Phase II Clinical Trial of Multiple Peptide Vaccination for Advanced Head and Neck Cancer Patients Revealed Induction of Immune Responses and Improved OS

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Abstract

Purpose: The peptides derived from ideal cancer-testis antigens, including LY6K, CDCA1, and IMP3 (identified using genome-wide cDNA microarray analyses), were used in immunotherapy for head and neck squamous cell cancer (HNSCC). In this trial, we analyzed the immune response to and safety and efficacy of vaccine therapy.

Experimental Design: A total of 37 patients with advanced HNSCC were enrolled in this trial of peptide vaccine therapy, and the OS, PFS, and immunologic response were evaluated using enzyme-linked Immunospot (ELISPOT) and pentamer assays. The peptides were subcutaneously administered weekly with IFA. The primary endpoints were evaluated on the basis of differences between HLA-A*2402-positive [A24(+) patients treated with peptide vaccine therapy and –negative [A24(–)] patients treated without peptide vaccine therapy among those with advanced HNSCC.

Results: Our cancer vaccine therapy was well tolerated. The OS of the A24(+) vaccinated group (n = 37) was statistically significantly longer than that of the A24(–) group (n = 18) and median survival time (MST) was 4.9 versus 3.5 months, respectively; P < 0.05. One of the patients exhibited a complete response. In the A24(+) vaccinated group, the ELISPOT assay identified LY6K-, CDCA1-, and IMP3-specific CTL responses in 85.7%, 64.3%, and 42.9% of the patients, respectively. The patients showing LY6K- and CDCA1-specific CTL responses demonstrated a longer OS than those without CTL induction. Moreover, the patients exhibiting CTL induction for multiple peptides demonstrated better clinical responses.

Conclusions: The immune response induced by this vaccine may improve the prognosis of patients with advanced HNSCC.

Introduction

Head and neck cancer (HNC) is the sixth most common type of cancer, representing approximately 6% of all cases and accounting for an estimated 650,000 new cancer cases and 350,000 cancer-related deaths worldwide every year (1–3); however, the disease carries a very poor prognosis. The 5-year survival among all stages of disease is approximately 60% (4). In the past decade, the treatment of locoregional advanced HNC has shifted from primary surgery to organ preservation using combination chemoradiotherapy (CRT). The current approach attempts to achieve both organ preservation and function with outcomes superior to radiotherapy alone or surgery with postoperative radiotherapy (5–11). Despite the use of aggressive treatment modalities, such as surgical tumor resection with radical neck dissection and chemoradiotherapy, maintaining long-term disease control of advanced HNC remains difficult. Some chemoradiotherapy regimens have a higher treatment effect; however, the 5-year survival rates have not been extended (12). One reason for the poor prognosis of HNC is the limited availability of treatment options for advanced disease. Although various drugs are used in chemotheraphy and intensity modulated radiation therapy (IMRT), no...
Peptide Vaccine Therapy for Head and Neck Cancer

Translational Relevance
Cancer vaccination that induces cytotoxic T lymphocytes (CTL) to cancer-testis antigens is a potentially attractive option for the treatment of head and neck cancer (HNC). However, to date, immunotherapy using cancer-testis (CT) antigen-derived peptides has not demonstrated a correlation between the immune response and antitumor efficacy in clinical trials of advanced HNC. The peptides derived from three CT antigens used in this clinical trial are ideal targets for anticancer immunotherapy against HNC because they are specifically overexpressed in cancer cells, but not many normal tissues. In this phase II clinical trial of 37 patients with head and neck squamous cell cancer (HNSCC), we investigated the safety and of clinical and immunologic responses to these peptide vaccines. Our results showed peptide-specific CTLs in the peripheral blood of the patients with advanced HNSCC and increased CD8+ T cell infiltration in the tumors following peptide vaccination. In addition, significantly, a CR was obtained after peptide vaccination in 1 patient who showed no effects after chemoradiotherapy or surgery. Furthermore, this is the first study to demonstrate that the peptide-specific CTL frequency is correlated with the overall survival in patients with HNSCC receiving peptide vaccination. These findings promote the use of peptide vaccination for the treatment of HNSCC.

