Phase II clinical trial of multiple peptide vaccination for advanced
head and neck cancer patients revealed induction of immune
responses and improved OS

Yoshihiro Yoshitake,¹,² Daiki Fukuma,¹ Akira Yuno,¹,³ Masatoshi
Hirayama,¹,³ Hideki Nakayama,¹ Takuya Tanaka,¹ Masashi Nagata,¹,⁴
Yasuo Takamune,¹ Kenta Kawahara,¹ Yoshihiro Nakagawa,¹ Ryoji
Yoshida,¹ Akiyuki Hirosue,¹ Hidenao Ogi,¹ Akimitsu Hiraki,¹ Hirofumi
Jono,⁵ Akinobu Hamada,⁵,⁶ Koji Yoshida,⁷,⁸ Yasuharu Nishimura,³
Yusuke Nakamura,⁹ and Masanori Shinohara¹

Author affiliations

¹Department of Oral and Maxillofacial Surgery, Graduated School of
Medical Sciences, Kumamoto University, Kumamoto, Japan.

²Itoh Dent-Maxillofacial Hospital, Kumamoto, Japan

³Department of Immunogenetics, Graduated School of Medical
Sciences, Kumamoto University, Kumamoto, Japan.

⁴Minamata City General Hospital and Medical Center

⁵Department of Pharmacy, Kumamoto University Hospital & Department
of Clinical Pharmaceutical Sciences, Graduate School of Pharmaceutical
Sciences, Kumamoto University, Kumamoto, Japan.
6Department of Clinical Pharmacology Group for Translational Research Support Core National Cancer Center Research Institute, Tokyo, Japan.

7Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

8OncoTherapy Science Incorporation, Research and Development Division, Kanagawa, Japan.

9Department of Medicine, University of Chicago, Chicago, Illinois, United States of America

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Corresponding author: Yoshihiro Yoshitake, D.D.S., Ph.D., Department of Oral and Maxillofacial Surgery, Graduated School of Medical, Science, Kumamoto University, Honjo 1-1-1 Chuo-ku, Kumamoto 860-8556, Japan. Phone: +81-96-373-5288; FAX: +81-96-373-5286; E-mail: yyoshi326@gmail.com

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Conflicts of interest
Yusuke Nakamura is a stockholder and scientific advisor for OncoTherapy Science, Inc. and Koji Yoshida is a current employee of OncoTherapy Science, Inc. Yasuharu Nishimura is supported by funding from OncoTherapy Science, Inc.

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Translational Relevance

Cancer vaccination that induces cytotoxic T lymphocytes (CTLs) 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 head and neck squamous cell cancer (HNSCC) patients, we investigated the safety of and clinical and immunological responses to these peptide vaccines. Our results showed peptide-specific CTLs in the peripheral blood of the advanced HNSCC patients and increased CD8+ T cell infiltration in the tumors following peptide vaccination. In addition, significantly, a CR was obtained after peptide vaccination in one 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 HNSCC patients receiving peptide vaccination.
These findings promote the use of peptide vaccination for the treatment of HNSCC.
Abstract

**Purpose:** The peptides derived from ideal cancer-testis antigens, including LY6K, CDCA1 and IMP3 (identified using genome-wide cDNA microarray analyses), were utilized 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 immunological response were evaluated using enzyme-linked ImmunoSpot (ELISPOT) and pentamer assays. The peptides were subcutaneously administered weekly with IFA. The primary endpoints were evaluated based on 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) 4.9 vs. 3.5 month, 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 deaths worldwide every year [1,2,3]; 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 chemotherapy and intensity modulated radiation therapy (IMRT), no molecular targeting agents against HNC
have been developed, except for cetuximab. Therefore, the development of novel treatment modalities, such as immunotherapy, is eagerly awaited.

