgaller et al. reported no dose-dependency in the capacity of the gp100 peptide to enhance immunogenicity in humans (at doses 1.0 - 10 mg) (31). In contrast, our data indicate dose-dependency in CTL induction, consistent with a previous report using a mouse model (20).

Ten of the 25 patients who received a dose higher than 1.0 mg did not exhibit GPC3-specific CTL frequencies ≥ 50. There was no significant difference in the clinical background of patients with GPC3-specific CTL frequencies ≥ 50 and those with <50. However, GPC3-specific CTL frequency tended to correlate with the serum level of AFP or summed intrahepatic tumor size (Supplement Table 2). In this study, several patients with advanced HCC exhibited a poor immunological response to GPC3 peptide vaccination. There are several possible explanations for this poor immunogenicity. HCC is frequently accompanied by cirrhosis, which creates an immunosuppressive environment. There is impairment of the function and maturation of dendritic cells, which has been shown to be related to an imbalance in the extracellular amino acid profile (32). In progressive HCC, the induction of CTL may be suppressed by regulatory T cells or immunosuppressive cytokines (33). It has been reported that
GPC3-specific CTLs become exhausted in HCC, and that this exhausted state cannot be reversed by blocking the CTLA-4 and PD-1 inhibitory co-stimulation pathways (34). Further studies will be necessary to increase the clinical efficacy of immunotherapy for advanced HCC.

The primary endpoint of this study was assessment of the safety of vaccination, but we also showed that tumor antigen-specific CTLs had a crucial role in the immunotherapy against GPC3. GPC3-specific CTL frequency was correlated with OS in this study. Peptide-specific IgG and delayed-type hypersensitivity post-vaccination have been reported as potential predictive makers of prolonged survival in advanced cancer patients vaccinated with peptides (35, 36). However, correlations between immune responses and OS have not been reported in other immunotherapy trials for HCC (7-9, 37). We found that patients with GPC3-specific CTL frequencies ≥ 50 had a longer survival than those with GPC3-specific CTL frequencies < 50. There was no significant difference in the clinical backgrounds of patients with GPC3-specific CTL frequencies ≥ 50 and those with < 50.

We clearly demonstrated the presence of GPC3 peptide-specific CTLs in peripheral blood, and showed that many CD8-positive T cells infiltrated tumors after GPC3 peptide vaccination. The evidence in this study serves as a proof-of-concept for immunotherapy using tumor antigen-specific CTLs. However, we did not confirm that the tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific CTLs. We are currently initiating a pilot study of liver biopsies performed before and after GPC3 peptide vaccination for advanced HCC to determine whether tumor-infiltrating lymphocytes are indeed GPC3 peptide-specific CTLs.

No complete responses were observed when GPC3 peptide vaccination was used as the sole therapy for advanced HCC. To-date, there has been no report of an adequate antitumor efficacy of immunotherapy in clinical trials involving advanced HCC patients; however,
immunotherapy, as an adjuvant after surgical resection, is expected (38). On the basis of this study, we have begun a phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for HCC patients, and have also planned combinatorial approaches with chemotherapy.

In conclusion, this phase I clinical trial of a GPC3-derived peptide vaccine showed the vaccination to be safe, and indicated a plethora of immunological responses. This study also showed that GPC3-specific CTL frequency was correlated with OS in patients with advanced HCC who received the GPC3 peptide vaccine.

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FIGURE LEGENDS

**Fig. 1.** Immunological monitoring of GPC3 peptide-specific T cell responses. (A) *Ex vivo* IFN-γ ELISPOT assay for GPC3 in $5 \times 10^5$ PBMCs was carried out before and after vaccination in case 32. The Δ spot number indicates the number of GPC3 peptide-specific CTLs. The number of IFN-γ-positive spots increased from 0 to 441 in the wells preincubated with GPC3 peptide. (B) Median spot number in *ex vivo* IFN-γ ELISPOT assay for GPC3 for each peptide dosage. GPC3-specific CTL frequency increased in a peptide dose-dependent manner. (C) *Ex vivo* GPC3 Dextramer staining before and after vaccination in case 32. GPC3 peptide-specific CTL frequency is indicated as the percentage of Dextramer-positive CTLs among PBMCs. The frequency of GPC3 peptide-specific CTLs increased from 0 to 0.12% in case 32. (D) Immunohistochemical staining showing CD8-positive lymphocytes infiltrating tumors before and after vaccination. In cases 8 and 11, CD8-positive T cells (brown) did not infiltrate the tumors before vaccination; in contrast, many CD8-positive T cells infiltrated the tumor after vaccination. Magnification = 200×.

