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.

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Disclosure Statement

The authors have no conflict of interest.

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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.

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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 1.5×10^4 peptide-pulsed DCs, 3×10^5 $CD8^+$ 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 (1×10^4 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 5×10^4 CTLs was cultured with 5×10^6 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

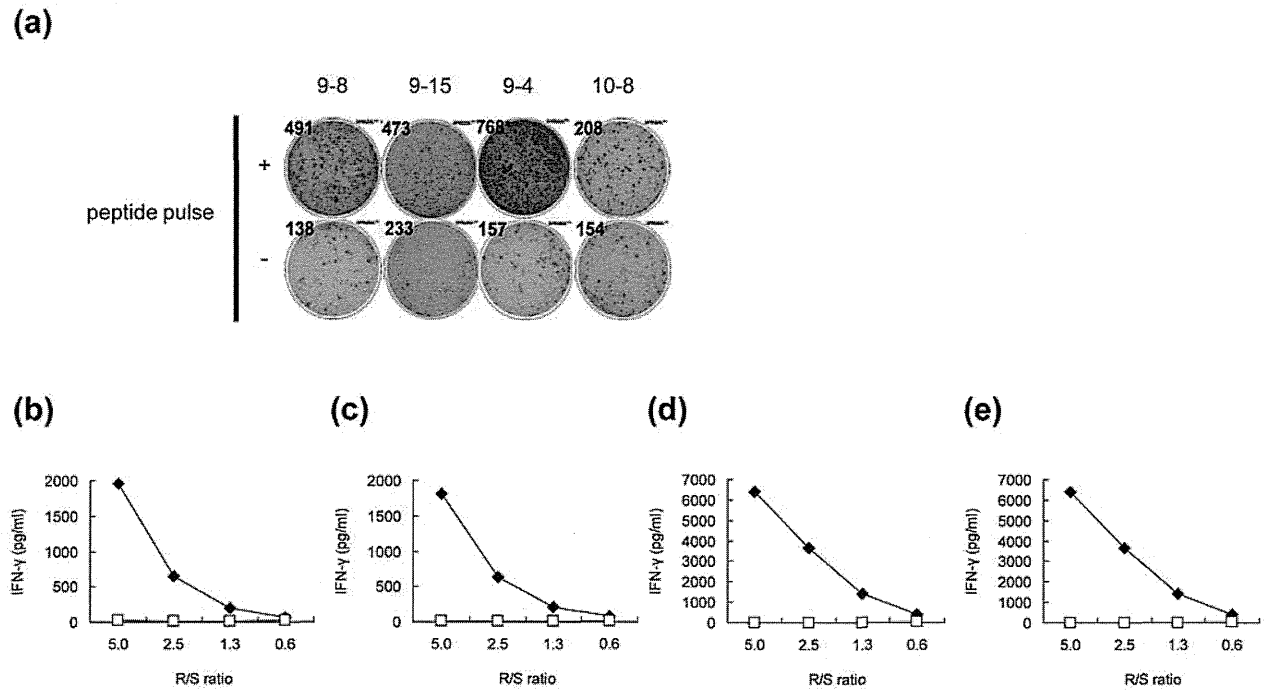

Figure 1. The IFN-γ production in response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide. (a) The IFN-γ production from cells induced by the indicated peptide-pulsed DCs was examined by an ELISPOT assay using T2 cells. "+" indicates the wells in which cells were stimulated with T2 cells pulsed 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 1×10^4 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.

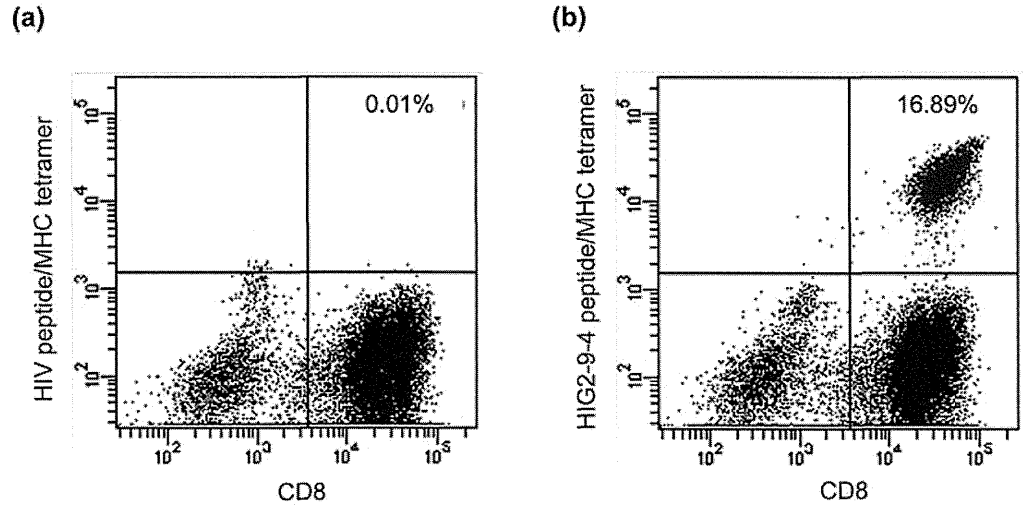

Figure 2. The expression of a HIG2-9-4 peptide-specific T cell receptor on CD8+ T cells. The expression of the HIG2-9-4 peptide-specific T cell receptor was examined on $CD3^+CD4^-$ cells following CTL expansion culture of HIG2-9-4 peptide-induced CTLs. (a) A quadrant gate was set based on the staining 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 (1×10^4 cells/well) or gene-transfected cells (5×10^4 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).