Materials and Methods

Study design
The present study is a phase II, open-label, nonrandomized clinical cancer vaccination trial conducted in an exploratory setting. The endpoints were evaluated on the basis of differences between the HLA-A24:02-positive [A24(+)] and -negative [A24(−)] groups as a biologic marker for the subgroup analysis. Vaccination with a mixture of multiple peptides derived from LY6K, CDCA1, and IMP3 and incomplete Freund’s adjuvant (IFA; Montanide ISA51, SEPP1/C) was performed in patients with HNSCC (n = 37) with locally advanced, recurrent, and/or metastatic tumors resistant to standard therapy. HLA-A genotyping was performed in all enrolled patients at the HLA Laboratory (Kyoto, Japan) according to the middle resolution genotyping method.

The primary endpoint in this study was overall survival (OS). The secondary endpoints were progression-free survival (PFS), immunologic responses, and adverse effects. Toxicities caused by the vaccination therapy were assessed according to the Common Terminology Criteria for Adverse Events version 3 (CTCAE). Immunologic monitoring was performed at the central laboratory using both enzyme-linked immunosorbent assay (ELISpot) and HLA-A24/TAA peptide pentamer assays with the in vitro culture of lymphocytes derived from PBMCs at the pre- and postvaccination periods, as described below. The OS, which was measured as the period until death from the day on which the patient received a terminal prognosis, was analyzed according to the Kaplan–Meier method, and the PFS was calculated to assess disease progression.

The assessment of the endpoints was performed using an intention-to-treat analysis. This trial was approved by the institutional review board of Kumamoto University (Approval number at Kumamoto University of Principal Investigator, No. 841) and registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) number, 00008379 (CTR-8379). Written informed consent was obtained from all participants. The trial was carried out in accordance with the Helsinki declaration regarding experimentation on human subjects.

Patient eligibility
The eligibility criteria for the patients participating in the clinical trial were as follows: (i) patients with HNSCC with locally advanced, recurrent and/or metastatic tumors who had failed to respond to standard therapy; (ii) adequate bone marrow, cardiac, pulmonary, hepatic and renal functions, including a WBC of ≥7,500/μL, a platelet count of ≥1.2 of the institutional upper limit of normal, AST, ALT, and ALP levels of less than 2.5 times the institutional upper limits of normal and a creatinine level of ≤1.5 of the institutional upper limit of normal; (iii) no history of therapy within the 4 weeks before the initiation of the trial; (iv) an ECOG performance status of 0–2; and (v) a determination of unsuitableness by the principal investigator or physician-in-chief.
In this study, none of the patients were excluded from these criteria.

Treatment protocol
Each of the three peptides (1 mg each) was emulsified in 1 mL of IFA and injected into the bilateral armpits. The vaccination was given subcutaneously once a week for 8 weeks. After that, they were vaccinated at every 4 weeks based on the detection of PD or the doctor’s assessment. For the immunologic evaluation, PBMCs were obtained from the patients at the prevaccination period and after the fourth and eighth vaccinations. For the imaging analysis, CT was performed during the prevaccination period (within 1 month before vaccination) and at every four vaccinations.

Peptides
Peptides derived from LY6K-177 (RycnLEGPPPI), CDCA1-64 (YcGIRLEH), and IMP3-508 (KTVNELQNL) able to induce tumor-reactive and HLA-A24 (A24:02)-restricted CTLs were synthesized as described elsewhere (14). The purity (>97%) of the peptides was determined using analytic-high performance liquid chromatography (HPLC) and mass spectrometry. The endotoxin levels and bioburden of the peptides were tested and determined to be within acceptable ranges of the GMP grade for vaccination (Neomips, Inc.).

