Efficacy of glypican-3-derived peptide vaccine therapy on the survival of patients with refractory ovarian clear cell carcinoma

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**ABSTRACT**

Compared with other epithelial ovarian carcinoma subtypes, ovarian clear cell carcinoma (OCCC) has been recognized to show chemoresistance. Therefore, new treatment modalities are required for patients with OCCC that is refractory to chemotherapy. The carcinoembryonic antigen glypican-3 (GPC3) is expressed by approximately half of OCCC and is a promising immunotherapeutic target. The purpose of this study was to evaluate the effect of GPC3 peptide vaccine against refractory OCCC patients. We conducted a phase II trial with a GPC3-derived peptide vaccine in OCCC patients. Immunological responses were analyzed by ex vivo IFN-γ ELISPOT assay. We also evaluated control subjects, who received best supportive care without vaccinations during the same period.

Thirty-two patients with refractory OCCC were enrolled between July 2010 and September 2015, and underwent GPC3 peptide vaccination. Fifteen patients were vaccinated less than six times because their general condition progressively deteriorated, and 17 patients were vaccinated at least six times. Three patients showed a partial response as the best overall response. The GPC3 peptide vaccine induced a GPC3-specific CTL response in 15 out of 24 patients who had PBMCs collected three times or more. The prognosis of palliative care patients without GPC3 peptide vaccinations was significantly poorer than that of those with GPC3 peptide vaccinations (post cancer-treatment survival: \( p = 0.002 \)). Although the disease control rate was not high, our results suggest that GPC3 peptide vaccinations may hold a significant impact to prolong survival of patients with refractory OCCC, allowing them to maintain quality of life with no serious toxicities.

**Abbreviations:** BSC, best supportive care; CR, complete response; CT, computed tomography; CTL, cytotoxic T lymphocyte; DCR, disease control rate; ELISPOT, enzyme-linked immunospot; EOC, epithelial ovarian carcinoma; FIGO, International Federation of Gynecology and Obstetrics; GPC3, glypican-3; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; IFA, incomplete Freund's adjuvant; IFN, interferon-γ; OCCC, ovarian clear cell carcinoma; OS, overall survival; PBMC, peripheral blood mononuclear cell; PET, positron emission tomography; PR, partial response; PS, performance status; RR, response rate; SD, stable disease; TAA, tumor-associated antigen

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**INTRODUCTION**

Epithelial ovarian carcinoma (EOC) is the most lethal gynecological malignancy. Ovarian clear cell carcinoma (OCCC) is a comparatively rare tumor, depending on the geographic location. In western countries, OCCC represents <10% of all EOC. In contrast, the incidence of OCCC was reportedly 15–25% of EOC in Japan. Compared with other EOC subtypes, OCCC is associated with greater chemoresistance and a poorer prognosis. Particularly for recurrent OCCC, the response rate (RR) to salvage chemotherapy was extremely low. Even in patients who achieved a response when they received conventional anticancer cytotoxic drugs, progression-free survival was less than 6 mo. In addition, we previously reported approximately two in three recurrent OCCC patients died within 12 mo of recurrence. There is currently no well-established chemotherapeutic regimen for OCCC. Therefore, novel and innovative strategies are required to improve outcomes for patients with OCCC that is refractory to chemotherapy.

Immunotherapy offers a promising therapeutic strategy for EOC. Following the demonstration of EOC immunogenicity, multiple immunotherapeutic approaches have been developed. A cancer vaccine that induces cytotoxic T lymphocytes (CTLs) to tumor-associated antigens (TAAs) is a potentially attractive option for EOC. Several TAAs identified in EOC are potential antigens for peptide vaccines. In peptide-based vaccine trials, occasional marked tumor regressions have been observed after peptide vaccination; however, peptide vaccines have shown limited efficacy as monotherapy in patients with progressive recurrent EOC.

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Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans. Previous studies showed that GPC3 was overexpressed in several malignant tumors, including hepatocellular carcinoma (HCC) and OCCH.13-18 GPC3 is useful not only as a carcinoma embryonic antigen for immunothera-
py but also as a novel tumor marker.

We identified the human leukocyte antigen (HLA)-A24:02-restricted GPC3298-306 (EYILSLEEL) and HLA-A2:02:01-restricted GPC3144-152 (FVGEFFTDV) peptides, both of which can induce GPC3-reactive CTLs without inducing autoim-
nunity.19 Furthermore, we confirmed that HLA-A2:02:01-
restricted GPC3144-152 peptide can bind to HLA-A2:02:06 and HLA-A1:02:07 by conducting a binding assay. HLA-A24 and A2 are common HLA-A alleles within the Japanese population.

