from the day of registration to the day of death from any cause or 1.5 years afterwards. Progression-free survival was counted from the day of registration to the day of progressive disease by clinical evaluation or imaging diagnosis, whichever was earlier.

Adverse events were evaluated at each hospital visit and graded according to the Common Toxicity Criteria version 3 (CTCAE v3). Adverse events which could not be ruled out as being related to the trial therapy were reported as adverse drug reactions (ADRs). For each adverse event, we documented the worst grade for each patient, and confirmed the incidence of each by grade.

### Exploratory assessment

Induction of VEGFR-2-specific CTLs and serum concentrations of VEGFR-2 were analyzed only in subjects who provided specific consent to receive these assessments at some of the participating medical institutions.

The induction of VEGFR-2-specific CTLs was evaluated by an enzyme-linked immunospot assay. CTL positivity was defined as when the calculated value (average spot number in the peptide pulse group - average spot number in the negative control group/average spot number in the peptide pulse group × 100) by time was greater than that of day 1, and further when the average spot number in the peptide pulse group was greater than the average spot number and standard deviation range in the negative control group.

Serum concentrations of VEGFR-2 were measured before drug administration on day 1, day 8, and day 29, using Quantikine® Human Soluble VEGFR-2 Immunoassay (R&D Systems, Inc).

### Statistical analysis

Overall survival, 1-year survival and progression-free survival were estimated with the Kaplan-Meier method. To assess differences in overall survival between the elpamotide and historical control groups [5, 6], log-rank tests and the Harrington-Fleming, in which time is weighted and was used in anticipation that the effects of the vaccine would present with time, were used.

Calculation of sample size was based on an additional treatment effect of 15 % in the elpamotide group compared with the 1-year survival rate in the historical control group, which was derived from previous reports [5, 6]. The null hypothesis was "no extension of 1-year survival" to achieve a one-sided type I error of <10 % and a power of >80 %. We estimated that the 1-year survival rate of the historical control group based on patients with BTC was 15–30 %, and expected elpamotide to add a treatment effect of 15 %. When the historical control group was set at 200 patients, the sample size needed for the elpamotide group was calculated to be 45–60 patients. Accordingly, we aimed to select a total of 50 patients.

Serum concentrations of VEGFR-2 were analyzed by post-hoc test. All statistical analyses were conducted with SAS software, version 9.1.3 (SAS Institute).

## Results

### Patient characteristics

Of the 55 patients registered from October 2009 to June 2011, 54 who underwent the trial therapy were included in the full analysis set and safety analysis set. Patient characteristics are summarized in Table 2. Compared to the historical control group, the present trial had higher proportions of patients without gallbladder cancer (66.7 % vs. 45–50.7 %) and those having a performance status of 0 (90.7 % vs. 60 %).

### Survival and response rate

Fourteen patients (25.9 %) survived ≥1.5 years, and two completed the 1.5-year trial therapy. The median number of courses of study treatment was 4.5 (range: 1–20), and the dose intensity of elpamotide and Gem was 90.0 and 82.7 %,

**Table 2** Patient characteristics (*N* _54)*

| Characteristics | No. of patients | % |
|---|---|---|
| Age, years | | |
| <65 | 27 | 50 |
| ≥65 | 27 | 50 |
| Sex | | |
| Male | 30 | 55.6 |
| Female | 24 | 44.4 |
| Primary tumour site | | |
| Intrahepatic bile duct | 20 | 37 |
| Gallbladder | 18 | 33.3 |
| Extrahepatic bile duct | 13 | 24.1 |
| Ampulla of vater | 3 | 5.6 |
| Extent of disease | | |
| Metastatic | 34 | 63 |
| Locally advaced | 20 | 37 |
| Resection | | |
| No | 37 | 68.5 |
| Yes | 17 | 31.5 |
| Lymphocyte | | |
| ≥18 % | 45 | 83.3 |
| <18 % | 9 | 16.7 |
| PS (ECOG) | | |
| 0 | 49 | 90.7 |
| 1 | 5 | 9.3 |

Clinical characteristics of the 54 patients who received elpamotide+GEM
*PS* (*ECOG*) Performance status (Eastern Cooperative Oncology Group)

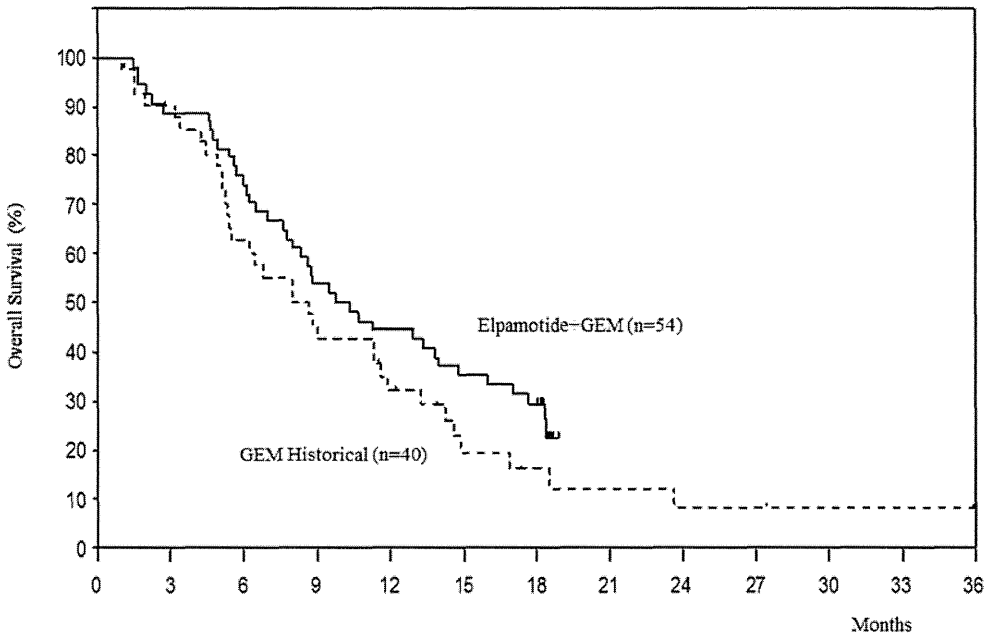


**Fig. 1** Overall survival

respectively. Main reasons for discontinuation were exacerbation of primary disease (34 cases) and adverse event-related reasons (6 cases).

Median survival was 10.1 months (95 % confidence interval (CI): 8.0–14.0 months), which was longer than that of the historical control (7.6 months) ($P$=0.079; Harrington-Fleming method; $P$=0.043, log-rank test; Fig. 1). One-year survival rate was 44.4 %, and median progression-free survival was 4.5 months (95 % CI: 2.8–7.1 months).

Median overall survival by site of origin was as follows: intrahepatic bile duct (11.6 months), extrahepatic bile duct (18.3 months), gallbladder (8.4 months), and vater papilla (9.8 months). These were superior to the 8.7, 10.1, 6.5, and 9.3 months, respectively, in the historical control.

None of the patients achieved complete response, while 10 achieved partial response, with the imaging response rate of 18.5 %. Stable disease was maintained for ≥6 months in 8 of 28 patients (14.8 %).

## Toxicity

Major hematologic ADRs included decreased white blood cell counts (75.9 %), decreased platelet counts (72.2 %), and decreased neutrophil counts (64.8 %). Major non-hematologic ADRs included injection site reaction (68.5 %), induration and erythema (64.8 and 27.8 %), nausea (51.9 %), and decreased appetite and malaise (37.0 %). Severe adverse effects were observed in five patients as follows: pneumocystis pneumonia, loss of appetite, thrombotic microangiopathy, interstitial lung disease, and fever. ADRs of grade 3 or higher are summarized in Table 3. There were no treatment-related deaths.

## Subgroup analysis

Among 37 patients who developed injection site reactions (ulcer, induration, or erythema), tumor regression was observed in 10 (27 %) during the study period. Moreover, the median overall survival of the 37 patients was significantly longer (14.8 months) compared to that of the remaining 17 who developed no injection site reactions (5.7 months; Table 4 and Fig. 2).

**Table 3** Adverse drug reactions

| Adverse drug reactions | Grade 3 | | Grade 4 | |
|---|---|---|---|---|
| | *N* | % | *N* | % |
| Hematological | | | | |
| Decreased neutrophil count | 16 | 29.6 | 3 | 5.6 |
| Decreased lymphocyte count | 9 | 16.7 | 0 | 0.0 |
| Decreased white blood cell count | 5 | 9.3 | 0 | 0.0 |
| Decreased platelet count | 4 | 7.4 | 1 | 1.9 |
| Anemia | 2 | 3.7 | 0 | 0.0 |
| Non-hematological | | | | |
| Pneumocystis jiroveci pneumonia | 1 | 1.9 | 0 | 0.0 |
| Thrombotic microangiopathy | 1 | 1.9 | 0 | 0.0 |
| Decreased appetite | 1 | 1.9 | 0 | 0.0 |
| Interstitial lung disease | 1 | 1.9 | 0 | 0.0 |
| Elevated alanine aminotransferase level | 1 | 1.9 | 0 | 0.0 |
| Elevated aspartate aminotransferase level | 1 | 1.9 | 0 | 0.0 |
| Elevated blood glucose level | 1 | 1.9 | 0 | 0.0 |
| Elevated gamma-glutamyltransferase level | 1 | 1.9 | 0 | 0.0 |
| Elevated hepatic enzyme level | 1 | 1.9 | 0 | 0.0 |

**Table 4** Relationship between the efficacy and injection site reactions

| | With ISR ($n$=37) $N$ (%) | Without ISR ($n$=17) $N$ (%) | $P$-value |
|---|---|---|---|
| Response | | | |
| Complete response (CR) | 0 (0.0) | 0 (0.0) | |
| Partial response (PR) | 10 (27.0) | 0 (0.0) | |
| Stable disease (SD) | 20 (54.1) | 8 (47.1) | |
| Progressive disease (PD) | 7 (18.9) | 7 (41.2) | |
| Not evaluable (NE) | 0 (0.0) | 2 (11.8) | |
| Overall survival | | | |
| Median survival (95 % CI) | 14.8 months (9.8, 18.4) | 5.7 months (4.6, 8.6) | 0.002 (H-F), <0.001 (log-rank) |

*CI* confidence interval, *ISR* injection site reaction

Exploratory analysis

The induction of VEGFR2-specific CTLs was assessed in nine patients; six were positive (66.7 %). There was no clear association between CTL positivity with treatment survival, response rate, or ADRs.

Serum concentrations of VEGFR-2 were evaluated in 43 patients, and found to be significantly increased from baseline (day 1) to day 8 ($P$=0.015), and significantly decreased from day 8 to day 29 ($P$=0.010); there was no significant difference from baseline to day 29. Response rate in the 31 patients (72 %) with an elevated serum VEGFR-2 concentration at day 8 was 19 %, and median survival was 13.3 months. There was no apparent association between serum VEGFR-2 concentration and efficacy or ADRs.

## Discussion

Tumor immunotherapy has recently gained much attention, and there are currently more than 100 clinical studies in progress around the world. As a results, some immunotherapeutic drugs already approved [7, 8], and such approval reflects the findings that immunotherapy activates the immune response in cancer patients and is clinically effective.