Lymphocyte preparation for immunologic monitoring
The protocol for the immunologic assay performed at the central laboratory was periodically standardized and validated according to the Clinical Laboratory Improvements Amendments (CLIA) and International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guidelines (20, 21).

PBMCs were obtained from the patients during the prevaccination period and after the fourth and eighth vaccinations. Peripheral blood was obtained via venipuncture, collected in EDTA tubes, and transferred to the central laboratory at room temperature. Within 24 hours of blood collection, PBMCs were isolated using the ficoll–paque plus (ge healthcare bio-sciences) density-gradient solution and stored at −80°C in cell stock media (liquid pool) without serum at 5 × 10^6 cells/mL. After thawing, the degree of cell viability was confirmed to be more than 90% according to a trypan blue dye exclusion assay.

For the in vitro culture, the PBMCs were thawed simultaneously, and 5 × 10^6 cells per well were incubated in medium containing 100 units/mL of recombinant IL2 (rIL2; Novartis) with peptide combinations. The purity (>97%) of the peptide antigens was verified using trypan blue dye exclusion assay.

ELISPOT assay
To monitor the antigen-specific immune response, an ELISPOT assay was performed using the human IFN-γ ELISPOT PLUS kit (Mabtech). Ninety-six-well plates with nitrocellulose membranes (millipore) were precoated with primary anti-IFN-γ antibodies (1-D1K) at 4°C overnight. The plates were then prereacted with RPMI-1640 medium containing 10% FBS (Invitrogen). Each vaccine peptide (10 µg/mL), HIV-specific peptide (ILKPEVPNG, 10 µg/mL), or CMV-specific peptide (RYLRDQQLL, 10 µg/mL)-pulsed TSI cells (2 × 10^4 per well) as stimulators, was incubated for 24 hours in triplicate with responder cells (from 2 × 10^4 per well to 2.5 × 10^5 per well) for a total of 200 µl/well in different responder/stimulator ratios, as indicated. Stimulation with phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma-Aldrich) + ionomycin (500 pmol/L; Sigma-Aldrich) was used as a positive control for T-cell activation. The cell mixtures were treated with biotinylated secondary anti-IFN-γ antibodies (7-B6-1) and incubated for 2 hours. The plates were then incubated with HRP reagent and stained with TMB (Mabtech). The spots were quantified using an auto-analyzing system, the ImmunoSPOT S4 (Cellular Technology Ltd). Positivity for an antigen-specific T-cell response was quantitatively defined according to our original evaluation tree algorithm (19). In brief, the number of peptide-specific spots was calculated as the average of triplicates by subtracting the number of spots in the HIV peptide-pulsed stimulator well from that observed in the immunized peptide-pulsed stimulator well. Positivity for an antigen-specific T-cell response was classified into four grades (−, +, ++, and ++++) depending on the number and variability of peptide-specific spots at different responder/stimulator ratios. When the algorithm indicated +, ++, or +++ at either the fourth or eighth vaccination point, we judged the case to be positive.

Pentamer staining and flow cytometry analysis
The in vitro cultured T cells were subjected to a pentamer assay to confirm peptide specificity. HLA-A24/LY6K-177, CDCA1-64 or IMP3 peptides-pentamer (Proimmune) staining in combination with anti-CD8 and anti-CD3 mAb staining was performed, and the results were analyzed using flow cytometry. Analysis of the frequency of Treg cells in the peripheral blood cells was performed using a FACSCalibur (Becton Dickinson). Frozen PBMC samples derived from patients before vaccination and at 7 days after the fourth and eighth vaccinations were thawed and directly used for detection of Treg cells. In this experiment, CD4+ CD25highFoxp3+ cells were judged as Treg cells. The antibodies used to detect Treg cells were as follows: CD4-fluorescein isothiocyanate, Foxp3-phycocerythrin, and CD25-Allophycocyanin (ebioscience).