We previously reported the safety and immunological and clinical responses to a GPC3-derived peptide vaccine in a phase I trial for advanced HCC patients. This trial demonstrated that GPC3 peptide vaccine-related OS. The median duration of wash-out period from the last anticancer treatments including radiation and surgery for metastatic sites) was 42.5 d and ranged from 15 to 741 d. Fifteen patients who received six or more vaccinations (≥6th vaccination group), seven patients (46.7%) had PS 2 and eight (53.3%) had PS 0 or 1. Patients in the <6th vaccination group had a worse PS than patients in the ≥6th vaccination group (p < 0.011). According to laboratory data at the first vac-
nation, no significant differences were observed in the neutrophil to lymphocyte ratio (p = 0.149). On the other hand, significant differences were noted in CA125 (p < 0.046) and albumin levels (p = 0.014) between the two groups. Major lesions of metas-
tases in 21 of 32 patients were carcinomatous peritonitis. Ten patients had received more than three prior treatments excluding primary surgery (including chemotherapy, radiation and surgery for metastatic sites).

Clinical responses and safety

Clinical responses are summarized in Table 2. Some patients could not undergo computed tomography (CT) or FDG posi-
tron emission tomography (PET)-CT scans after vaccination because of clinical cancer progression. These patients were judged to have disease progression, but were not removed from the analyses. Disease control rate (DCR) at 6 and 12 mo were 9.4% (partial response [PR] 2 + stable disease [SD] 13/32) and 6.3% (PR 2/32), respectively. The 12-mo GPC3 peptide vac-
cine-related OS rate in all patients was 20.6%. When 17 patients who received six or more GPC3 vaccines were selected, DCR at 6 and 12 mo increased to 17.6% (3/17) and 11.8% (2/17), respectively, and levels of the tumor markers (CA125 and/or CA19-9) temporarily decreased in 10 out of 17 patients. The 12-mo GPC3 peptide vaccine-related OS rate in these 17 patients was 38.8%.

We examined pre-vaccine clinical prognostic factors (Table S2). Univariate analysis indicated that PS (p = 0.028), albumin levels at first vaccination (p = 0.005) and metastatic major lesions (p = 0.032) were prognostic factors for GPC3 peptide vaccine-related OS. According to prior treatments, there was no significant difference between number of prior treatments and GPC3 peptide vaccine-related OS (p = 0.784). In addition, we analyzed whether types of prior treatments (chemotherapy alone versus chemotherapy plus other cancer treatments including radiation and surgery for metastatic sites) and the duration of wash-out period from the last anticancer treatment (45 vs. 45 < d) showed any correlation with GPC3 peptide vaccine-related OS. The median duration of wash-out period was 42.5 d and ranged from 15 to 741 d. Fifteen patients received the first GPC3 peptide vaccination following wash-out period of more than 45 d. Neither types of prior treatments

Figure 1. Trial profile. Fifteen patients stopped treatment. Worsening of PS: 13, brain infarction: 2, adverse effects: 0 (treatment related). Seventeen patients received six vaccinations or more.
We investigated whether any difference existed in the survival of patients who received GPC3 peptide vaccines or best supportive care (BSC) only. Patient characteristics according to the last anticancer treatment before GPC3 peptide vaccine are presented in Table S3. As shown in Fig. 2, patients who received GPC3 peptide vaccine showed significantly improved post-cancer-treatment survival.

We have previously presented two patients (case 4 and case 6) with refractory OCCC who experienced a PR in this trial of a GPC3 peptide vaccine.22 Clinical and immunological response of the other clinical responder are shown in Fig. 3.

No vaccine-related CTC grade 3 or higher adverse events were observed, and most patients experienced local skin reactions at the injection site, consistent with previous studies.20,23

### Immunological responses

The results are presented in Table 2 and Fig. S1. Based on results from ex vivo interferon-γ (IFNγ) enzyme-linked immunospot (ELISPOT) assays using collected PBMCs until the 3rd vaccination, the GPC3 peptide vaccine induced a GPC3-specific CTL response in 15 out of these 24 patients (62.5%). In the primary tumors that could be obtained, expression of GPC3 was detected in 8 (42.1%) of 19 patients. A less than 50% reduction in the expression of HLA class I was observed in 6 (31.6%) of these 19 patients.