Immunotherapy is a potentially attractive treatment option for HNC. Some tumor-associated antigens (TAAs) identified in HNC cells have the potential to be used in peptide-based vaccines. However, immunotherapy using TAA-derived peptides has not demonstrated adequate antitumor efficacy in clinical trials of advanced HNC. In this clinical trial, we used peptides derived from LY6K, CDCA1 and IMP3. All antigens used in this study were cancer-testis antigens, which are ideal targets for anticancer immunotherapy because they are particularly overexpressed in cancer cells and testis, a site of immune privilege, but not in other normal tissues, and promote the proliferation of cancer cells [13-16]. We identified LY6K 177-186 (RYCNLEGPI), CDCA1 56-64 (VYGIRLEHF) and IMP3 508-516 (KTVNELQNL) peptides that can induce peptide-reactive and HLA-A24 (A*24:02)-restricted cytotoxic T lymphocytes (CTLs) without stimulating autoimmunity. HLA-A24 is the most common HLA class I allele in the Japanese population, and 60% of Japanese individuals (95% of whom have an A*24:02 genotype), 20% of Caucasians and 12% of Africans are positive for HLA-A24 [17, 18]. A
phase II clinical cancer vaccination trial using a combination of multiple peptides derived from LY6K, TTK and IMP3 in HLA-A*24:02(+) patients with advanced esophageal squamous cell carcinoma (ESCC) refractory to standard ESCC therapy was recently performed [14, 19], and the evidence from which encouraged us to develop this therapy against HNSCC for an evaluation in a phase II trial.

In this study, we evaluated the immunological responses to and safety and survival benefits of cancer vaccination in a phase II trial of advanced HNSCC patients refractory to standard therapy.
Materials and Methods

Study design

The present study is a phase II, open-label, non-randomized clinical cancer vaccination trial conducted in an exploratory setting. The endpoints were evaluated based on differences between the HLA-A*24:02-positive (A24(+)) and -negative (A24(−)) groups as a biological 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, SEPPIC) was performed in HNSCC patients (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), immunological responses and adverse effects. Toxicities caused by the vaccination therapy were assessed according to the Common Terminology Criteria for Adverse Events version 3 (CTCAE). Immunological monitoring was performed at the central laboratory using both enzyme-linked immunospot (ELISPOT) assays and
HLA-A24/TAA peptide pentamer assays with the *in vitro* culture of lymphocytes derived from PBMCs at the pre- and post-vaccination 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, 000008379 (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: A) HNSCC patients with locally advanced, recurrent and/or metastatic tumors who had failed to respond...
to standard therapy; B) adequate bone marrow, cardiac, pulmonary, hepatic and renal functions, including a WBC of \( \geq 2,000/\mu l \), a platelet count of \( \geq 75,000/\mu l \), a total bilirubin level of \( \leq 2.0 \) 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 \( \leq 1.5 \) of the institutional upper limit of normal; C) no history of therapy within the four weeks prior to the initiation of the trial; D) an ECOG performance status (PS) of 0–2; and E) an age of 18–85 years. The exclusion criteria were as follows: A) pregnancy (including the refusal or inability to use effective means of contraception among females of childbearing potential); B) currently breastfeeding; C) serious bleeding disorders; D) serious infections requiring antibiotics; E) concomitant treatment with steroids or immunosuppressive agents; and F) a determination of unsuitableness by the principal investigator or physician-in-charge.

In this study, none of the patients were excluded by 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 eight weeks. After that, they were vaccinated at every four weeks based on the detection of PD or the doctor’s assessment. For the immunological evaluation, PBMCs were obtained from the patients at the pre-vaccination period and after the fourth and eighth vaccinations. For the imaging analysis, CT was performed during the pre-vaccination period (within one month before vaccination) and at every four vaccinations.

**Peptides**

Peptides derived from LY6K-177 (RYCNLEGPI), CDCA1-64 (VYGIRLEHF) and IMP3-508 (KTVNELQNL) able to induce tumor-reactive and HLA-A24 (*A*24:02)-restricted CTLs were synthesized as described elsewhere [14]. The purity (>97%) of the peptides was determined using analytical 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 (NeoMPS, Inc., San Diego).
Lymphocyte preparation for immunologic monitoring

The protocol for the immunological 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 pre-vaccination 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, Piscataway, NJ) density gradient solution and stored at −80°C in cell stock media (Juji field) without serum at 5 x 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 x 10^5 cells per well were incubated in medium containing 100 units/ml of recombinant IL-2 (rIL-2; Novartis) with peptide stimulation.
(10 μg/ml) performed twice on days 1 and 8 in combination with HIV-specific peptide (ILKEPVHG, 10 μg/ml) as a negative control and CMV-specific peptide (RYLRDQQL, 10 μg/ml) as a positive control. On day 15, the cultured lymphocytes were subjected to an ELISPOT assay and a flow cytometry analysis after a depletion of CD4+ cells using magnetic beads (Invitrogen, Grand Island, NY). A conventional ELISPOT assay using TISI cells, a human B-lymphoblastoid cell line expressing HLA-A24, pulsed with the relevant peptide as a target in combination with an irrelevant HIV-specific peptide as a negative control was performed, followed by the HLA-A24/TAA peptide pentamer assay, as described below.

