acceptable levels as Good Manufacturing Practice grade for vaccines. These peptides and the epitope peptide derived from the human immunodeficiency virus-envelope (HIV-Env) protein restricted with HLA-A*2402 (RYLRDQQLL) were used to measure the CTL response.

## Study design and End-points

### Study 1

This study was a phase I clinical trial with dose-escalation of the five peptides. This study was primarily conducted to evaluate the safety and to find the recommended dose (RD) of these peptides, and secondarily to evaluate immunological and antitumor effects. Dose escalation was performed in 3 patients' cohort with doses of 0.5 mg, 1 mg, and 3 mg for each peptide. Each peptide was mixed with 0.5 ml of incomplete Freund's adjuvant (IFA) (Montanide ISA51; Seppic, Paris, France) administered to patients.

### Study 2

Since the theoretical binding affinities of the 5 epitope peptides to HLA-A*2402 were not so different (Table 1), within one order, a single injection of the cocktail of five peptides could be expected to induce immune responses at the same level as separate injections of each of the five peptides. This study was conducted to evaluate the safety as well as immunological and antitumor effects. The cocktail of 5 peptides at the dose of 3 mg was mixed with 1.5 ml of IFA and administered to 6 patients.

### Study 1 & 2

The peptides were administered subcutaneously into the thigh or axilla regions on days 1, 8, 15, and 22 in a 28-day treatment course. Administration of the peptides was performed repeatedly for at least eight weeks for the evaluation of the safety. Vaccination was continued after 8 weeks or even after the progression of the disease when a patient wished and a primary doctor who provided best supportive care or additional chemotherapies, recommended. From the fourth courses of treatment, the vaccination schedule was changed to be biweekly, and from the seven courses, it was reduced to once a month.

A complete blood count and serum chemistry tests were performed every 2 weeks. Signs of toxicity were assessed according to CTCAE. Dose-limiting toxicity was defined as a hematological toxicity of grade 4 or greater and non-hematological toxicity of grade 3 or greater. Fifty milliliters of blood was drawn before each course, and then peripheral-blood mononuclear cells (PBMCs) and blood plasma were isolated. PBMCs and plasma were preserved in liquid nitrogen tank until examination. The vaccinated patients (n = 18) were assessed for immunological and clinical responses according to the Response Evaluation Criteria in Solid Tumors version1.0 (RECIST) as well as serum Carcinoembryonic antigen (CEA). All known sites of disease were evaluated on a monthly basis by computed tomography (CT) or magnetic resonance imaging (MRI) before vaccination and after each course.

### Estimation of local skin reactions at the vaccinated sites

Local skin reactions at injected site of vaccine were assessed according to CTCAE grading.

### Measurement of the peptide-specific IFN-γ response

Antigen-specific T cell response was estimated by enzyme-linked ImmunoSpot (ELISPOT) assays following in vitro sensitization as described previously [21,22]. The number of peptide-specific spots was calculated by subtracting the spot number in the control well from the spot number of a well with vaccinated peptide-pulsed stimulator cells. Antigen specific T cell response was classified into four grades (−, +, ++, or +++) according to the algorithm flow chart described in our previous report (+++: IFN-γ producing cell is contained more than 0.2%, ++: 0.02 - 0.2%, +: 0.01 - 0.02%, −: less than 0.01% in the sample applied for ELISPOT) (Additional file 1: Figure S1 [21]). Sensitivity of our ELISPOT assay was estimated at approximately average level by the ELISPOT panel of the Cancer Immunotherapy Consortium [23].

### Statistical analysis

Overall survival (OS) rates and progression free survival (PFS) rates were analyzed by the Kaplan-Meier method, and survival was measured in days from the first vaccination to succumbing to the disease. P-values were assessed using a log-rank test. Cox regression model was used for multivariate analysis of biomarkers for overall survival.

**Table 1 Binding score of each peptide**

| Epitope peptides | Sequence | Binding score* to HLA-A*24:02 | Expression in colorectal cancer (%) | Remarks on function and tumor relevance | References |
|---|---|---|---|---|---|
| RNF43 - 721 | NSQPVWLCL | 10 | 90% | Growth of cancer cells | [4,17] |
| TOMM34 - 299 | KLRQEVKQNL | 13.4 | 80% | Growth of cancer cells | [5] |
| KOC1 - 508 | KTVNELQNL | 14.4 | 77% | Metastasis and invasion | [6,7,18] |
| VEGFR1 - 1084 | SYGVLLWEI | 66 | 100%** | Tumor angiogenesis,growth through autocrine | [14,15,19,20] |
| VEGFR2 - 169 | RFVPDGNRI | 22 | | | |

*The binding affinities were estimated using the BioInformatics and Molecular Analysis Section websites.
**100% in tumor cells as well as tumor associated neovascularity.

Student's t-test was used for the analysis of peptide specific immune responses. All statistical analyses were performed with SPSS statistics 17.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

## Results

From February 2007 to March 2009, nineteen CRC patients were enrolled in this study and received vaccine treatment. One patient (case 7) who refused continuation of the vaccine therapy after a single administration of peptides was excluded from this analysis. Characteristics of eighteen patients are summarized in Table 2. Most of the patients except two cases had received chemotherapy regimens including fluorouracil, irinotecan or oxaliplatin prior to the vaccine treatment; one (case 11) of the two exceptions was due to pelvic abscess after the low anterior resection. The other exception (case 16) was because this patient refused chemotherapy. Another patient (case 18) had refused surgical treatment because she was afraid of the high complication risk of the second operation of the recurrent tumor in the pelvis. We provided vaccination with the written informed consent, but she accepted to receive curative resection after 8 weeks of vaccination. This case was included in the immunological analysis, but excluded from the analysis of survival.

### Study 1: dose escalation study

#### *Safety, peptides-specific immune responses and recommended dose*

The vaccination was well tolerated without any high-grade systemic adverse reactions in any of the 19 patients at any doses as shown in Table 2. However, all patients revealed injection site reactions with grade 1–2 swelling with/without inflammation except two cases with grade 2 ulceration. In this cohort of 19 patients, no dose-limiting toxicity was detected in any patients.

ELISPOT assay (Additional file 2: Figure S2) was performed using the samples obtained before and every 4 weeks after the beginning of vaccinations to evaluate induction of peptide specific CTL by measuring the IFN-γ secretion as biomarkers. The average numbers of specific CTL induction against each of five peptides per each patient within 12 vaccinations (3 courses) at the doses of 0.5 mg, 1.0 mg, and 3.0 mg were 2.0 (6 peptides/3 patients), 2.3 (7/3), and 3.2 (19/6), respectively (Table 3). The CTL responses within 3 courses (12 weeks) of vaccinations were highest in the group who received the dosage of 3.0 mg. We therefore decided that the RD was 3.0 mg/body.

### Study 2: peptide cocktail study

#### *Safety and peptides specific IFN-γ response*

Since the weekly injection at five independent loci for each peptide is painful to patients and the binding affinities of these 5 epitope peptides to the HLA molecules were suspected to be not so different (Table 1), we examined whether the mixture of five peptides in one shot (cocktail peptide) can induce peptide-specific CTLs at same or similar levels, compared with the five independent shots of each peptide. The cocktail of 3 mg each of 5 peptides was mixed with IFA and tested in 6 patients.

This vaccination protocol was well tolerated without any treatment-associated adverse events except the grade 1 or 2 injection site reaction. We measured peptide-specific CTL responses in these cocktail-treated patients and compared with those in patients who received injection at five independent loci (Table 3). In six "cocktail"-treated patients, we observed induction of CTLs in 5 patients for RNF43, 3 patients for TOMM34, 4 patients for KOC1, 4 patients for VEGFR1, and 5 patients for VEGFR2 (total of 21 peptide-specific CTL inductions in 6 patients) within 3 courses of the vaccination. Among the patients who were injected 3 mg each of the five peptides independently revealed induction of CTLs for RFN43, TOMM34, KOC1, VEGFR1, and VEGFR2 in 3, 4, 5, 4, and 3 patients, respectively (total of 19 peptide-specific CTL inductions in 6 patients). There was no significant difference to induce the immune responses between the multiple injections of "each peptide" and the single injection of "the peptide cocktail" ($p = 0.694$, t-test). These results indicated that the single injection of "the peptide cocktail" is likely to induce the similar immune responses to the multiple injections of "each peptide".

### Clinical evaluation

One patient (case 1) achieved a complete response in lymph node metastasis in the hepato-duodenal ligament and lasted for over 5 years (Figure 1A&B). In addition, 6 patients maintained stable disease condition for 4 to 7 months. In case 3, the massive liver metastases as well as lung metastases (data not shown) were kept stably for 5 months as shown in Figure 1C.

### Survival

Median PFS and OS periods of the 17 patients were 2.3 months (95% CI: 2.0-2.6) and 13.5 months (95% CI: 1.4-25.6), respectively (Figure 2A). The 2-year survival rate was 41.2% (95% CI: 17.9-64.5). Significant improvement of OS after 6 months from the first vaccination has implied the delayed response of the vaccination [24].

Patients with strong induration and redness, or those with ulceration (CTCAE grade2) survived significantly longer than patients without these reactions (Figure 2B). Patients with CTL responses to two or more peptides within 3 courses of the vaccination trended to have a longer survival (Figure 2C). Patients who revealed positive CTL responses against three or more peptides within 6 courses (24 weeks) of the vaccination showed