We also investigated immunological parameters in relation to GPC3 peptide vaccine-related OS (Table S4). Expression of GPC3, HLA class I and TILs in the primary tumors was not a predictive marker of the effects of GPC3 peptide vaccination. Although the GPC3 peptide vaccine-related OS of patients with a negative GPC3-specific CTL response was poorer than that of patients with a positive response, it was not significant (p = 0.074).

### Discussion

Recurrent or persistent OCCC has been reported as having a potentially chemoresistant phenotype against conventional cytotoxic agents, leading to poorer prognosis. Thus, novel treatment approaches must be adopted for OCCC. With compelling evidence that EOC is an immunogenic tumor, immunotherapeutic approaches are currently being evaluated and should be optimized based on histology-specific features.

For platinum-resistant OCCC, RRs and DCRs for several regimens in the setting of second- or higher-line chemotherapy
Abbreviation: TIL, tumor-infiltrating lymphocyte.

| Case | No. of vaccination | Clinical response | TIL | Immunohistochemical analysis |
|------|-------------------|-------------------|-----|-----------------------------|
|      |                   |                   | GPC3 | HLA class I | CD8+ T cells | Increased GPC3-specific CTL | Timing of initial GPC3-specific CTL increase |
| 1    | 8                 | PD                | +   | –   | 3+ | Negative | 1+ | After 5th vaccination |
| 2    | 8                 | PD                | –   | –   | 3+ | Negative | 2+ | After 1st vaccination |
| 3    | 7                 | PD                | –   | 3+ | 3+ | Negative | 3+ | After 1st vaccination |
| 4    | 27               | PR → PD           | +   | 2+ | 3+ | Positive  | 1+ | After 2nd vaccination |
| 5    | 7                 | PD                | NA  | NA | NA | –        | – | — |
| 6    | 27               | SD → PR → PD      | +   | – | 3+ | Negative | 3+ | After 2nd vaccination |
| 7    | 6                 | PD                | +   | 2+ | 2+ | Negative | – | — |
| 8    | 6                 | PD                | –   | –   | 2+ | Negative | 2+ | After 4th vaccination |
| 9    | 6                 | PD                | –   | –   | 3+ | Negative | 1+ | After 1st vaccination |
| 10   | 8                 | PD                | –   | 1+ | 2+ | Negative | 1+ | After 1st vaccination |
| 11   | 8                 | PD                | +   | –   | 2+ | Negative | 1+ | After 2nd vaccination |
| 12   | 8                 | PD                | NA  | NA | NA | 1+ | After 1st vaccination |
| 13   | 13               | PR → SD → PD      | +   | 2+ | 3+ | Negative | 1+ | After 4th vaccination |
| 14   | 8                 | PD                | –   | NA | NA | NA        | – | — |
| 15   | 6                 | PD                | +   | 2+ | 3+ | Negative | 2+ | After 3rd vaccination |
| 16   | 6                 | PD                | +   | NA | NA | NA        | 1+ | After 3rd vaccination |
| 17   | 8                 | PD                | –   | 3+ | Positive | – | — |
| 18   | 1 Brain infarction | NA                | –   | 3+ | Positive | NA | NA |
| 19   | 1 Brain infarction | PD                | NA  | NA | NA | NA        | NA | NA |
| 20   | 2 PD             | NA                | –   | 2+ | 2+ | Negative | NA | NA |
| 21   | 1 PD             | NA                | NA  | NA | NA | NA        | NA | NA |
| 22   | 1 PD             | NA                | NA  | NA | NA | NA        | NA | NA |
| 23   | 1 PD             | NA                | NA  | NA | NA | NA        | NA | NA |
| 24   | 4 PD             | –                 | 1+  | 3+ | Negative | – | — |
| 25   | 3 PD             | –                 | NA  | NA | NA | –        | — |
| 26   | 3 PD             | –                 | –   | 2+ | Negative | – | — |
| 27   | 4 PD             | –                 | NA  | NA | NA | –        | — |
| 28   | 1 PD             | NA                | NA  | NA | NA | NA        | NA | NA |
| 29   | 4 PD             | –                 | –   | 3+ | Positive | NA | NA |
| 30   | 2 PD             | –                 | –   | 3+ | Negative | – | — |
| 31   | 5 Brain infarction| –                 | –   | 3+ | Negative | – | — |
| 32   | 3 PD             | –                 | NA  | NA | NA | 1+ | After 1st vaccination |

Abbreviation: TIL, tumor-infiltrating lymphocyte.