The present trial was planned and conducted before Gem plus cisplatin therapy became the standard chemotherapy for BTC based on results of the ABC-02 [9] and BT-22 [10] trials. The reliable reference data at the time of planning this trial were only the retrospective data from two studies [5, 6]. Based on results from those studies, we set the threshold 1-year survival rate at 15–30 %, and expected to add a 15 % treatment effect. The result was a 44.4 % 1-year survival rate, which was in line with this prediction. However, the proportion of good performance status cases and of those without gallbladder cancer were high in this trial. Thus, in the comparison with the historical control, improved survival may have been related to patient background, rather than the vaccine's additive effects. Median survival with the standard Gem plus cisplatin

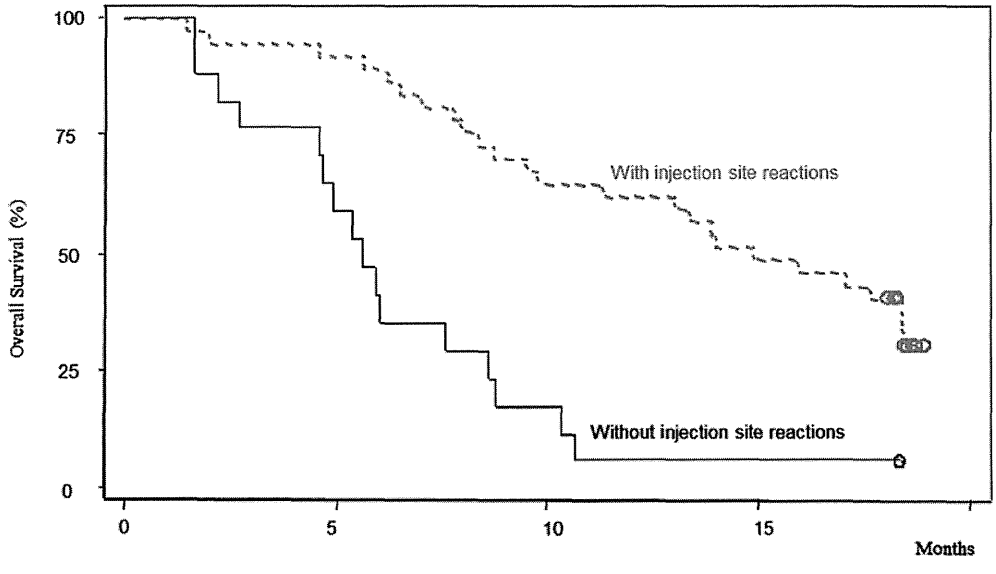


**Fig. 2** Overall survival with or without injection site reactions

therapy in the ABC-02 and BT22 trials was 11.7 and 11.2 months, respectively. Based on the median survival of 10.1 months in the present trial, single-agent Gem chemotherapy clearly lacks power as a platform for additive effects over elpamotide.

Survival curves for subgroups of patients who did and did not exhibit injection site reactions differed substantially. The fact that those who exhibited injection site reactions showed better long-term results suggests that it can be used as an indicator for early determination of those likely to benefit from therapy. This phenomenon was also observed in the Gem ± elpamotide trial (PEGASUS-PC Study), which targeted advanced pancreatic cancer patients, and although primitive, it may serve as a highly reliable indicator.

In conclusion, combined immunotherapy with Gem and elpamotide was well-tolerated and showed moderate antitumor effects. For future development of therapies, it will be necessary to optimize the target population for which therapeutic effects could be expected.

**Acknowledgments** Dr. Keisuke Aiba, Dr. Wasaburo Koizumi, Dr. Takuji Okusaka, and Dr. Yutaka Matsuyama of the Independent Data Monitoring Committee for their insightful comments.

Dr. Yukio Muramatsu and Dr. Hiroaki Onaya of the Independent Diagnostic Radiology Committee for their insightful comments.

Statcom Co., Ltd. for their assistance in preparing and editing this manuscript.

**Funding** This work was supported by OncoTherapy Science Inc., Fuso Pharmaceutical Industries Ltd., and Otsuka Pharmaceutical Co., Ltd.



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Cancer Science
Japanese Cancer Association JCA
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*Report*

# Guidance for peptide vaccines for the treatment of cancer

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**Key words**

Cancer vaccines, clinical study, guidance, non-clinical study, peptide vaccines

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The Japan Society for Biological Therapy has published Guidance for peptide vaccines for the treatment of cancer 2012 Dec; 2: (15 screens) [Cited 28 Apr 2014]. Available from URL: http://jsbt.org/guidance (in Japanese)[1]. This is the English version of that report.

**Funding information**

Japanese Society for Biological Therapy

Received April 8, 2014; Revised April 28, 2014; Accepted May 6, 2014

*Cancer Sci* **105** (2014) 924–931

doi: 10.1111/cas.12443

**Recent progress in fundamental understanding of tumor immunology has opened a new avenue of cancer vaccines. Currently, the development of new cancer vaccines is a global topic and has attracted attention as one of the most important issues in Japan. There is an urgent need for the development of guidance for cancer vaccine clinical studies in order to lead to drug development. Peptide vaccines characteristically have the effect of indirectly acting against cancer through the immune system – a mechanism of action that clearly differs from anticancer drugs that exert a direct effect. Thus, the clinical development of cancer peptide vaccines should be planned and implemented based on the mechanism of action, which differs significantly from conventional anticancer drug research. The Japanese Society for Biological Therapy has created and published Guidance for peptide vaccines for the treatment of cancer as part of its mission and responsibilities towards cancer peptide vaccine development, which is now pursued globally. We welcome comments from regulators and business people as well as researchers in this area.**

The molecular mechanism for the presentation and recognition of melanoma antigens was revealed through the identification of a cancer antigen gene by a Belgian group, van der Bruggen *et al.* in 1991.[2,3] Clinical research of peptide vaccines aginst melanoma using this molecular mechanism subsequently commenced in 1995.[4] Numerous studies have since been reported to show the immunological efficacy of vaccines such as inducing cytotoxic T lymphocytes (CTL);[5] however, the impact of cancer vaccines with limited tumor regression effects could not be proven in clinical study designs given that tumor regression effects are often used as an indicator of efficacy. As a result, Dr Rosenberg of the US National Cancer Institute (NCI) issued a negative report on the effect of cancer vaccines[5] in 2004. Since 2006, the inhibitory effect of cancer peptide vaccines administered as adjuvant therapy has been noted in successive reports with respect to lung cancer and breast cancer, and attention has been drawn to both the preventive effect of cancer vaccines and the subsequent improvement in survival rates.[6,7] In 2010, the cancer vaccine sipuleucel-T,[8] which demonstrated an extended effect on survival rates in cases of castration-resistant prostate cancer, was approved by the US FDA and cancer vaccines were re-unveiled as a new treatment. In 2009, prior to the approval of sipuleucel-T, the US FDA had issued guidance to companies engaged in the development of cancer vaccines, publishing important specifics on the development of cancer vaccines and seeking public comment on cancer vaccines.[9] Currently, the development of new cancer vaccines is a global topic and has

attracted attention as one of the most important issues in Japan. There is an urgent need for the development of guidance for cancer vaccine clinical studies in order to lead to drug development.

The Japanese Society for Biological Therapy is a group of researchers focused on the research of biological therapies to treat cancer. The Society was initially established in 1987, as a Research Group Meeting (a Kenkyukai) named The Society of Biological Response Modifiers to promote the exchange of information for the progress of new cancer treatments. The Society was renamed the Japanese BRM society in 1995, and subsequently in 1999, adopted its current name, the Japanese Society for Biological Therapy. This society has demonstrated its medical and social responsibility as the leader in this area by assembling Japanese and international researchers to discuss results pertaining to state-of-the-art biological treatment and by publishing the results of these conferences. As part of its mission and responsibilities towards cancer peptide vaccine development, which is now pursued globally, the Japanese Society for Biological Therapy has created and published these Guidance for peptide vaccines for the treatment of cancer.[1]

## Characteristics of Cancer Peptide Vaccines

Cancer peptide vaccines are peptides that express pharmacological activity through utilization of the human immune system rather than being pharmacologically active themselves. Peptide vaccines administered subcutaneously reach the lymph nodes via host antigen-presenting cells and lymph flow, eventually inducing an immune response. This is accomplished through the following molecular mechanism: (i) the peptide binds to antigen-presenting cells, human leukocyte antigens (HLA) or major histocompatibility complex (MHC) molecules on the target cell surface; (ii) T-cell receptors (TCR) recognize the HLA-peptide complexes; and (iii) antigen-specific cytotoxic T-cells (specific CTL) are induced. Peptide vaccines characteristically have the effect of indirectly acting against cancer through the immune system – a mechanism of action that clearly differs from anticancer drugs and low-molecular-weight compounds that exert a direct effect. Thus, the clinical development of cancer peptide vaccines should be planned and implemented based on this mechanism of action, which differs significantly from conventional anticancer drug research. The guidances published by the US FDA Center for Biologics Evaluation and Research (CBER) in September 2009[9] were developed based on this idea. In addition, the following points should be considered in designing cancer peptide vaccine clinical research: (i) subjects allowing evaluation of the delayed effect of treatment initiated through the immune system should be selected; (ii) the study design should assume that long-term continuous administration is required and therefore focus both on survival rate and cytoreductive effects; and (iii) outcomes should be evaluated by a scientific method that allows the analysis of delayed effects.

## The Concept of Non-Clinical Safety Testing for Cancer Peptide Vaccines

**The purpose of conducting non-clinical safety testing.** Non-clinical studies aimed at clarifying the toxicological and pharmacological properties of target compounds are necessary in the development of new drugs. Particularly, describing the toxicological properties of novel treatments is essential to ensuring the safety of humans in clinical studies. Information determining the safe initial dose in clinical studies and predicting the toxic effects that may occur with administration of the test substance can be obtained from non-clinical safety testing that has been designed and implemented properly.

**Animal species selection in non-clinical safety testing.** In order to obtain useful results predicting the effect of the test substance in humans from non-clinical safety testing, a suitable animal species must be identified. A suitable animal species is defined as a species in which extrapolation of the effect of the test substance to humans has been confirmed. Currently, there are no known suitable animal species for the non-clinical safety testing of peptide vaccines.

As previously mentioned, peptide vaccines are simply peptides and, not being pharmacologically active themselves, they express pharmacological activity through utilization of the human immune system, namely, antigen presentation and recognition of HLA-peptide complexes by TCR on the surface of lymphocytes and the subsequent induction of CTL. The HLA structure differs significantly between animal species; therefore, no other animal species shares an identical HLA structure with humans. Peptides used in vaccines are not able to bind to the MHC of experimental animal species, which renders antigen presentation impossible to any animal model. This indicates that there are no animal species in which peptides demonstrate pharmacological activity with a mechanism similar to that observed in humans. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline S6 (Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals) proposes the use of transgenic animal models in non-clinical safety testing in light of the characteristics of peptide vaccines, since it is possible to recreate transgenic MHC molecules. However, it is difficult to reproduce the necessary human-type CTL recognition and activation in order to demonstrate drug efficacy and impossible to create an animal model with completely transgenic TCR. Accordingly, it is practically impossible to use a transgenic animal model to reproduce the pharmacological activity that occurs in the human body as a result of the administration of peptide vaccines.