**Table 2 Patients characteristics and outcomes**

| Case | Dose of peptide (mg) | Age | Sex | PS | Site of desease | Previous treatment* | IFN γ response to each peptide | | | | | Number of vaccination | Period of vaccinations (days) | Toxicity | | Response | Prognosis (days) | | | Treatments after vaccination |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | R | T | K | R1 | R2 | | | Injection site reaction | General | | PFS | OS | | |
| | | | | | | | Pre vaccinations | | | | | | | | | | | | | |
| | | | | | | | Within 3 courses of vaccinations | | | | | | | | | | | | | |
| | | | | | | | Within 6 courses of vaccinations | | | | | | | | | | | | | |
| 1 | 0.5 | 56 | M | 0 | LN | IRI, OX, FU | ++ | − | − | − | − | 85 | 2150 | 2 | None | CR | 2150 | 2150 | A | None |
| | | | | | | | − | − | + | ++ | − | | | | | | | | | |
| | | | | | | | + | ++ | + | ++ | − | | | | | | | | | |
| 2 | 0.5 | 72 | F | 1 | Lung, bone | IRI, OX, FU | − | − | − | − | − | 16 | 120 | 1 | None | PD | 60 | 191 | D | None |
| | | | | | | | + | − | − | − | + | | | | | | | | | |
| | | | | | | | + | + | − | − | + | | | | | | | | | |
| 3 | 0.5 | 75 | M | 1 | Liver, lung | IRI, OX, FU | − | − | ++ | − | − | 27 | 364 | 1 | None | SD | 158 | 406 | D | OX, FU, BEV |
| | | | | | | | ++ | − | − | − | + | | | | | | | | | |
| | | | | | | | ++ | − | + | − | + | | | | | | | | | |
| 4 | 1 | 59 | F | 2 | Dissemi. | IRI, OX, FU | − | − | − | − | − | 8 | 49 | 1 | None | PD | 36 | 80 | D | None |
| | | | | | | | ++ | − | − | − | − | | | | | | | | | |
| | | | | | | | ++ | − | + | − | + | | | | | | | | | |
| 5 | 1 | 68 | M | 0 | Lung | IRI, OX, FU | − | − | − | − | − | 25 | 329 | 2 | None | PD | 68 | 1009 | D | OX, FU, IRI, BEV, CETU |
| | | | | | | | ++ | + | ++ | + | ++ | | | | | | | | | |
| | | | | | | | ++ | + | ++ | + | ++ | | | | | | | | | |
| 6 | 1 | 69 | M | 2 | Lung, LN | IRI, OX, FU | − | − | + | − | − | 13 | 84 | 2 | None | PD | 62 | 110 | D | None |
| | | | | | | | − | ++ | − | − | − | | | | | | | | | |
| | | | | | | | − | ++ | − | − | − | | | | | | | | | |
| 8 | 3 | 85 | F | 1 | Dissemi. | FU | − | − | − | − | − | 11 | 70 | 2 | None | PD | 103 | 461 | D | IRI, FU |
| | | | | | | | − | − | − | + | − | | | | | | | | | |
| | | | | | | | − | − | − | + | − | | | | | | | | | |
| 9 | 3 | 59 | M | 0 | Lung | IRI, OX, FU | + | − | − | − | − | 45 | 777 | 2 | None | SD | 221 | 885 | D | None |
| | | | | | | | ++ | ++ | ++ | ++ | − | | | | | | | | | |
| | | | | | | | ++ | ++ | ++ | ++ | − | | | | | | | | | |
| 10 | 3 | 49 | F | 0 | Liver, lung | IRI, FU | ++ | − | − | − | − | 39 | 777 | 2 | Fever <38.0°C | PD | 69 | 834 | D | OX, FU, IRI, BEV |
| | | | | | | | +++ | − | ++ | ++ | + | | | | | | | | | |
| | | | | | | | +++ | − | ++ | ++ | + | | | | | | | | | |

**Table 2 Patients characteristics and outcomes** *(Continued)*

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 3 | 46 | F | 0 | Lung | none | – | – | – | – | – | 13 | 64 | 2 | Erythena (G1) | SD | 117 | 1029 | D | OX, FU, IRI, BEV |
| | | | | | | | – | + | ++ | +++ | ++ | | | | | | | | | |
| | | | | | | | – | + | ++ | +++ | ++ | | | | | | | | | |
| 12 | 3 | 71 | M | 0 | Local | OX, FU | – | – | – | – | – | 63 | 959 | 2 | None | SD | 120 | 1059 | D | IRI, FU, OX, BEV |
| | | | | | | | + | ++ | ++ | – | + | | | | | | | | | |
| | | | | | | | + | ++ | ++ | + | + | | | | | | | | | |
| 13 | 3 | 71 | M | 2 | Liver, lung, bone | IRI, OX, FU, RAD | – | – | – | – | – | 8 | 49 | 1 | None | PD | 57 | 133 | D | None |
| | | | | | | | – | ++ | ++ | – | – | | | | | | | | | |
| | | | | | | | – | ++ | ++ | – | – | | | | | | | | | |
| 14 | 3 (mix) | 65 | F | 2 | Dissemi. | IRI, OX, FU | – | – | – | – | – | 8 | 49 | 1 | None | PD | 50 | 342 | D | None |
| | | | | | | | + | + | ++ | + | ++ | | | | | | | | | |
| | | | | | | | + | + | ++ | + | ++ | | | | | | | | | |
| 15 | 3 (mix) | 71 | M | 2 | Liver, lung | IRI, OX, FU | – | – | – | – | – | 8 | 49 | 1 | None | PD | 36 | 102 | D | None |
| | | | | | | | + | – | – | +++ | ++ | | | | | | | | | |
| | | | | | | | + | – | – | +++ | ++ | | | | | | | | | |
| 16 | 3 (mix) | 57 | M | 1 | Local | none | – | – | ++ | – | – | 38 | 749 | 2 | None | SD | 771 | 1161 | D | None |
| | | | | | | | + | – | – | +++ | +++ | | | | | | | | | |
| | | | | | | | + | ++ | – | +++ | +++ | | | | | | | | | |
| 17 | 3 (mix) | 65 | M | 1 | Dissemi. | OX, FU | – | + | – | +++ | – | 16 | 126 | 2 | None | PD | 69 | 145 | D | None |
| | | | | | | | + | + | + | +++ | + | | | | | | | | | |
| | | | | | | | + | + | + | +++ | + | | | | | | | | | |
| 18 | 3 (mix) | 55 | F | 0 | Local | IRI, FU | – | + | + | – | – | 8 | 49 | 2 | None | SD | 56 | 56 | A | Curative resection |
| | | | | | | | – | – | + | – | – | | | | | | | | | |
| | | | | | | | – | – | + | – | – | | | | | | | | | |
| 19 | 3 (mix) | 58 | F | 2 | Liver, Dissemi. | OX, FU, BEV | – | ++ | + | – | + | 8 | 49 | 1 | None | PD | 37 | 52 | D | None |
| | | | | | | | ++ | + | ++ | – | + | | | | | | | | | |
| | | | | | | | ++ | + | ++ | – | + | | | | | | | | | |

PS: Eastern Cooperative Oncology Group performance status, LN: lymph nodes, Dessemi: peritoneal dissemination, PFS: progression free survival, OS: over all survival.
IRI: irinotican, OX: oxaliplatin, FU: fluoropyrimidine, RAD: radiation, BEV: bevacizumab, CETU: cetuximab, R: RNF43, T: TOMM34, K: KOC1, R1: VEGFR1, R2: VEGFR2, D: daed, A: alive.
CR:complete response, PR: partial response, SD: stable desease, PD: progression desease, *: operation was performed for all patients for the resection of primary lesions.

**Table 3 Number of patients responded to each peptide**

| Dose of peptide (mg) | Patients | R | T | K | R1 | R2 | Total | Average* |
|---|---|---|---|---|---|---|---|---|
| | n | Within 3 courses of vaccinations | | | | | | |
| 0.5 | 3 | 2 | 0 | 1 | 1 | 2 | 6 | 2.0 |
| 1 | 3 | 2 | 2 | 1 | 1 | 1 | 7 | 2.3 |
| 3 | 6 | 3 | 4 | 5 | 4 | 3 | 19 | 3.2** |
| 3 (cocktail) | 6 | 5 | 3 | 4 | 4 | 5 | 21 | 3.5** |

R: RNF43, T: TOMM34, K: KOC1, R1: VEGFR1, R2: VEGFR2.
*: The average numbers of specific CTL induction against each of five peptides per each patient.
**: There was no significant difference to induce the immune responses between the multiple injections of "each peptide" and the single injection of "the peptide cocktail". (p=0.694, t-test).

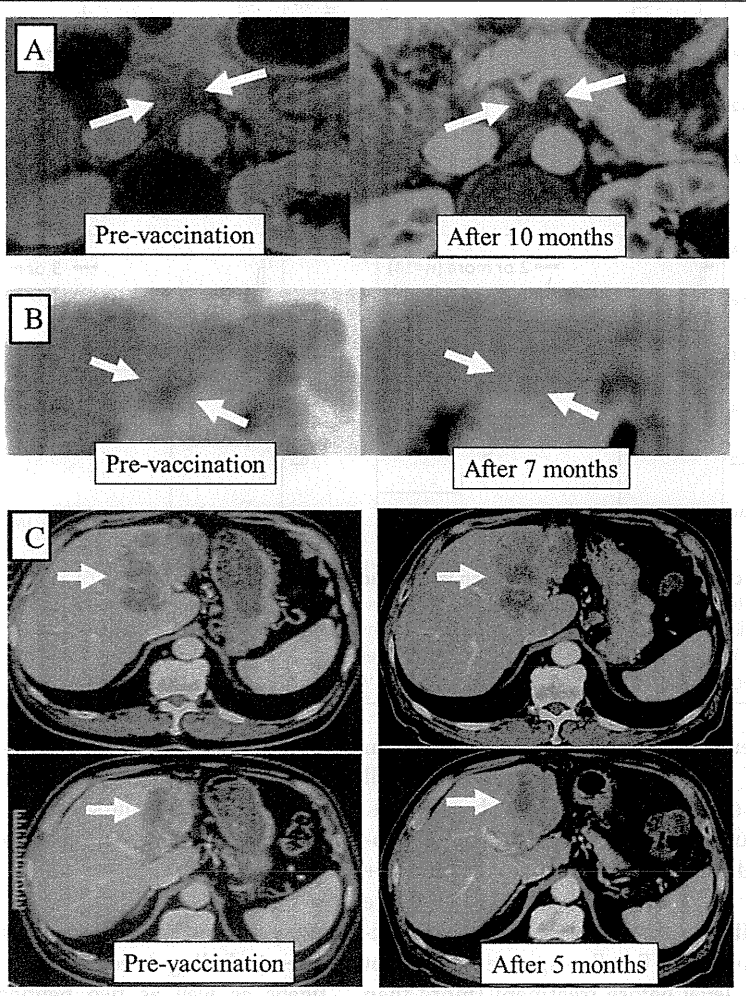


**Figure 1 Diagnostic images of case 1 and case 3. (A)** T1-weighted magnetic resonance imaging (MRI) of representative radiologic response to vaccination in case 1 who achieved complete response. **(B)** Positron Emission Tomography (PET) of case 1. Diffusely infiltrated lymph node metastases around the hepato-duodenal ligament by MRI (**A**, left side) as well as PET (**B**, left side) were confirmed their disappearance by MRI and PET analysis after 10 months of vaccination (**A** & **B**, right side). **(C)** Computed tomography in case 3 who achieved stable disease. The massive liver metastases were kept stably for 5 months.