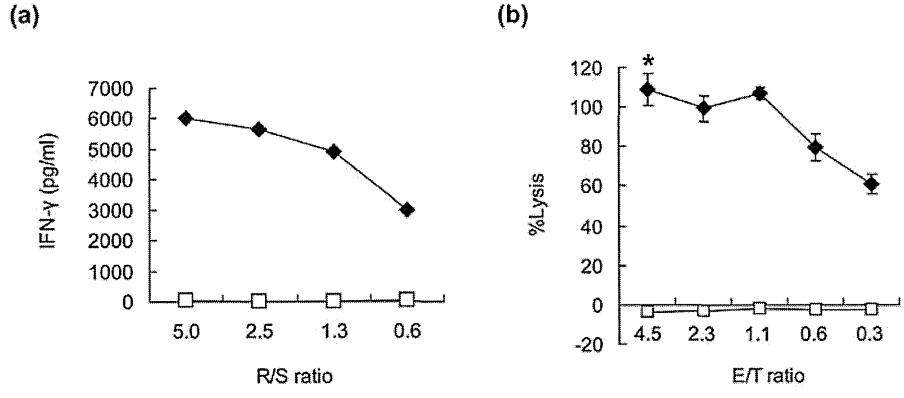

Figure 3. The IFN-γ production and cytotoxic activity of a HIG2-9-4 peptide-specific CTL clone. (a) An established CTL clone was stimulated with T2 cells pulsed with the HIG2-9-4 peptide (closed diamonds) or HIV peptide (open squares). The IFN-γ production in the culture supernatant was examined by ELISA. 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

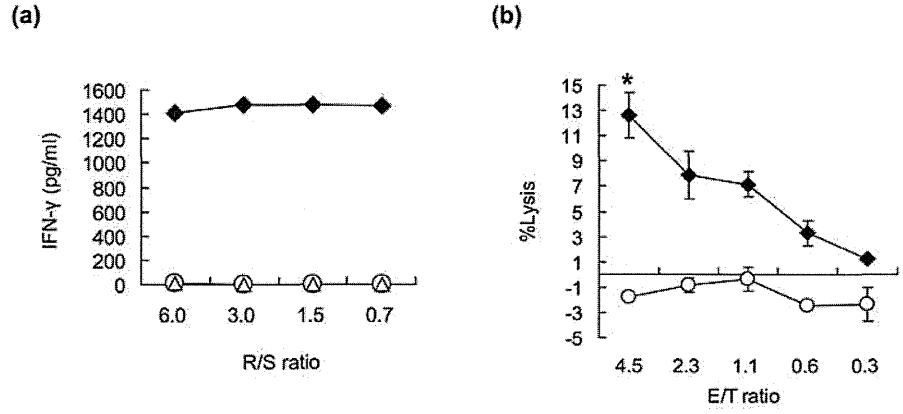

Figure 4. The recognition of HIG2 and HLA-A*02:01-expressing cells by a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:01 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:01 alone (open triangle), 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

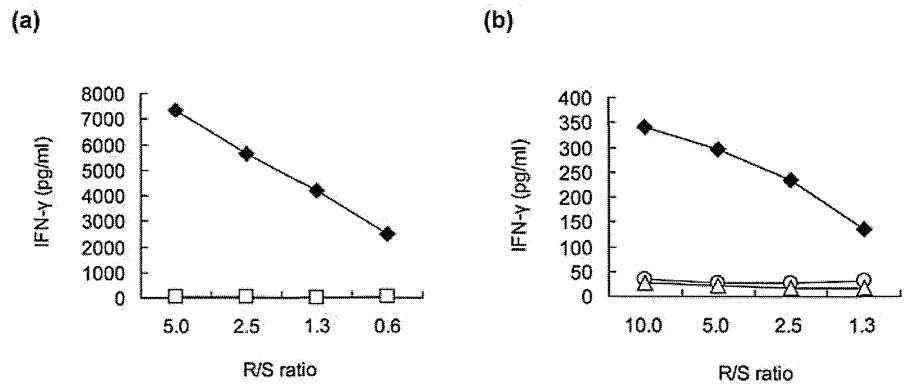

Figure 5. The HLA-A*02:06-restricted response of a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was induced from HLA-A*02:06-positive PBMCs, and stimulated with HLA-A*02:06-positive PSCCA0922 cells pulsed with the HIG2-9-4 peptide (close diamond) or HIV peptide (open 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

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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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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

cancer (12-17). Research has found that IL-17 promotes tumor growth through angiogenesis and inflammation (18), and several other studies have demonstrated that IL-17 contributes to reduced tumor growth by promoting dendritic cell, cytotoxic T cell (CTL), and NK cell trafficking to, and retention within, the tumor microenvironment (19,20). Therefore, whether these cells promote tumor growth or regulate antitumor responses remains controversial. In the case of human gastric cancer, there are several reports concerning the prevalence of Th17 cells in the tumor microenvironment, tumor-draining lymph nodes and peripheral blood (12,13). In our previous study, the expression level of IL-17 mRNA in gastric tumors was associated with the depth of tumor invasion, lymphovascular invasion and lymph node involvement suggesting that IL-17 is clearly associated with tumor progression (21). However, since no previous studies have been carried out concerning the proportion of IL-17 in the peritoneal cavity in human gastric cancer patients, we hypothesized that the expression level of IL-17 mRNA in peritoneal lavage may be involved in the development of peritoneal carcinomatosis in gastric cancer.