**Pharmacokinetic properties of peptides themselves.** It has been confirmed that peptides are rapidly degraded *in vivo* by dipeptidases into indigenous amino acids. Accordingly, the potential toxicity from metabolites is considered to be extremely low and non-clinical safety testing for peptide vaccines should take this characteristic of peptides into account.

**The situation concerning peptide vaccine non-clinical safety testing in Europe and the United States.** As described above, the requirements for non-clinical safety testing of peptide vaccines differ significantly from those required in the testing of other low-molecular-weight drugs. This is clearly shown in the guidance for non-clinical safety trials required by the regulatory authorities in Europe and the United States (the FDA and European Medicines Agency). Actually, clinical studies for peptide vaccines have been allowed to proceed in the absence of non-clinical safety testing when it has been demonstrated that information ensuring the safety of peptide vaccine administration to humans can only be obtained in humans. From the perspective of animal welfare, this avoids the unnecessary use of animals and reduces excess animal experimentation as much as possible.[10] In such cases, a logical explanation might be required as to why non-clinical safety testing is unnecessary.

**Matters to be considered in peptide vaccine non-clinical safety testing.** As previously mentioned, from the perspective of its mechanism expressing pharmacological activity, there are no

suitable experimental animal species on which non-clinical safety testing of peptide vaccines can be conducted. However, it is still necessary to consider testing in order to confirm the safety of investigational products. Impurities contained in the active ingredient or any other unintentional contamination may present safety issues when a test preparation is administered to humans. Negligible risk-based reference values have been set with respect to drug substance impurities and are listed in the guidelines; however, the possibility of unknown compounds not defined by guidelines or the unintentional contamination of compounds cannot be eliminated (ICH guideline Q3A "Impurities in New Drug Substances"[11] and ICH guideline Q3B "Impurities in New Drug Products"[12]). Therefore, chemical analysis of the peptide drug substance and animal studies to confirm any effect of exposure are useful in determining the presence or absence of adverse effects from impurities and contaminants. Finally, additional tests in experimental animal species to evaluate local irritation effects, route of administration and dosage form should also be devised when feasible.

## The Concept of Quality Assurance in the Research and Development of Peptide Vaccines for the Treatment of Cancer

This guidance illustrates the concept of quality assurance in the research and development of cancer vaccines composed of chemically synthesized peptides as their active ingredient. Quality assurance also refers to the appropriateness of the drug substance or drug product for its intended use. This guidance assumes the drug substance to be peptides and the drug product to be an injectable solution composed of peptides to which adjuvants have been added (including any adjustments made at the time of administration). Furthermore, this guidance summarizes the minimum important points with respect to the quality of peptide vaccines during clinical studies; whether further examination is required will depend on the nature of each peptide vaccine, particularly in cases where the clinical study is aimed at obtaining regulatory approval.

**Requirements of the laws and regulations pertaining to the quality of the test substance for clinical studies.** "Investigational drugs manufactured in a plant with appropriate methods of manufacturing control and quality control as well as the structural equipment necessary to ensure the quality of said investigational drug" is the standard adopted with respect to quality assurance of test substances to be used in clinical trials (Article 17 and 26-3 of the Ministerial Ordinance on Good Clinical Practice for Drugs[13]). Compliance with investigational drug Good Manufacturing Practices (GMP[14]) is required. However, there is no mention of test substances used in clinical studies other than clinical trials in the Ethical Guidance for Clinical Studies[15] and, as such, the quality of such test substances is left up to the researchers.

**The need for quality assurance during research and development.** The use of a drug substance or product manufactured with a certain quality is essential in clinical studies to ensure the reliability and reproducibility of the test results and to protect the safety of the subjects. Because of the chemical and biological nature of peptide vaccines, general non-clinical safety testing does not necessarily provide information that is useful with respect to human administration and some information can be obtained only after administering the test substance to humans. For this reason, the necessity of peptide vaccine non-clinical safety testing is debatable. Even in cases where non-clinical safety testing of the peptide (the active ingredient of the peptide vaccine) is deemed unnecessary (refer to the section on non-clinical safety testing), it is still necessary to ensure the safety of impurities in accordance with the amount and type of impurities contained in the drug substance or product (refer to the section on drug substance specifications and purity testing).

Continued quality control of the peptide vaccine from the initial stages of research is a prerequisite to guarantee the quality and the results of both non-clinical and clinical studies.

**The concept of quality assurance during research and development.** Quality assurance of drugs is accomplished through a combination of various methods, including thorough characteristic analysis of the drug, setting appropriate standards and test methods based on these characteristics, and GMP-based quality control assessments. Quality assurance during research and development is linked with development progress and by necessity the extent of quality assurance required will change depending on the methods used, making a uniform definition difficult. Accordingly, quality assurance should be carried out in a flexible phased manner, in line with development while still taking risk into account. This guidance specifically addresses the setting of appropriate specifications and the concept of GMP-based quality control assessments.

**The concept of peptide vaccine specification setting.** Specifications are a list composed of the test method, a description of analysis used in the test and appropriate acceptance criteria (limits, range and other criteria) for testing to be carried out in a prescribed manner. Specifications are a manner of controlling the drug substance or product to guarantee the quality and consistency of the test substance and are an important element of quality assurance. Each item included in the specifications is intended to ensure the proper quality of the drug substance or product and any characteristics of the test substance required to ensure safety and efficacy should be set. If these characteristics change during storage, this change should be examined and appropriate specifications or storage conditions set. The Guidance for stability testing[16] serve as a reference for test conditions when conducting storage-related tests.

*Drug substance specifications.* The following specifications (both test methods and criteria) can be applied to the quality assurance of almost all peptide vaccine drug substances during research and development:

1 *Description.* A qualitative statement about the shape and color is necessary (for example, "white to pale yellow solid").
2 *Identification testing.* The identification tests should be specific for the drug substance. Specificity may be guaranteed through the combination of two or more methods.
3 *Assay (content).* It is necessary to set a specific analysis method whereby there is no interference from impurities from degraded products that may appear during storage.
4 *Purity testing.* Purity testing is a test method for identifying organic and inorganic impurities and any residual solvent. Knowing the impurity profile of a test substance also assists in determining the necessity of any safety testing.

Organic impurities are those that occur during the manufacturing process and storage and may be substances with an unknown structure. Inorganic impurities are usually substances with a known structure resulting from the manufacturing process, such as a reagent. Solvents used in the manufacturing process are organic or inorganic liquids and their toxicity is usually known.

Structure determination of individual impurities and decisions on the necessity of safety testing should be carried out

based on ICH-Q3A (R2): Impurities in new drug substances.[11] In cases where subjects will intake 2 g or less of the drug substance per day, the threshold at which impurity structure determination is required is considered to be the lower of 0.10% or 1.0 mg daily intake; the threshold at which safety confirmation is required is considered to be the lower of 0.15% or 1.0 mg daily intake. The specifications with respect to residual solvent should be set with reference to ICH-Q3C (R3): Impurities: guideline for residual solvents.[17]

*Preparation specifications.* Specifications for description, identification testing, assay (content) and purity testing can be applied to the quality assurance of almost all peptide vaccine products during research and development. The purity testing of drug products should control for both organic impurities produced by the decomposition of the drug substance and for impurities produced in the manufacturing process of the drug product. Impurities resulting from the manufacturing process of the drug substance are usually governed by drug substance specifications and, as such, do not need to be dealt with in drug product specifications. Decisions on the necessity of safety testing and structure determination of drug product impurities should be carried out based on ICH-Q3B (R2): Impurities in new drug products.[12]

As peptide vaccines are injectable solutions, it is also necessary to set test methods and criteria to evaluate sterility before human administration. Sterility can be evaluated through management of the sterilization process and by testing the sterility of the final product. In the event the drug product requires reconstitution at the time of administration, the method of reconstitution must be examined and confirmation must be made that the final product retains the necessary characteristics.

Any specifications necessary for either characteristics of the drug substance or product (such as moisture content) in addition to sections Drug substance specifications and Preparation specifications above can be set with reference to ICH-Q6A: Test procedures and acceptance criteria for new drug substances and new drug products.[18]

*Adjuvant specifications.* Peptide vaccines are usually mixed with an adjuvant at the time of administration; however, adjuvant specifications should be set independently from the specifications for the target compound. Specifications for description, identification testing, assay (content) and purity testing can be applied to the quality assurance of adjuvants as they are to drug substances.

**The concept of GMP-based manufacturing control and quality control.** The purpose of GMP is to create a mechanism to minimize human error, to prevent contamination and degradation of quality and to maintain quality. In order to implement this objective of GMP, manufacturing control and quality control must be carried out as a series of operations. These operations include the creation of instructions for the manufacturing method and testing method, manufacture and testing according to the instructions, and the creation and storage of records. To ensure the safety of subjects and the reliability of clinical studies, all records related to the manufacturing control and quality control of the test substance must be stored in a manner that facilitates checking at a later date. Investigational drug GMP[12] and its Q&A[19] may be referred to in the implementation of GMP-based control of the investigational drug.

## Clinical Studies

**The concept of early exploratory studies and late-stage confirmatory studies.** The main purpose of early exploratory clinical studies on cancer peptide vaccines is to clarify the recommended dose, the recommended dosing schedule, the presence or absence of biological activity and the safety profile. In late-stage confirmatory studies, the purpose of peptide vaccine clinical trials is also to clarify the vaccine's efficacy and safety in a given population.

The following clinical points should be considered in connection with early exploratory studies and late-stage confirmatory studies:

*Early or advanced-stage cancer.* Many early stage clinical studies on conventional cytotoxic anticancer drugs with the purpose of determining the optimal dose, dosing schedule and maximum tolerated dose (MTD) are performed on subjects with various forms of advanced stage cancer. Because the disease progresses relatively quickly in such advanced-stage subjects, the activity of the target drugs must be observed and evaluated in a short period of time in these early stage exploratory studies. Subsequent late-stage confirmatory studies are performed as large-scale, randomized, controlled studies on subjects with a single type of cancer to determine clinical efficacy and safety. If clinical efficacy and safety are observed in studies on advanced-stage cancer patients, clinical development progresses targeting earlier stage patients and the implementation of clinical studies on adjuvant therapy is also possible.

However, if clinical studies on cancer peptide vaccines target advanced-stage subjects similar to clinical studies on conventional cytotoxic anticancer drugs, there may not be sufficient time for immune response-mediated antitumor activity to appear due to the relatively short period from the commencement of drug administration to disease progression. In addition, advanced-stage cancer subjects often undergo multiple treatments, which can damage their immune system and possibly weaken the response of the cancer peptide vaccine. Evaluating cancer peptide vaccines in earlier-stage subjects ensures enough time for the vaccine to induce an immune response and manifest effects; therefore, earlier-stage subjects are considered more suitable for the study of cancer peptide vaccines than late-stage subjects. The disadvantage of studies on earlier-stage subjects is that it generally takes a long time for a conclusion to be reached. Therefore, the pros and cons of the stage of the subjects (early stage or advanced stage) must be considered when conducting clinical studies of cancer peptide vaccines.

If a standard treatment exists, it is necessary to determine the optimal timing of cancer peptide vaccine introduction: prior to, during or after the completion of the standard treatment and, in the case of treatment during the same period, as monotherapy or combination therapy. It is also necessary to ensure the safety and biological activity of any combined treatment regimen and provide for appropriate evaluation.