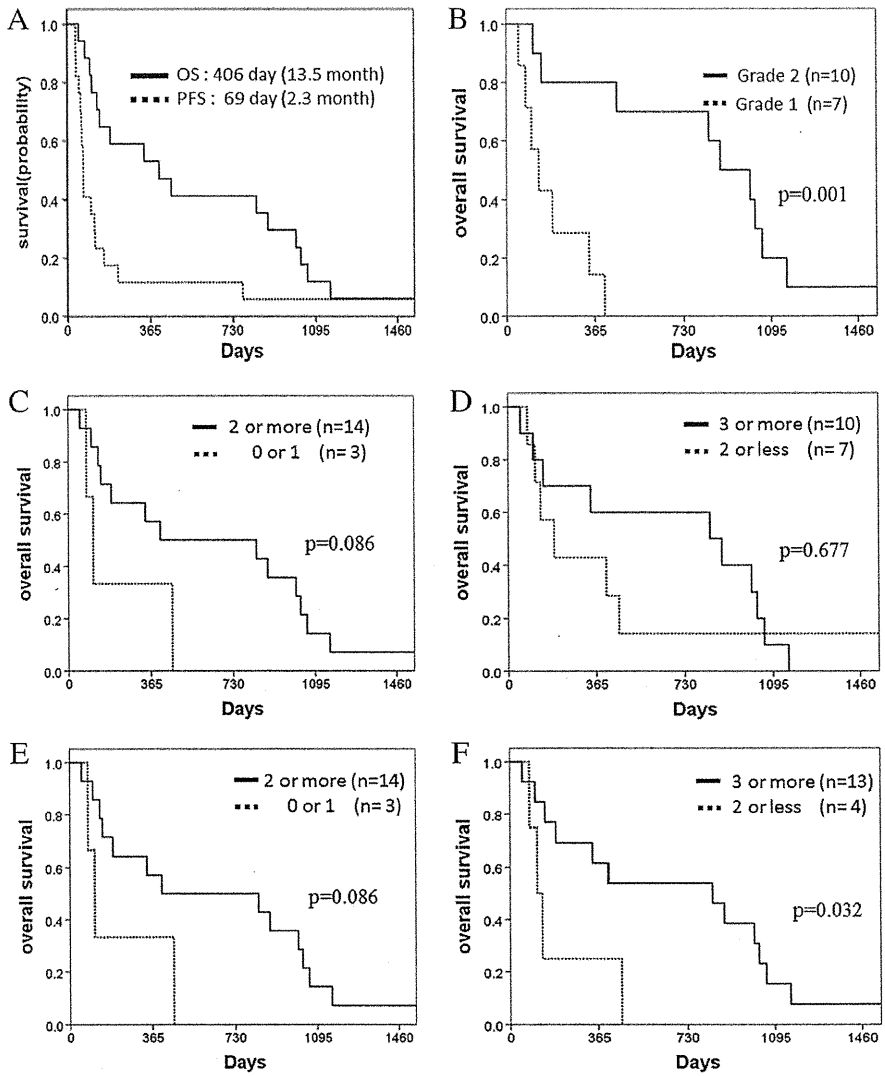


**Figure 2 Kaplan-Meier curves for survival of seventeen vaccinated patients. (A)** Overall survival (OS) and progression free survival (PFS) of seventeen vaccinated patients. **(B)** Overall survival according to the skin reactions. Local skin reactions at injected site of vaccine were assessed according to CTCAE grading. **(C, D)** Overall survival according to the number of peptide specific responses within 3 courses of the vaccination (C: 2 or more versus 1 or less, D: 3 or more versus 2 or less). **(E, F)** Overall survival according to the number of peptide specific responses within 6 courses of the vaccination (E: 2 or more versus 1 or less, F: 3 or more versus 2 or less).

a significantly longer survival (Figure 2F). In addition to the numbers of detected peptide specific T cell responses, strength of T cell responses such as +, ++, and +++ were evaluated in terms of efficacy of peptide vaccination and clinical outcome, for example, + versus ++ or more, or, ++ or less versus +++. There was no significant difference in the clinical outcome according to the strength of T cell responses (data not shown). Serum CEA level before treatment (more than 100 ng/ml or less) was also the predictive marker for the prognosis of these patients ($p = 0.003$, data not shown). Next we performed multivariate analysis of biomarkers for overall survival using Cox regression model (Table 4). For overall survival, multivariate analysis indicated that CTL responses to two or more peptides within 3 courses and strong skin reactions at injected site were significant predictors.

## Discussion

We performed phase I study using five novel epitope peptides, including three peptides derived from three oncoantigens as well as two peptides targeting VEGFR1 and VEGFR2, for colorectal cancer. Vaccinations of five peptides in three different doses as well as cocktail treatment of five peptides in metastatic CRC patients were well tolerated without any severe systemic adverse events. Although

**Table 4 Multivariate analysis of biomarkers for overall survival using Cox regression model**

| | Variable | Hazard ratio | 95% CI | P value |
|---|---|---|---|---|
| Step 1 | CEA: < 100 (ng/ml) versus ≥100 | 0.209 | 0.017 to 2.542 | 0.220 |
| | Local skin reaction: grade2 versus grade1 | 0.171 | 0.013 to 2.190 | 0.175 |
| | CTL response: 2 or more versus 1 or less (within 3 courses) | 0.078 | 0.011 to 0.548 | 0.010 |
| Step 2 | Local skin reaction: garde2 versus garde1 | 0.048 | 0.007 to 0.326 | 0.002 |
| | CTL response: 2 or more versus 1 or less (within 3 courses) | 0.101 | 0.016 to 0624 | 0.014 |

CI, confidence interval.

two patients revealed ulceration at the skin injection site, they were able to continue the vaccine treatment. Hence, the safety of our study was confirmed as previous reports using the same class of peptides [21,22,25].

The average numbers of specific CTL induction against these five peptides after 12 vaccinations of the doses of 0.5 mg, 1.0 mg, and 3.0 mg were calculated to be 2.0, 2.3, and 3.2 peptides/patient, respectively. In this study, the maximum tolerated dose was not observed. Moreover, there was no sign of immunosuppression due to excessive administration at the doses of 3.0 mg. Although the number of the patients enrolled in this study was very limited, the higher dose of peptide seems to induce peptide-specific CTLs more effectively than the lower dose. Hence, we would like to the recommended dose of each peptide for further study to be 3.0 mg.

We also attempted vaccinations of the 5-peptide cocktail that was also well tolerated without any serious systemic toxicity. Although there were some concerns that the mixture of multiple peptides in a single injection might reduce the immune response due to the different affinity of each peptide to an HLA molecule, we observed that the average numbers of peptide-specific CTL inductions per patient was 3.5 within 3 courses of vaccinations. Our results demonstrated that the cocktail of multiple peptides is non-inferiority to the separate injection of each of multiple peptides. A previous report described that the frequency of CTL induction specific to vaccinated peptides in advanced CRC was approximately 33% [26]. However, we observed the peptide-specific CTL induction in more than 60% of the patients after the 12-week of vaccination at the dose of 3.0 mg/week, indicating that our peptide epitopes might have higher immunogenicity than peptides used previously.

Among the 18 patients we evaluated clinically, one patient showed the complete response and six patients kept stable conditions for 4–7 months. Hence, the response rate (RR) was calculated to be 5.6% and the disease control rate (DCR) to be 38.9%. One review article reported that the RR of active cancer vaccines for CRC was 0.9% and the DCR was 11.1% [26]. Hence, our clinical data has also implied the possible higher immunogenicity of our vaccines.

Although our peptide vaccination did not show obvious superiority in the aspect of RR or DCR, compared to the worldwide standard therapies for CRC [27,28], the median overall survival time (MST) of our patients was 13.5 months which seemed longer than other presently-available standard therapies; for example, the MST was 6.1 months and 6.2 months in a phase III trial of Cetuximab [27] or Panitumumab [28] in patients with chemotherapy-refractory metastatic CRC, respectively. As US Food and Drug Administration described in "The guidance for therapeutic cancer vaccine", this kind of vaccine treatment usually takes a few to several months to show clinical benefit due to the time lag to induce the sufficient number of effecter cells and is expected to demonstrate delayed effects [24]. The relation between peptide specific responses and OS was shown in Figure 2. Patients who revealed the CTL induction to two or more peptides within 3 courses (MST: 13.5 months) trended to survive longer (P = 0.086) than patients with CTL induction to one or no peptide (MST: 3.7 months). Moreover, patients with CTL induction against three or more peptides within 6 courses of the vaccination (MST: 27.8 months) had significantly longer survival (P = 0.032) than the remaining patients (MST: 3.7 months), suggesting the more CTLs were induced, the better prognosis was expected and supporting the use of multiple peptides for advanced cancer patients. Similarly, local reactions at peptides-injected sites could be possibly a good predictive biomarker(s) for longer survival; Patients with grade 2 local skin reactions at injected site of vaccine survived significantly (P = 0.001) longer (MST: 29.5 months) than patients with grade 1 reactions (MST: 4.4 month). These data suggested that the monitoring of the CTL responses and the skin reactions might become good predictive markers during the treatment for the efficacy of vaccination. Although it is very difficult at present, the prior selection of patients who are likely to respond well and induce CTLs effectively to vaccination is also very important. The presence of a higher number of infiltrated T cells in tumor microenvironment was suggested as a predictive biomarker for the response to immunotherapies and the selection of patients with the better treatment outcome of vaccinations [29].

In conclusion, although the number of patients in this early-phase trial is very limited, our peptides vaccine therapy was demonstrated to be safe, effectively induce peptide-specific immune responses and possibly improve the prognosis of advanced colorectal cancer. It is certain

that although we need to verify this preliminary result by a much larger double-blind study, we believe that further stages of clinical trials should be worth doing.

## Conclusions

This study indicated that the combination of five novel peptides can induce strong peptide specific immune responses in the group who received the dosage of 3.0 mg, and that the single injection of "the peptide cocktail" is likely to induce the similar immune responses to the multiple injections of "each peptide", and that the overall survival of patients treated with our peptides was prolonged obviously after 6 months from the first vaccination, which has implied the delayed response of the vaccination. Moreover, the induction of peptide specific immune responses had significant relevance to longer survival. Although we need to verify this preliminary result by a much larger double-blind study, we believe that these findings surely lead to the novel therapeutic strategy for advanced colorectal cancer.

## Additional files

**Additional file 1: Figure S1.** Positivity of antigen-specific T cell response was quantitatively defined according to the evaluation tree algorithm. In brief, the peptide-specific spots (SS) were the average of triplicates by subtracting the HIV peptide-pulsed stimulator well from the immunized peptide-pulsed stimulator well. The %SS means the percentage of SS among the average spots of the immunized peptide pulsed stimulator well. The positivity of antigen-specific T cell response were classified into four grades (–, +, ++, and +++) depending on the amounts of peptide-specific spots and invariability of peptide-specific spots at different responder/stimulator ratios. SS, peptide-specific spots; R1, responder/stimulator ratio = 1; R2, responder/stimulator ratio = 0.5; R3, responder/stimulator ratio = 0.25; R4, responder/stimulator ratio = 0.125.

