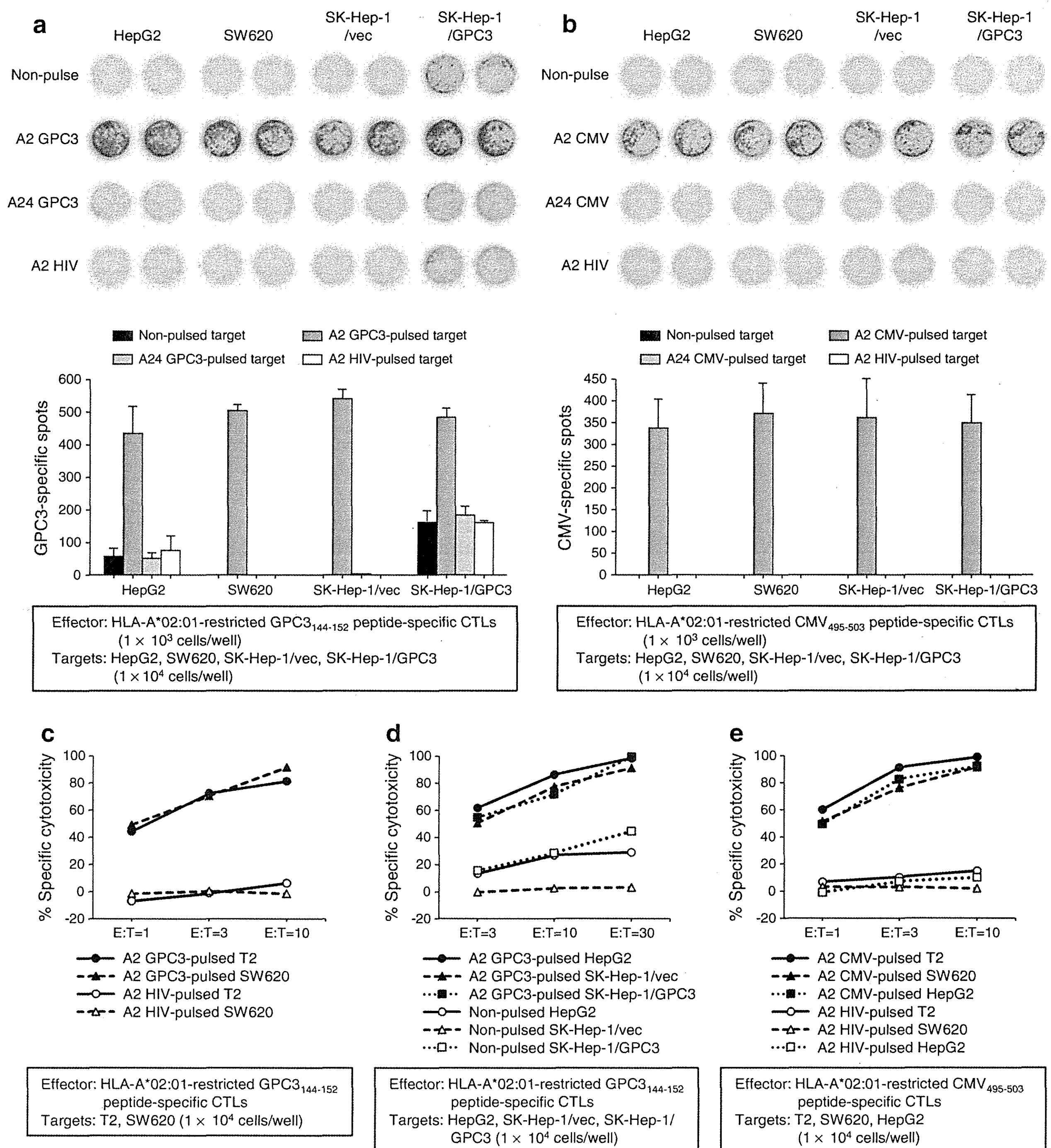

Fig. 1 In vitro CTL activity against the peptide-pulsed targets. (**a** and **b**) IFN-γ ELISPOT assay. (**c**, **d**, and **e**) Cytotoxicity assay. HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs (**a**, **c**, and **d**) and HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTLs (**b** and **e**) showed activity depending on the peptide density of tumor cells. Data are representative of three independent experiments, and bar graphs represent mean values of three independent experiments (SD) in (**a** and **b**)

Intratumoral injection of a combination of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide and its specific CTLs resulted in statistically significant tumor growth inhibition ($P < 0.05$) (Fig. 3c). Similarly, this treatment was effective against SK-Hep-1/vec (Fig. 3d), SK-Hep-1/GPC3 (Fig. 3e), and HepG2 (Fig. 3f) tumors. Intratumoral injection of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs alone against GPC3-expressing tumors, SK-Hep-1/GPC3 and HepG2, was only partially effective, suggesting that the HLA-A*02:01-restricted $GPC3_{144–152}$