Immunohistochemical analysis of p16INK4A expression in cancer tissues
Immunohistochemical analysis of HPV p16INK4A expression was performed on formalin-fixed paraffin-embedded oral cancer tissue sections derived from 28 independent patients investigated in this clinical trial using the CINnet p16INK4A assay (ventana medical systems, inc.), according to the manufacturer’s instructions. Cervical cancer tissue sections known to be HPV-positive were used as a positive control, and an omission of primary anti-HPV antibody was used as a negative control.

Statistical analysis
The OS and PFS were analyzed according to the Kaplan–Meier method, and statistical differences were assessed using the log-rank test. All statistical analyses were performed using the SPSS statistics 21.0 software package (SPSS, Inc.).
Results

Patient characteristics

We recruited 55 eligible patients with HNSCC between December 1, 2008 and December 5, 2012. A total of 37 patients were enrolled in this study (Supplementary Table S1). The background characteristics of the patients were not statistically different between the HLA-A*24:02-positive group (n = 37) treated with peptide vaccination and the HLA-A*24:02-negative group (n = 18) that received best supportive care. The median follow-up period was 4.3 months (range, 0.3–54.2 months). Of the 55 patients, 39 were male. The average age was 65 years (range, 36–85 years). A total of 3 patients had a PS of 2, 44 patients had a PS of 1, and 8 patients had a PS of 0. Staging was performed according to the TNM classification for HNC; 54 patients were diagnosed with stage IV disease and 1 patient was diagnosed with stage III disease. A total of 38 patients had undergone conventional chemotherapy, radiotherapy, and surgery, 13 patients had undergone chemotherapy and radiotherapy, 1 patient had undergone chemotherapy only, 1 patient had undergone radiotherapy only, and 2 patients had not received any treatment before the peptide vaccine therapy.

We investigated the expression of HPV-associated protein in oral squamous cell cancer tissues derived from 28 patients investigated in this study. We performed immunohistochemical staining of p16INK4A, which is the most reliable surrogate marker for HPV infection, as reported by Vermorken and colleagues (22, 23). Among the 28 cancer patients investigated, the 4 cases were maxillary gingival cancer, 11 cases were mandibular gingival cancer, 9 cases were tongue cancer, 3 cases were buccal mucosal cancer, and 1 case was oropharyngeal cancer. A positive staining of p16INK4A was observed in only 1 patient (case 24) of tongue cancer. The other 27 cases were negative for p16INK4A expression. Therefore, we suggest that there is no correlation between HPV infection status of oral cancer cells and the effects of peptides vaccination at least in our present study.

Clinical response, OS, and PFS

The characteristics and clinical responses of the patients treated with peptide vaccination (n = 37) are shown in Table 1. Among...
OS was 4.9 months. The median time to PFS was 1.9 months. The median disease control rate (CR + SD) was 27.0% after 3 months, and nine were found to have stable disease (SD) for 3 months, according to the RECIST criteria. The disease control rate (CR+SD) was 27.0% after 3 months. The median time to PFS was 1.9 months. The median OS was 4.9 months.

When the patients were classified into A24(+) and A24(−) groups, the OS of the A24(+) group was statistically significantly longer than that of the A24(−) group (4.9 vs. 3.5 months at MST, respectively; \( P < 0.05 \); Fig. 1A). The PFS of the A24(+) group was not significantly better than that of the A24(−) group (1.9 vs. 1.0 months at MST, respectively; \( P = 0.13 \); Fig. 1B).