In the present study, we quantitatively investigated expression of IL-17 messenger RNA (mRNA) in the peritoneal lavage of gastric cancer patients who underwent curative resection. The association of IL-17 expression levels with clinicopathological factors and prognosis was also assessed. Since IL-2 and IL-12 have been used for intraperitoneal immunotherapy in patients with various types of cancers, including gastric cancer (22-25), the possibility of IL-17 as a therapeutic target for patients with gastric cancer was also investigated.

Materials and methods

Patients. Included in the present study was a series of 114 patients (80 men and 34 women) with gastric cancer who underwent surgery at Wakayama Medical University Hospital (WMUH) from 2003 to 2006. At the beginning of the operation, we examined tumor metastases in the abdominal cavity. When it was thought that curative resection was possible, we performed gastrectomy with lymphadenectomy. We performed gastrectomy in 114 patients with gastric cancer. Seventy-nine patients underwent surgically curative resection and 35 underwent non-curative resection. None of the patients received anticancer therapy prior to surgery. Individuals with autoimmune disease, inflammatory bowel disease or viral infections were excluded. The clinicopathological characteristics of the 114 patients are summarized in Table IA. Clinical stages of the tumors were determined according to the International Union Against Cancer's TNM classification for gastric cancer. After surgery, all patients underwent a follow-up, with the median follow-up at analysis being 61 months (range, 1.3-98.5) for all patients. Every 3 to 6 months, physical examination, blood chemistry, including carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9, and computed tomography were performed for each patients. Written informed consent was obtained from all patients before participation in the present study. In addition, the local ethics committee of WMUH approved the study.

Preoperative peritoneal wash examination. At the beginning of each operation, 100 ml saline was introduced into the Douglas cavity and aspirated by gentle stirring. These washes were centrifuged at 1,800 rpm for 5 min to collect intact cells. A part of each peritoneal wash was examined cytopathologically after conventional Papanicolaou staining.

RNA extraction and DNA synthesis. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) followed by RNase-Free DNase Set treatment (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using a reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Samples were stored at -80°C until use.

Quantitative real-time RT-PCR. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with isolated total RNA (1 μg) on the LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany). The following oligonucleotide primers and hybridization probes were used: human IL-17 (GenBank accession no. NM 002190; 53-231 bp): sense, 5'-CTGGGAAGACCTCA TTGG-3'; antisense, 5'-CCTTTTGGGATTGGTATTGG-3'; fluorescein-labeled probe, 5'-TCCTCAGAATTTGGGC ATCCTGGATTTC-3'; and LC Red 640-labeled probe, 5'-TGGGATTGTGATTCCTGCCTTCACTATGG-3'; human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GenBank accession no. NM 002046; 746-1052 bp): sense, 5'-TGAACGGGAAGCTCACTGG-3'; antisense, 5'-TCC ACCACCCTGTTGCTGTA-3'; fluorescein-labeled probe, 5'-TCAACAGCGACACCCACTCCT-3'; and LC Red 640-labeled probe, 5'-CACCTTTGACGCTGGGGCT-3'. Primers and probes were designed by Nihon Gene Research Laboratories, Inc. (Miyagi, Japan). After 10 min of initial denaturation at 95°C, the cycling protocol entailed 40 cycles of denaturation at 95°C (10 sec), annealing at 62°C (15 sec) and elongation at 72°C (8 sec). For GAPDH, the thermocycling protocol was the same, except that annealing was performed at 55°C (15 sec) and 50 cycles were run. On each run, all samples were quantified according to the LightCycler software program, version 3.8 (Roche Molecular Biochemicals). The levels of mRNA for IL-17 were corrected with GAPDH housekeeping control amplifications. We used the following for quantitative RT-PCR analysis: IL-17 ratio = IL-17 value/ GAPDH value x 10^4.

Determination of the cut-off value. The cut-off value of the IL-17 mRNA ratio was determined as the median value based on the quantified values of 114 samples in the present study. The cut-off value was 1.22.

Immunohistochemistry and quantitative microscopy. Sections (4 μm) were prepared from paraffin-embedded blocks derived from gastric tumors. Sections were deparaffinized in xylene and graded alcohols, and rinsed in phosphate-buffered saline. Antigen retrieval from the tissues was carried out by autoclaving the tissues in 0.01 M citrate buffer (pH 6.0) at 100°C for 10 min. The antibody used was goat anti-IL-17 (dilution at 10 μg/ml; R&D Systems, Minneapolis, MN, USA). The antibodies were incubated overnight at 4°C. The immunocomplex was visualized by a polymer envision method, EnVision™+ Kit (DakoCytomation, Glostrup, Denmark). For quantification