*Target cancer (limited to a single type of cancer or multiple types of cancer?).* Phase I clinical studies of cytotoxic anticancer drugs typically targeting subjects with various types of cancer at various stages. While it is possible that the investigational drug will exhibit a different reaction in different subject populations, this is not usually a major barrier to determining the main objectives of phase I clinical studies, which are to determine the MTD and safety profile of the investigational drug. If the toxicity of the cytotoxic anticancer drug is proven to be within the allowable range in the phase I clinical study, a phase II study will be subsequently carried out on subjects with specific types of cancer.

However, in studies targeting patients with differing cancers of differing stages and differing prior treatment, this diversity

may significantly affect the cancer peptide vaccine-induced reaction. When targeting a variety of subject populations in an early exploratory study of cancer peptide vaccines, there is a high possibility that the safety and efficacy results will vary more widely than the respective results obtained in cytotoxic anticancer drug testing, which renders interpretation of the results difficult. Therefore, the diversity of the subject population should be considered when selecting the subject population for cancer peptide vaccine clinical studies.

*Human leucocyte antigen.* It is considered reasonable to measure subjects' Human leucocyte antigen (HLA) considering the molecular immunological background in which cancer peptide vaccines have been developed. As a general rule, it is common to design a study that examines subjects possessing the HLA that matches with the relevant peptide. However, the development of peptide vaccines that include the possibility of non-matching HLA as a next-generation vaccine has also commenced. Therefore, researchers are required to specify in their study design whether to measure HLA or, alternatively, whether to administer the peptide vaccine to subjects with non-matching HLA and to both specify the rationale for their decision in the study protocol and explain the possible advantages and disadvantages to the subjects.

*Antigen expression.* As a rule, expression of the antigen targeted by the cancer peptide vaccine in cancer tissues should be confirmed prior to the commencement of the study and its relationship with efficacy and safety data should be analyzed in detail.

*Multiple antigen peptide vaccines.* Cases where cancer peptide vaccine preparations contain multiple tumor-associated antigens are envisioned. In such cases, the vaccine is expected to induce multiple tumor-specific immune responses and respond to tumor heterogeneity. Generally, it is not considered necessary to evaluate the safety and activity of each component of peptide vaccine preparations containing multiple tumor-associated antigens; however, a case-by-case examination will be required.

**Early exploratory studies.** The main purpose of cancer peptide vaccine early exploratory studies is to clarify the safety profile of the preparation, set the recommended dose and the recommended dosing schedule, clarify potential biological activity and present scientific data to serve as the basis for future drug development.

*Determination of safety—the initial dose and dosing schedule.* In early exploratory studies, it is important to determine the safety of the drug and optimize the dosing schedule. To do this, the initial dose and dose escalation, followed by the recommended dose and recommended dosing schedule must all be attained. These matters are generally determined based on the data obtained via *in vitro* and *in vivo* non-clinical studies. However, as mentioned in the non-clinical safety testing section, useful data concerning the pharmacological activity and safety of the peptide vaccine preparation is unlikely to be obtained in animal studies and may only be obtained after human administration. In contrast, multiple cancer peptide vaccine clinical studies have been carried out on humans as early exploratory studies as translational researches (TR); at the present point in time, no significant toxicity has been reported. Researchers should keep this in mind and consider the need for further safety testing in humans. For clinical studies conducted with the purpose of applying for regulatory approval, even studies based on existing TR analysis, it is necessary to plan early exploratory studies to reconfirm safety in a minimum number of subjects. While implementation using a conventional "3 + 3 design" as described below is possible, a cohort of subjects can be added if necessary. If safety is confirmed, an early exploratory study for the purpose of analyzing the recommended dose, recommended dosing schedule and survival rates should subsequently be planned.

*Dose escalation testing.* So far, in the development of cancer treatment, a "3 + 3 design" has been used as the standard approach with respect to the dose escalation schedule. Once three subjects are registered, testing begins. If dose limiting toxicity (DLT) is not observed in any of the subjects, three additional subjects are registered and given a higher dose and the test continues. If DLT is observed in any one of three subjects, three new subjects are registered and administered with the same dose. If DLT is observed in two or more out of the six subjects administered with this dose, the maximum tolerated dose (MTD) is deemed to have been exceeded and no higher doses will be administered.

The "3 + 3 design" is used in many cancer peptide vaccine clinical studies; however, it is reportedly difficult to identify the MTD if the expression of dose-dependent toxicity is not observed. A possible recommended dose may be prescribed with consideration given to constraints in cancer peptide vaccine preparation, procedural or technical problems in administration or anatomical issues with respect to the administration site.

Accordingly, consideration of a study design other than the standard "3 + 3 design" in order to gather useful dose escalation-related information is also recommended in cancer peptide vaccine clinical studies. For example, the possibility of an approach whereby the dosage is increased in the same subject has been suggested.

In contrast, the standard "3 + 3 design" is a sure way to obtain cancer peptide vaccine safety information when administration involves combinations with other drugs, an invasive technique or a site where anatomical consideration of safety is required.

*Continuous administration.* In routine clinical practice for cancer, the current treatment is generally discontinued in the event of disease progression or recurrence. However, as time is required to induce an antigen-specific immune response in the administration of a cancer peptide vaccine, continuous administration of the drug with consideration of the possibility of late-onset effects is desirable. Alternatively, continuous administration of a cancer peptide vaccine even after disease progression or recurrence could also result in drawbacks: the subject losing the opportunity to undergo other treatments, an increase in adverse events or mortality during the treatment period, or deterioration in the quality of the clinical study. Accordingly, it is necessary to fully consider the criteria for continuation and discontinuation of the vaccine and formulate a study plan when conducting clinical studies of cancer peptide vaccines.

*Early exploratory studies: single-arm studies and randomized controlled studies.* In cancer peptide vaccine early exploratory studies, similar to clinical studies of typical anticancer drugs, the design of a study must be able to: (i) obtain data that demonstrates the cancer peptide vaccine proof of concept; (ii) validate the vaccine's relationship with the standard therapy (positioning); and (iii) clarify the recommended dose and recommended dosing schedule.

In the development of typical cancer drug treatments, the primary objective of phase II clinical studies is to demonstrate the cytoreductive effect. This is because the cytoreductive effect is considered the most appropriate surrogate for the extension of a

vital prognosis. However, an extended vital prognosis can be obtained with cancer peptide vaccines even in cases where a cytoreductive effect cannot be obtained. Such fact should be considered in the design of early exploratory studies on cancer peptide vaccines. Therefore, in the development of cancer peptide vaccines it is important for the design of clinical studies, even early exploratory studies, to primarily focus on vital prognosis indicators. In cases where it is necessary to design an early exploratory study to analyze the recommended dose and recommended dosing schedule, the primary objective of inducing a cancer antigen-specific immune response – the cancer peptide vaccine proof of concept – is assumed. Ideally, the primary objective is directly specified in the protocol.

When planning early exploratory studies, the advantages and disadvantages of a single-arm study versus a randomized controlled study (Table 1)[20] should be carefully considered. The results obtained from single-arm studies must be compared against historical data, which introduces bias and other confounding variables, such as time. Since the cytoreductive effect of cancer peptide vaccines is limited, overall survival and relapse-free survival/disease-free survival become important effect indicators; however, these indicators may produce even greater variations from the differences in historical data because of evolving subject background, etc. In contrast, while randomized controlled studies are too small in size to statistically verify efficacy, they can provide feasibility information (outcome predictions, protocol adherence and sample size determination), which is useful in the design of full randomized controlled trials.

*Pharmacokinetic and immune response monitoring.* In general, analysis of pharmacokinetics (PK) and pharmacodynamics (PD) is required in early exploratory studies of drug development. This is because the accumulation of scientific data concerning blood concentration, tissue distribution, metabolism and excretion of a drug is considered to contribute to the understanding of the drug's efficacy. However, a cancer peptide vaccine administered subcutaneously is intended to exert an immune system-mediated effect through lymph flow and considering this mode of action it is difficult to find any meaning in measuring the concentration of the drug in the blood. In addition, because PK analysis itself is assumed to be difficult, as peptides are rapidly degraded *in vivo* by dipeptidases, etc. (refer to section Pharmacokinetic properties of peptides themselves), it is considered unlikely for useful new data to be obtained by measuring the concentration of the drug in the blood in early exploratory studies. Researchers should bear this in mind and, after examining the data obtained in non-clinical studies, scientifically and logically examine the need for pharmacokinetic analysis[21] in human studies.

It is possible to monitor the immune response expected to be induced by the cancer peptide vaccine over time. As cancer peptide vaccines are believed to cause antitumor activity by inducing a cancer antigen-specific immune response as their mechanism of action, monitoring the immune response is extremely important in PD analysis for the following reasons:

1 The dose and schedule are optimized and a determination made as to whether the cancer peptide vaccine induces its intended immune response in early exploratory studies. These results form the basis for further development of the cancer peptide vaccine and planning of future confirmatory trials.
2 The relationship between indicators of clinical efficacy and the type and strength of immune response are important in confirmatory studies and useful in analysis.

Multiple monitoring methods are required to identify an important immune response. An assay method to measure the most important and relevant immune response with respect to antitumor effect must be developed and validated. Where possible, it is recommended to use at least two immunological assay methods in order to monitor the cancer antigen-specific immune response envisioned from the research hypothesis. Methods such as cancer peptide vaccine delayed type-hypersensitivity reaction testing, peptide-specific cytotoxic testing, Interferon-γ Enzyme-Linked Immunospot peptide-specific assay and peptide-specific multimeric flow cytometry are recommended. The reproducibility of results must be validated for each measurement. The assay conditions, positive and negative controls, positive and negative cut-off values and the statistical procedure used to analyze the results should be specified in the clinical study protocol prior to the commencement of a clinical study.

*Concurrent cancer peptide vaccine and target antigen test development.* In the case of drugs from which a specific antigen response is expected as the mechanism of action, it is important to concurrently develop a method of measuring expression of the target antigen in the cancer tissue of individual subjects, etc. and consider the possibility of using this data in immune reaction monitoring and subject selection.

If seeking regulatory approval and a new measurement method will be developed in a clinical study, the applicant must work with the regulatory agency to propose a plan for

**Table 1. Differences between single-arm exploratory studies and randomized controlled exploratory studies**

| | Single-arm exploratory studies | Randomized controlled exploratory studies |
|---|---|---|
| Advantages | More information about adverse events related to the new treatment can be obtained | Control group information can be obtained at the same time |
| | There is a chance to implement the new treatment to all participating subjects | The randomization increases reliability with respect to the response rate end-point |
| | Simple end-points can be set and results obtained quickly | The randomization also increases reliably with respect to overall survival and progression-free survival |
| Disadvantages | A historical control is required | Statistical analysis is difficult with the low number of cases in early exploratory studies |
| | The response rate does not necessarily reflect the survival time | Subjects in the terminal stages of cancer may not accept randomization |
| | It is difficult to obtain reliable results with respect to overall survival and progression-free survival | Not as much information about adverse events related to the new treatment can be obtained |
| | | Implementation of confirmatory studies may be difficult if satisfactory results are obtained |

the concurrent development of the assay method together with the cancer peptide vaccine. This plan must be done prior to submitting the application to the agency. At the presubmission conference, the regulatory authorities will provide scientific and institutional advice with respect to the development of *in vitro* diagnostics and medical equipment.