Prolonged OS in the A24(+) group correlated with specific CTL responses

In the A24(+) group, in vitro cultured T cells were subjected to ELISPOP and pentamer assays, and positive CTL responses specific for the LY6K-, CDCA1-, and IMP3 peptides after vaccination were observed in 85.7%, 64.3%, and 42.9% of the patients, respectively. When the OS was compared between the A24(+) patients in the CTL response-positive and-negative groups, the patients showing a CTL response specific to the LY6K peptide exhibited a significantly longer OS than those without an LY6K-specific CTL response (Fig. 2A). Similarly, the patients demonstrating a positive response specific to the CDCA1 peptide exhibited a significantly longer OS than those without a CTL response (Fig. 2B). The OS of the patients with an IMP3-specific CTL response tended to be longer than that of the patients without a CTL response, although the difference was not statistically significant. The PFS of the patients with LY6K-, CDCA1-, and IMP3-specific CTL responses tended to be longer than that of the patients without CTL responses (Fig. 2C and D). Interestingly, when the patients were divided into four groups according to the number of antigenic peptides to which they showed a positive CTL response, the OS was longer in the groups in which the patients demonstrated a positive CTL response to a larger number of peptides (Fig. 3); the MST of the patients exhibiting CTL responses to three peptides was longer (19.5 months) than that observed in the other patient groups (Fig. 3). These observations indicate that the immunologic response induced by the peptide vaccination contributed to improving the prognosis of these patients.
The HLA-A24(+) patients were classified into four groups according to the number of peptide antigens (0, 1, 2, or 3) inducing a CTL response. A, the OS tended to increase as the number of peptides inducing a CTL response increased according to the number of peptide antigens (0, 1, 2, or 3) inducing a CTL response. B, the PFS exhibited a similar tendency to the OS.

The detailed chronological changes of CTL responses checked at MST are shown in Supplementary Fig. S2A. In 20 cases investigated, 7 cases, 12 cases, 3 cases, and 2 cases had TAAs before and after the vaccination, respectively. As a result, the expression level was not changed much, expression loss of TAAs has not been observed.

The results of the representative ELISPOT and pentamer assays specific to the LY6K peptide are shown in Fig. 4. The ELISPOT assay indicated substantial T-cell responses specific to the LY6K peptide in comparison with the irrelevant peptide (Supplementary Fig. S1A), according to the criteria described in Materials and Methods. This LY6K-specific T-cell response was further confirmed by the LY6K-pentamer assay, as shown in Supplementary Fig. S1C, with a proportion of 27.0% of pentamer(+) CD8(+) cells among CD3(+) T cells. Moreover, the infiltration of CD8(+) T cells into the tumor tissue increased after vaccination (Supplementary Fig. S1D). Tumor biopsies were performed with informed written consent before vaccination and at the time of recurrence after vaccination.

The detailed chronological changes of CTL responses checked at before vaccination, the fourth and the eighth vaccinations among 24 cases investigated are shown in Supplementary Fig. S2A. In 20 cases out of 22 cases, CTL responses increased depending on increased vaccinations, whereas CTL response decreased in 2 cases. In 1 of those cases, CTL response decreased after the eighth vaccination, but the strong CTL response was observed after the 12th and the 16th vaccinations (data not shown). This observation may be due to the timing of blood sampling when the patients' physical condition became worse after the eighth vaccination. We thought that CTL response is accidentally undetectable only at the eighth vaccination, thereafter induction of CTL in this patient has been observed after repeated vaccinations. In terms of the correlation between positive pentamer responses and positive ELISPOT responses, positive correlation was observed in 2 cases. We showed the results of the case 24 in Supplementary Fig. S2B.

There is only 1 case from whom tumor tissue specimens can be collected after vaccination. We confirmed the expression of HLA class I before and after the vaccination. As a result, the expression level was not changed much, expression loss of TAAs has not been observed.

Adverse reactions
The peptide vaccine therapy was well tolerated without any treatment-associated adverse events of grade 3 or higher. Twenty-eight of the 37 patients developed grade 1 or 2 local skin reactions with redness, induration, swelling, and pruritus at the injection site. No high-grade fevers, fatigue, diarrhea, headaches, rashes, or itching were observed in any of the patients, and no hematologic, cardiovascular, hepatic, or renal toxicity was observed during or after vaccination. The adverse events observed in this trial are listed in Table 2.