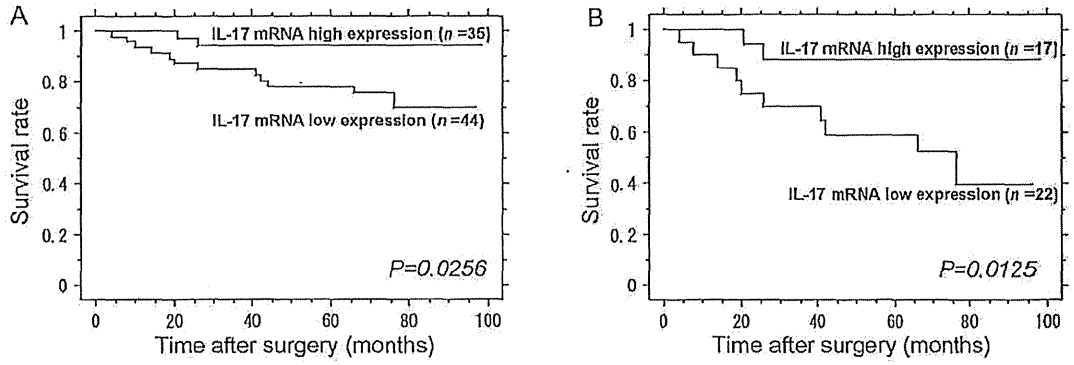

Figure 1. (A) Overall survival curves of the 79 gastric cancer patients who underwent curative R0 resection stratified according to IL-17 mRNA high or low expression in peritoneal lavage as determined by real-time RT-PCR. A significant difference in survival was noted between the IL-17 mRNA high expression and IL-17 mRNA low expression groups (P=0.0256; log-rank test). (B) The overall survival of the 79 patients who underwent curative resection with clinical stage II/III tumors according to IL-17 mRNA high or low expression in peritoneal lavage as determined by real-time RT-PCR. The IL-17 mRNA low expression group had a significantly poorer prognosis than the high expression group among the clinical stage II/III patients (P=0.0125, log-rank test).

of IL-17-positive cells, highly positive areas were initially identified by scanning tumor sections using light microscopy. Data were obtained by manually counting positively stained cells in five separate areas of intratumoral regions under x400 high power magnification. The regions were counted by a pathologist who had no knowledge of the other clinicopathological features and survival outcomes.

Flow cytometry. For intracellular molecule measurements, cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 5 h in the presence of GolgiPlug (BD Biosciences, San Diego, CA, USA). Then, the cells were harvested and stained with PerCP-Cy5.5-conjugated anti-CD3 mAb, FITC-conjugated anti-CD4 mAb, FITC-conjugated anti-CD8 mAb and FITC-conjugated anti-γδ TCR mAb (BD Biosciences) for 30 min on ice. For intracellular staining, after fixation and permeabilization using BD Cytofix/Cytoperm (BD Biosciences), the cells were stained with PE-conjugated anti-IL-17 mAb (BD Biosciences) for 30 min at 4˚C. After washing, the cells were analyzed by FACSCalibur, using CellQuest (BD Biosciences).

Statistical analysis. The Mann-Whitney test and the Kruskal-Wallis test were used to determine statistical significance between the covariates. The Cox proportional hazards model was used to compute the univariate and multivariate hazards ratios for the study parameters. Survival curves were computed using the Kaplan-Meier method and compared by means of the log-rank test. The survival curve was calculated from the date of surgery. In Fig. 3, we used the Spearman rank correlation coefficient. All statistical analyses were performed with StatView 6.0 (Abacus Concepts, Inc., Berkeley, CA, USA) statistical software program. A value of $P<0.05$ was considered to indicate a statistically significant result.

Results

Relationship between IL-17 mRNA and clinicopathological factors. To evaluate the biological significance of IL-17 expression in peritoneal lavage from patients with gastric cancer, the association between mRNA expression levels of IL-17 and clinicopathological factors was investigated. In all patients, the IL-17 mRNA expression level in the peritoneal lavage increased according to the depth of tumor invasion and peritoneal metastases, while the expression level was not associated with cytologic examination (Table IA). In the patients who underwent curative resection, IL-17 mRNA expression levels in peritoneal lavage increased according to the depth of tumor invasion (Table IB). On the other hand, no significant association was recognized between the expression level of IL-17 mRNA and histological type, lymph node metastases, lymphatic invasion, vessel invasion, clinical stage or tumor size.

Correlation between patient survival and IL-17 mRNA expression in peritoneal lavage. Kaplan-Meier survival curves indicated the overall survival of gastric carcinoma patients stratified according to the results of the IL-17 mRNA expression status in peritoneal lavage. The survival curves of all 114 patients displayed no significant difference between the IL-17 mRNA low expression group and the IL-17 mRNA high expression group (data were not shown). Importantly, however, based on the survival curves, among the 79 patients who underwent curative R0 resection, the patients in the IL-17 mRNA low expression group (n=44) had a significantly poorer prognosis when compared with the patients in the IL-17 mRNA high expression group (n=35) (Fig. 1A; $P<0.05$). During the median 61 months of postoperative surveillance, 14 (17.8%) of the 79 patients who underwent curative resection died, and 12 (85.7%) of these 14 patients developed peritoneal metastasis. Regarding peritoneal recurrence, 10 (22.7%) of the 44 cases in the IL-17 mRNA low expression group developed peritoneal metastases, while 2 (5.7%) of the 35 cases in the IL-17 mRNA high expression group developed peritoneal metastases.