**Additional file 2: Figure S2.** Representative immunologic monitoring assays detecting antigen-specific T-cell responses in patient 10 **(A)** and 16 **(B, C)**, which were induced interferon-g (IFN-g)-producing cells. Positivity of antigen-specific T-cell response was quantitatively defined according to the evaluation tree algorithm (Additional file 1: Figure S1).

### Abbreviations

RNF43: Ring finger protein 43; TOMM34: 34 kDa-translocase of the outer mitochondrial membrane; KOC1: Insulin-like growth factor–II mRNA binding protein 3; VEGFR: Vascular endothelial growth factor receptor; CRC: Colorectal cancer; ELISPOT: Enzyme-linked immunospot; PBMC: Peripheral blood mononuclear cells; CTL: Cytotoxic T lymphocytes; CR: Complete clinical response; SD: Stable disease; PD: Progressive disease; PFS: Progression free survival; OS: Overall survival; HLA: Human leukocyte antigen; MST: Median overall survival time; ECOG: Eastern cooperative oncology group; RECIST: Response evaluation criteria in solid tumors; TIL: Tumor infiltrating cells; CTCAE: Common Terminology Criteria for Adverse Events version3.0; PS: Performance status; HPLC: high performance liquid chromatography; HIV-Env: The human immunodeficiency virus-envelope; IFA: Incomplete Freund's adjuvant; CT: Computed tomography; MRI: Magnetic resonance imaging; IFN: Interferon.

### Competing interests

Yusuke Nakamura is a stock holder and a scientific advisor of OncoTherapy Science, Inc. The other authors have no potential conflicts of interest to disclose.

### Authors' contributions

SH designed, performed and evaluated clinical study, and wrote the manuscript. YN and MO participated in the design, review and revision of the manuscript. KT participated as the main coordinator and investigator regarding the immunological data analysis and evaluation. HT, NS, RT, YI YT, NI, SY, HS, AK, and HF assisted to perform clinical study and data analysis. All authors participated in the data acquisition and discussion of the manuscript and approved the final manuscript.

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# Analysis of cytotoxic T lymphocytes from a patient with hepatocellular carcinoma who showed a clinical response to vaccination with a glypican-3-derived peptide

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**Abstract.** Glypican-3 (GPC3), which is a carcinoembryonic antigen, is overexpressed in human hepatocellular carcinoma (HCC). Previously, we performed a phase I clinical trial of GPC3-derived peptide vaccination in patients with advanced HCC, and reported that GPC3 peptide vaccination is safe and has clinical efficacy. Moreover, we proposed that a peptide-specific CTL response is a predictive marker of overall survival in patients with HCC who receive peptide vaccination. In this study, we established GPC3-derived peptide-specific CTL clones from the PBMCs of an HLA-A*02:07-positive patient with HCC who was vaccinated with an HLA-A2-restricted GPC3 peptide vaccine and showed a clinical response in the phase I clinical trial. Established CTL clones were analyzed using the IFN-γ ELISPOT assay and a cytotoxicity assay. GPC3 peptide-specific CTL clones were established successfully from the PBMCs of the patient. One CTL clone showed cytotoxicity against cancer cell lines that expressed endogenously the GPC3 peptide. The results suggest that CTLs have high avidity, and that natural antigen-specific killing activity against tumor cells can be induced in a patient with HCC who shows a clinical response to vaccination with the $GPC3_{144-152}$ peptide.

## Introduction

Primary liver cancer, which is frequently hepatocellular carcinoma (HCC), is the sixth most common cancer and third most frequent cause of cancer-related death worldwide, and it is becoming more prevalent not only in East Asia, South-East Asia, and Africa but also in Western countries (1-3). Recently, the multikinase inhibitor sorafenib was demonstrated to prolong overall survival (OS) in patients with advanced HCC, and it has become the standard drug for first-line systemic treatment (4-6). However, based on the Response Evaluation Criteria in Solid Tumors (RECIST), the response rate for sorafenib is rather low, and the incidence of adverse events is relatively high, especially in elderly patients (7). Therefore, the generation of a novel effective therapy for HCC is a priority.

Immunotherapy is an attractive option for treating HCC. Many of the tumor antigens associated with HCC are potential candidates for peptide vaccines (8,9). The carcinoembryonic antigen Glypican-3 (GPC3), which is a 65-kDa protein of 580 amino acids, belongs to the family of glycosyl-phosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans (HSPG) (10,11). GPC3 is specifically overexpressed in HCC (72-81% of cases) and correlates with poor prognosis (12-16). This suggests that GPC3 is an ideal target for anti-HCC immunotherapy.

We have previously demonstrated the antigenicity of GPC3, and that the HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) peptide and the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide can induce GPC3-reactive CTLs without inducing autoimmunity (17-21).

HLA-A2 is the most frequent HLA-A type in all ethnic groups (22). HLA-A2 is also expressed in about 40% of Japanese persons (23,24) and in about 50% of Caucasians (25). Among Caucasians, >90% of HLA-A2-positive individuals carry the HLA-A*02:01 allele (25), whereas among the Japanese, there are multiple common and well-documented (CWD) allelic variants, including HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 (26). The frequencies of the HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 alleles in the Japanese population are 19, 14 and 7%, respectively (26). Therefore, we confirmed that the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide could also bind to HLA-A*02:06 and HLA-A*02:07 using a binding assay (unpublished data).

On the basis of these results, we conducted a phase I clinical trial of a GPC3-derived peptide vaccine in 33 patients with advanced HCC. The HLA-A*24:02-restricted $GPC3_{298-306}$ peptide was used for HLA-A*24:02-positive patients and

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the HLA-A*02:01-restricted $GPC3_{144-152}$ peptide was used for HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07-positive patients. We found that GPC3 vaccination was well-tolerated, and that the GPC3 peptide vaccine induced a GPC3-specific CTL response in almost all of the patients (27-30). Moreover, the vaccination-induced GPC3-specific CTL response correlated with overall survival (OS); the OS was significantly longer in patients with high GPC3-specific CTL frequencies than in those with low GPC3-specific CTL frequencies (27). In terms of clinical responses, one patient showed a partial response (PR) and 19 patients showed stable disease 2 months after initiation of treatment. One patient with HCC who showed a PR was HLA-A*02:07-positive. In addition, several HLA-A*02:01-restricted GPC3 peptide-specific CTL clones with cytotoxic activities against GPC3 were established from the peripheral blood mononuclear cells (PBMCs) of patients vaccinated in this trial (27).

The aims of the present study were: i) to establish GPC3-derived, peptide-specific CTL clones from the PBMCs of an HLA-A*02:07-positive patient with HCC who showed a PR in the phase I clinical trial; and ii) to analyze the functions of these CTL clones.

## Materials and methods

*Ethics information.* 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. All the patients gave written informed consent before entering the study at the National Cancer Center Hospital East (Chiba, Japan). The trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR no. 000001395).

*PBMCs collection.* Peripheral blood samples were obtained pre- and post-vaccination from the patient with HCC who was HLA-A*02:07-positive. Post-vaccination, blood samples were collected from the patient every 2 weeks. The GMP-grade peptide $GPC3_{144-152}$ (FVGEFFTDV) (American Peptide Co., Sunnyvale, CA, USA) was emulsified in IFA (Montanide ISA-51 VG; SEPPIC, Paris, France) and injected intradermally at 30 mg/body three times at 14-day intervals (27,28). PBMCs were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and frozen in liquid nitrogen until use.

*Cell lines.* The human lung cancer cell line 1-87 ($GPC3^-$, HLA-A*02:07$^+$/A*11:01$^+$) and hepatitis B virus (HBV)-integrated human hepatocellular carcinoma cell line JHH-7 ($GPC3^+$, HLA-A*24:02$^+$/A*31:01$^+$) were conserved in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Company, St. Louis, MO, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA).

*Plasmids and transfection.* The expression vectors pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pcDNA3.1 that contained the HLA-A*02:07 cDNA were used for the transfection experiments. The pcDNA3.1 construct that contained HLA-A*02:07 was kindly provided by Dr Ryo Abe (Tokyo University of Science, Chiba, Japan). The JHH-7/HLA-A*02:07 cell line was obtained by transfection of JHH-7 cells with the expression vector using FuGENE HD (Roche Applied Science, Mannheim, Germany). JHH-7/mock and JHH-7/HLA-A*02:07 cells were cultured in DMEM that was supplemented with 10% heat-inactivated FBS and 1 mg/ml G418 (Calbiochem, Darmstadt, Germany).

*Induction of $GPC3_{144-152}$ peptide-specific CTLs from PBMCs.* The PBMCs were cultured ($2x10^6$ cells/well) with the $GPC3_{144-152}$ peptide in RPMI-1640 (Sigma Chemical Company) that was supplemented with 10% heat-inactivated FBS, 100 IU/ml recombinant human IL-2 (Nipro, Osaka, Japan), and 10 ng/ml recombinant human IL-15 (PeproTech Inc, Rocky Hill, NJ, USA) for 14 days.

*CD107a staining and flow cytometry analysis.* $CD8^+$ T cells were isolated using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs that were stimulated with the $GPC3_{144-152}$ peptide for 14 days. The $CD8^+$ T cells were incubated with $GPC3_{144-152}$-pulsed or $HIV_{19-27}$-pulsed 1-87 cells at a ratio of 2:1 for 3.5 h at 37°C. CD107a-specific antibodies (BD Biosciences, San Jose, CA, USA) were included in the mixture during the incubation period.

*Generation of CTL clones.* $CD8^+$ $CD107a^+$ cells were sorted using a FACSAria cell sorter (BD Biosciences). Sorted CTLs were stimulated and the CTL clones were established as previously described (28).

*Cytotoxicity assay.* Cytotoxic capacity was analyzed with the Terascan VPC system (Minerva Tech, Tokyo, Japan). The CTL clone was used as the effector cell type. Target cells were labeled in calcein-AM solution for 30 min at 37°C. The labeled cells were then co-cultured with the effector cells for 4-6 h. Fluorescence intensity was measured before and after the culture period, and specific cytotoxic activity was calculated as previously described (28).

*IFN-γ ELISPOT assay.* Specific secretion of IFN-γ from human CTLs in response to stimulator cells was assayed using the IFN-γ ELISPOT kit (BD Biosciences), according to the manufacturer's instructions. Stimulator cells were pulsed with or without peptide for 1.5 h at room temperature and then washed three times. Responder cells were incubated with stimulator cells for 20 h. The resulting spots were counted using an ELIPHOTO counter (Minerva Tech).