Skin reactions, OS, and PFS
Skin reactions were observed after vaccination in 75.7% of all enrolled patients. The OS and PFS of the skin reaction–positive and negative patients are shown in Fig. 5A and B. The OS of the skin reaction–positive group was statistically significantly longer than that of the skin reaction–negative group (7.1 vs. 1.4 months at MST, respectively; \( P < 0.01 \); Fig. 5A). Moreover, the PFS of the skin reaction–positive group was statistically significantly longer than that of the skin reaction–negative group (2.3 vs. 1.2 months at MST, respectively; \( P < 0.01 \); Fig. 5B).

A case of CR following peptide vaccination
The patient in case 18 developed tongue cancer recurrence and lymph node metastasis on the right side of her neck 15 months after surgery. Initially, she received S-1 chemoradiotherapy at a daily dose of 80 mg for 14 days and irradiation (a total of 40 Gy) for 20 days in addition to two cycles of chemotherapy with docetaxel (80 mg) and cisplatin (90 mg) as adjuvant therapy. Despite receiving these treatments, the patient's tumors did not disappear. Therefore, we decided to administer our peptide vaccine therapy. The amount of purulent discharge and swelling on the right side of the neck decreased to a normal state after 12 vaccinations, and the symptoms of tumor recurrence and neck lymph node metastasis disappeared after 16 vaccinations (Fig. 4C). The frequency of pentamer-positive CTL increased after vaccination (from 0.01% to 6.0%), and an ELISPOT assay showed that the LY6K peptide-specific CTL response increased after 16 vaccinations (Fig. 4A and B). At present, 37 months after the initiation of peptide vaccination, the patient has been remained free of tumor recurrence and metastasis.

Discussion
In the present phase II clinical study, we demonstrated that the CT antigenic peptide-based vaccination-induced immune response is positively correlated with a better prognosis in patients with advanced and inoperable HNSCC. In addition, cancer vaccination using a combination of multi-epitope peptides as monotherapy may provide a clinical benefit for patients. To our knowledge, this study is the first to show a promising result indicating that
therapeutic cancer vaccination with multiple peptides can potentially improve the prognosis of patients with advanced HNSCC refractory to standard therapy.

Several phase II and III clinical trials have recently demonstrated promising and therapeutic results of cancer vaccination (19, 24–32). However, most of these studies were performed using single antigen-based vaccination with several modifications, and the clinical benefits appeared to be limited. To further improve the clinical response to cancer vaccination, it is necessary to consider the application of a combination of multiple peptide vaccines derived from different TAAs, as such therapy may overcome problems associated with the heterogeneity of tumor cells and escape of tumor cells from the peptide-specific immune response due to the loss of an antigen expression (28, 33). In general, the preferable characteristics of target molecules for the development of cancer vaccines include (i) a high level of immunogenicity, (ii) a common and high expression in cancer cells, (iii) a specific expression in cancer cells, testis or fetal tissues only, and (iv) the presence of essential molecules for cell division and survival (to prevent a loss of expression; refs. 28, 33). In this regard, the LY6K, CDCA1, and IMP3 molecules used in the present trials are considered to be the most appropriate because they have already been proven to be cancer–testis antigens satisfying all four ideal characteristics described above (13–16, 34–36) and are expressed in the majority of HNSCC cells. This study was the first report of peptide vaccine therapy for patients with HNSCC. We administrated mixed peptide vaccine, so CTL was more inducible as compared with using only one peptide. OS was significantly prolonged, the patient who responded to the three peptides than one peptide. In the future, we want to develop the peptide vaccine therapy that can reject the cancer cells more strongly.