**Fig. 2.** Response assessment in case 24. (A) CT imaging, showing liver, pleura and supraclavicular lymph node metastases before vaccination. (B) CT imaging after vaccination was judged as an indicator of a PR. The supraclavicular lymph node metastasis and multiple liver tumors regressed markedly. The pleura metastasis was necrotic. (C) We biopsied the remaining liver tumor after vaccination. Immunohistochemical staining showed expression of GPC3 and HLA class I on tumor cells. There was massive infiltration of CD8-positive T cells. Magnification = 200×.
**Fig. 3.** Kaplan-Meier curves for overall survival. Patients with GPC3-specific CTL frequencies $\geq 50$ had a longer survival than those with GPC3-specific CTL frequencies $< 50$ ($p = 0.033$). MST, median survival time.
A

GPC3 peptide

Δspot number in 5×10⁵ PBMCs

The rate of peptide-specific CTLs in PBMCs

HIV peptide

B

R² = 0.8428

GPC3-specific spot number

Dose of peptide (mg)

0 10 20 30

0 20 40 60

0 40 80

0 80 120

C

Pre-vaccination

2 weeks after 2nd vaccination

1 month after 3rd vaccination

GPC3-Dextramer

0%

0.11%

0.12%

HIV-Dextramer

0%

0%

0%

D

Case 8
Hepatocellular carcinoma

Pre-vaccine

Post-vaccine

Case 11
Lymph node metastasis

Pre-vaccine

Post-vaccine

Figure 1
Research.
A  Before vaccination

B  1 month after 3rd vaccination

C

Figure 2
CTL frequencies ≥ 50 (N=15) MST: 12.2m

CTL frequencies < 50 (N=18) MST: 8.5m

P=0.033

Figure 3
Table 1. Patient characteristics, clinical response and GPC3 specific CTL response

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/Sex</th>
<th>Stagea (UICC/LCSGJ)</th>
<th>PSb</th>
<th>Child-Pugh</th>
<th>Hepatic Virus infectionc</th>
<th>Prior therapyd</th>
<th>Tumor responsee (months)</th>
<th>PFSf (months)</th>
<th>OSg (months)</th>
<th>HLA-A</th>
<th>The spot number of GPC3 specific CTL</th>
<th>Expression in the primary tumorh</th>
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<td>SD</td>
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aStage: Staging was performed 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).
bPS, performance status. cHepatic virus infection B. HBsAg was examined by radioimmunoassay. C: HCV was detected by RT-PCR. dPrior 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. eTumor response. Tumor responses were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) guidelines and modified RECIST (mRECIST) assessment. fPFS, progression free survival. gOS, overall survival. hNumber of GPC3-specific CTL spots. The number of GPC3 peptide-specific CTL spots (post-vaccination) was the maximum number of spots in an ex vivo IFN-γ ELISPOT assay for GPC3 peptide, performed after vaccination and using 5×10^5 PBMCs. iExpression in the primary tumor. 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.
### Table 2. The incidence of adverse event

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<th>Adverse event</th>
<th>Total (%)</th>
<th>Grade 1 (%)</th>
<th>Grade 2 (%)</th>
<th>Grade 3 (%)</th>
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<td>Any event</td>
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<td>9 (27.3)</td>
<td>20 (60.6)</td>
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<td>Any immune-related event</td>
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<td>27 (81.8)</td>
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<td>Drug fever</td>
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<td>Rash or Flushing</td>
<td>27 (81.8)</td>
<td>24 (72.7)</td>
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<td>Injection site reaction</td>
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<td>Blood</td>
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<td>3 (9.1)</td>
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<td>5 (15.2)</td>
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<td>Increase in PT-INR*</td>
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<td>6 (18.2)</td>
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<td>Increase in alanine aminotransferase</td>
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<td>10 (30.3)</td>
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<td>Renal</td>
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<td>Increase in creatinine</td>
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<td>Proteinuria</td>
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*PT-INR, prothrombin time: international normalized ratio
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<th>P univariate</th>
<th>P multivariate</th>
<th>Hazard Ratio (95%CI)</th>
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<td>Age (≥65 / &lt;65)</td>
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<td>Performance Status (0/1)</td>
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<td>Child-Pugh (A/B)</td>
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<td>Virus infection (+/-)</td>
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<td>Distant metastasis (+/-)</td>
<td>0.032</td>
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<td>1.71 (0.64-4.54)</td>
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<tr>
<td>Invasion of IVC or PV (+/-)</td>
<td>0.040</td>
<td>0.706</td>
<td>1.21 (0.45-3.30)</td>
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<tr>
<td>AFP (≥100ng/ml / &lt;100ng/ml)</td>
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<td>0.004</td>
<td>4.66 (1.61-13.19)</td>
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<tr>
<td>Tumor sizea (≥10cm /&lt;10 cm )</td>
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<td>0.005</td>
<td>4.36 (1.58-12.05)</td>
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<td>GPC3 specific CTL b (50 ≥/ &lt;50)</td>
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<td>0.032</td>
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<td>HLA (A2/A24)</td>
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<td>Vaccinec (≥1mg/&lt;1mg)</td>
<td>0.053</td>
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*aTumor size estimated by the Response Evaluation Criteria in Solid Tumors(RECIST)
*b The GPC3 peptide specific CTL frequency examined with Ex vivo IFN-γ ELISPOT assay in 5 × 10⁵ PBMCs
*c The dosage of one vaccine
Phase I trial of glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma showed immunological evidence and potential for improving overall survival

Yu Sawada, Toshiaki Yoshikawa, Daisuke Nobuoka, et al.

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