ELISPOT assay

In order to monitor the antigen-specific immune response, an ELISPOT assay was performed using the human IFN-γ ELISPOT PLUS kit (Mabtech, Nacka Strand, Sweden). Ninety-six-well plates with nitrocellulose membranes (Millipore, Molshelm, France) were pre-coated with primary anti-IFN-γ antibodies (1-D1K) at 4°C overnight. The plates were then pre-reacted with RPMI medium containing 10% FBS (Invitrogen). Each vaccine peptide (10 μg/ml)-, HIV-specific peptide
(ILKEPVHG, 10 μg/ml)- or CMV-specific peptide (RYLRDQQLL, 10 μg/ml)-pulsed TISI cells (2 × 10^4/well) as stimulators, was incubated for 24 hours in triplicate with responder cells (from 2 × 10^4/well to 2.5 × 10^3/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, St. Louis MO) + ionomycin (500 pM, 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 two 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 in order to confirm peptide specificity. HLA-A24/LY6K- , /CDCA1- 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, San Jose, CA, USA). Frozen PBMC samples derived from patients before vaccination and at 7 days after the 4th and 8th vaccinations were thawed and directly used for detection of Treg cells. In this experiment, CD4⁺CD25^{high}Foxp3⁺ cells were judged as Treg cells. The antibodies used to detect Treg cells were as follows: CD4-fluorescein isothiocyanate, Foxp3-phycoerythrin, and CD25-Allophycocyanin (e-Bioscience, San Diego, CA, USA).

**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 CINtec® p16INK4A assay (Ventana Medical Systems, Inc., Tucson, Arizona), 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 HNSCC patients between December 1, 2008 and December 5, 2012. A total of 37 patients were enrolled in this study (Supplementary Table 1). 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 three patients had a PS of 2, 44 patients had a PS of 1 and eight 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 one 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, one patient had undergone chemotherapy only, one patient had undergone radiotherapy only and two patients had not received any treatment prior to 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 et al. [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 one 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 the 37 patients, one (case 18) was judged to have achieved a complete response (CR) for 37 months and nine were found to have stable disease (SD) for three months, according to the RECIST criteria. The disease control rate
(CR+SD) was 27.0% after three months. The median time to progression-free survival 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, \( p < 0.05 \), Figure 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, \( p = 0.13 \), Figure 1B).

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

In the A24 (+) group, *in vitro* cultured T cells were subjected to ELISPOT 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 (Figure 2A). Similarly, the patients
demonstrating a positive response specific to the CDCA1 peptide exhibited a significantly longer OS than those without a CTL response (Figure 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 (Figure 2C, 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 (Figure 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 (Figure 3). These observations indicate that the immunological response induced by the peptide vaccination contributed to improving the prognosis of these patients.

The results of the representative ELISPOT and pentamer assays specific to the LY6K peptide are shown in Figure 4. The ELISPOT assay indicated substantial T cell responses specific to the LY6K peptide in comparison to the irrelevant peptide (Supplementary Figure 1A),
according to the criteria described in the Materials and Methods section. This LY6K-specific T cell response was further confirmed by the LY6K-pentamer assay, as shown in Supplementary Figure 1C, 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 Figure 1D). 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 4th and the 8th vaccinations among 24 cases investigated are shown in Supplementary Figure 2A. In 20 cases out of 22 cases, CTL responses increased depending on increased vaccinations, whereas CTL response decreased in 2 cases. In one case of them, CTL response decreased after the 8th 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 8th vaccination. We thought that CTL response is accidentally undetectable only at the 8th 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 Figure S2B.