*Determination of recognition efficiency.* Calcein-AM-labeled target cells were pulsed with various concentrations of peptide, starting at $10^{-6}$ M and decreasing in log steps to $10^{-14}$ M. The CTL clones were incubated with the target cells at an effector:target (E/T) ratio of 10:1 for 4 h. The recognition efficiencies of the CTL clones were defined as previously described (28).

*RNA interference.* Human GPC3-specific siRNAs were chemically synthesized as double-strand RNA (Invitrogen). A non-silencing siRNA, AllStras Neg. Control siRNA, was obtained from Qiagen (Valencia, CA, USA). The following

GPC3-specific siRNA sequences were used: GPC3-siRNA (#4149), 5'-UUAUCAUUCCAUCACCAGAGCCUCC-3'; GPC3-siRNA (#4150), 5'-GGAGGCUCUGGUGAUGGAAU GAUAA-3': and GPC3-siRNA (#4151), 5'-UAUAGAUGACUG GAAACAGGCUGUC-3'. Synthetic siRNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocols.

*RT-PCR*. Using the TRIzol reagent (Invitrogen), we extracted total cellular RNA from untreated or siRNA (GPC3-siRNA or negative-siRNA)-treated JHH-7/HLA-A*02:07. cDNA was synthesized using the PrimeScript II 1st Strand cDNA Synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The cDNA was added to a reaction mix that contained 10X Ex Taq Buffer (Takara), 2.5 mM dNTP mixture (Takara), 5 units Ex Taq (Takara), and 10 $\mu$M of the *GPC3*- or *β-actin*-specific PCR primers. The following primer sequences (sense and antisense, respectively) were used: for *GPC3*, 5'-AGCCAAAAGGCAGCAAGGAA-3' and 5'-AAGA AGAAGCACACCACCGA-3'; and for *β-actin*, 5'-CCTCGCCT TTGCCGATCC-3' and 5'-GGATCTTCATGAGGTAGTC AGTC-3'. PCR was performed using the 96-well Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). PCR was performed for 20 cycles of 98°C for 10 sec, 64°C for 30 sec and 72°C for 30 sec, followed by a step of 72°C for 10 sec.

*Sequence analysis of TCR-β gene*. Using the TRIzol reagent (Invitrogen), total cellular RNA was extracted from established CTL clones. The cDNA of the *TCR-β* gene was synthesized using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions, with the modification that we used 200 nM of the primer specific for the TCR-β chain constant region. The cDNA products were subjected to 2-step PCR, as previously described by Yukie Tanaka-Harada (35,36), and the PCR products were purified and sequenced in the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). The *TCR-β* variable (*TRBV*) gene, *TCR-β* joining (*TRBJ*) gene, *TCR-β* diversity (*TRBD*) alleles, and complementarity-determining region 3 (*CDR3*) sequences were identified using the IMGT databases (http://www.imgt.org/).

## Results

*$GPC3_{144-152}$ peptide-specific CTLs in the peripheral blood of the patient exert a clinical effect*. We analyzed the immune responses of the patient who showed a PR following $GPC3_{144-152}$ peptide vaccination. In this patient, the supraclavicular lymph node metastases markedly regressed, two liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (27). The levels of DCP decreased in the patients over the 2-month period. We evaluated the $GPC3_{144-152}$-specific immune responses in the peripheral blood using the *ex vivo* IFN-γ ELISPOT assay. For the HLA-A*02:07-positive patient with advanced HCC, the number and area of the spots increased after two rounds of vaccination, as compared with the pre-vaccination values, and the peak values were noted 10 weeks after the start of the treatment (Fig. 1A).

*Establishment of $GPC3_{144-152}$-specific CTL clones from the PBMCs of the patient*. To investigate the ability of the $GPC3_{144-152}$-specific CTLs induced by peptide vaccination to recognize antigen, we established CTL clones from the PBMCs of this patient 10 weeks after the start of treatment. The PBMCs were stimulated with the $GPC3_{144-152}$ peptide *in vitro* for 14 days. $CD8^+$ T cells were isolated from the stimulated PBMCs, and then incubated with peptide-pulsed 1-87 cells. $CD8^+$ $CD107a^+$ cells that reacted with the $GPC3_{144-152}$-pulsed 1-87 cells were sorted to the single-cell level. Thus, we established $GPC3_{144-152}$ peptide-specific CTL clones.

Three established CTL clones were analyzed for function using the IFN-γ ELISPOT assay and cytotoxicity assay. All of the CTL clones released IFN-γ in response to the $GPC3_{144-152}$-pulsed 1-87 cells, but not in response to non-pulsed 1-87 cells (Fig. 1B). Moreover, these CTL clones showed cytotoxicity against $GPC3_{144-152}$-pulsed 1-87 cells, but not against non-pulsed or HIV19-27-pulsed 1-87 cells (Fig. 1C). These results indicate that the CTL clones 24-4-2, 24-4-7 and 24-2-10 have specificity for the $GPC3_{144-152}$ peptide.

*Functional avidity of the $GPC3_{144-152}$-specific CTL clones*. We evaluated the cytotoxicity profiles of the CTL clones for 1-87 cells pulsed with a decreasing concentration series (from $10^{-6}$ to $10^{-14}$ M) of the $GPC3_{144-152}$ peptide. The peptide concentration at which the curve reached 50% cytotoxicity was defined as the recognition efficiency of the clone. The recognition efficiencies of CTL clones 24-4-2, 24-4-7 and 24-2-10 were $10^{-11}$, $10^{-9}$ and $10^{-8}$ M, respectively (Fig. 2). This result suggests that CTL clone 24-4-2 has a higher avidity than the other two clones and, conversely, that CTL clone 24-2-10 has a lower avidity than the other two clones.

*A $GPC3_{144-152}$-specific CTL clone recognizes cancer cells that endogenously express GPC3*. Next, we tested the reactivities of these CTL clones against cancer cell lines that expressed GPC3 and HLA-A*02:07. We used the JHH-7/mock ($GPC3^+$, HLA-A*02:07-) and JHH-7/HLA-A*02:07 ($GPC3^+$, HLA-A*02:07$^+$) transfectants as the target cells (Fig. 3A). The CTL clone 24-4-2 (with high avidity) produced IFN-γ and was cytotoxic for JHH-7/HLA-A*02:07 cells but not for JHH-7/mock cells (Fig. 3B and C). The other clones did not produce IFN-γ and did not exhibit cytotoxicity for the two target cell lines. These results suggest that only high-avidity CTLs recognize cancer cells that express GPC3 peptide endogenously.

*CTL clone 24-4-2 shows specificity for GPC3*. To ascertain the GPC3 antigen-specific response of CTL clone 24-4-2, we created a GPC3 knockdown via siRNA treatment of the JHH-7/HLA-A*02:07 cells. GPC3 expression by the JHH-7/HLA-A*02:07 cells was clearly decreased by the GPC3-siRNA, as assessed by RT-PCR (Fig. 4A). We examined the IFN-γ production levels of CTL clone 24-4-2 against JHH-7/HLA-A*02:07 cells treated with GPC3-siRNA. IFN-γ production by CTL clone 24-4-2 was significantly decreased by the GPC3-siRNA (Fig. 4B). These results indicate that the HLA-A2-restricted $GPC3_{144-152}$ peptide is processed naturally by cancer cells, and that both HLA-A*02:07 and HLA-A*02:01 can present the $GPC3_{144-152}$ peptide on the surfaces of cancer cells.

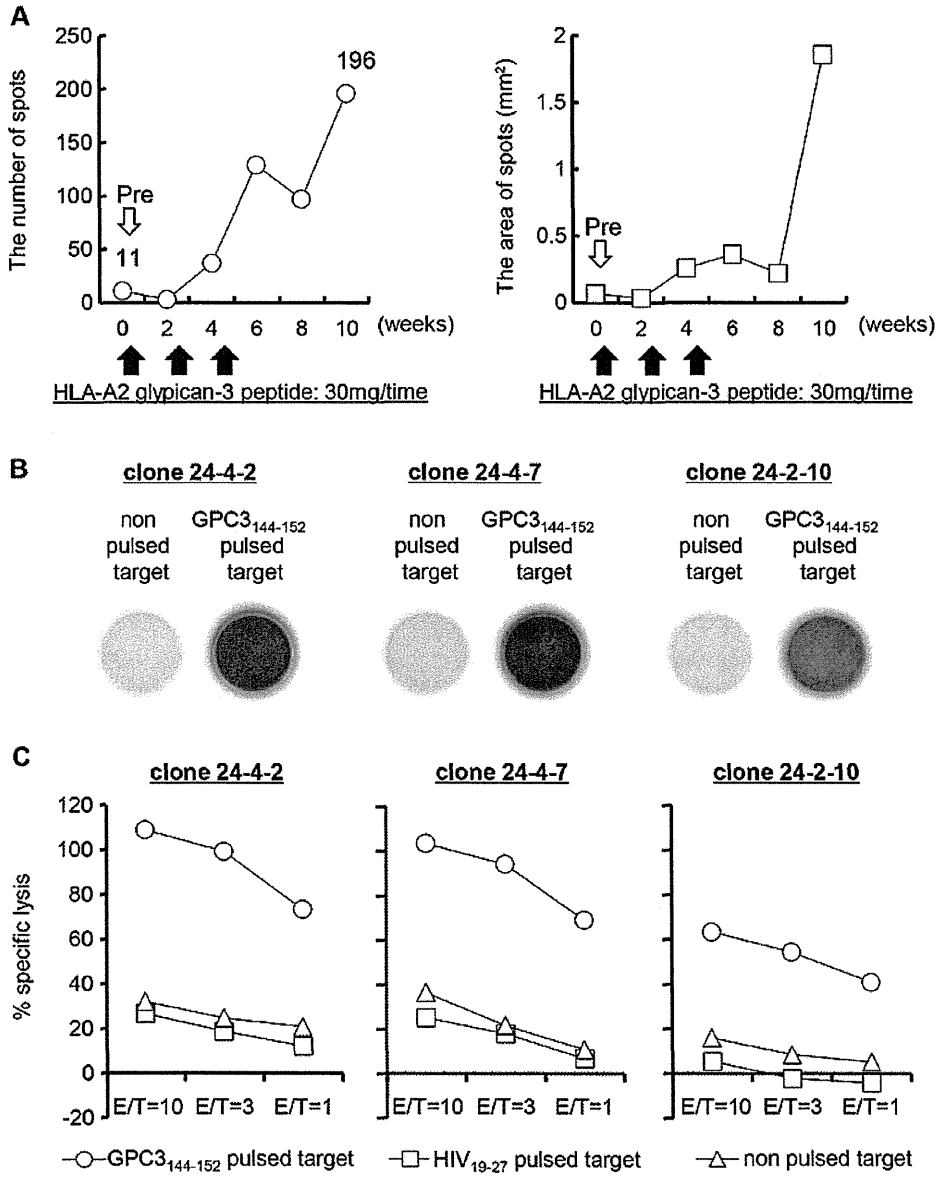


Figure 1. GPC3 peptide-specific CTL clones established from the PBMCs of a patient following GPC3 peptide vaccination. (A) Changes in the frequencies of $GPC3_{144\text{-}152}$ peptide-specific CTLs before and after vaccination in a patient who showed a PR post-vaccination. Changes in the GPC3 peptide-specific CTLs are observed as differences in the number (left) and the area (right) of spots in an *ex vivo* IFN-γ ELISPOT assay. (B) Results of the IFN-γ ELISPOT assay against peptide-pulsed target. HLA-A*02:07$^{+}$ cancer cell line 1-87 was used as the target. The target was pulsed with the $GPC3_{144\text{-}152}$ peptide. A non-pulsed target was used as the negative control. The ratio of effector cells to target cells (E/T) is 1. (C) Results of the cytotoxicity assay against peptide-pulsed target. The 1-87 cells were used as the target. Non-pulsed and $HIV_{19\text{-}27}$ peptide-pulsed targets were used as negative controls. E/Ts are 10, 3 and 1, respectively. A representative of three experiments is shown.

