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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

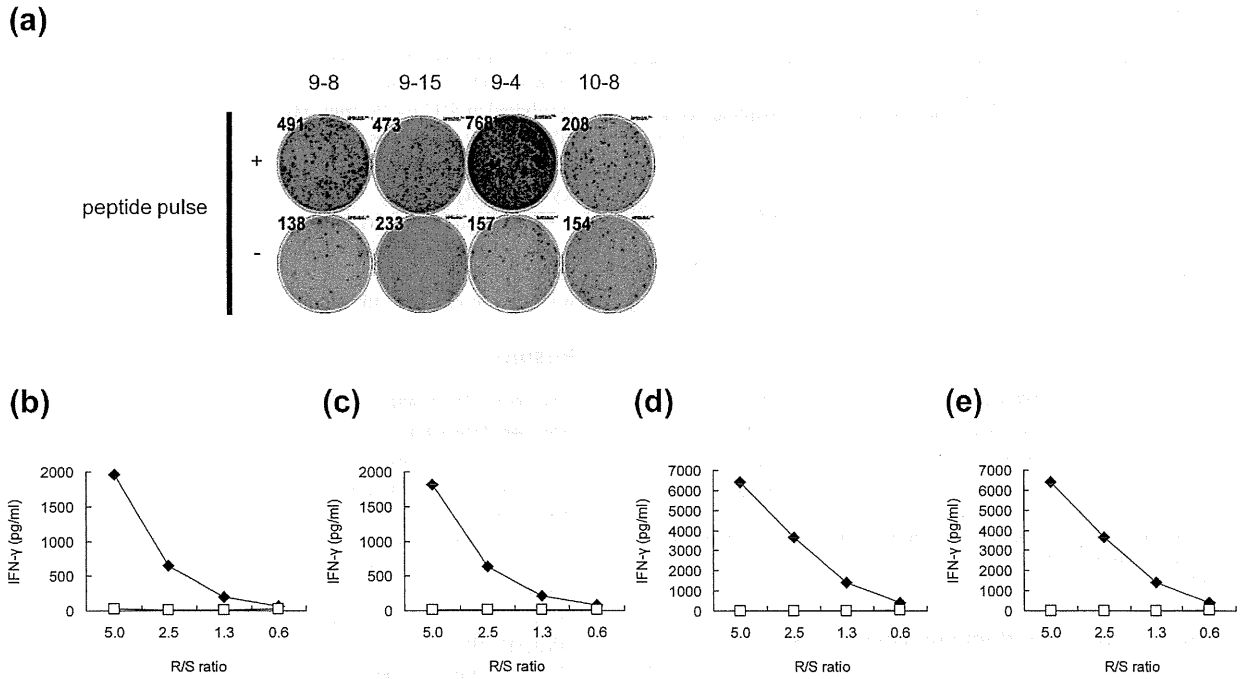

Figure 1. The IFN-γ production in response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide. (a) The IFN-γ production from cells induced by the indicated peptide-pulsed DCs was examined by an ELISPOT assay using T2 cells. "+" indicates the wells in which cells were stimulated with T2 cells pulsed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