**Verification studies.** As peptide vaccines are included as drug treatments for cancer, the implementation of confirmatory studies in line with the concept of cancer drug treatment is required. Importantly, it is important to design a clinical study with an understanding of the characteristics of the cancer peptide vaccine.

Verification studies are carried out in order to establish a standard therapy and to verify the efficacy of the new treatment based on phase I and II early exploratory clinical studies. It is necessary to set an appropriate objective for the treatment in line with the subject. The purpose of many cancer drug treatments is to prolong life and mitigate symptoms.

Overall survival (OS), progression-free survival (PFS) and disease-free survival (DFS) are used as the primary end-points in validation testing. The primary end-points will differ according to the disease and pathological condition (for example, postcurative resection or unresectable, etc.). They will also differ according to whether the peptide vaccine is administered as monotherapy or in combination with antineoplastic agents. For instance, it is extremely difficult to judge progression if the peptide vaccine is administered as monotherapy and, in such cases, it is more appropriate to adopt OS or DFS as end-points. However, if the peptide vaccine is administered in combination with antineoplastic agents, it is possible to adopt PFS in addition to OS and DFS.

As objective evaluation of the symptom mitigation effect and quality of life (QOL) is difficult, and there is no established method for measuring these indicators. The end-point of quality-adjusted life year – life-years weighted by QOL – has been introduced. Evaluation of cost-effectiveness taking into account the cost of medical care must also be considered.

Safety evaluation is also an important purpose of confirmatory studies and is carried out through comparison with a control treatment. As confirmatory tests generally take the form of large-scale randomized studies and implementation of a high-quality study is required, it is necessary to prepare a sufficient study implementation system including a data center that monitors the test and manages data centrally.

*Study design.* The objective of the study design is to verify the non-inferiority or superiority of the developed treatment based on its efficacy and safety. Because cancer peptide vaccines, in principle, target difficult-to-cure diseases with poor prognosis, a study of superiority is considered desirable. Nevertheless, a study of non-inferiority is acceptable in the event there are safety issues with the current standard treatment. If the non-inferiority hypothesis of the test treatment is validated and not rejected (non-inferiority is demonstrated), it is possible to design a subsequent study to verify superiority or concurrent non-inferiority and superiority. In this subsequent study, superiority is concluded only if it can be demonstrated. If superiority cannot be proven, at least non-inferiority can be concluded.

Appropriate controls must be put in place to avoid bias that affects analysis of the test results and activities. As a rule, the control group in a confirmatory study is administered with the standard treatment at the time. A comparison is made with untreated subjects for diseases or pathological conditions if no standard treatment is available. In these cases, a placebo-controlled trial is desirable. Studies involving a placebo must be carefully considered and planned, because treatment with a placebo alone brings about a risk of serious adverse events such as death or irreversible morbidity through the suspension of treatment.

Necessary information, such as stratification factors, is determined and the number of subjects determined from the setting of non-inferiority or superiority, significance level, detection power and the difference to be detected.

*End-points.* End-points differ with respect to unresectable advanced cancer subjects (including recurrence) and post-total lesion excision subjects (adjuvant therapy).

1 Unresectable advanced cancer. As the main purpose of the treatment is to prolong life and mitigate symptoms, the main primary end-points of OS and PFS are used. It is also possible to adopt PFS under some circumstances and the setting of these primary end-points is determined by the disease and treatment (see above).
2 Postoperative adjuvant therapy. As many excisions are performed for the purpose of healing, the main purpose of adjuvant therapy is to improve the healing rate. Accordingly, the primary end-points of OS and DFS are used.

*Safety evaluation.* Even if the conclusion of safety was obtained in early exploratory studies, the verification study must also carefully evaluate safety through the monitoring of appropriate subjects.

In verification studies, safety is evaluated by comparison with the control group and is generally set as a secondary end-point. Arrangements must also be made in the event of unexpected adverse events and serious adverse events with respect to the reporting requirements as well as evaluations such as the relationship between the treatment and the appropriate response.

Safety is evaluated in accordance with criteria such as the Common Terminology Criteria for Adverse Events (CTCAE), which is based on adverse events, blood biochemical testing and physiological test results. Adverse events that are not listed in the CTCAE are generally evaluated by severity as mild, moderate, severe or life-threatening.

As evaluation of safety and timely feedback as to the appropriateness of study continuity is required during the study, it is necessary to establish an independent evaluation committee.

*Efficacy evaluation and statistical analysis.* Efficacy is evaluated mainly by the primary end-points OS or DFS. In this analysis, the survival rate is generally calculated using the Kaplan–Meier method and a comparison between treatment groups is performed using a log rank test or Wilcoxon test. The log rank test has a high detection power in cases where the hazard ratio of the test group compared with the control group is constant during the observation period. Meanwhile, the Wilcoxon test has a higher detection power than the standard log-rank test in cases where the test treatment induces a location shift for the density function of event occurrence. Of note, late-onset effects are assumed to be due to the antigen-specific immune response-mediated pharmacological efficacy of cancer peptide vaccines. Bearing this in mind, the need for analysis using new statistical methods, such as a method that weights the late period of observation as proposed in the Harrington–Fleming method,[22] is also envisioned. The statistical analysis method must be specified in the protocol along with the significance criteria.

While PFS and response rate are sometimes set as secondary efficacy end-points, it is important that the secondary efficacy end-points are set according to the characteristics of the peptide vaccine. Reduction in the lesion size and progression are

other important points for objective evaluation and, as a rule, are evaluated by an independent evaluation committee based on Response Evaluation Criteria in Solid Tumors, etc.

## Conclusion

The active promotion of clinical studies is essential in the development of cancer peptide vaccines and the creation of appropriate clinical study guidance is necessary for the active promotion of these clinical studies. This Guidance for peptide vaccines for the treatment of cancer has been published by the Japanese Society for Biological Therapy. Needless to say, periodic review of this guidance may be necessitated with the advancement of cancer vaccine research in the future. The Japanese Society for Biological Therapy welcomes comments from regulators and business people as well as researchers in this area.

## Acknowledgments

This work was supported by the Japanese Society for Biological Therapy.

## Disclosure Statement

The authors have no conflict of interest.

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PLOS ONE

# Identification of an HLA-A2-Restricted Epitope Peptide Derived from Hypoxia-Inducible Protein 2 (HIG2)

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## Abstract

We herein report the identification of an HLA-A2 supertype-restricted epitope peptide derived from hypoxia-inducible protein 2 (HIG2), which is known to be a diagnostic marker and a potential therapeutic target for renal cell carcinoma. Among several candidate peptides predicted by the HLA-binding prediction algorithm, HIG2-9-4 peptide (VLNLYLLGV) was able to effectively induce peptide-specific cytotoxic T lymphocytes (CTLs). The established HIG2-9-4 peptide-specific CTL clone produced interferon-γ (IFN-γ) in response to HIG2-9-4 peptide-pulsed HLA-A*02:01-positive cells, as well as to cells in which HLA-A*02:01 and HIG2 were exogenously introduced. Moreover, the HIG2-9-4 peptide-specific CTL clone exerted cytotoxic activity against HIG2-expressing HLA-A*02:01-positive renal cancer cells, thus suggesting that the HIG2-9-4 peptide is naturally presented on HLA-A*02:01 of HIG-2-expressing cancer cells and is recognized by CTLs. Furthermore, we found that the HIG2-9-4 peptide could also induce CTLs under HLA-A*02:06 restriction. Taken together, these findings indicate that the HIG2-9-4 peptide is a novel HLA-A2 supertype-restricted epitope peptide that could be useful for peptide-based immunotherapy against cancer cells with HIG2 expression.

**Citation:** Yoshimura S, Tsunoda T, Osawa R, Harada M, Watanabe T, et al. (2014) Identification of an HLA-A2-Restricted Epitope Peptide Derived from Hypoxia-Inducible Protein 2 (HIG2). PLoS ONE 9(1): e85267. doi:10.1371/journal.pone.0085267

**Editor:** Rachel Louise Allen, University of London, St George's, United Kingdom

**Received** September 20, 2013; **Accepted** November 25, 2013; **Published** January 8, 2014



**Funding:** *The authors have no support or funding to report.*

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Renal cell carcinoma (RCC) comprises approximately 2–3% of all human malignancies [1]. Although patients with localized RCC can be curable by radical nephrectomy, approximately 30% of patients are observed to have metastasis at the time of diagnosis, and the median survival is only 1.5 years. Furthermore, 30% of patients experience a relapse after initial surgery, and no adjuvant treatment has yet been established [2–4]. Several molecular targeting agents, including the recently approved VEGFR tyrosine kinase inhibitor [5], were developed as novel therapeutics for RCC, but the majority of patients eventually develop treatment-resistant disease [6–13]. It is notable that RCC is one of the most immune responsive cancers. IL-2 based immunotherapy is currently the only curative treatment for metastatic RCC, but it is poorly tolerated, with significant side effects, and the efficacy has been limited to a 20% response rate, including a 5–10% complete response rate [14–17]. This limited success poses further challenges to improve the efficacy of immunotherapies for RCC. While therapeutic vaccines that induce immunity in response to tumor antigens have been under investigation for decades, the number of antigens identified in RCC and the efficacy in clinical trials have been limited [18–21].

Hypoxia-inducible protein 2 (*HIG2*) was first annotated as a novel gene induced by hypoxia and glucose deprivation [22]. A recent functional analysis revealed that HIG2 is a novel lipid droplet protein that stimulates intracellular lipid accumulation [23]. We reported HIG2 upregulation in RCC, and suggested its usefulness as a diagnostic biomarker for RCC [24]. Our findings also implied that HIG2 might be a good molecular target for the development of novel cancer treatment, because its expression was hardly detectable in normal organs except for the fetal kidney. Importantly, significant growth suppression of RCC cells occurred when endogenous *HIG2* was suppressed by *HIG2*-specific RNAi, suggesting that HIG2 has an essential role in the proliferation of RCC cells. An additional study revealed that HIG2 expression was found in 86% of human RCC tissue samples (80/93) and also correlated with the clinicopathological characteristics and survival of RCC patients [25].

In the present study, we focused on HIG2 as a novel tumor antigen, which induces antigen-specific cytotoxic T lymphocytes (CTLs) against RCC cells. We investigated the HIG2-derived epitope peptide restricted to HLA-A*02:01, the most common HLA class I type in Caucasians and the second most common type in the Japanese population [26,27], and demonstrate that this epitope peptide can also be presented by another HLA-A2 supertype allele. Thus, this epitope peptide would be applicable for peptide-based immunotherapies for RCC patients with HLA-A2.

### Ethics statement

The study protocol was approved by the Institutional Review Board of OncoTherapy Science, Inc. and written informed consent was obtained from all subjects, in accordance with the guidelines of the Ethical Committee on Human Research of Wakayama Medical University, School of Medicine, OncoTherapy Science, Inc., The University of Tokyo, Juntendo University School of Medicine, The University of Tokushima and University of Chicago.

## Materials and Methods

### Peptides

HIG2-derived 9-mer and 10-mer peptides that have high binding affinity (binding score >10) to HLA-A:*02:01 were predicted by the binding prediction software "BIMAS" (http://www-bimas.cit.nih.gov/molbio/hla_bind), and the homologous sequences were examined by the homology search program "BLAST" (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Selected high affinity peptides and the HLA-A*02:01-restricted HIV-derived epitope peptide (ILKEPVHGV) [28] were synthesized by Sigma (Ishikari, Japan). The purity (>90%) and the sequences of the peptides were confirmed by analytical HPLC and a mass spectrometry analysis, respectively. Peptides were dissolved in dimethylsulfoxide at 20 mg/ml and stored at −80°C.