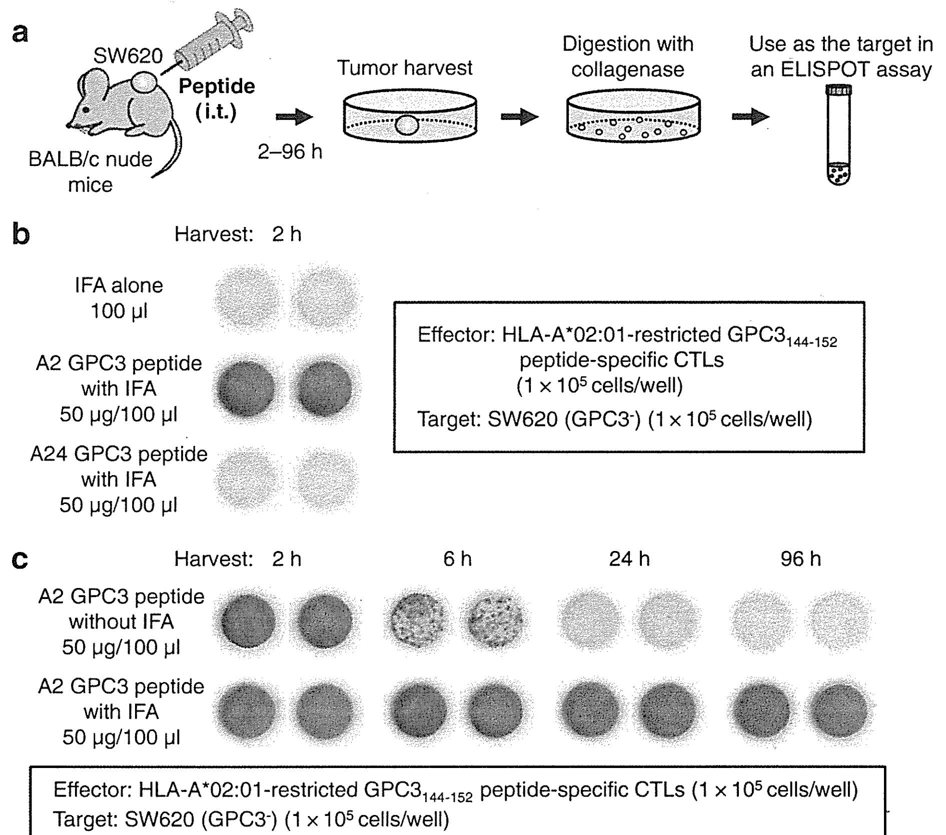

Fig. 2 IFN-γ ELISPOT assay for loading of injected peptide onto HLA class I molecules of tumor cells in vivo. **a** Experimental schematic representation. **b** HLA-A*02:01-restricted $GPC3_{144-152}$ or -A*24:02-restricted $GPC3_{298-306}$ peptide emulsified with IFA was intratumorally injected, and the tumors were harvested after 2 h. IFA alone: no antigenic peptide; 50 μl of 7 % $NaHCO_3$ was mixed with an equal volume of IFA. **c** HLA-A*02:01-restricted $GPC3_{144-152}$ peptide with or without IFA was injected, and tumors were harvested at various times. Data are representative of three independent experiments

peptide endogenously presented on SK-Hep-1/GPC3 and HepG2 tumor cells was not sufficiently dense. However, intratumoral injection of HLA-A*02:01-restricted $GPC3_{144-152}$ peptide increased the peptide density and markedly enhanced CTL activity. Similarly, intratumoral injection of HLA-A*02:01-restricted $CMV_{495-503}$ peptide followed by its specific CTLs resulted in statistically significant tumor growth inhibition ($P < 0.05$) (Fig. 3g). Intratumoral injection of a combination of antigen peptide and its specific CTLs had a significant antitumor effect.

Therapeutic advantage of intratumoral peptide injection as an option for antigen-specific cancer immunotherapy

After the induction of $OVA_{257-264}$ peptide-specific CTLs by peptide vaccination (Fig. 4a) or after the adoptive transfer of $OVA_{257-264}$ peptide-specific CTLs (Fig. 4c), intratumoral injection of $OVA_{257-264}$ peptide was effective against RMA cells, which are OVA-negative tumor cells. The RMA tumors cells that were injected intratumorally with $OVA_{257-264}$ peptide demonstrated significant tumor growth inhibition, compared with mice without intratumoral injection of $OVA_{257-264}$ peptide ($P < 0.05$). The survival rate in the treatment group was significantly better than that in the control groups ($P < 0.05$) (Fig. 4b, d). The group that did not receive $OVA_{257-264}$ peptide vaccine but that received intratumoral peptide injection showed a partial treatment effect (Fig. 4b).

To obtain direct evidence that intratumoral peptide injection leads to local accumulation of antigen-specific CTLs, an OVA tetramer assay was performed using an adoptive cell transfer model (Fig. 4e). Two RMA tumors were bilaterally implanted per mouse. One tumor was injected with the $OVA_{257-264}$ peptide plus IFA, and the other tumor with IFA alone (Fig. 4f). As shown in Fig. 4g, the tumor that underwent both adoptive cell transfer of activated OT-I CTLs and intratumoral injection of the OVA peptide contained more OVA-specific CTLs than the other tumors. Local accumulation of OVA-specific CTLs after intratumoral injection of the $OVA_{257-264}$ peptide was confirmed by OVA tetramer assay.

Neither toxic signs nor death due to intratumoral injection of the $OVA_{257-264}$ peptide was observed. Moreover, to evaluate the risk of autoaggression by intratumoral peptide injection, the tissues of treated mice in an adoptive cell transfer model were pathologically examined. The spleen, brain, lung, heart, liver, kidney, and tumor were critically scrutinized, and the findings were compared with those from