There is only one 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 Figures 5A and 5B. 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, $p<0.01$, Figure 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, $p<0.01$, Figure 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 (Figure 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 (Figure 4A.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. In order 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 tumor-associated antigens, 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 (1) a high level of immunogenicity, (2) a common and high expression in cancer cells, (3) a specific expression in cancer cells, testis or fetal tissues only and (4) the presence of essential molecules for cell division and survival (to prevent a loss of expression) [28, 33]. In this regard, the LY6K, CDCA1 and IMP3 molecules used in the present trials are considered to be 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 first report of peptide vaccine therapy for head and neck squamous cell cancer patients. 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 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 to 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], we herein 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 iSBSTe-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers [38-40], we carried out immunological 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. Since the peptides used in the present study exhibited strong
immunogenicity, *in vitro* immunological 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 et al. 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 a 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 to 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 further 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 Figure 3). In two patients (cases 1 and 3), the high proportions of Treg cells were observed at pre- and post-vaccination, and CTL was induced by only 1 peptide. On the other hand, in 2 patients (cases 2 and 4) the low proportions of Treg cells were observed at pre- and post-vaccination, and CTLs were induced by all 3 peptides. Furthermore, in one patient (case 5) who
showed increased proportion of Treg cells at post-vaccination, 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 one 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 couldn’t 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, one patient (case 18) achieved a clinical CR. This finding demonstrates that our peptide vaccination can yield an excellent response in some HNC patients. The patient had received adjuvant chemotherapy with limited systemic chemotherapy for three months prior to 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 five months after the
start of peptide vaccination, and the LY6K-peptide specific CTL response increased to 6% by \textit{in vitro} stimulation approximately eight 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 three years. An ELISPOT assay was used to detect a CTL response against the LY6K peptide two 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 \cite{44}. In this clinical study, we experienced a patient who was vaccinated 61 times during an approximately four-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.
Since 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 HNC patients in the adjuvant setting. However, even if the patient is at an advanced stage of 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 advanced HNSCC patients.
**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.
Acknowledgements

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July 2013.

Figure Legends

Figure 1. Overall and progression-free survival of the HLA-A24(+) and HLA-A24(−) groups. The HLA-A24(+) patients received peptide vaccination, while the HLA-A24(−) patients did not. The OS (A) and PFS (B) were evaluated in the HLA-A24 (HLA-A*24:02)-positive patients treated with peptide vaccination and the HLA-A24-negative patients treated without peptide vaccination for the subgroup analysis. MST, median survival time. The OS and PFS were analyzed according to the Kaplan-Meier method, and statistical differences were assessed using the log-rank test.

Figure 2. A prolonged OS and PFS in the HLA-A24(+) group were correlated with a CTL response specific to each of the LY6K and CDCA1 peptides. In the HLA-A24(+) group, in vitro cultured T cells were subjected to ELISPOT assays. Positive CTL responses specific to LY6K- and CDCA1 peptides after vaccination were observed in 85.7% and 64.3% of the patients, respectively. The OS was compared between the patients with a positive CTL response (CTL (+)) and those with a negative CTL response (CTL (−)) to each peptide, and the patients with a positive CTL response specific to the LY6K peptide were found to
exhibit a significantly longer OS and PFS than those without a CTL response (A, C). Similarly, the patients with a positive CTL response specific to the CDCA1 peptide showed a significantly longer OS and PFS than those without a CTL response (B, D).

Figure 3. OS of the four subgroups of HLA-A24(+) patients receiving peptide vaccination classified according to the number of peptides inducing a positive CTL response. 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. B, The PFS exhibited a similar tendency to the OS. MST, median survival time.

Figure 4. CT imaging of the recurrent and metastatic tumors in case 18, in which the clinical response to vaccination was judged to be a complete response (CR). A B, PBMCs obtained from the patient in case 18 (HLA-A*24:02-positive) after the 12th and 16th vaccinations were cultured in rIL-2 for 14 days with two episodes of LY6K peptide stimulation. The cultured lymphocytes were subjected to an ELISPOT.
assay following the depletion of CD4-positive cells using magnetic beads. The results of immunological monitoring assays using an ELISPOT assay and flow cytometry with the HLA-A24/LY6K-pentamer in combination with anti-CD8 and anti-CD3 mAbs are presented at the time points after 12 (A) and 16 (B) vaccinations in case 18. The LY6K-specific CTL response increased after 16 vaccinations. C, CT imaging showed tumor recurrence and lymph node metastasis before vaccination. After 16 cycles of vaccination, the recurrent and metastatic tumors disappeared, and the patient was judged to have exhibited a CR on CT imaging by a radiologist. It has been three years since the tumor and metastatic lymph node lesions disappeared.