Correlation between survival and IL-17 mRNA in peritoneal lavage in advanced gastric cancer. Among the patients who underwent curative resection with clinical stage II/III tumors, the prognosis of the IL-17 mRNA low group was significantly

Table I. Clinicopathological data and IL-17 mRNA expression of the 114 patients and the 79 patients who underwent curative resection.

A, Data of the 114 patients

Factor	No. of patients	Expression of IL-17 mRNA[a]	P-value
Age (years)			
≤65	50	2.11±0.648	0.448
>65	64	1.57±0.399	
Gender			
Male	80	1.75±0.415	0.718
Female	34	1.93±0.724	
Depth of tumor invasion			
T1	30	1.00±0.523	0.023[c]
T2	13	1.49±0.130	
T3	31	2.44±0.809	
T4	40	2.51±0.582	
Lymph node metastasis			
N0	43	2.25±0.681	0.477
N1	28	1.26±0.581	
N2	21	1.61±0.708	
N3	22	1.84±0.894	
Histological type			
Differentiated	54	2.08±0.601	0.387
Undifferentiated	60	1.55±0.423	
Lymphatic invasion			
Negative	37	1.90±0.659	0.338
Positive	77	1.76±0.432	
Vessel invasion			
Negative	57	1.83±0.544	0.292
Positive	57	1.78±0.479	
Peritoneal metastasis			
Negative	100	2.07±0.439	0.031[d]
Positive	14	7.21±0.290	
Cytologic examination			
Negative	91	1.22±0.591	0.801
Positive	23	1.89±0.404	
Stage[b]			
I	40	1.95±0.660	0.412
II	18	2.29±0.109	
III	21	1.38±0.784	
IV	33	1.64±0.531	
Tumor size (cm)			
≤5	60	1.75±0.516	0.059
>5	54	1.87±0.506	

Table I. Continued.

B, Data of the 79 patients who underwent curative resection

Factor	No. of patients	Expression of IL-17 mRNA[a]	P-value
Age (years)			
≤65	35	2.01±0.781	0.329
>65	44	1.72±0.527	
Gender			
Male	58	1.80±0.517	0.975
Female	21	1.93±0.724	
Depth of tumor invasion			
T1	30	1.00±0.523	0.020[c]
T2	13	1.49±0.130	
T3	23	2.39±0.927	
T4	13	2.90±0.124	
Lymph node metastasis			
N0	43	2.25±0.681	0.307
N1	20	1.48±0.700	
N2	10	1.90±0.106	
N3	6	3.26±0.284	
Histological type			
Differentiated	41	2.31±0.719	0.613
Undifferentiated	38	1.33±0.524	
Lymphatic invasion			
Negative	34	1.72±0.690	0.118
Positive	45	1.95±0.610	
Vessel invasion			
Negative	49	1.72±0.597	0.087
Positive	30	2.05±0.708	
Stage[b]			
I	40	1.95±0.660	0.497
II	18	2.29±0.109	
III	21	1.44±0.819	
Tumor size (cm)			
≤5	54	1.62±0.509	0.097
>5	25	2.36±0.943	

[a]Expression of mRNA for IL-17 was corrected with GAPDH housekeeping control amplifications. Values represent mean ± SEM. [b]Stage according to the TNM classification for gastric cancer (UICC). [c]P-value of Kruskal-Wallis test as appropriate. [d]P-value of Mann-Whitney test as appropriate.

poorer than that of the patients in the IL-17 mRNA high group (Fig. 1B; P<0.05). From the point of view of the depth of the invasion, patients in the IL-17 mRNA low group had significantly poorer outcome than those in the IL-17 mRNA high group for patients in the T2/3/4 subgroups (Fig. 2A; P<0.05). In the T4 subgroup, patients with IL-17 mRNA low expression in peritoneal lavage had a significantly poorer survival than those with IL-17 mRNA high expression (Fig. 2B; P<0.05).

Preoperative peritoneal wash assay as an independent prognostic factor. We evaluated prognostic factors in the 79 patients who underwent curative R0 resection. With the overall

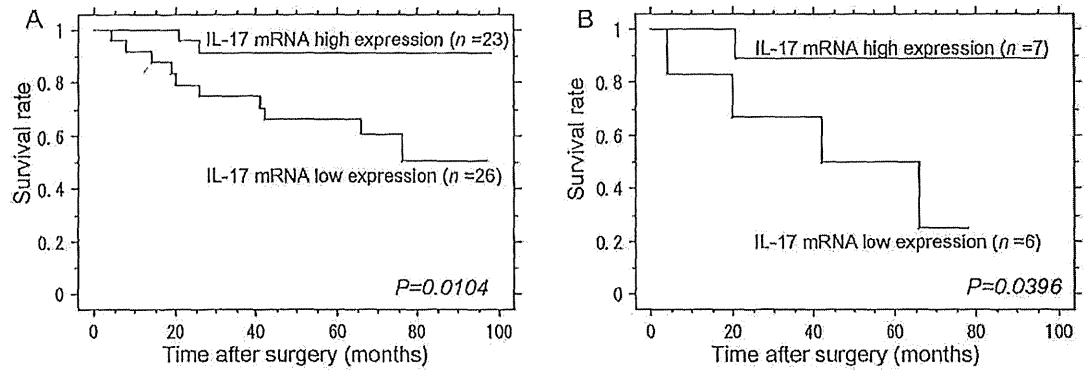

Figure 2. Overall survival curves of the 79 gastric cancer patients who underwent curative resection with pT2/3/4 stage tumors stratified according to IL-17 mRNA high or low expression in peritoneal lavage. (A) Overall survival of the 79 patients subdivided according to IL-17 mRNA high or low expression in peritoneal lavage with pT2/3/4 stage tumors. The IL-17 mRNA low expression group had a significantly poorer prognosis than the high expression group in the combined pT2/3/4 subgroup (P=0.0104; log-rank test). (B) Overall survival of the 79 patients with pT4 stage tumors subdivided according to IL-17 mRNA high or low expression in peritoneal lavage. The IL-17 mRNA low expression group had a significantly poorer prognosis than the high expression group among the pT4 patients (P=0.0396; log-rank test).