In the present phase II clinical trial, we compared the OS and PFS between a A24(+) group treated with peptide vaccination and an A24(−) group treated without peptide vaccination. The OS
Table 2. The incidence of adverse events

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<th>Total (%)</th>
<th>Any events</th>
<th>Any immune-related events</th>
<th>Drug fever</th>
<th>Rash or flushing</th>
<th>Injection site reaction (redness, induration, ulceration)</th>
<th>Pruritus</th>
<th>Blood disorders</th>
<th>Leukopenia</th>
<th>Neutropenia</th>
<th>Anemia</th>
<th>Thrombopenia</th>
<th>Increase in PT-INR*</th>
<th>Hepatic disorders</th>
<th>Hyperbilirubinemia</th>
<th>Increase in serum aspartate aminotransferase</th>
<th>Increase in serum alanine aminotransferase</th>
<th>Proteinuria</th>
<th>Increase in serum creatinine</th>
<th>Proctitis</th>
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<tr>
<td>28 (75.7)</td>
<td>28 (75.7)</td>
<td>3 (8.1)</td>
<td>0 (0)</td>
<td>25 (67.6)</td>
<td>27 (73.0)</td>
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<td>0 (0)</td>
<td>Increase in serum AST (0)</td>
<td>Increase in serum ALT (0)</td>
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*PT-INR, prothrombin time-international normalized ratio.

and, less significantly, PFS in the A24(+) group treated with peptide vaccination were longer than those observed in the A24(−) group treated without peptide vaccination, suggesting that therapeutic cancer vaccine treatment using peptides inducing HLA-A24-restricted CTLs may provide a survival benefit in patients with advanced HNSCC. Furthermore, as demonstrated in the A24(+) group, specific CTL responses to two or three peptides may improve the OS in comparison with that observed in patients with CTL induction to no or only a single peptide. Although treatment with cancer vaccination has been shown to result in increased levels of circulating tumor antigen-specific T cells (37), herein we provided direct evidence of a positive correlation between the extent of the peptide-specific CTL response and a longer OS. Therefore, the findings of the present study support the hypothesis that peptide vaccination-induced immune responses contribute to improving the prognosis of patients with advanced HNSCC.

According to the recommendation by the iSBSTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers (38–40), we carried out immunologic monitoring in the A24(+) group treated with peptide vaccination using two different assays, ELISPOT and pentamer assays, at three different time points at a central laboratory. Because the peptides used in the present study exhibited strong immunogenicity, in vitro immunologic monitoring in the A24(+) group was successfully performed in a reliable fashion.

In this clinical trial, we used a vaccine containing 3 mg of peptides in total, among which 1 mg of each of three peptides was mixed, in an attempt to activate CTL responses against tumor cells. Previously, Nakatsura and colleagues reported the use of Glypican-3–derived peptide vaccination (Glypican-3 is an oncofetal antigen that is overexpressed in hepatocellular carcinoma cells). The authors were able to induce Glypican-3–specific CTL response dose-dependently (41). In addition, considering the amount and effect of intradermal administration, good results were obtained with 3 or 10 mg of peptides. Therefore, in performing our peptide vaccination trial using mixed peptides derived from tumor-specific antigens, we prepared 3 mg of peptides in total. As a result, the patients exhibiting a CTL response against three and two peptides demonstrated an extended OS in comparison with those exhibiting a CTL response against none or only one peptide. This finding suggests that the effects of peptide vaccination are observed in patients with some precursor T cells against TAAs. In 13 of the 15 patients who were not evaluated for a CTL response, we were unable to obtain blood samples for the CTL analysis because they received less than four peptide vaccinations. In addition, it was observed that 15 of the 37 patients exhibited CTL induction against two or three types of peptides, suggesting that the individual peptides used for vaccination do not inhibit the CTL induction of each other. There is a possibility that some patients will exhibit induced CTL responses to all peptides used for vaccination if the number of patients tested is increased. On the other hand, in the peripheral blood collected from patients vaccinated with these peptides, increased responses of CD4+ T cells reactive to the same and other TAA-derived peptides have been observed (42, 43). These phenomena may be explained by the activation of CD4+ T cells exposed to TAAs released from tumor cells killed by CTLs in the presence of dendritic cells that can uptake and process TAAs into antigenic peptides recognized by CD4+ T cells.