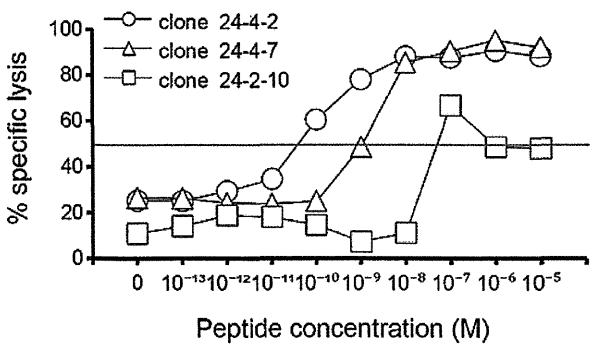


Figure 2. $GPC3_{144\text{-}152}$ peptide-specific avidity of the established CTL clones. The established CTL clones were tested for avidity using 1-87 cells that were pulsed with various concentrations of the $GPC3_{144\text{-}152}$ peptide. The peptide concentration at which the curve crossed the 50% cytotoxicity mark was defined as the recognition efficiency of that clone. E/T is 10. A representative of three experiments is shown.

*Established CTL clones have different sets of TCR-β alleles.* We analyzed the *TCR-β* gene sequences of the established CTL clones. The *TRBV*, *TRBJ* and *TRBD* alleles were identified using the IMGT databases. Thus, we identified the *TRBV*, *TRBD* and *TRBJ* alleles of the CTL clones (Table I). Each of the established CTL clones had different allele sets.

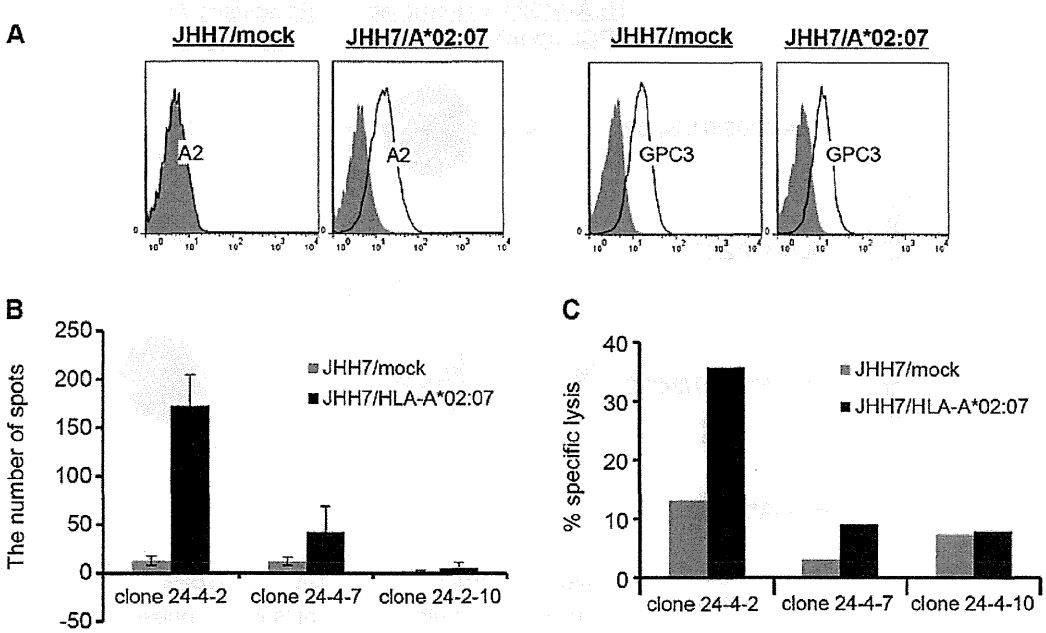


Figure 3. Recognition of GPC3$^+$ cancer cells by the established CTL clones. (A) Expression of HLA-A2 (left panel) and GPC3 (right panel) on established GPC3$^+$ HLA-A*02:07$^+$ cancer cells and control cells. (B) Results of the IFN-γ ELISPOT assay for the GPC3$^+$ cancer cell line. The HLA-A*02:07-overexpressing GPC3$^+$ cancer cell line, JHH7/HLA-A*02:07, was established and used as the target. JHH7/mock cells were used as the negative control. E/T ratio, 1. Data are presented as mean ± SD of three independent batches. (C) Results of the assay for cytotoxicity against the GPC3$^+$ cancer cell line. JHH7/HLA-A*02:07 cells were used as the target. JHH7/mock cells were used as the negative control. E/T is 3. A representative of three experiments is shown.

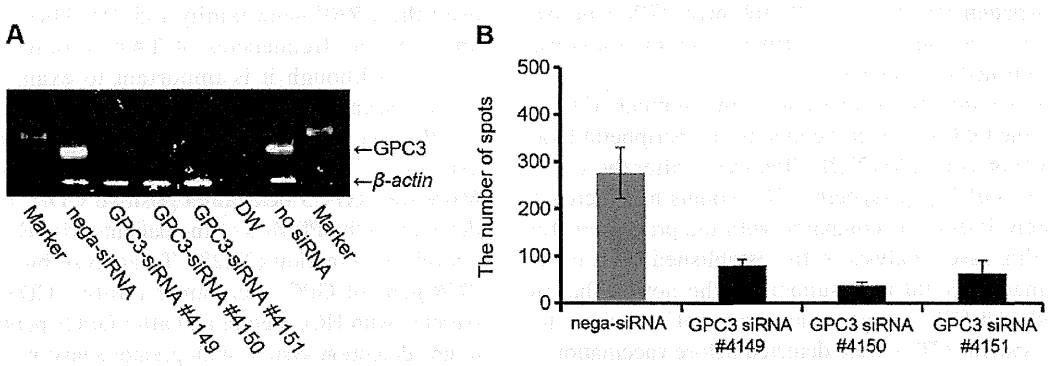


Figure 4. GPC3 specificity of CTL clone 24-4-2. (A) GPC3 expression levels on JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or negative (nega)-siRNA for 48 h, as determined by RT-PCR. (B) Results of the IFN-γ ELISPOT assay for JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or nega-siRNA. E/T is 1. Data are presented as mean ± SD of three independent batches.

*CTL clone 24-4-2 is subject to HLA-A*02:07 restriction.* We investigated whether CTL clone 24-4-2 recognized the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex and the $GPC3_{144-152}$ peptide-HLA-A*02:06 complex, as well as the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex. Healthy donor PBMCs with HLA-A*02:01, HLA-A*02:06, HLA-A*02:07 and HLA-A*24:02 were used as the targets, and an HLA-A*02:01-restricted, GPC3-specific CTL clone, which is a previously established CTL clone (26), was used as the control. The HLA-A*02:01-restricted CTL clone recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex, and CTL clone 24-4-2 recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex (Fig. 5). These outcomes indicate that CTL clone 24-4-2 has HLA-A*02:07 restriction.

Table I. TCR-β chain sequencing for established CTL clones.

| No. | TRBV | TRBJ | TRBD |
|---|---|---|---|
| Clone 24-4-2 | 18*01 | 1-2*01 | 1*01 |
| Clone 24-4-7 | 7-3*01 | 2-7*01 | 1*01 |
| Clone 24-2-10 | 7-6*01 | 2-1*01 | 2*01 |

## Discussion

Clinical trials of peptide-based vaccines are underway in several parts of the world. However, the monitoring of individual CTL post-vaccination has scarcely been reported in

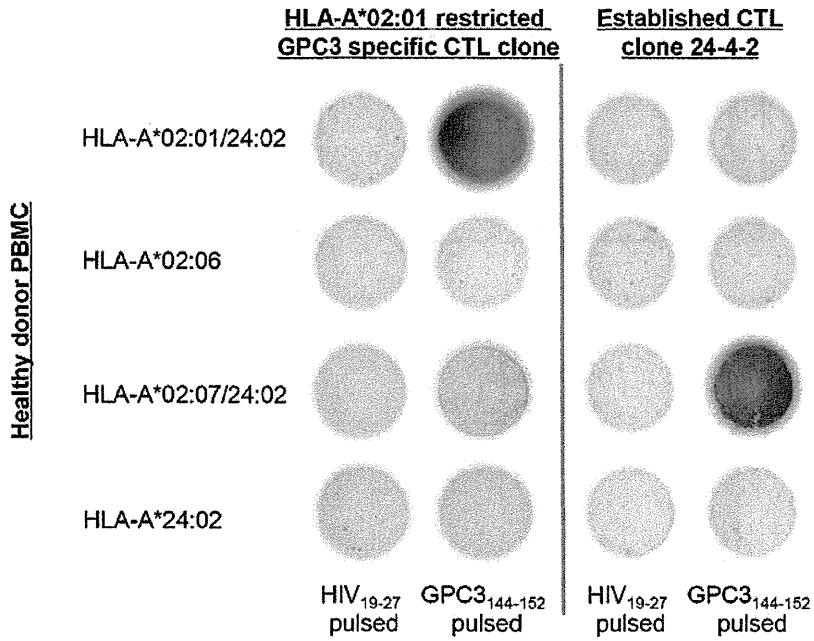


Figure 5. CTL clone 24-4-2 shows HLA-A*02:07 restriction. Results of the IFN-γ ELISPOT assay for healthy donor PBMCs with HLA-A2. The established CTL clone 24-4-2 and the HLA-A*02:01-restricted, GPC3-specific CTL clone were used as effectors. E/T is 0.2. A representative of two experiments is shown.

immunotherapy trials. In the present study, we established HLA-A*02:07$^+$ $GPC3_{144-152}$-specific CTL clones from the PBMCs of a patient who showed a PR following GPC3-derived peptide vaccination and we performed functional analyses against established CTL clones.