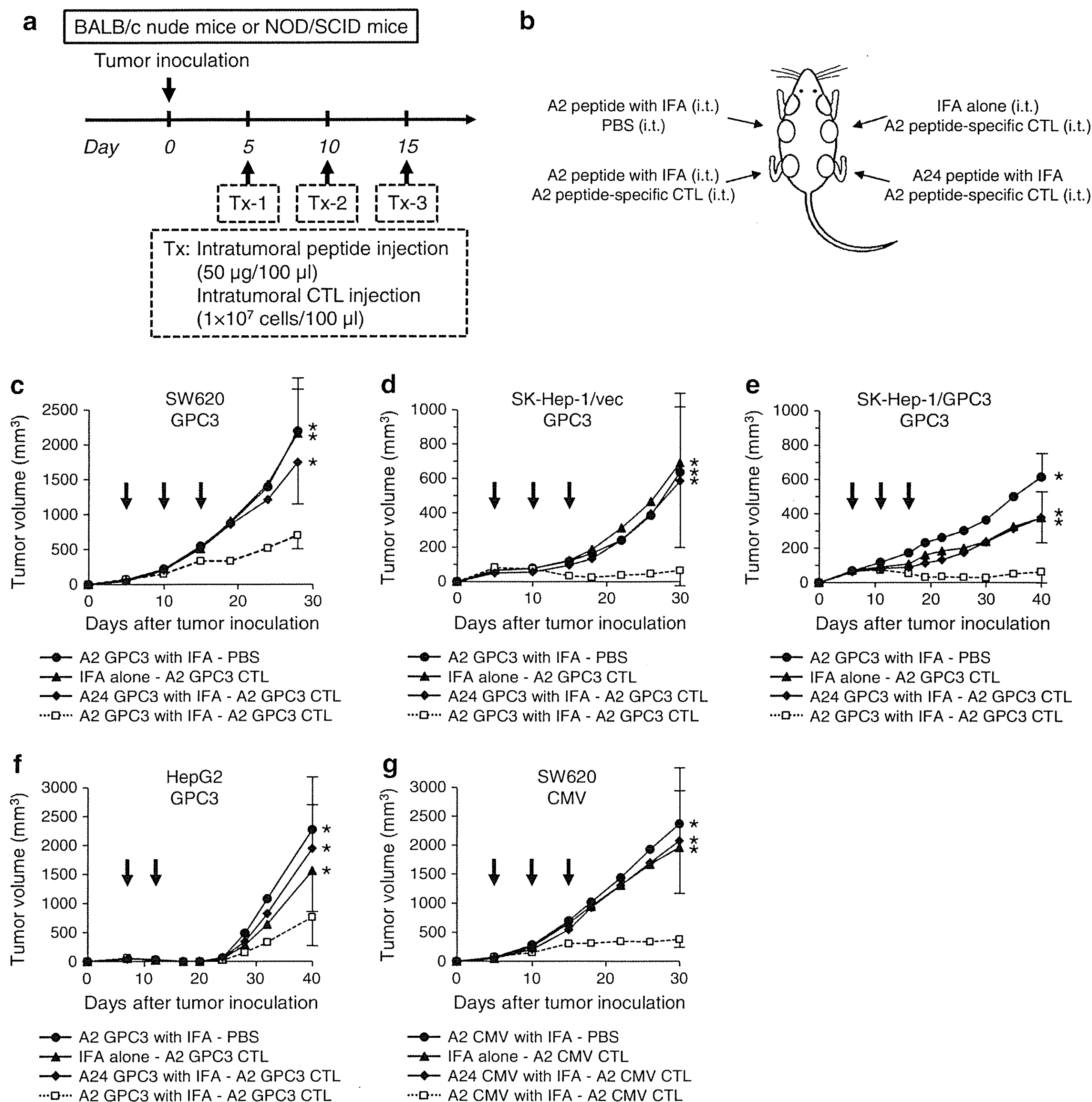

Fig. 3 Antitumor effect of intratumoral peptide injection in an immunodeficient mouse model. Intratumoral injection of a combination of antigen peptide and its specific CTLs had a significant antitumor effect. **a** Treatment schedule. **b** Experimental schematic representation. BALB/c nude mice or NOD-SCID mice were inoculated subcutaneously on their *back* with SW620, SK-Hep-1/vec, SK-Hep-1/GPC3, or HepG2 tumor cells. Four tumors were implanted per mouse, and HLA-A*02:01-restricted $GPC3_{144-152}$ or $CMV_{495-503}$ peptide emulsified with IFA (50 μg/100 μl) and HLA-A*02:01-restricted $GPC3_{144-152}$ or $CMV_{495-503}$ peptide-specific human CTLs (1×10^7 cells/100 μl) were injected into each tumor. (**c**, **d**, **e**, **f**, and **g**) Tumor volume. Tumor growth was expressed by mean tumor volume; *bars* (SD). Seven mice were used in each experiment. *Arrows* indicate the days when treatment was performed. $*P < 0.05$ compared with treatment group (Mann–Whitney U test)

mice that had intratumoral injection with IFA alone. In mice treated with intratumoral injection of $OVA_{257-264}$ peptide, a larger number of $CD8^+$ T-cells had infiltrated the RMA tumor 24 days after the transfer of OT-I CTLs and 10 days after the last intratumoral injection of $OVA_{257-264}$ peptide. However, the simultaneous infiltration of normal tissues by

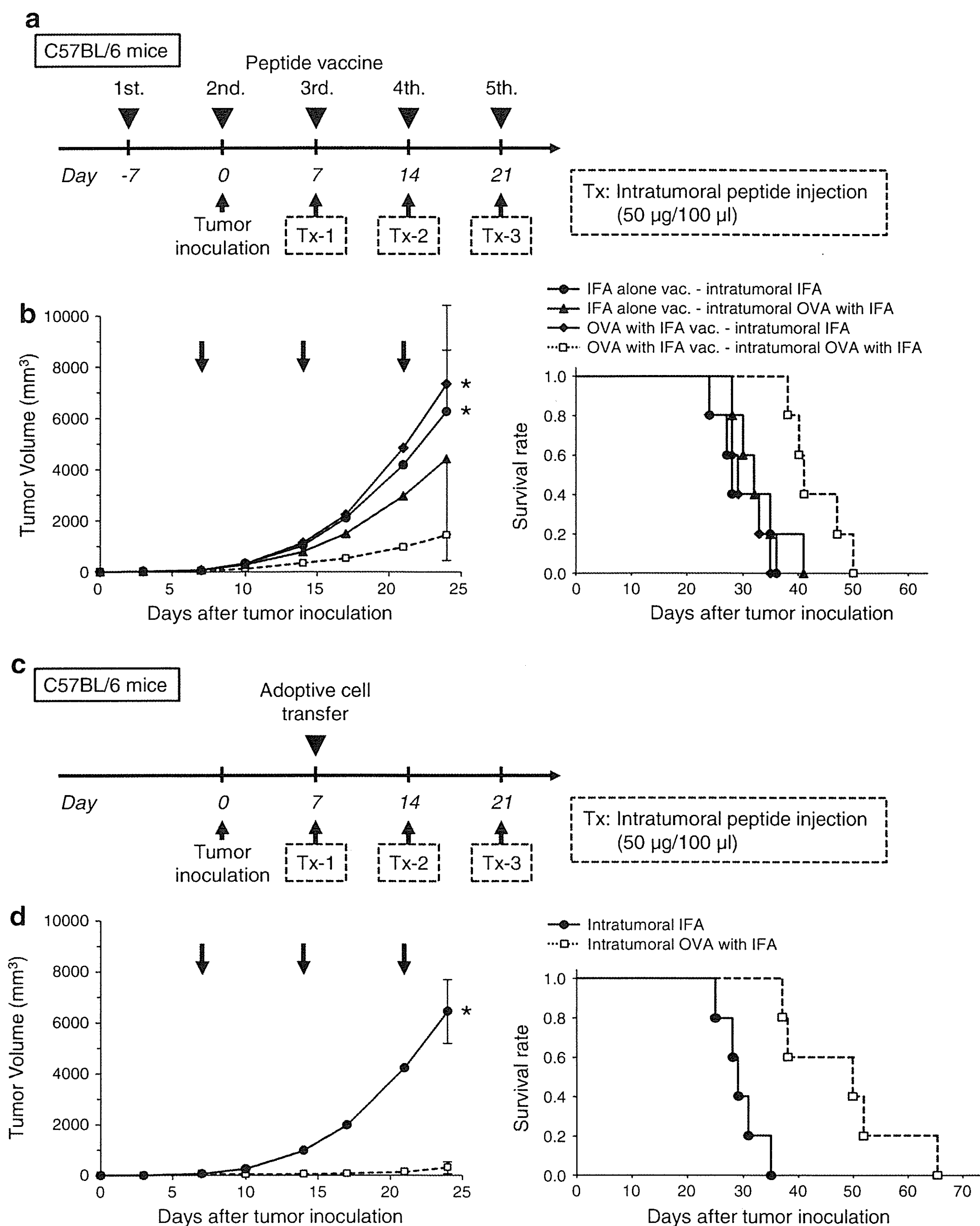

Fig. 4 Therapeutic advantage of intratumoral peptide injection as an option for antigen-specific cancer immunotherapy. (**a** and **b**) Peptide vaccine model. (**c** and **d**) Adoptive cell transfer model. (**a** and **c**) Treatment schedule. (**b** and **d**) Tumor growth and Kaplan–Meier survival *curves*. Tumor growth was expressed by mean tumor volume; *bars* (SD). $^{*}P < 0.05$ compared with the treatment group (Mann–Whitney U test). The survival of mice in the treatment group was significantly better than that in the control groups ($P < 0.05$) (log-rank test). Five mice were used in each group. **e** Schedule for analysis of local accumulation of OVA-specific CTLs in an adoptive cell transfer model. **f** Experimental schematic representation. Two tumors were implanted per mouse (5×10^4 cells/100 µl). One tumor was injected with the OVA peptide plus IFA, and the other with IFA alone. **g** OVA tetramer assay. Local accumulation of OVA-specific CTLs was confirmed in a tumor injected with the OVA peptide plus IFA. Data are representative of three independent experiments. **h** Immunohistochemical staining of CD8 in tumor and normal tissues. Spleen was used as positive control. *Scale bars*, 50 µm