Table II. Univariate and multivariate analysis of the overall survival for the 79 patients who underwent R0 curative resection.

	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (years)						
≤65 vs. >65	1.62	0.542-4.840	0.388	-	-	-
Gender						
Male vs. female	1.07	0.335-3.438	0.906	-	-	-
Lymph node metastasis						
Negative vs. positive	9.98	1.296-76.95	0.027	2.94	0.175-49.39	0.454
Serosal invasion						
Negative vs. positive	9.04	2.014-40.53	0.004	1.56	0.215-11.35	0.659
Lymphatic invasion						
Negative vs. positive	9.98	1.296-76.95	0.027	1.05	0.046-23.79	0.976
Vessel invasion						
Negative vs. positive	10.38	2.320-46.42	0.0022	4.16	0.459-37.68	0.205
Histological type						
Differentiated vs. undifferentiated	2.16	0.720-6.497	0.169	-	-	-
Tumor size (cm)						
≤5 vs. >5	7.32	2.289-23.40	0.0008	4.61	1.19-17.78	0.027
IL-17 mRNA expression						
Low expression vs. high expression	4.69	1.049-20.99	0.043	7.91	1.65-38.03	0.0098

CI, confidence interval.

survival as an endpoint, lymph node metastasis, serosal invasion, lymphatic invasion, vessel invasion, tumor size and IL-17 mRNA expression were found to be significant as prognostic factors by univariate analysis. Moreover, when multivariate analysis was performed with these six covariates and the same endpoint, IL-17 mRNA low expression in peritoneal lavage and tumor size were found to be independent significant predictive factors for prognosis (Table II; HR, 7.91; 95% CI, 1.65-38.03; P=0.0098).

Correlation between IL-17-positive cells in primary tumor tissues and IL-17 mRNA expression in peritoneal lavage. To examine the correlation in IL-17 production between the level in peritoneal lavage and in the primary tumor tissues,

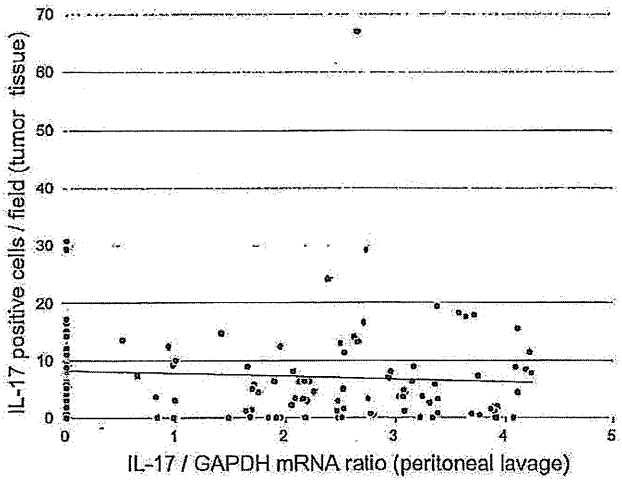

Figure 3. Correlation between IL-17-positive cells in tumor tissues and IL-17 mRNA expression in peritoneal wash. Immunohistochemical staining for IL-17 in primary tumor tissues was performed in the same patient samples whose peritoneal lavage was examined for IL-17 mRNA. The longitudinal axis shows IL-17-positive cells per field in the tumor tissues. The horizontal axis shows the IL-17/GAPDH mRNA ratio in peritoneal lavage.

we performed immunohistochemical staining for IL-17 in the primary tumors in the same patient samples. In the primary tumor tissues, IL-17 immunoreactive cells were detected in the cytoplasm of mononuclear cells; however, none of the tumor cells were stained for IL-17. IL-17-producing cells in the tumor tissues were 7.30±0.82 (mean ± SE) per field. There was no correlation between the number of IL-17-positive cells in the tumor tissues and IL-17 mRNA expression in the peritoneal wash (r=0.092; P=0.329) (Fig. 3).

Analysis of IL-17-producing cells in peritoneal lavage. Immunohistochemical staining of peritoneal wash revealed that mononuclear cells were stained for IL-17. However, neither tumor cells nor mesothelial cells were stained for IL-17. To identify which mononuclear cells produced IL-17 in the peritoneal lavage, we performed flow cytometric analysis using anti-IL-17, -CD3, -CD4, -CD8 and -γδ TCR antibodies. $CD3^+$ T cells produced IL-17, while γδ T cells did not produce IL-17 (Fig. 4A). $CD4^+$ T cells mainly produced IL-17, and a small population of $CD8^+$ T cells also produced IL-17. The mean percentage of IL-17-positive $CD8^+$ T cells among the total IL-17-positive cell population was only 27.6±4.85% (n=5), and in contrast, IL-17-positive $CD4^+$ T cell population was 72.2±4.86% (n=5). Representative flow cytometry analysis is shown in Fig. 4B.