We analyzed the frequencies of Treg cells before and after vaccinations in 5 patients (Supplementary Fig. S3). In 2 patients (cases 1 and 3), the high proportions of Treg cells were observed pre- and postvaccination, and CTL was induced by only one
peptide. On the other hand, in 2 patients (cases 2 and 4), the low proportions of Treg cells were observed pre- and postvaccination, and CTLs were induced by all the three peptides. Furthermore, in 1 patient (case 5) who showed increased proportion of Treg cells postvaccination, peptides-specific CTLs were not induced by the vaccination. Considering from these results, it is suggested that it may be difficult to induce CTL in patients with high proportion of Treg cells before vaccination. On the other hand, in patients with low proportion of Treg cells before vaccination, it may be possible to induce CTL. Because the peptide-specific CTLs were not induced in 1 patient who showed significant increase of Treg cells after vaccination even though Treg cell proportion was not so high before vaccination, there may be a possibility that CTL could not be well induced in the presence of increased Treg cells. In any case, we have to investigate Treg cell status in more patients to make a conclusion of this question in future.

In the present study, 1 patient (case 18) achieved a clinical CR. This finding demonstrates that our peptide vaccination can yield an excellent response in some patients with HNC. The patient had received adjuvant chemotherapy with limited systemic chemotherapy for 3 months before vaccination. She was evaluated to have a PS of 1 according to the ECOG classification, with a WBC count of 6,000/μL and a lymphocyte level of 1,300/μL. In this case, the number of LY6K-specific CD8+ T cells finally increased at 5 months after the start of peptide vaccination, and the LY6K-peptide specific CTL response increased to 6% by in vitro stimulation approximately 8 months later. At the same time, disappearance of the patient’s tumor recurrence and neck lymph node metastasis was confirmed on a CT examination. Since then, no tumor recurrence has been observed for 3 years. An ELISPOT assay was used to detect a CTL response against the LY6K peptide 2 years after the start of vaccination, and we considered the patient to have obtained the successful induction of memory T cells against the peptides.

Recently, there was a report that persisting peptide/IFA vaccine depots can induce specific T-cell sequestration, dysfunction, and deletion at the site of vaccination in mice (44). In this clinical study, we experienced a patient who was vaccinated 61 times during an approximately 4-year period, with good induction of peptide-specific CTL responses. The patient was in a tumor-bearing state and received a significant dose of peptide vaccination. This may be why the patient demonstrated a peptide-specific CTL response for such a long period. In addition, the degree of T-cell sequestration and dysfunction may differ depending on the type of tumor-associated antigen or based on differences between humans and mice.

Because available cancer vaccines are likely to be applied as adjuvant treatment in patients at a high risk of recurrence following surgical resection of the primary tumor (28, 33), we are planning to develop a cancer peptide vaccine for use in the treatment of patients with HNC in the adjuvant setting. However, even if the patient is at an advanced stage of the disease and has received intensive treatment with chemotherapy and/or radiotherapy, the present study indicates that cancer peptide vaccination may provide some clinical benefit as monotherapy without severe adverse effects. In general, there is no curative therapy for HNSCC associated with inoperable tumors or recurrence after surgery. Hence, we believe that our protocol is promising for improving the prognosis and quality of life, at least for some fraction of patients with advanced HNSCC.

Conclusion
To our knowledge, this study is the first to show the proof of concept that CT antigenic peptide-based vaccination-induced immune responses are associated with better prognoses in patients with advanced HNSCC, implying that cancer vaccination with multiple peptides as monotherapy may provide hope for patients with advanced and/or inoperable HNSCC refractory to standard therapy.

Disclosure of Potential Conflicts of Interest
K. Yoshida is an employee of OncoTherapy Science, Inc. Y. Nishimura reports receiving a commercial research grant from OncoTherapy Science, Inc. Y. Nakamura reports receiving a commercial research grant from and has ownership interest (including patents) in OncoTherapy Science, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
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