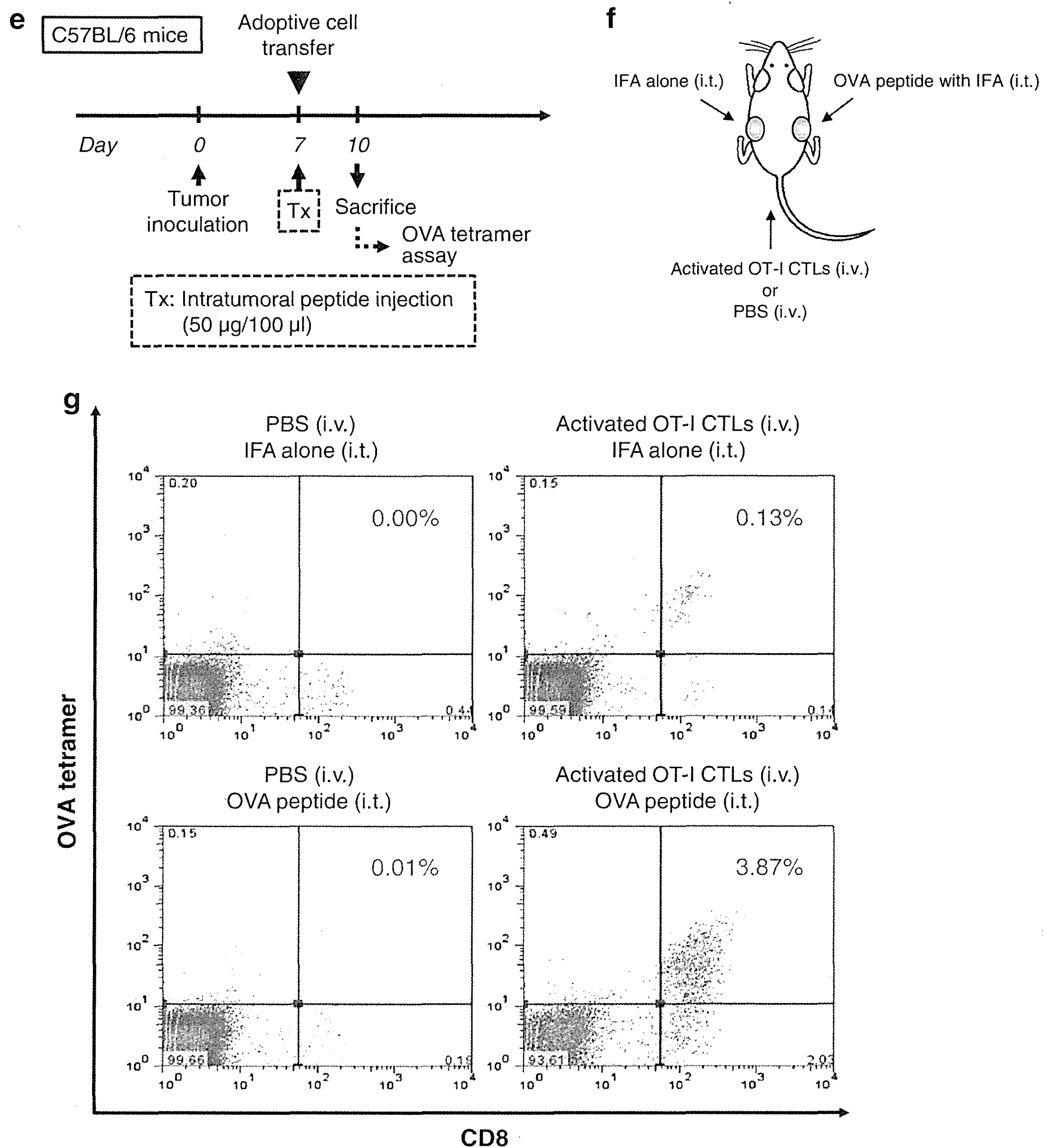

Fig. 4 continued

$CD8^+$ T-cells was not observed (Fig. 4h). These results suggest that peptide from intratumoral injection did not spread into normal tissues.

The effect of antigen spreading to another tumor after intratumoral peptide injection

Using an adoptive cell transfer model, we assessed the possibility of antigen-spreading effect after intratumoral peptide injection, as depicted in Fig. 5a. Two RMA tumors were bilaterally and metachronously implanted per mouse, and only the first tumors received intratumoral injection of the $OVA_{257–264}$ peptide. The sizes of the second tumors were compared with those from mice that received intratumoral injection of IFA alone (Fig. 5b). Whereas the second tumors were established 14 days after the second tumor inoculation in three out of four control mice, all four peptide-loaded mice that had received intratumoral $OVA_{257–264}$ peptide injection into their first tumor completely rejected the challenge of the second tumor, which did not receive intratumoral $OVA_{257–264}$ peptide injection itself (Fig. 5c).

To confirm the hypothesis of antigen spreading, an IFN-γ ELISPOT assay was performed. RMA tumor-bearing