This patient showed an increase in the number of CTLs specific for the GPC3-derived peptide in the peripheral blood after vaccination (Fig. 1A) (27,28). Ten weeks after the start of treatment, the $GPC3_{144-152}$-specific CTL counts had increased approximately 18-fold, as compared with the pre-vaccination counts. In this case, analysis of the established CTL clones after vaccination could lend support to the notion that the vaccine-induced CTLs exert an antitumor effect, since few $GPC3_{144-152}$-specific CTLs were detected before vaccination.

In the present study, we confirmed that $GPC3_{144-152}$-specific CTL clones are cytotoxic for both $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that express GPC3 peptide endogenously. Confirming that the GPC3 peptide-specific CTL clones kill cancer cells that express endogenously the antigen peptide is important because antigen-derived and CTL-inducible peptides are not necessarily presented by cancer cells that endogenously express the antigen (31-33). Three established CTL clones showed cytotoxic activities related to their avidity for $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that expressed the GPC3 peptide endogenously. These results show that although CTLs with different avidity can be isolated, only those CTLs with high avidity can kill cancer cells that express the antigen peptide endogenously. Several investigators have demonstrated a correlation between T-cell avidity and target recognition by T-cell populations that recognize murine tumor models and human cancers (34). Our results strongly support this observation.

The TCR usage of antigen-specific T cells is thought to be influenced by the affinity of the TCR for the antigen peptide-HLA class I complex. Several studies on the TCR usage of tumor-associated antigen (TAA)-specific T cells have used the *TRBV* gene family (35-41). These studies mainly analyzed the frequencies of TAA tetramer positive CD8$^+$ T cells. Although it is important to examine quantitative aspects, such as the frequencies of TAA tetramer positive CD8$^+$ T cells, the cytotoxicity of these T cells against cancer cells that express the TAA peptide endogenously cannot be confirmed. Moreover, GPC3 dextramer positive CD8$^+$ T cells were not detected in the PBMCs of the patients with HCC before GPC3 peptide vaccination (27,28). To analyze biased usage of the *TCR* gene of GPC3 dextramer positive CD8$^+$ T cells in the patients with HCC before and after GPC3 peptide vaccination, a new detection system with greater sensitivity *ex vivo* will be required. In the present study, we analyzed the *TCR-β* genes of the established $GPC3_{144-152}$-specific CTL clones, to confirm that these CTL clones have different TCRs. Our experiments show that the established CTL clones have different TCR-β-chain allele sets, i.e., *TRBV*, *TRBD* and *TRBJ* alleles (Table I), and different CDR3 sequences (data not shown). These results suggest that various GPC3-specific CTLs are induced by $GPC3_{144-152}$ peptide vaccination.

A*HLA-A*02:07 differs from HLA-A*02:01 by a single non-conservative change (Y to C) at residue 99. X-ray crystallographic data have identified position 99 as one of the residues forming the D secondary pocket, which engages the residue at position 3 on peptide ligands (42-44). Although hHLA-A*02:07 was originally not included in the HLA-A2 supertype, cross-reactivity between HLA-A*02:07 and other A2 subtypes was detected at the functional level (44,45). Moreover, this HLA molecule indeed binds a subset of the peptide repertoire bound by other A2 subtypes (44). For these reasons, HLA-A*02:07 should also be included in the A2 supertype (46). Ito *et al* (47) and Nonaka *et al* (48) reported that an HLA-A2-restricted

CTL line established from the tumor-infiltrating lymphocytes (TIL) of an HLA-A*02:07-positive patient showed significant cytotoxicities for HLA-A*02:01-, HLA-A*02:06- and HLA-A*02:07-positive cancer cells. Therefore, we examined whether the $GPC3_{144-152}$-specific CTL clone 24-4-2, which was established from the PBMCs of an HLA-A*02:07- positive patient with HCC, could recognize HLA A-A*02:01 or HLA-A*02:06. However, this CTL clone failed to recognize HLA-A*02:01 or HLA-A*02:06.

We have reported previously on the detection via immunohistochemical staining of massive infiltration of CD8-positive T cells into the remaining liver tumor of this patient (27). It was difficult to confirm that these tumor-infiltrating $CD8^+$ T cells have specificity for GPC3. Currently, we are conducting clinical testing of liver biopsies taken before and after GPC3 peptide vaccination of patients with advanced HCC. Our aim is to reveal the GPC3 peptide-specific immune responses induced by the GPC3-derived peptide vaccine in both the peripheral blood and the tumor. We are analyzing the *TCR* gene sequences of CD8 or GPC3 dextramer positive T cells in both the peripheral blood and tumor. Already in this trial, a remarkable clinical effect has been observed for an HLA-A*02:07-positive patient with HCC who received $GPC3_{144-152}$ peptide vaccination (49).

HLA-A*02:07 is present in the populations of East Asia, South-East Asia (7%), and northern India (11.5%) (26,50-52). In southern China, the frequency of the HLA-A*02:07 allele is reported to be even higher than the frequency of the HLA-A*02:01 allele (53,54). In addition, about 75% of liver cancer cases occur in South-East Asia, including China, Hong Kong, Taiwan, Korea, India and Japan (55). Taking together these previous reports and our results, it appears that HLA-A*02:07-positive patients with HCC are good candidates for $GPC3_{144-152}$ peptide vaccination. Further studies will be necessary to prove the clinical efficacy of GPC3 peptide vaccination for advanced HCC.

In conclusion, we present substantial evidence that $GPC3_{144-152}$-specific CTLs with different TCR allele sets that are induced in patients with HCC who show a PR following $GPC3_{144-152}$ peptide vaccination indicate not only high avidity but also natural antigen-specific killing activity against tumor cells.

## Acknowledgements

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# Identification of HLA-A2 or HLA-A24-restricted CTL epitopes for potential HSP105-targeted immunotherapy in colorectal cancer

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**Abstract.** We previously reported that heat shock protein 105 (HSP105) is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer and has proven to be a novel biomarker for the immunohistochemical detection of these cancers. In the present study, we used HLA-transgenic mice (Tgm) and the peripheral blood mononuclear cells (PBMCs) of colorectal cancer patients to identify HLA-A2 and HLA-A24-restricted HSP105 epitopes, as a means of expanding the application of HSP105-based immunotherapy to HLA-A2- or HLA-A24-positive cancer patients. In addition, we investigated by *ex vivo* IFN-γ ELISPOT assay whether the HSP105-derived peptide of cytotoxic T cells (CTLs) exists in PBMCs of pre-surgical colorectal cancer patients. We found that four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYKQDL) and HSP105 A24-7 (EYVYEFRDKL), are potential HLA-A2 or HLA-A24-restricted CTL HSP105-derived epitopes. HSP105-specific IFN-γ-secreting T cells were detected in 14 of 21 pre-surgical patients with colorectal cancer in response to stimulation with these four peptides. Our study raises the possibility that these HSP105 peptides are applicable to cancer immunotherapy in patients with HSP105-expressing cancer, particularly colorectal cancer.

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*Key words:* cancer immunotherapy, cytotoxic T cell, colorectal cancer, heat shock protein 105, HLA-transgenic mice

## Introduction

Colorectal cancer is one of the most prevalent cancers and a major cause of mortality worldwide (1). Although adjuvant systemic chemotherapy or chemoradiation can confer a limited but significant survival advantage, novel and more effective therapies are needed. To improve survival rates, new therapeutic agents have been investigated. Immunotherapy for colorectal cancer is a promising candidate treatment, and there is evidence that host immune responses can influence survival (2). Ideal targets for immunotherapy are gene products overexpressed in cancer cells but silenced in normal tissues, with the exception of immune-privileged tissues, such as that of the testis.

We previously reported that heat shock protein 105 (HSP105), identified by SEREX, is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer, but with little to no expression in normal tissues aside from the testis (3,4). HSP105 is a stress protein induced by various stressors and belongs to the HSP105/110 family and plays an important role as a chaperone under physiological conditions (5). Using immunohistochemical analysis, we previously found that HSP105 was specifically overexpressed in 44 of 53 (83.0%) colorectal cancer patients (4). It has also been reported that DNA vaccination with both HSP105 and bone marrow-derived dendritic cells (BM-DCs) pulsed with HSP105 led to tumor rejection of colorectal cancer but did not induce an autoimmune reaction in mice (6-8).

This suggests that HSP105 presents a useful tumor-specific antigen target for immunotherapy. However, HSP105-derived epitope peptides of $CD8^+$ T cells have not been identified. The gene frequency of HLA-A24 (A*24:02) is relatively high in Asian populations, especially the Japanese, but low in Caucasians. On the other hand, the gene frequency of HLA-A2 (A*02:01) is high among several ethnic groups, including Asians and Caucasians (9). Therefore, HLA-A2 or HLA-A24-restricted cytotoxic T cell (CTL) HSP105 epitopes could be extremely

useful for immunotherapy in a large portion of patients worldwide. In the present study, we identified human HSP105-derived CTL epitopes restricted by HLA-A2 or HLA-A24 using HLA-transgenic mice (Tgm) and examined whether these epitope-based peptides could activate HSP105-reactive CTLs in peripheral blood mononuclear cells (PBMCs) of patients with colorectal cancer.

## Materials and methods

*Mice*. HLA-A2.1 (HHD) Tgm, H-2D$^{b-/-}$β2m$^{-/-}$ double-knockout mice introduced with the human β2m-HLA-A2.1(α1 α2)-H-2D$^{b}$ (α3 transmembrane cytoplasmic) (HHD) mono-chain gene construct were generated in the Departmente SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France (10,11) and were kindly provided by Dr F.A. Lemonier. HLA-A24.2 (HHD) Tgm were purchased from Japan SLC, Inc. (Shizuoka, Japan). Female 6- to 8-week-old BALB/c mice (H-2K$^{d}$) and BALB/c nude mice, purchased from Charles River Japan (Yokohama, Japan), were maintained and handled in accordance with animal care policy.