Clinical responses were evaluated according to RECIST v1.0.

Tumor marker: CA125 and/or CA19-9.

Expression of GPC3 and HLA class I, and the number of CD8+ T cells in the primary tumors were determined by immunohistochemistry. The extent of staining of tumor cells for GPC3 and HLA class I: 0, no reactivity; 1, <10%; 2, 10–49%; 3, ≥50%; NA, not analyzed. Quantification of TIL: positive, ≥10 counts of CD8+ T cells/high-power fields (HPF); negative, <10 counts/HPF.

The maximum number of GPC3 peptide-specific CTL spots was scored as 0 (none), +1 (≤50), +2 (50–99) or +3 (≥100) in an ex vivo IFNγ ELISPOT assay per 5 × 10⁵ PBMCs.

are reported to be in the range of 1–10% and 4–20%, respectively. In this clinical trial, DCR for the primary endpoint was not enough (3/32; 9.4%). However, the 32 enrolled patients were heavily pre-treated with a mean of 3 (1–8) previous treatments prior to GPC3 vaccine and poor general condition because of loose eligibility criteria compared with patients who received salvage chemotherapy. Therefore, when 17 patients who were in comparatively good pre-vaccine condition (better PS, lower CA125 levels and higher Alb levels subjects, Table S1) and vaccinated at least six times (10 weeks or more) were selected, DCR increased to 17.6%. As clinical trials for HCC patients have previously reported, we also confirmed that there were no serious vaccine-related adverse events for OCCC patients. These results are significant for refractory OCCC patients, allowing them to maintain quality of life without significantly sacrificing efficacy. In addition, we investigated whether any difference existed in the survival of patients who received GPC3 peptide vaccines or control patients who received BSC only during the same period. Patients who received GPC3 peptide vaccines showed significantly improved post cancer-treatment survival. This result should be specially mentioned; however, there was a limitation in our ability to evaluate the efficacy of GPC3 peptide vaccines, because this study was not a randomized-controlled study. The establishment of a biomarker to predict the antitumor response of GPC3 vaccines in refractory OCCC is promising.

We investigated pre-vaccine clinical parameters in relation to GPC3 peptide vaccine-related OS. Poor PS and carcinomatous peritonitis (metastatic major lesions) indicated an independently worse prognosis in a multivariate analysis. It may be important to select appropriate OCCC patients for GPC3 peptide vaccine therapy based on pre-vaccine general condition, including the degree of carcinomatous peritonitis.

In the present study, to identify predictive immunological biomarkers for the antitumor effect of GPC3 peptide vaccine, we analyzed the expression of GPC3 and HLA class I in the
primary tumor and induction of GPC3-specific T cell response by ex vivo IFNγ ELISPOT assay. However, none of them were enough biomarkers.

We evaluated GPC3 expression in the available primary tumors from 19 of the 32 patients treated with vaccination by immunohistochemical analysis, and GPC3 expression was detected in 8 of these 19 patients (42.1%). We previously reported that the GPC3 peptide vaccine improved the 1-y recurrence rate in HCC patients.23 However, GPC3 expression status (positive versus negative) was not a significant factor ($p = 0.361$) (Table S4). The frequency of GPC3 expression in OCCC was lower than that in HCC. In addition, the intratumor heterogeneity of GPC3 expression was observed at different levels in our preliminary research depending on the locations and timing of biopsies. Case 6 showed PR in spite of undetectable GPC3 expression in the primary tumor. Although the present study was limited by the small sample size, it may be difficult to predict the clinical response against metastatic tumors based on the strength of GPC3 immunohistochemical expression in just one part of primary OCCC tissue. Furthermore, the level of TAA protein expression by immunohistochemical analysis does not always reflect the amount of displayed antigenic peptide on the cell surface. We consider it possible to predict the antitumor response of peptide vaccine therapy based on HLA class I/peptide complex expression on the cell surface in pre-vaccine biopsy specimens. Thus, we have attempted to prepare monoclonal antibodies against the HLA-A24/GPC3298-306 peptide complex and HLA-A2/GPC3144-152 peptide complex.