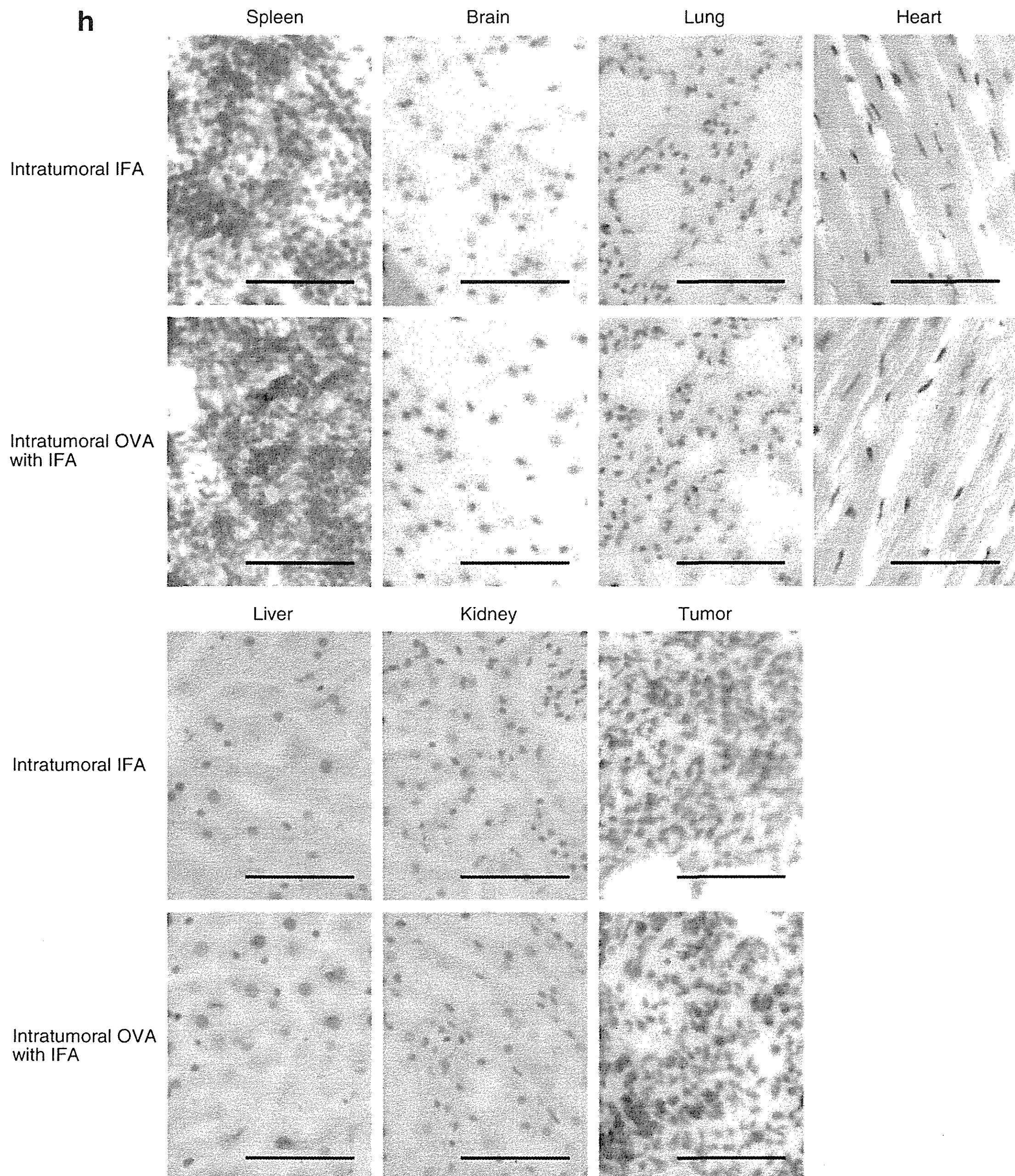

Fig. 4 continued

C57BL/6 mice that had received adoptive transfer of OT-I CTLs and intratumoral injection of $OVA_{257-264}$ peptide were killed, and their spleens were obtained 21 days after adoptive transfer and 7 days after the last intratumoral injection. $CD8^+$ T-cells, isolated from the spleen cells using anti-CD8a magnetic beads, were incubated with irradiated RMA cells for 3 days. $CD8^+$ T-cells were separated from RMA cells using anti-CD8a magnetic beads before the assay. An IFN-γ ELISPOT assay was performed in duplicate using $CD8^+$ T-cells as effector cells and RMA cells as target cells (Fig. 5d). The mice that had received intratumoral injection of $OVA_{257-264}$ peptide showed a significant response to OVA-negative RMA tumor cells compared with control mice that had received intratumoral injection of IFA alone ($P < 0.05$). The observed induction of RMA-derived antigen-specific CTLs provides evidence that antigen spreading occurred by treatment with intratumoral $OVA_{257-264}$ peptide and intravenous OT-I CTLs (Fig. 5e).