Figure 5. The occurrence of skin reactions was correlated to the prolongation of OS and PFS. The patients with skin reactions (+) exhibited longer OS (A) and PFS (B) with statistical significance than those without skin reactions (−). The MST of the OS of the patients with skin reactions (+) vs. those without (−) was 7.1 vs. 1.4 months, respectively. The MST of the PFS of the patients with skin reactions (+) vs. those without (−) was 2.3 vs. 1.2 months, respectively.
Figure 1

A

OS (%)

HLA-A24(+)  n=37
Peptide Vaccine Therapy
MST: 4.9 mo

HLA-A24(-)  n=18
Best Supportive Care
MST: 3.5 mo

P < 0.05

B

PFS (%)

HLA-A24(+)  n=37
Peptide Vaccine Therapy
MST: 1.9 mo

HLA-A24(-)  n=18
Best Supportive Care
MST: 1.0 mo

P = 0.13
Figure 2

A

**LY6K  CTL (+) : 85.7% (21/24 cases)**

Overall survival in peptide vaccination group

- CTL (+)  n=21 MST: 8.1 mo
- CTL (-)  n=3 MST: 1.4 mo

$P < 0.01$

B

**CDCA1  CTL (+) : 64.3% (9/14 cases)**

Overall survival in peptide vaccination group

- CTL (+)  n=9 MST: 11.3 mo
- CTL (-)  n=5 MST: 4.6 mo

$P < 0.05$

C

**LY6K  CTL (+) : 85.7% (21/24 cases)**

Progression free survival in peptide vaccination group

- CTL (+)  n=21 MST: 2.3 mo
- CTL (-)  n=3 MST: 1.4 mo

$P < 0.01$

D

**CDCA1  CTL (+) : 64.3% (9/14 cases)**

Progression free survival in peptide vaccination group

- CTL (+)  n=9 MST: 7.0 mo
- CTL (-)  n=5 MST: 1.7 mo

$P < 0.01$
Figure 3

A

OS (%)

0 5 10 15 20 25 30 35 40 45 50 55

MST

CTL 3 Ag (n=6) 19.5
CTL 2 Ag (n=9) 8.1
CTL 1 Ag (n=7) 4.6
CTL (-) (n=2) 1.4

B

PFS (%)

0 5 10 15 20 25 30 35 40

MST

CTL 3 Ag (n=6) 12.0
CTL 2 Ag (n=9) 2.3
CTL 1 Ag (n=7) 1.7
CTL (-) (n=2) 1.3
Figure 4

A  After 12 times vaccination

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B  After 16 times vaccination

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C  Pre vaccination

- Mandibular bone
- recurrence
- LN meta

C  After 16 times vaccination
**Figure 5**

**A**

Skin reaction (+) : 75.7% (28/37 cases)

- Skin reaction (+) (n=28)  
  MST: 7.1 mo
- Skin reaction (-) (n=9)  
  MST: 1.4 mo

**B**

Skin reaction (+) (n=28)  
MST: 2.3 mo
Skin reaction (-) (n=9)  
MST: 1.2 mo

Statistical significance: $P < 0.01$
Table 1.
HNSCC patient characteristics, clinical response, and immune response of CTL

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Abbreviations: CR, complete response; SD, stable disease; PD: progressive disease; PFS, progression free survival; OS, overall survival; PS: performance status

*a*Stage: staging was carried out according to the TNM classification for Head and Neck cancer (World Health Organization; WHO).

*b*Prior therapy: Ope, surgery; S-1, tegafur, gimeracil, oteracil potassium; CDDP, *cis*-diamminedichloroplatinum; DTX, docetaxel; 5FU, fluorouracil; RT, radiotherapy.

*c*Clinical response were evaluated according to RECIST guidelines.

*d*CTL responses were classified into four subgroups by the number of peptides inducing the positive CTL responses in patients.

*e*(x), Completion of last treatment; (y), Start of vaccination.
Table 2.

The incidence of adverse events

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<tr>
<th>Event</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any events</td>
<td>28 (75.7)</td>
</tr>
<tr>
<td>Any immune-related events</td>
<td>28 (75.7)</td>
</tr>
<tr>
<td>Drug fever</td>
<td>3 (8.1)</td>
</tr>
<tr>
<td>Rash or Flushing</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Injection site reaction (redness, induration, ulceration)</td>
<td>25 (67.6)</td>
</tr>
<tr>
<td>Pruritus</td>
<td>27 (73.0)</td>
</tr>
<tr>
<td>Blood disorders</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anemia</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Thrombopenia</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Increase in PT-INR*</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hepatic disorders</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hyperbilirubinemia</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Increase in serum aspartate aminotransferase</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Increase in serum alanine aminotransferase</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Renal disorders</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Increase in serum creatinine</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*PT-INR, prothrombin time-international normalized ratio
Phase II clinical trial of multiple peptide vaccination for advanced head and neck cancer patients revealed induction of immune responses and improved OS

Yoshihiro Yoshitake, Daiki Fukuma, Akira Yuno, et al.

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