### Cell lines

T2 (HLA-A*02:01, lymphoblast), Jiyoye (HLA-A32, Burkitt's lymphoma), EB-3 (HLA-A3/Aw32, Burkitt's lymphoma), *Cercopithecus aethiops*-derived COS7 and A498 (HLA-A*02:01, kidney carcinoma) cells were purchased from the American Type Culture Collection (Rockville, MD). PSCCA0922 (HLA-A*02:06/A*31:01, a B cell line) was provided by the Health Science Research Resources Bank (Osaka, Japan). Caki-1 (HLA-A*24:02/A*23:01, renal clear cell carcinoma) cells were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University. The HIG2 expression in A498 and Caki-1 cells was confirmed by a Western blotting analysis [24]. T2, Jiyoye, EB-3 and PSCCA0922 cells were maintained in RPMI1640 (Invitrogen, Carlsbad, CA), A498 and Caki-1 cells were maintained in EMEM (Invitrogen) and COS7 cells were maintained in DMEM (Invitrogen). Each medium was supplemented with 10% fetal bovine serum (GEMINI Bio-Products, West Sacramento, CA) and 1% antibiotic solution (Sigma-Aldrich, ST. Louis, MO).

### Gene transfection

The plasmid encoding *HLA-A*02:01* was a generous gift from Dr. Kawakami (Keio University, Tokyo Japan). cDNA fragments encoding *HLA-A*02:06* or *HIG2* (GenBank Accession Number NM_013332) were cloned into the pcDNA3.1/myc-His vector (Invitrogen). Plasmid DNAs containing *HLA-A*02:01*, *HLA-A*02:06* and/or *HIG2* were transfected into COS7 cells using Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. COS7 cells were incubated with the transfection mixture at 37°C overnight prior to use as stimulator cells. The introduction of the targeted proteins was confirmed by a Western blotting analysis.

### *In vitro* CTL induction

$CD8^+$ T cells and monocyte-derived dendritic cells (DCs) were prepared from peripheral blood of healthy volunteers (either HLA-A*02:01 or HLA-A*02:06 positive) with written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and $CD8^+$ T cells were harvested by positive selection with a Dynal CD8 Positive Isolation Kit (Invitrogen). Monocytes were enriched from the $CD8^-$ cell population by adherence to a tissue culture dish (Becton Dickinson, Franklin Lakes, NJ) and were cultured in AIM-V (Invitrogen) containing 2% heat-inactivated autologous serum (AS), 1,000 U/ml of GM-CSF (R&D Systems, Minneapolis, MN) and 1,000 U/ml of interleukin (IL)-4 (R&D Systems) on day 1. On day 4, 0.1 KE/ml of OK-432 (Chugai Pharmaceutical Co., Tokyo, Japan) was added in the culture to induce the maturation of DCs. On day 7, DCs were pulsed with 20 μg/ml of the respective synthesized peptides in the presence of 3 μg/ml of β2-microglobulin (Sigma-Aldrich, ST. Louis, MO) in AIM-V at 37°C for 4 h [29]. These peptide-pulsed DCs were then incubated with 30 μg/ml of mitomycin C (MMC) (Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) at 37°C for 30 min. Following washing out the residual peptide and MMC, DCs were cultured with autologous $CD8^+$ T cells on 48 well plates (Corning, Inc., Corning, NY) (each well contained $1.5\times10^4$ peptide-pulsed DCs, $3\times10^5$ $CD8^+$ T cells and 10 ng/ml of IL-7 (R&D Systems) in 0.5 ml of AIM-V/2% AS). Two days later, these cultures were supplemented with IL-2 (CHIRON, Emeryville, CA) (final concentration: 20 IU/ml). On days 14 and 21, T cells were further re-stimulated with the autologous peptide-pulsed DCs, which were freshly prepared every time. On day 28, the CTL activity against peptide-pulsed T2 or PSCCA0922 cells was examined by an interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assay.

### IFN-γ enzyme-linked immunospot (ELISPOT) assay

The human IFN-γ ELISPOT kit and AEC substrate set (BD Biosciences) were used to analyze the T cell response to the respective peptides. The ELISPOT assay was performed according to the manufacturer's instructions. Briefly, T2 or PSCCA0922 cells were pulsed with 20 μg/ml of the respective peptides at 37°C for 20 h, and the residual peptide that did not bind to cells was washed out to prepare peptide-pulsed cells as the stimulator cells. After removing 500 μl of supernatant from each well of *in vitro* CTL-inducing cultures, 200 μl of cell culture suspensions were harvested from each well and distributed to two new wells (100 μl each) on Multiscreen-IP 96 well plates (Millipore, Bedford, MA). The cells were co-incubated with peptide-pulsed cells ($1\times10^4$ cells/well) at 37°C for 20 h. HIV peptide-pulsed cells were used as a negative control. Spots were captured and analyzed by an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology Ltd, Shaker Heights, OH) and the ImmunoSpot Professional Software package, Version 5.0 (Cellular Technology Ltd).

### CTL expanding culture

The peptide-specific CTLs harvested from ELISPOT-positive wells after *in vitro* CTL induction were expanded by a modified protocol based on the previously described methods [30,31]. A total of $5\times10^4$ CTLs was cultured with $5\times10^6$ MMC-inactivated Jiyoye or EB-3 cells (30 μg/ml at 37°C for 30 min treatment) in 25 ml of AIM-V/5% AS containing 40 ng/ml of anti-CD3 monoclonal antibody (BD Biosciences, San Diego, CA) on day 0. IL-2 was added 24 h later (final concentration: 120 IU/ml), and fresh AIM-V/5% AS containing 30 IU/ml of IL-2 was provided on days 5, 8 and 11. On day 14, CTLs were harvested and the CTL activity was examined by an IFN-γ enzyme-linked immunosorbent assay (ELISA).

### Establishment of CTL clones

CTL clones were established by the limiting dilution method. Briefly, CTLs were diluted to 0.3, 1 or 3 cells per well in 96 well

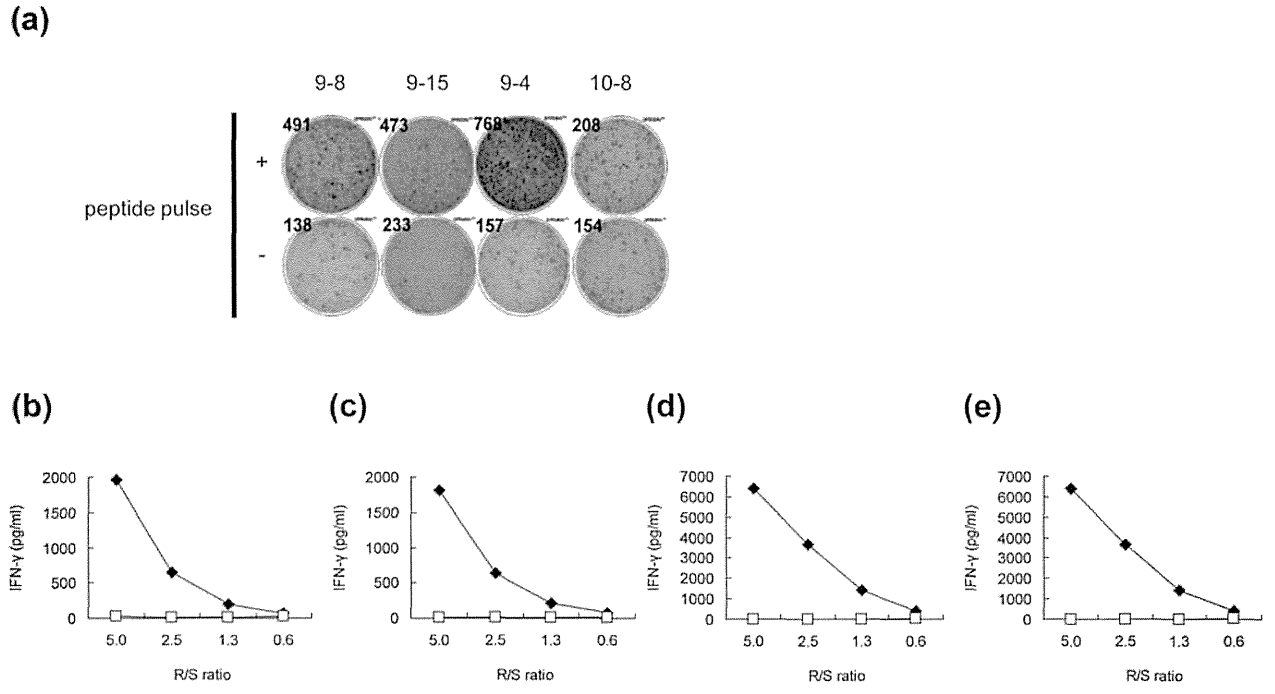


**Figure 1. The IFN-γ production in response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide.** (a) The IFN-γ production from cells induced by the indicated peptide-pulsed DCs was examined by an ELISPOT assay using T2 cells. "+" indicates the wells in which cells were stimulated with T2 cells pulsed with the indicated peptide and "−" indicates the wells in which cells were stimulated with HIV peptide-pulsed T2 cells. The IFN-γ production from cells induced with HIG2-9-8 (b), HIG2-9-15 (c), HIG2-9-4 (d) or HIG2-10-8 (e) peptide stimulation after CTL expanding culture was examined by ELISA. Cells were stimulated with T2 cells pulsed with the corresponding peptide (closed diamonds) or HIV peptide (open squares) at the indicated responder/stimulator ratio (R/S ratio). Similar results were obtained from three independent experiments.
doi:10.1371/journal.pone.0085267.g001

round bottom plates (Corning, Inc.), and were cultured with MMC-treated $1\times10^4$ Jiyoye and EB-3 cells in 125 µl AIM-V containing 5% AB serum and 30 ng/ml of an anti-CD3 monoclonal antibody on day 0. IL-2 was added to each well on day 10 (final concentration: 125 IU/ml). On day 14, an IFN-γ ELISPOT assay was performed to measure the CTL activity of each clone.