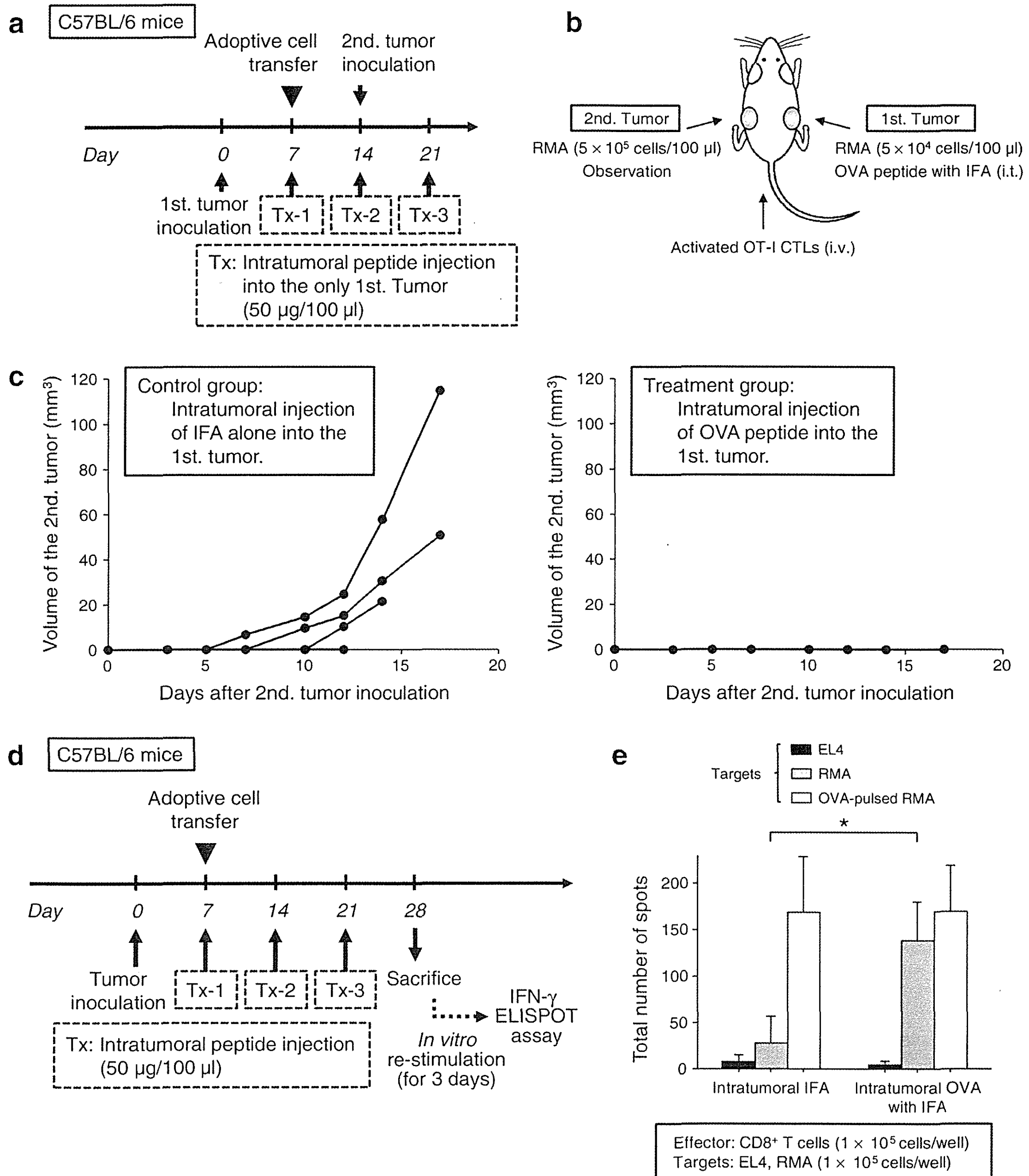

Fig. 5 Effect of antigen-spreading to another tumor after intratumoral peptide injection. **a** The schedule for the experiment on antigen-spreading effect in an adoptive cell transfer model. **b** Experimental schematic representation. Two tumors were metachronously implanted per mouse (first tumor: 5×10^4 cells/100 μl, second tumor: 5×10^5 cells/100 μl), and only the first tumor (*right back*) received intratumoral peptide injection. The second tumor (*left back*) was not treated, but was observed. **c** The growth of the second inoculated RMA tumor. *Four lines* indicate the tumor growth of each mouse. All four mice in the treatment group completely rejected the second tumor challenge. **d** The experiment schedule to confirm antigen spreading. **e** IFN-γ ELISPOT assay. EL4 cells were used as negative control targets. The data are expressed as mean values of three mice (SD). $*P < 0.05$ compared with control (Mann–Whitney U test)

Discussion

We demonstrated that intratumoral peptide injection leads to additional peptide loading onto MHC class I molecules of tumor cells, causing enhanced CTL recognition of tumor cells. It is likely that a larger number of antigen-specific CTLs infiltrate the tumors after this procedure, and tumor cells are killed more easily because CTL activity depends

on the peptide density of tumor cells in an HLA class I-restricted manner. In other words, intratumoral peptide injection enhances the antigenicity of tumor cells, regardless of whether the tumor cells originally expressed the antigen. To the best of our knowledge, this is the first study to show the efficacy of intratumoral peptide injection in detail. A previous report demonstrated that peptide injection around a tumor assisted the activity of low-avidity CTLs in an immunodeficient mouse model [21]. In addition, we demonstrated the advantage as a therapeutic modality combined with antigen-specific cancer immunotherapy without any adverse reactions associated with this procedure in mice. Intratumoral peptide injection can strengthen the efficacy of every kind of antigen-specific cancer immunotherapy and may be a useful therapeutic option.

This is the first study to describe anticancer treatment with CMV-derived peptide-specific CTLs. Virus-derived antigens, which are exogenous antigens, usually have stronger antigenicity than tumor-associated autoantigens. Therefore, virus-derived antigen-specific CTLs are easier to induce [22]. Theoretically, every kind of antigen is applicable to our procedure unless it is expressed in healthy human cells. However, it is unclear whether post-CMV-infected lesions are safe from CMV-specific CTL cytotoxicity. Further investigations are necessary regarding the possible clinical use of exogenous antigens, such as CMV-derived peptides.

We used $NaHCO_3$, which is known to have therapeutic effects against tumors [23, 24], as a peptide diluent. However, our data demonstrated the efficacy of intratumoral peptide injection, because control animals which underwent intratumoral injection of IFA alone or IFA plus an irrelevant peptide also received $NaHCO_3$.

In an in vivo tumor growth inhibition assay using a peptide vaccine model, the group that did not receive the $OVA_{257-264}$ peptide vaccine but that received intratumoral peptide injections showed a partial treatment effect. This indicates that intratumoral or peritumoral antigen-presenting cells recognized intratumorally injected $OVA_{257-264}$ peptide and induced $OVA_{257-264}$ peptide-specific CTLs after three intratumoral peptide injections. However, we showed in this study that intratumoral peptide injection attracted more $OVA_{257-264}$ peptide-specific CTLs and was more effective when combined with peptide vaccines or adoptive cell transfer therapies.

A limitation of intratumoral peptide injection is its delivery method. First, immunotherapy is expected to contribute toward cancer therapy especially in the early stages or in the prevention of recurrence, in which cancer sites, the so-called "micro lesions," are undetectable by imaging modalities. However, intratumoral peptide injection must be limited to the tumors, which are detectable by imaging modalities, and can be approached with a needle. Second, it is difficult to spread the peptides over the whole tumor by intratumoral injection, especially against large tumors. Moreover, it is difficult to approach all of the multiple tumors. This procedure might limit the ability of immunotherapy as a systemic therapy. If a novel method of delivering peptides to tumor cells selectively through a systemic route is established in the future due to advances

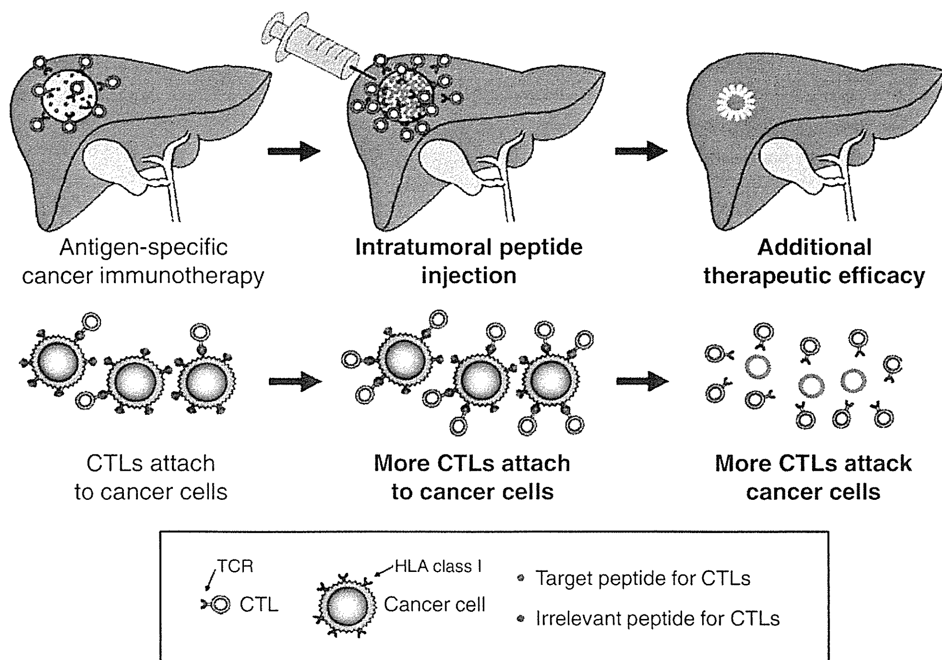

Fig. 6 A proposed mechanistic model of intratumoral peptide injection for improvement in antigen-specific cancer immunotherapy of solid tumors

in drug-delivery technologies, this method will become more suitable for clinical application.

Another limitation is that it requires the presence of MHC class I molecules. The potential loss of MHC class I expression in tumors would lead theoretically to the failure of this approach. Previous reports have indicated that 61–85 % of breast cancers had loss of or decreased HLA class I expression [25–27]. On the other hand, the down-regulation of HLA class I was less frequently observed in other cancers [27–30]. Before clinical application, it is necessary to select cancers in which HLA class I expression is sufficiently high.