The number of patients in whom a GPC3-specific CTL response was induced in our trial for refractory OCCC was lower than that in previous trials for HCC. And although the GPC3 peptide-specific CTL frequency in PBMCs after vaccination by ex vivo IFNγ ELISPOT assays was correlated with OS in the previous phase I trial for patients with advanced HCC,20,21 we found no correlation between GPC3 peptide-specific CTL frequency and GPC3 peptide vaccine-related OS in this study. Questions remain as to why most of the vaccinated patients show increased numbers of GPC3-specific CTLs in the absence of a clinical response. According to induced GPC3-specific CTLs, it is important not only to evaluate the quantity but also to assess the quality. Indicators of the quality of CTLs include antigen-specific killing activity against tumor cells that naturally process and present the peptide epitope, high affinity for antigen and multifunctionality such as the production of not just IFNγ, TNF-α, perforin and granzyme B. We tried
to establish GPC3 peptide-specific CTL clones from PBMCs of some OCCC patients vaccinated with GPC3 peptide by single-cell sorting using Dextramer. The established CTL clones from PBMCs of patients without clinical response had low avidity and were not capable of killing cancer cells expressing GPC3 (data not shown). These results may suggest that vaccine-induced CTLs were partially exhausted. This is one of potential reasons why clinical outcomes do not necessarily correlate with induction of GPC3-specific T cell response. Regarding clinical responders in this trial, it is difficult to confirm whether tumor regression was actually induced by GPC3-specific CTLs or other mechanisms. Antigen spreading may have occurred following the GPC3 peptide-specific CTL response after the vaccination and contributed to tumor regression. The difference in effectiveness may have been caused by the heterogeneity associated with immune-escape mechanisms, including the down-regulation of cancer-specific antigens and/or HLA class I in tumor cells and activation of immune checkpoint pathways. Further studies are necessary to determine whether a GPC3-specific immune response in PBMCs was associated with the clinical outcome.

Among different immunotherapeutic approaches, short peptide-based vaccinations have several advantages such as good immunological efficacy, low production, and administration costs and safety. On the other hand, there is remarkable progress in cancer immunotherapy with anti-programmed death-1 (PD-1) or anti-programmed death-ligand 1 (PD-L1) antibodies for advanced stages of cancers, including melanoma, lung cancer, renal cancer and ovarian cancer, and the targeting of further inhibitory lymphocyte receptors (checkpoints) is explored in an increasing number of pre-clinical studies. However, a single pathway inhibitor or activator is unlikely to have a dramatic effect due to the complexity of malignancy, and the opposing immunogenic and immunosuppressive forces at play. Thus, the challenge is to examine rational combinations with other immunotherapeutic/targeted/cytotoxic agents that offer maximal clinical benefits at the lowest cost. Recently, we reported that a PD-1-blocking antibody augmented GPC3-specific CTL clones that degranulate against HCC cells evaded by the CTLs due to PD-L1 expression in vitro. Moreover, various types of next-generation peptide vaccines (multi-peptide cocktail vaccines, multivalent long peptide vaccines, personalized peptide vaccines and neoantigen-derived peptide vaccines) are under development.

In conclusion, our results indicate that GPC3 peptide vaccines demonstrate antitumor effects in certain patient populations with refractory OCCC, and may be a beneficial treatment option for refractory OCCC patients who are in comparatively good general condition. Further studies are essential for providing more insight into how to maximize the potential of GPC3 peptide vaccines as monotherapy, and proving the clinical benefits of combination therapies with this peptide vaccine and immune checkpoint blockades.

Materials and methods

Patient eligibility

This clinical trial was approved and monitored by the Institutional Review Board at Nagoya University School of Medicine. Thirty-two patients with refractory OCCC were enrolled between July 2010 and September 2015. All patients gave written informed consent before treatment. The following eligibility criteria were used: diagnosis of OCCC on the basis of histologic examinations; aged between 20 and 80 y; an Eastern Cooperative Oncology Group PS of 0-2; HLA-A*24:02- or HLA-A2 (HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07)-positive status, as determined using commercially available genomic DNA typing tests; no expectation of response to other anticancer therapies and adequate organ function (white blood cell count ≥2,000/mm³, platelets ≥50,000/mm³, serum creatinine ≤2.1 mg/dL, aspartate aminotransferase ≤165 IU/L, total bilirubin ≤3.6 mg/dL, alkaline phosphatase ≤1795 IU/L). The following exclusion criteria were applied: other active malignancy; clinically serious infection; severe cardiac insufficiency; active gastrointestinal bleeding; severe interstitial pneumonitis; massive ascites and/or hydrothorax; concurrent treatment with systemic steroids or immunosuppressive agents; or unsuitability for this trial, based on clinical judgment. This trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number: 00003696).