*Cell lines*. The human colorectal cancer cell line SW620 (endogenously expressing HSP105 and HLA-A*02:01, 24:02) and human liver cancer cell line HepG2 (HSP105-low expressing and HLA-A*02:01, 24:02), were kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). Murine colorectal cancer cells, Colon26 (C26) (endogenously expressing HSP105 and H-2K$^{d}$) were kindly provided by Dr Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Osaka, Japan). T2 cells (a TAP-deficient and HLA-A*02:01-positive cell line) were provided by Kyogo Ito of Kurume University. Cells were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS.

*RNA interference*. Small interfering RNAs targeting human HSP105 were chemically synthesized by Dharmacon Research (HSP105-siRNA and luciferase; Lafayette, CO, USA) as previously described (12), with the following siRNA sequences: HSP105-siRNA, UUGGCUGCAACUCCGAUU GTT and luciferase, CGUACGCGGAAUACUUCGATT. The transfection of siRNA oligonucleotides was carried out using Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines.

*Peptides*. Human HSP105-derived peptides, identical in amino acid sequence with mouse HSP105 and expressing the binding motifs for HLA-A*02:01- and HLA-A*24:02-encoded molecules, were designed with BIMAS software (BioInformatics and Molecular Analysis Section; Center for Information Technology, NIH, MD, USA). We purchased a total of 16 versions of peptides carrying the HLA-A2 (A*0201)-binding motifs and 9 versions of peptides carrying the HLA-A24 (A*2402)-binding motifs from Biologica (Tokyo, Japan) (Table I).

*Induction of HSP105-reactive CTLs in Tgm*. Peptide immunizations in mice were performed as previously described (13). In brief, bone marrow (BM) cells (2x10$^{6}$) from HLA-A2 or HLA-A24 Tgm were cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (5 ng/ml) and 2-mercaptoethanol (0.8 ng/ml) for 7 days in 10-cm plastic dishes. These BM-DCs were pulsed with the two HSP105 peptide mixtures (1 μmol/l each peptide) for 2 h at 37˚C. We primed the HLA-A2 or HLA-A24 Tgm with the syngeneic BM-DC vaccine (5x10$^{5}$/mice) into the peritoneal cavity twice, once per week. Seven days following the last immunization, the spleens were collected and CD4$^{-}$ spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production from the CD4$^{+}$ spleen cells co-cultured with the BM-DCs. The CD4$^{-}$ spleen cells (2x10$^{6}$/well) were stimulated with syngeneic BM-DCs (2x10$^{5}$/well) that had been pulsed with each peptide *in vitro*. After 6 days, the frequency of cells producing IFN-γ/2x10$^{4}$ CD4$^{-}$ spleen cells upon stimulation with syngeneic BM-DCs (1x10$^{4}$/well), pulsed with or without each peptide, was assayed using an enzyme-linked immunospot (ELISPOT) assay as previously described (13).

*Identification of a CTL epitope in BALB/c mice*. The peptide immunizations in mice were performed as previously described (14). Splenocytes removed from mice 7 days following the last immunization were harvested and cultured in 24-well culture plates (2.5x10$^{6}$/well) in 45% RPMI, 45% AIMV, 10% FCS and supplemented with recombinant human interleukin 2 (100 U/ml), 2-mercaptoethanol (50 μmol/l) and each peptide (10 μmol/l). After 5 days, the cytotoxicity of these cells against target cells was assayed using standard 6-h $^{51}$Cr release assays (15).

*Blood samples*. Blood samples from cancer patients were collected during routine diagnostic procedures after obtaining formal consent from patients at the Kumamoto University Hospital, from April to September 2006 and from patients at the National Cancer Center Hospital East, from December 2006 to March 2007. The study was approved by the local ethics committee, and informed consent was obtained from all patients.

*Induction of HSP105-reactive human CTLs*. We isolated PBMCs from heparinized blood of HLA-A24$^{+}$ and/or HLA-A2$^{+}$ Japanese patients with colorectal cancer using Ficoll-Conray density gradient centrifugation; peripheral monocyte-derived dendritic cells (DCs) were generated as previously described (16,17). CD8$^{+}$ T cells were isolated with CD8 microbeads (Miltenyl Biotec, Bergisch Gladbach, Germany) from PBMCs of the same donor and peptide-reactive CD8$^{+}$ CTLs were generated. Five days following the last stimulation, the cytotoxic activities of the CTLs against cancer cell lines were measured by $^{51}$Cr-release assay as previously described (15). For these assays, CTLs were co-cultured with each cancer cell line, as the target cells (5x10$^{3}$/well), at the indicated effector/target ratio.

*In vivo tumor challenge*. Subcutaneous tumors were induced in mice by injecting 1x10$^{4}$ SW620 cells suspended in 100 μl PBS or Hanks' balanced salt solution (Gibco, Grand Island, NY, USA) into the backs of BALB/c nude mice. Tumor incidence and volumes were assessed weekly using calipers and tumor areas were measured. Results are presented as mean tumor areas ± SD.

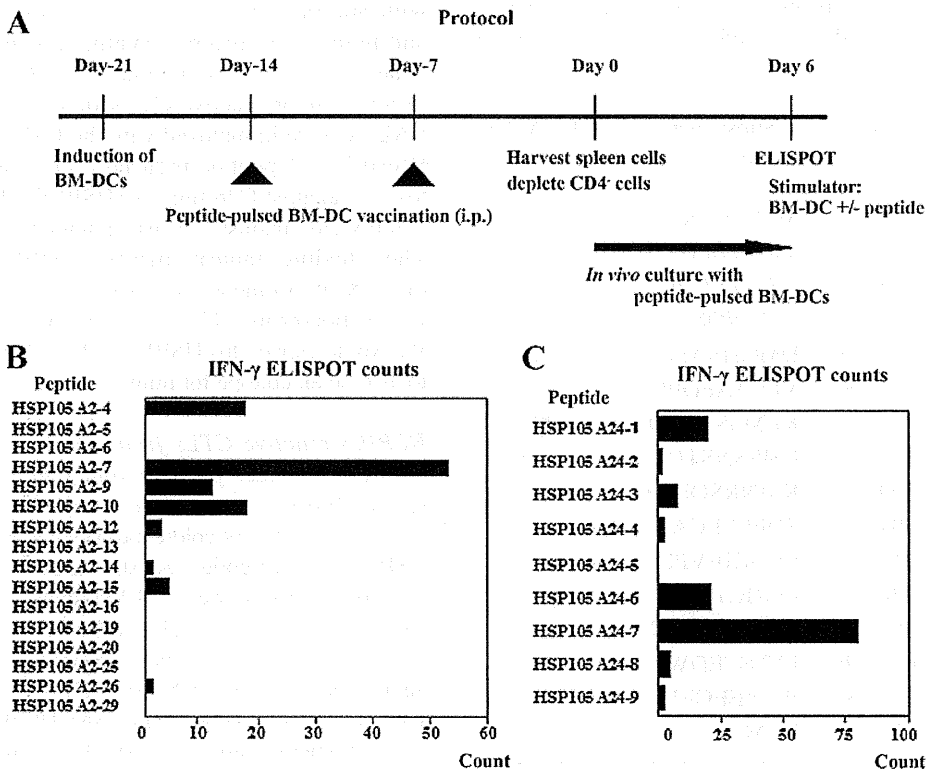


Figure 1. Identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 using HLA-A2.1 Tgm and HLA-A24 Tgm. (A) The protocol used for identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 is shown. We primed the HLA Tgm with BM-DCs ($5x10^5$) pulsed with the mixture of HSP105-derived peptides carrying the HLA-A2 or HLA-A24 binding motif into the peritoneal cavity once a week for 2 weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells ($2x10^6$/well) were stimulated with syngeneic BM-DCs ($2x10^5$/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in the IFN-γ ELISPOT assay. (B) The bar graphs show the IFN-γ ELISPOT counts per $2x10^4$ $CD4^-$ spleen cells co-cultured with HLA-A2-restricted peptide-pulsed BM-DCs after normalization to counts from cells co-cultured with BM-DCs without peptide loading. (C) The bar graphs show the IFN-γ ELISPOT counts in the HLA-A24-restricted peptides. The columns represent the means from duplicate assays.

*Ex vivo IFN-γ ELISPOT assay in peripheral blood in pre-surgical colorectal cancer patients. Ex vivo* IFN-γ ELISPOT assays were performed to determine tumor-specific interferon-γ (IFN-γ)-secreting T cells. The 96-well plates were coated with anti-human IFN-γ (BD Biosciences Co., Ltd., USA). After an overnight incubation at 4˚C, the wells were washed and blocked with complete medium for 2 h at room temperature. A total of $1x10^6$ unfractionated PBMCs were added in duplicate wells and incubated at 37˚C for 18-20 h with or without peptides at 0.2 μl/well (1-10 μM). The plate was washed and then incubated with 5 μg/ml biotinylated anti-human IFN-γ antibody for 2 h at room temperature. After washing away the antibodies, streptavidin-HRP was added for 1 h. Finally, the plate was washed and replaced with fresh substrate solution and the reaction was terminated by washing with distilled water. The HLA-A2-restricted CMV peptide (NLVPMVATV) and HLA-A24 restricted CMV peptide (QYDPVAALF), which includes an epitope derived from the CMV pp65 protein, were used as positive controls.

*Histological and immunohistochemical analysis.* To investigate whether $CD8^+$ T cells infiltrated normal tissues triggered by the HSP105-derived peptide vaccine, we performed immunohistochemical staining with a monoclonal antibody against CD8 (1:100; LifeSpan BioSciences, Inc., Seattle, WA, USA) in tissue specimens from HLA-A2 Tgm immunized with the HSP105 peptides, as previously described (7). Immunohistochemical staining with rabbit polyclonal antibodies against HSP105 (1:200; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) was performed according to the manufacturer's instructions.

## Results

*Identification of HLA-A2-or HLA-A24-restricted CTL epitopes in HLA Tgm.* We designed pools of HSP105 peptides possessing amino acid sequences conserved between humans and mice that have a highly predicted binding score to HLA-A2 (pool of 16 different peptides) or HLA-A24 (A*24:02) (pool of 9 different peptides) (Table I). $CD4^-$ spleen cells were obtained from Tgm immunized twice i.p. with BM-DCs that had been pulsed with each peptide mixture; the spleen cells were then stimulated *in vitro*, again with the BM-DCs pulsed with each peptide mixture (Fig. 1A).

The IFN-γ ELISPOT counts, normalized to those of spleen cells co-cultured with BM-DCs without peptide loading, clearly indicated a HSP105 A2-7 peptide-specific response in the $CD4^-$ spleen cells (Fig. 1B). These $CD4^-$ spleen cells ($2x10^4$/well) showed 55±29.7 spot counts/well in response to the BM-DCs pulsed with the HSP105 A2-7 peptide, whereas they showed 23±31.1 spot counts/well in the presence of