Antigen-spreading effects have been observed following anticancer immunotherapy [31–34]. The second tumor challenge is easily rejected due to immunological memory. Therefore, we fixed the number of implanted tumor cells as the second tumors could be established. In this study, we report evidence of an antigen-spreading effect after intratumoral peptide injection. If this antigen-spreading effect is sufficiently steady and reliable, intratumoral peptide injection may even be effective against imaging-invisible or unapproachable tumors.

In conclusion, intratumoral peptide injection is an attractive strategy for enhancing tumor cell antigenicity. It can induce additional peptide loading onto tumor cells, making tumor cells more antigenic for antigen-specific CTL activity against tumor cells. Moreover, it may be a useful option for improvement in antigen-specific cancer immunotherapy against solid tumors (Fig. 6).

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Conflict of Interest The authors declare that they have no conflicts of interest.

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Clinical Cancer Research

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Clinical Cancer Research

Cancer Therapy: Clinical

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 ≥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 ≥3,000/μL, hemoglobin ≥8.0 g/dL, platelets ≥50,000/μL, total bilirubin ≤3.0 mg/dL, aspartate aminotransferase ≤200 IU/L, alanine aminotransferase ≤200 IU/L, and serum creatinine ≤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)

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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

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Table 2. The incidence of adverse event

Adverse event	Total (%)	Grade I (%)	Grade II (%)	Grade III (%)
Any event	33 (100)	9 (27.3)	20 (60.6)	4 (12.1)
Any immune-related event	33 (100)	27 (81.8)	6 (18.2)	0
Drug fever	8 (24.2)	4 (12.1)	4 (12.1)	0
Rash or flushing	27 (81.8)	24 (72.7)	3 (9.1)	0
Injection site reaction	33 (100)	33 (100)	0	0
Pruritus	6 (18.2)	6 (18.2)	0	0
Blood	15 (45.4)	6 (18.2)	9 (27.3)	0
Leukopenia	6 (18.2)	2 (6.1)	4 (12.1)	0
Neutropenia	8 (24.2)	5 (15.2)	3 (9.1)	0
Anemia	5 (15.2)	2 (6.1)	3 (9.1)	0
Thrombopenia	3 (9.1)	1 (3.0)	2 (6.1)	0
Increase in PT-INR	2 (6.1)	2 (6.1)	0	0
Hepatic	23 (69.7)	10 (30.3)	9 (27.3)	4 (12.1)
Hyperbilirubinemia	9 (27.3)	3 (9.1)	4 (12.1)	2 (6.1)
Increase in aspartate aminotransferase	14 (42.4)	4 (12.1)	6 (18.2)	4 (12.1)
Increase in alanine aminotransferase	12 (36.4)	10 (30.3)	1 (3.0)	1 (3.0)
Renal	9 (27.3)	6 (18.2)	3 (9.1)	0
Increase in creatinine	4 (12.1)	2 (6.1)	2 (6.1)	0
Proteinuria	6 (18.2)	4 (12.1)	2 (6.1)	0
Other laboratory				
Increase in alkaline phosphatase	9 (27.3)	4 (12.1)	4 (12.1)	1 (3.0)
Hypoalubuminemia	10 (30.3)	7 (21.2)	3 (9.1)	0
Hyponatremia	13 (39.4)	12 (36.4)	1 (3.0)	0
Hyperkalemia	4 (12.1)	4 (12.1)	0	0

Abbreviation: PT-INR, prothrombin time-international normalized ratio.

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 I or II 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 5 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

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 second vaccination, the number of GPC3 peptide–specific CTLs in 5×10^5 PBMCs was increased from 0 to 441 in case 32 (Fig. 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 (Fig. 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-positice cells before and after vaccination, as shown in Fig. 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 3 times, the GPC3-specific CTL frequency decreased within 2 months after the third vaccination. We could vaccinate 4 or more times in 12 cases. In 9 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 carried out (with informed consent) in 7 patients to evaluate the therapeutic effect after vaccination. We evaluated infiltration of CD8-positive T cells by

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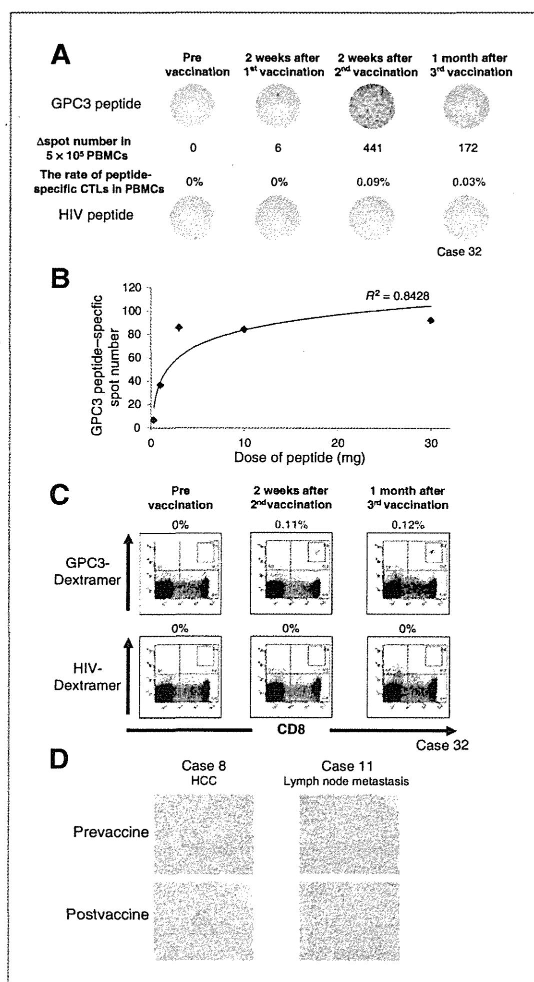

Figure 1. Immunologic monitoring of GPC3 peptide–specific T-cell responses. A, *ex vivo* IFN-γ ELISPOT assay for GPC3 in 5×10^5 PBMCs was carried out before and after vaccination in case 32. The Δspot number indicates the number of GPC3 peptide–specific CTLs. The number of IFN-γ–positive spots increased 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.

immunohistochemical staining. In case 8, liver biopsy was carried out 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 by a diagnostic biopsy that this patient underwent before 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 (Fig. 1D). In 5 of 7 cases, infiltration of CD8-positive T cells into the tumor was increased after vaccination.

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 2 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 2 months. The median time to tumor progression (TTP) was 3.4 months [95% confidence interval (CI), 2.1–4.6]. The median OS was 9.0 months (95% CI, 8.0–10.0).

In case 24, supraclavicular lymph node metastases markedly regressed, 2 liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (Fig. 2A and B). We carried out 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 (Fig. 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 α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are useful tumor markers of HCC (24). The levels of AFP or DCP decreased temporarily at least once in 9 of the 33 patients during the 2-month period (Supplementary Table S1). In 7 of these 9 patients, the levels of DCP fell to less than 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.