Vaccination schedule and endpoints

HLA-A*24:02-restricted GPC3298-306 peptide (EYILSLEEL) (American Peptide Company) was used in HLA-A24-positive patients and HLA-A*02:01-restricted GPC3144-152 peptide (FVGGEFTDV) (American Peptide Company) in HLA-A2-positive patients in principle. Patients received the intradermal injection of GPC3 peptide emulsified with incomplete Freund’s adjuvant (IFA) (Montanide ISA-51VG, SEPPIC) near the bilateral axillary lymph nodes. The peptides and IFA were synthesized according to Good Manufacturing Practice guidelines. The dose of GPC3 peptide injected was 3 mg per body. Vaccinations were carried out biweekly from the first until the 6th, and repeated at 6-week intervals after the 7th according to the trial schedule. Patients who remained non-progressive after eight vaccinations were allowed to continue treatment until disease progression (Fig. S2).

The primary endpoint was the 6-mo DCR. DCR was defined as complete response (CR) plus PR plus SD. Secondary endpoints were safety and survival. GPC3 peptide vaccine-related OS was measured from the date of first vaccination until death or final follow-up contact. The data were fixed at the end of March 2016.

Post cancer-treatment survival

To investigate the survival effects of the GPC3 peptide vaccine, post cancer-treatment survival, defined as the time interval between the last date of anticancer treatment before GPC3 peptide vaccine after recurrence/progression (non-CR) and death from the disease, was also analyzed. For the BSC group, 33 patients with refractory OCCC were registered and treated by the Tokai Ovarian Tumor Study Group, consisting of Nagoya University Hospital and affiliated hospitals during the same trial period. Control patients were eligible if they fulfilled the following: (1) had histologically confirmed OCCC, (2) recurrence/progression was diagnosed by radiologic and/or physical findings, (3) the last date of anticancer treatment was identified.
and (4) excluded from this analysis if they were lost to follow-up. Data were collected from medical records and clinical follow-up visits.

**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. Blood and urine examinations were performed before each vaccination. Tumor regression was assessed on CT or FDG PET-CT before vaccination, and then approximately every 3 mo after the first vaccination. Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (v1.0). All time estimates except post cancer-treatment survival were recorded with the date of first vaccination as the baseline.

**Ex vivo IFNγ ELISPOT assay**

An ex vivo IFNγ ELISPOT assay was conducted to measure the antigen-specific CTL response, as described previously. Briefly, peripheral blood (30 mL) was obtained before each vaccination and centrifuged with a Ficoll-Paque gradient. PBMCs were frozen before immunologic analysis. Non-cultured PBMCs (2.5–5 × 10^5 per well) were added to plates in the presence of peptide antigens (10 mg/mL) and incubated for 20 h at 37°C in 5% CO2. The GPC3 antigen was the HLA-A2-restricted GPC3_144-152 peptide or HLA-A2’24:02-restricted GPC3_298-306 peptide. PBMCs plus HLA-A2-restricted HIV19–27 (TLNAWKVV) peptide (PromImmune) or HLA-A’24:02-restricted HIV583–591 (RYLKDOQQL; PromImmune) were used as negative controls. The assays were conducted in duplicate.

**Immunohistochemical analysis**

Surgical specimens were stained with monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8+ (clone 1A5; dilution 1:80; Novocastra) and HLA class I (clone EMR8/5; dilution 1:1,000; Hokudo), according to the manufacturers’ directions. Concerning quantification of GPC3 and HLA class I expressions, the extent of staining was scored as 0 (0%), +1 (<10%), +2 (10–49%) or +3 (≥50%) according to the percentage of the positive staining areas in relation to the total cancer areas. Membrane immune reactivity levels for HLA class I were evaluated. We counted intraepithelial infiltrated CD8+ T cells in high-power fields (HPF; ×400) and calculated their averages. On the basis of the average counts of infiltrated lymphocytes, we classified them into two groups: T-cell infiltration—positive group with ≥10 counts/HPF or T-cell infiltration—negative group with <10 counts/HPF.

**Statistical analysis**

GPC3 peptide vaccine-related OS and post cancer-treatment survival rates were analyzed by the Kaplan–Meier method. Prognostic factors were evaluated using the log-rank test and Cox proportional hazard models. Significance was defined by a value of $p$ less than 0.05. All statistical analyses were conducted using SPSS version 23.

**Disclosure of potential conflicts of interest**

T.N. is a scientific advisor for Ono Pharmaceutical co, Ltd. T.N. is supported by a fundamental research funding from Ono Pharmaceutical co, Ltd. The other authors have no potential conflicts of interest to declare with regard to this study.

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