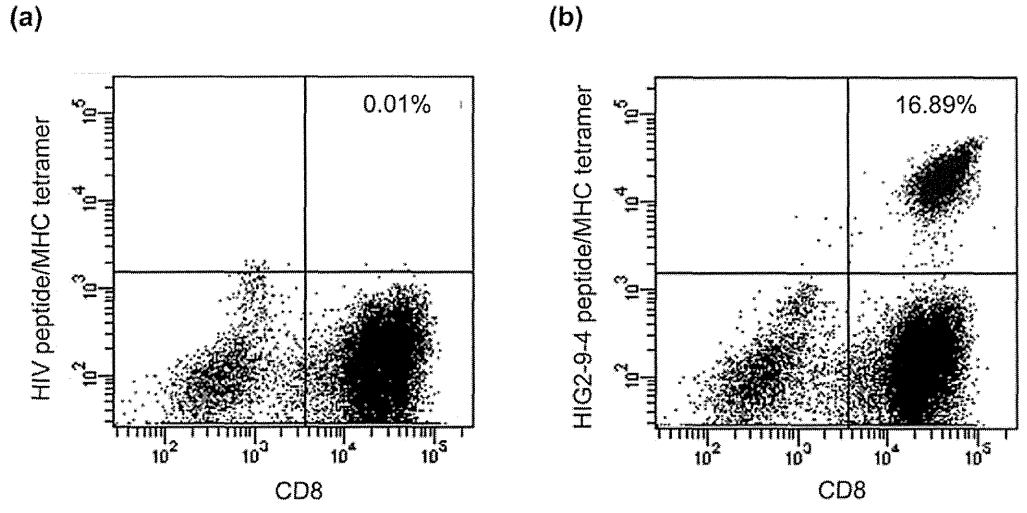


**Figure 2. The expression of a HIG2-9-4 peptide-specific T cell receptor on CD8+ T cells.** The expression of the HIG2-9-4 peptide-specific T cell receptor was examined on $CD3^+CD4^-$ cells following CTL expansion culture of HIG2-9-4 peptide-induced CTLs. (a) A quadrant gate was set based on the staining results with the HIV peptide/HLA-A*02: 01 tetramer. (b) $CD8^+$ T cells expressing the HIG2-9-4 peptide/HLA-A*02: 01-specific T cell receptor were detected. Similar results were obtained from three independent experiments.
doi:10.1371/journal.pone.0085267.g002

**Table 1.** Candidate peptides derived from HIG2 restricted with HLA-A*02:01.

| Peptide name | Amino acid sequence (mer) | Binding Score |
|---|---|---|
| HIG2-9-8 | YLLGVVLTL (9) | 836.253 |
| HIG2-9-13 | VLTLLSIFV (9) | 650.311 |
| HIG2-9-15 | TLLSIFVRV (9) | 488.951 |
| HIG2-9-4 | VLNLYLLGV (9) | 271.948 |
| HIG2-9-9 | LLGVVLTLL (9) | 83.527 |
| HIG2-9-22 | RVMESLEGL (9) | 31.957 |
| HIG2-9-6 | NLYLLGVVL (9) | 28.027 |
| HIG2-10-8 | YLLGVVLTLL (10) | 836.253 |
| HIG2-10-29 | GLLESPSPGT (10) | 113.047 |
| HIG2-10-4 | VLNLYLLGVV (10) | 14.495 |
| HIG2-10-15 | TLLSIFVRVM (10) | 13.174 |
| HIG2-10-18 | SIFVRVMESL (10) | 12.248 |

The binding score was obtained from the BIMAS website (http://www-bimas.cit.nih.gov/molbio/hla_bind).
doi:10.1371/journal.pone.0085267.t001

### IFN-γ enzyme-linked immunosorbent assay (ELISA)

The CTL activity was examined by IFN-γ ELISA. Peptide-pulsed cells ($1\times10^4$ cells/well) or gene-transfected cells ($5\times10^4$ cells/well) were used to stimulate CTLs at several responder/stimulator ratios in 200 μl of AIM-V/5% AS on 96 well round bottom plates (Corning Inc.). After 24 h of incubation, cell-free supernatants were harvested, and the IFN-γ production was examined by an IFN-γ ELISA kit (BD Biosciences) according to the manufacturer's instructions.

### Flow cytometry

The expression of peptide-specific T cell receptors was examined on FACS-Canto II (Becton Dickinson, San Jose, CA) using PE-conjugated peptide/MHC tetramer (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions. Briefly, *in vitro* expanded CTLs were incubated with peptide/MHC tetramer at room temperature for 10 min, and then a FITC-conjugated anti-human CD8 mAb, APC-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb and 7-AAD (BD Biosciences) were added and incubated at 4°C for 20 min. HIV peptide (ILKEPVHGV)/HLA-A*02: 01 tetramer was used as a negative control.

### Cytotoxicity assay

The cytotoxic activity of the induced CTL clones was tested by a 4 h $^{51}Cr$ release assay as described previously [32]. Data are presented as the means ± SD of triplicate samples. Student's t test was used to examine the significance of the data.

## Results

### CTL induction with HLA-A*02:01-binding peptides derived from HIG2

We synthesized twelve 9-mer and 10-mer peptides, corresponding to parts of the HIG2 protein that had been suggested to bind to HLA-A*02:01 by the prediction with the BIMAS program (Table 1). After *in vitro* culture to induce CTLs, IFN-γ production was observed specifically when cells were stimulated with T2 cells that had been pulsed with the HIG2-9-8 peptide (YLLGVVLTL), HIG2-9-4 peptide (VLNLYLLGV), HIG2-9-15 peptide (TLLSIFVRV) or HIG2-10-8 peptide (YLLGVVLTLL) among all of the candidate peptides shown in Table 1 (Fig. S1 showing all 12 wells of one experiment and Fig. 1a showing representative wells). After CTL-expanding culture, cells still produced IFN-γ in response to the respective peptides in a responder/stimulator ratio-dependent manner, and HIG2-9-4 peptide-specific CTLs produced a higher amount of IFN-γ than CTLs stimulated with other peptides (Figs. 1b–e). In the independent experiments using PBMCs from other 2 donors, HIG2-9-4 peptide-specific CTLs produced the highest amount of IFN-γ (data not shown). We confirmed the existence of HIG2-9-4/HLA-A*02:01-specific $CD8^+$ T cells by tetramer staining. A significant population of $CD3^+CD4^-CD8^+$ cells expressed the HIG2-9-4/HLA-A*02:01-specific T cell receptor after the expansion of cells obtained by *in vitro* CTL induction (Fig. 2).

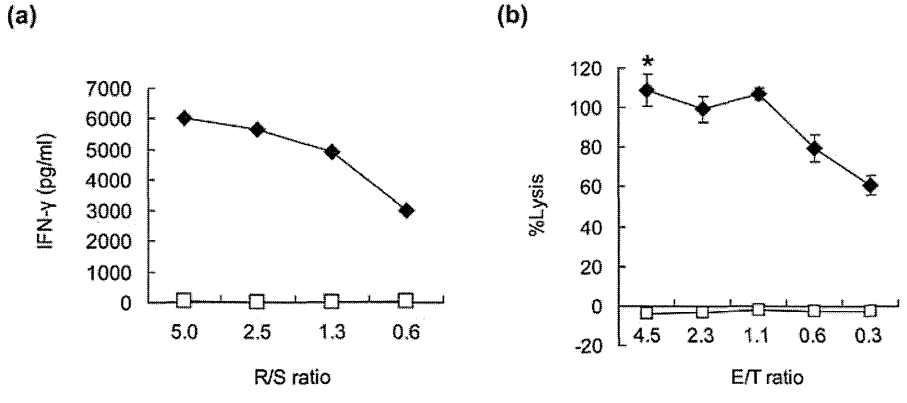


**Figure 3. The IFN-γ production and cytotoxic activity of a HIG2-9-4 peptide-specific CTL clone.** (a) An established CTL clone was stimulated with T2 cells pulsed with the HIG2-9-4 peptide (closed diamonds) or HIV peptide (open squares). The IFN-γ production in the culture supernatant was examined by ELISA. R/S ratio; responder/stimulator ratio. (b) The cytotoxic activity of the HIG2-9-4 peptide-specific CTL clone was examined against peptide-pulsed T2 cells (close diamond) or T2 cells pulsed with the HIV peptide (open square). E/T ratio; effector/target ratio. All experiments were performed in triplicate. The representative results from three independent experiments are shown. *$P<0.001$
doi:10.1371/journal.pone.0085267.g003

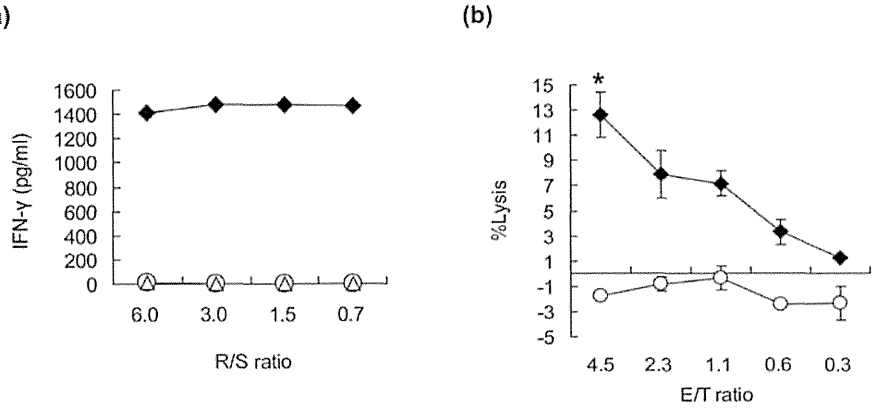


**Figure 4. The recognition of HIG2 and HLA-A*02:01-expressing cells by a HIG2-9-4 peptide-specific CTL clone.** (a) A HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:01 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:01 alone (open triangle), then the IFN-γ production was examined by ELISA. R/S ratio; responder/stimulator ratio. (b) The cytotoxic activity of the HIG2-9-4 peptide-specific CTL clone was examined against HLA-A*02:01-positive HIG2-expressing A498 cells (closed diamond) or HLA-A*02:01-negative HIG2-expressing Caki-1 cells (open circle). E/T ratio; effector/target ratio. All experiments were performed in triplicate. Representative results from three independent experiments are shown. *; $P<0.001$.
doi:10.1371/journal.pone.0085267.g004

## Establishment of HIG2-9-4 peptide-specific CTL clones

We subsequently established HIG2-9-4 peptide-specific CTL clones by the limiting dilution of induced CTLs. The established HIG2-9-4 peptide-specific CTL clone produced a large amount of IFN-γ when it was stimulated with HIG2-9-4 pulsed-T2 cells, while no IFN-γ production was detected when they were stimulated with HIV-peptide-pulsed-T2 cells (Fig. 3a). Furthermore, the HIG2-9-4 peptide-specific CTL clone exerted substantial cytotoxic activity against T2 cells pulsed with the HIG2-9-4 peptide, but not those pulsed with the HIV peptide (Fig. 3b). However, we failed to establish any CTL clones that reacted with HIG2-9-8, HIG2-9-15 or HIG2-10-8 peptides, even after several attempts using multiple donors (data not shown). In addition, we found no homologous sequence to the HIG2-9-4 peptide by a homology search using the BLAST algorithm (data not shown), indicating that the HIG2-9-4 peptide is a unique epitope peptide among the candidate peptides predicted by the BIMAS program that can induce potent and stable CTLs.