Discussion

In the present study, we demonstrated that in patients who underwent R0 resection, the prognosis of patients in the IL-17 mRNA low expression group was significantly poorer than those in the high expression group. This is the first study evaluating the prognostic value of IL-17 detection by real-time RT-PCR in peritoneal lavage as a valuable prognostic factor in gastric cancer.

IL-17 was originally identified as a proinflammatory cytokine that induces neutrophils, and previous studies also have shown that inflammation is linked to cancer development and progression. It has recently been reported that the levels of IL-17-producing cells are significantly increased in tumor tissues, peripheral blood, malignant ascites fluid, and malignant pleural effusion from a variety of cancer patients (13,14,26-28). Despite recent advances in our understanding of the function of Th17 cells in humans, very little is known about their prevalence and tumor immunosurveillance.

In mice, overexpression of IL-17 by gene transduction into tumor cells promoted tumor growth through angiogenesis (18), but seemingly in contrast, IL-17 also suppressed tumor growth

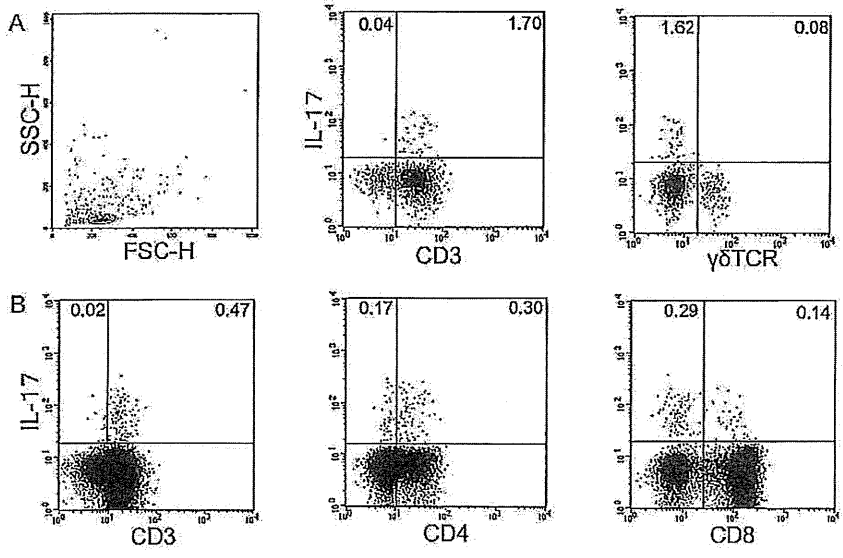

Figure 4. Flow cytometric analysis using anti-IL-17, -CD3, -CD4, -CD8 and -γδ TCR antibodies to identify IL-17-producing cells in peritoneal washes. (A) Cells were stained with PE-anti-IL-17 mAb, PerCP-anti-CD3 mAb and FITC-anti-γδ TCR mAb after stimulation with PMA and ionomycin for 5 h. (B) Cells were stained with PE-anti-IL-17 mAb, PerCP-anti-CD3 mAb, FITC-anti-CD4 mAb and FITC-anti-CD8 mAb after stimulation with PMA and ionomycin for 5 h. Representative flow cytometry plots using peritoneal washes from subjects with cytology class II.

via a T-cell-dependent mechanism (19). Whether IL-17 promotes tumor growth or regulates antitumor responses remains controversial.

In humans, there are several reports concerning intratumoral expression of IL-17 and its prognostic role in several cancer types such as hepatocellular carcinoma (HCC), colon, esophageal and gastric cancer. In HCC, colon, and prostate cancer patients, intratumoral IL-17-positive cells were found to be correlated with poor survival (17,29,30). Our previous study showed that Th17 cells infiltrated the tumor and secreted IL-17 in the tumor microenvironment, leading to tumor progression through angiogenesis and neutrophil infiltration in patients with gastric cancer. In the present study, we hypothesized that IL-17 promotes tumor progression in the peritoneal cavity, based on our previous study suggesting that IL-17 is related to tumor progression in the tumor microenvironment. We quantitatively analyzed the expression levels of IL-17 mRNA in peritoneal lavage from gastric cancer patients. Based on the survival curves, among the 79 patients who underwent R0 resection, the patients in the IL-17 mRNA low expression group had a significantly poorer prognosis than the patients in the IL-17 mRNA high expression group. This result was contradictory to our hypothesis. This discrepancy may be explained by the difference in the impact of IL-17 on tumor progression in the thoracoabdominal cavity and in tumor tissue. In fact, in the present study, there were no correlations noted between primary tumor tissues and peritoneal wash in terms of IL-17 expression. In lung cancer, Ye *et al* (26) reported that patients with a higher proportion of Th17 cells in malignant pleural effusion exhibited significantly longer overall survival than patients with a lower proportion of Th17 cells. Similarly, in ovarian cancer, the expression of IL-17 in ascites was analyzed, and patients with a higher IL-17 expression in ascites had a significantly lower death hazard than those with a lower IL-17 expression (28).