OS 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 conducted 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 to elucidate prognostic factors. Univariate analysis indicated that distant metastasis (−; $P = 0.032$), invasion of the inferior vena cava (IVC) or portal vein (PV;

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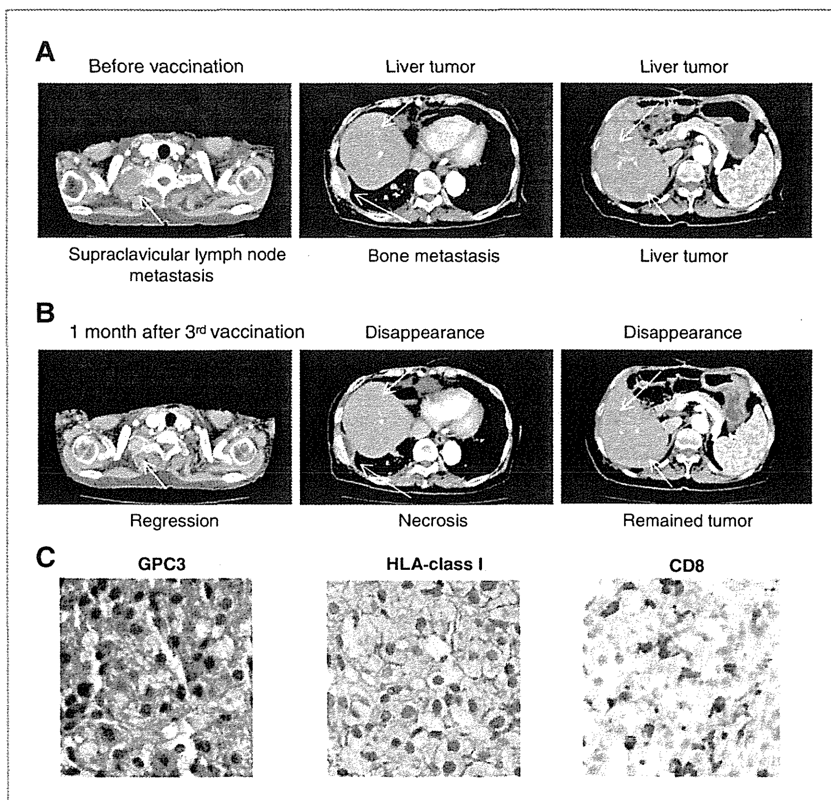

Figure 2. Response assessment in case 24. A, CT imaging, showing liver, pleura, and supraclavicular lymph node metastases before vaccination. B, CT imaging after vaccination was judged as an indicator of a PR. The supraclavicular lymph node metastasis and multiple liver tumors regressed markedly. The 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.

$P = 0.040$), AFP $\geq$ 100 ng/mL ($P = 0.003$), tumor size $\geq$ 10 cm ($P = 0.003$), and GPC3-specific CTL frequency < 50 were prognostic factors for OS. Furthermore, AFP $\geq$ 100 ng/mL ($P = 0.004$; HR = 4.66; 95% CI, 1.61–13.19), tumor size $\geq$ 10 cm ($P = 0.003$; HR = 4.36; 95% CI, 1.58–12.05), and GPC3-specific CTL frequency < 50 ($P = 0.032$; HR = 2.71; 95% CI, 1.09–6.72) were prognostic factors for OS in a multivariate analysis. We showed that GPC3-specific CTL

Table 3. Prognostic factors of OS

	P univariate	*P* multivariate	HR (95% CI)
Sex (male/female)	0.991		
Age (≥65/<65)	0.608		
Performance status (0/1)	0.707		
Child–Pugh (A/B)	0.063		
Virus infection (+/−)	0.956		
Distant metastasis (+/−)	0.032	0.284	1.71 (0.64–4.54)
Invasion of IVC or PV (+/−)	0.040	0.706	1.21 (0.45–3.30)
AFP (≥100/<100 ng/mL)	0.003	0.004	4.66 (1.61–13.19)
Tumor size[a] (≥10/<10 cm)	0.003	0.005	4.36 (1.58–12.05)
GPC3-specific CTL[b] (≥50/<50)	0.033	0.032	2.71 (1.09–6.72)
HLA (A2/A24)	0.091		
Vaccine[c] (≥1/<1 mg)	0.053		

[a]Tumor size estimated by the RECIST.
[b]The GPC3 peptide–specific CTL frequency examined with *ex vivo* IFN-γ ELISPOT assay in 5×10^5 PBMCs.
[c]The dosage of one vaccine.

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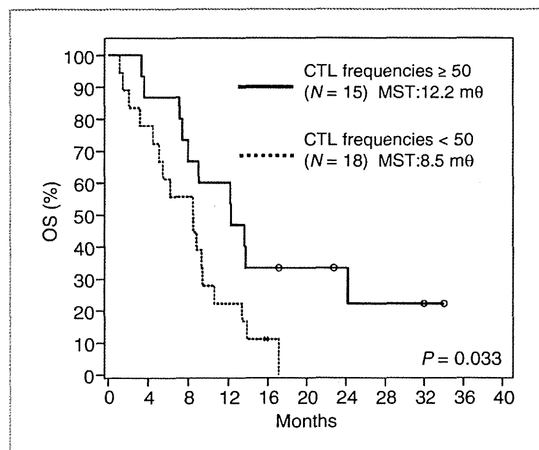

Figure 3. Kaplan–Meier curves for OS. Patients with GPC3-specfic CTL frequencies ≥50 had a longer survival than those with GPC3-specfic CTL frequencies <50 ($P = 0.033$). MST, median survival time.

frequency could be a predictive marker of the effects of GPC3 peptide vaccination. We compared patients with GPC3-specfic 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 (≥1.0 vs. <1.0 mg; Supplementary Table S2). Analysis of all 33 patients showed that the median OS was 12.2 months (95% CI, 6.5–18.0) in patients with GPC3-specfic CTL frequencies ≥50, compared with 8.5 months (95% CI, 3.7–13.1) in those with GPC3-specfic CTL frequencies <50 ($P = 0.033$; Fig. 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 Fig. S2). 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 PR, and 3 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 immunologic responses using an *ex vivo* IFN-γ 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 patients with HCC in another vaccination trial (9). Differences in tumor antigen may account for the differences in immune response between the 2 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 and colleagues reported no dose dependency in the capacity of the gp100 peptide to enhance immunogenicity in humans (at doses 1.0–10 mg; ref. 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-specfic 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 (Supplementary Table S2). In this study, several patients with advanced HCC exhibited a poor immunologic 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 costimulation 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 postvaccination have been reported as potential predictive makers of prolonged survival in patients with advanced cancer 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-specfic CTL frequencies ≥ 50 had a longer survival than those with GPC3-specfic CTL frequencies <50. There was no significant difference in the clinical backgrounds of patients with GPC3-specific CTL frequencies ≥50 and those with <50.

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