## Specific CTL response to HIG2 and HLA-A*02:01-expressing cells

To further verify the recognition of HIG2-expressing cells with HLA-A*02:01 by the HIG2-9-4-specific CTL clone, we prepared COS7 cells in which either or both of two plasmids designed to express the full-length of HIG2 and HLA-A*02:01 were transfected. The HIG2-9-4-specific CTL clone produced IFN-γ when the cells were exposed to the COS7 cells expressing both HIG2 and HLA-A*02:01, while no IFN-γ production was observed when they were exposed to COS7 cells expressing either HIG2 or HLA-A*02:01 (Fig. 4a). Furthermore, the HIG2-9-4 peptide-specific CTL clone demonstrated cytotoxic activity against A498 cells expressing both HLA-A*02:01 and HIG2, while no

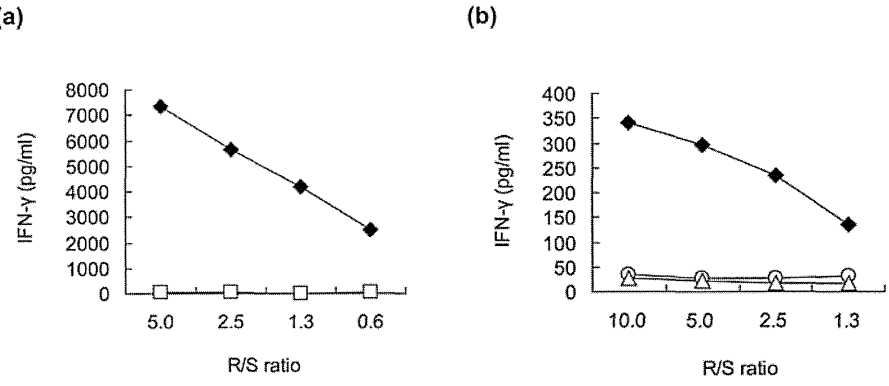


**Figure 5. The HLA-A*02:06-restricted response of a HIG2-9-4 peptide-specific CTL clone.** (a) A HIG2-9-4 peptide-specific CTL clone was induced from HLA-A*02:06-positive PBMCs, and stimulated with HLA-A*02:06-positive PSCCA0922 cells pulsed with the HIG2-9-4 peptide (close diamond) or HIV peptide (open square). (b) The HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:06 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:06 alone (open triangle). The IFN-γ production in the culture supernatant was examined by ELISA. R/S ratio; responder/stimulator ratio. The representative results from three independent experiments are shown.
doi:10.1371/journal.pone.0085267.g005

cytotoxicity was observed against HIG2-expressing Caki-1 cells without HLA-A*02:01 expression (Fig. 4b).

### The HIG2-9-4 peptide cross-reacts with HLA-A*02:06

We additionally evaluated the cross-reactivity of the HIG2-9-4 peptide with HLA-A*02:06, since HLA-A*02:06 differs from HLA-A*02:01 by a single amino acid, and some reports have indicated the presentation of HLA-A*02:01-restricted peptides on HLA-A*02:06 [33,34]. Similar to the HLA-A*02:01 experiments, potent CTL clones were established from the PBMCs of HLA-A*02:06-positive donors by stimulation with the HIG2-9-4 peptide. An established CTL clone showed potent IFN-γ production when it was exposed to HIG2-9-4 peptide-pulsed HLA-A*02:06-positive PSCCA0922 cells (Fig. 5a). Furthermore, this CTL clone recognized COS7 cells that expressed both HIG2 and HLA-A*02:06 and produced IFN-γ, while no IFN-γ production was observed when stimulated with COS7 cells that expressed either HIG2 or HLA-A*02:06 (Fig. 5b). These results suggested that the HIG2-9-4 peptide is cross-reactive with HLA-A*02:06 to induce CTLs that show CTL activity against HLA-A*02:06- and HIG2-expressing cells.

## Discussion

The recent FDA approvals of the cellular immunotherapy, Sipuleucel-T (Provenge), and immunomodulatory antibody, ipilimumab (Yervoy), have provided a proof of concept that the immune system can be used as a new approach to treat cancer [35,36]. Immunization with HLA-restricted epitope peptides derived from tumor antigens is a strategy that has been vigorously pursued to activate the immune system [37-40]. Unfortunately, many of the vaccine trials using epitope peptides failed to demonstrate clinical efficacy due, at least in part, to the potential immune escape mechanisms, which are attributed to the loss of tumor antigen expression by tumor cells [41-43]. Accordingly, the selection of tumor antigens which play a key role in tumor cell proliferation or survival is considered to be important to overcome immune escape. If a targeted tumor antigen is essential for tumor growth, the downregulation of this tumor antigen as a form of immune escape is expected to impair tumor progression.

Correspondingly, in the guidelines from the FDA (Guidance for Industry: Clinical Considerations for Therapeutic Cancer Vaccines), multi-antigen vaccines which contain multiple tumor antigens in order to generate multiple tumor-specific immunological responses were mentioned to effectively hinder escape mechanisms. We therefore consider that the identification of epitope peptides derived from multiple tumor antigens which are involved in tumor progression or survival can contribute to the development of multi-antigen vaccines, and can improve the efficacy of peptide vaccine therapies. We have previously identified epitope peptides derived from various tumor antigens, each of which plays a key role in tumor progression, and some of these peptides have been applied for clinical trials as multi-peptide vaccines [44–46].

In this study, we identified an HLA-A2 supertype-restricted epitope peptide derived from HIG2. HIG2 was upregulated in RCC and hardly detectable in normal organs except for the fetal kidney, and importantly, HIG2 expression was found to be directly associated with the proliferation of RCC cells [24]. Hence, RCC cells are thought to maintain HIG2 expression even under immunoselective pressure, or to otherwise exhibit tumor growth suppression resulting from the loss of HIG2 expression.

IFN-γ-producing stable CTL clones specific to the HIG2-9-4 peptide (VLNLYLLGV) were established from HLA-A2 (either A*02:01 or A*02:06)-positive PBMCs, and these clones responded specifically to COS7 cells that expressed both HIG2 and HLA-A2 (A*02:01 or A*02:06). We also revealed that HIG2-9-4-specific HLA-A*02:01-restricted CTLs exerted cytotoxic activity against RCC cells that were positive for both HIG2 and HLA-A*02:01, but not against negative cells. These results suggested that HLA-A2 (A*02:01 or A*02:06)-restricted HIG2-9-4 peptide-specific CTLs are inducible and stable, and these CTLs substantially respond to HIG2-expressing cells through the endogenous processing of the HIG2-9-4-peptide and the subsequent presentation with the HLA-A2 (A*02:01 or A*02:06) molecule on the cell surface. In addition, HIG2 is an oncofetal antigen, as described above, and no homologous sequence to the HIG2-9-4 peptide was demonstrated by a homology search using the BLAST algorithm. Thus, HIG2-9-4 peptide-specific CTLs should not induce unintended immunological responses to normal cells, such as those associated with autoimmune diseases, even if this novel and unique peptide induces strong immune responses against HIG2-expressing RCC.

HIG2 expression was found in the majority of RCC patients (86%) [25], and additionally, the HLA-A2 supertype is the most common HLA class I type in Caucasians and the second most common type in the Japanese population [26,27]. Therefore, identification of HLA-A2 supertype-restricted epitope peptides derived from HIG2 could be applicable for immunotherapies in a wide variety of RCC patients. As well as finding novel tumor antigens which are widely expressed in cancer patients, finding epitope peptides restricted to major HLA Class I types will facilitate further development of cancer immunotherapies. We are now conducting clinical trials to examine the immunogenicity and safety of a HIG2-9-4 peptide vaccine in RCC patients.

## Supporting Information

**Figure S1 Response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide detected by IFN-γ ELISPOT assay.** The IFN-γ production from cells induced by the indicated peptide-pulsed DCs in 12 wells for each peptide was examined by an ELISPOT assay. "+" indicates the wells in which cells were stimulated with T2 cells pulsed with the indicated peptide and "−" indicates the wells in which cells were stimulated with HIV peptide-pulsed T2 cells. The wells in which the difference between peptide-pulsed cells and HIV peptide-pulsed cells were over 50 spots are indicated by squares.
(TIF)

## Acknowledgments

The authors thank Dr. Kawakami (Keio University, Tokyo, Japan) for providing the expression vector, and the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University for providing the cell lines.

## Author Contributions

Conceived and designed the experiments: TT RO HY. Performed the experiments: SY MH TW TH. Analyzed the data: SY MH TW TH. Wrote the paper: SY. Scientific advise: MK MM MT MI. Support to draft the manuscript: KT TK YN.

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# Prognostic significance of IL-17 mRNA expression in peritoneal lavage in gastric cancer patients who underwent curative resection

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Received October 23, 2013; Accepted November 11, 2013

DOI: 10.3892/or.2013.2911

**Abstract.** Peritoneal dissemination is frequently detected in patients with advanced gastric cancer. The peritoneal cavity is a compartment in which an immunologic host-tumor interaction can occur. There are no reports on the relationship between IL-17 expression in peritoneal lavage and prognosis in gastric cancer patients. Therefore, we investigated the expression of IL-17 mRNA in peritoneal lavage from gastric cancer patients and assessed the association of its expression with clinicopathological parameters and prognosis. Peritoneal lavage was obtained from 114 patients with gastric cancer at initial surgery. Seventy-nine patients underwent curative resection. Among these 79 patients, IL-17 mRNA expression was associated with the depth of tumor invasion ($P<0.05$). Twelve of the 79 patients who underwent curative resection died, and 9 of those 12 developed peritoneal metastasis. Notably, among the 79 patients who underwent curative resection, those with high expression of IL-17 mRNA in peritoneal lavage had significantly prolonged survival when compared to these patients with low expression of IL-17 mRNA in peritoneal lavage ($P<0.05$) as evidence by the survival curves. In a multivariate analysis, low expression of IL-17 mRNA in peritoneal lavage and tumor size were found to be independent significant predictive factors for prognosis (HR, 7.91; 95% CI, 1.65-38.03) in the patients who underwent curative resection. IL-17 mRNA expression in peritoneal lavage is a reliable prognostic factor for patients undergoing curative resection for gastric cancer. Low IL-17 expression in the peritoneal cavity may correlate with cancer development in the peritoneal cavity in patients with gastric cancer.

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*Key words:* gastric cancer, interleukin-17, peritoneal lavage, prognostic factor

## Introduction

Although the prognosis of gastric cancer has improved with the development of early diagnosis and new therapeutic strategies, it remains one of the main causes of cancer-related mortality worldwide (1), with peritoneal carcinomatosis, often associated with malignant ascites, being the most frequent cause of death in patients with advanced gastric cancer. Peritoneal dissemination is considered to arise from free cancer cells in the peritoneal cavity exfoliated from the serosal surface of the stomach penetrated by the primary tumor (2). Therefore, cytologic examination of peritoneal washes has been performed at laparotomy to detect free cancer cells in patients with advanced gastric cancer, and it is recognized as one of the most important prognostic factors (3-7). Since 1999, it has been reported that the presence of free cancer cells in the peritoneal cavity should be considered as an independent prognostic factor in patients with gastric cancer by the Japanese Gastric Cancer Association. Moreover, in the International Union Against Cancer's TNM classification 7th edition for gastric cancer, positive peritoneal cytology is defined as stage IV.

Therefore, detection of free cancer cells in peritoneal washes is a standard method for the assessment of risk for peritoneal carcinomatosis. However, patients who are diagnosed as having no free cancer cells in the peritoneal cavity by conventional cytology sometimes develop peritoneal recurrence after curative resection. This may occur since cytology is considered to have low sensitivity. In fact, recently, real-time RT-PCR techniques have been developed in order to increase the sensitivity of conventional peritoneal lavage cytology (8,9). Concerning another cause for the development of recurrent peritoneal disease, the cytokine network may play an important role in the immunosuppressive and immunostimulatory properties of cancer-related ascites fluid (10,11). However, the role of cytokines produced by cells in the peritoneal cavity on tumor growth in gastric cancer patients is still unclear.

Th17 cells have recently been identified as having a distinct Th cell lineage and were found in an experimental animal model of cancer and in human cancers. Th17 cells have been found in several types of human cancers, such as ovarian, prostate, colorectal, and other malignancies, as well as gastric