Most recently, it has been reported that $CD8^+$ T cells that produce IL-17 (Tc17 cells) are abundant in gastric cancer tissue, and the percentage of Th17 cells is relatively lower than that of Tc17 cells in tumors. The intratumoral Tc17 cell percentage was significantly associated with tumor progression and poor prognosis (31). In the present study, flow cytometric analysis showed that $CD4^+$ Th17 cells predominantly produced IL-17 in the peritoneal lavage; however, the percentage of Tc17 cells was lower than that of Th17. This suggests that IL-17-producing T cells are different between tumor tissue and the abdominal cavity, and the potential role of IL-17 could also be different in the tumor microenvironment between tumor tissue and the abdominal cavity.

There is another reason why the results of the present study were in conflict with our expectations. This may be because the role of IL-17 is different before and after the tumor is established. In the present study, the expression levels of IL-17 were significantly higher in peritoneal carcinomatosis-positive cases than those of negative cases, while they were not associated with cytologic examination (Table IA). Once tumor cells attach to the peritoneum, IL-17 may play a role as a tumor growth cytokine through angiogenesis to a greater exent than its role in regulating antitumor responses. Our results suggest that endogenous IL-17 plays different roles before tumor attachment versus in established tumor growth. Furthermore, in the abdominal cavity, previous studies indicate that peritoneal mesothelial cells secrete various cytokines and growth factors, such as IL-6, IL-8, IL-1α and β, granulocyte colony stimulating factor (G-CSF), as well as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2. These results indicate that peritoneal mesothelial cells are one of the central elements of the cytokine network controlling disease processes in the abdominal cavity. Kryczek *et al* suggested that, in the peritoneal cavity, IL-17 is positively associated with INF-γ effector T cells and Th1-type chemokines, CXCL9 and CXCL10, but not with Th2-type chemokines, CXCL12 and CCL22, in ovarian cancer ascites. Mechanistically, Th17 cell-derived IL-17 and INF-γ were found to synergistically induce the production of CXCL9 and CXCL10 and in turn promote effector T-cell migration (28). Thus, IL-17 may function as a polyfunctional cytokine profile in human tumors. There is no doubt that the role of IL-17 is highly complicated, and it remains controversial whether IL-17 promotes tumor growth or regulates the antitumor response.

In conclusion, IL-17 mRNA expression in peritoneal lavage detected by real-time RT-PCR is a reliable prognostic factor for patients with curative resection in gastric cancer. Low IL-17 gene expression in the peritoneal cavity may correlate with cancer development in the peritoneal cavity and poor prognosis in patients with gastric cancer.

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ORIGINAL ARTICLE

Clinical benefits of thoracoscopic esophagectomy in the prone position for esophageal cancer

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Abstract
Purposes The clinical benefits of thoracoscopic radical esophagectomy in the prone position compared to conventional open esophagectomy have not been fully documented.
Methods Forty-six patients with esophageal cancer who underwent MIE in the prone position (MIE-P group) were enrolled, and 46 case-matched controls that underwent open esophagectomy (OE group) were identified using propensity score methods to achieve a valid comparison of outcomes between MIE and open esophagectomy.
Results The duration of systemic inflammatory response syndrome was shorter in the MIE-P group than in OE group ($P = 0.005$). The time to first walking was earlier in the MIE-P group ($P < 0.001$). Although the vital capacity ratio (%VC) declined after the operation in both groups, the change ratio of the %VC was 85.3 % in the MIE-P group and 69.6 % in the OE group ($P < 0.001$). No mortality occurred in either group. The postoperative morbidity rate was lower in the MIE-P group (13 %) than in the OE group (30.4 %) ($P = 0.020$). Two patients (4.3 %) in the OE group and one patient in the MIE-P group (2.2 %) had pneumonia.
Conclusions MIE in the prone position was associated with less impairment of the pulmonary function, earlier recovery of activity and lower subsequent morbidity compared to open esophagectomy. Further investigation of the long-term outcomes is, therefore, needed.

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Keywords Minimally invasive esophagectomy · Thoracoscopic esophagectomy · Esophageal cancer · Prone position · Postoperative pulmonary function · Postoperative morbidity

Introduction

A number of studies have demonstrated the safety and possible advantages of minimally invasive esophagectomy (MIE) in selected cohorts of patients [1–6]. MIE is, therefore, being performed with increasing frequency [7], and evidence of the short-term benefits of MIE over traditional open procedures with a similar oncological outcome is accumulating. Most comparative studies have demonstrated clinical advantages of MIE, such as less blood loss, a shorter intensive care unit (ICU) stay and similar survival. Nevertheless, systemic reviews of studies involving MIE have been equivocal and have failed to draw definitive conclusions [8]. A population-based national study in England has shown that there were no significant benefits demonstrated in the mortality and overall morbidity [9]. Most recently, a randomized controlled trial has shown the benefits of MIE in terms of a lower incidence of pulmonary infection and better quality of life compared to open esophagectomy [10].

Various types of MIE for patients with esophageal cancer have been described, and the most generally performed technique involves thoracoscopic mobilization of the esophagus in the left lateral decubitus position [1, 2, 4, 11]. Recently, the advantages of thoracoscopic esophageal mobilization in the prone position have also been reported [12, 13]. Compared to the left lateral decubitus position, the prone position allows better operative exposure and improved surgeon ergonomics, resulting in reduced