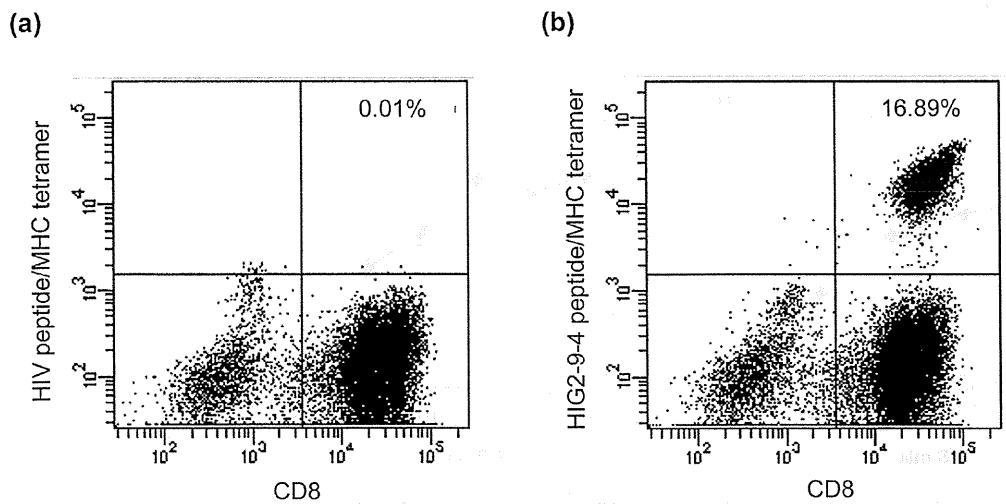

Figure 2. The expression of a HIG2-9-4 peptide-specific T cell receptor on CD8+ T cells. The expression of the HIG2-9-4 peptide-specific T cell receptor was examined on $CD3^+CD4^-$ cells following CTL expansion culture of HIG2-9-4 peptide-induced CTLs. (a) A quadrant gate was set based on the staining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

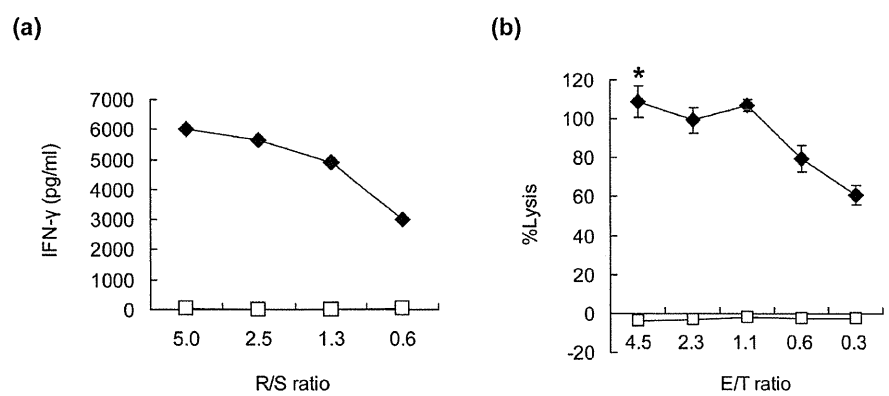

Figure 3. The IFN-γ production and cytotoxic activity of a HIG2-9-4 peptide-specific CTL clone. (a) An established CTL clone was stimulated with T2 cells pulsed with the HIG2-9-4 peptide (closed diamonds) or HIV peptide (open squares). The IFN-γ production in the culture supernatant was examined by ELISA.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

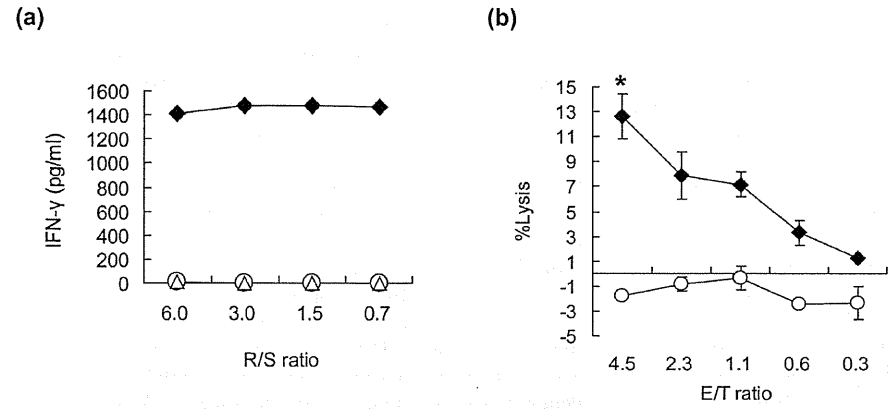

Figure 4. The recognition of HIG2 and HLA-A*02:01-expressing cells by a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:01 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:01 alone (open triangle),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

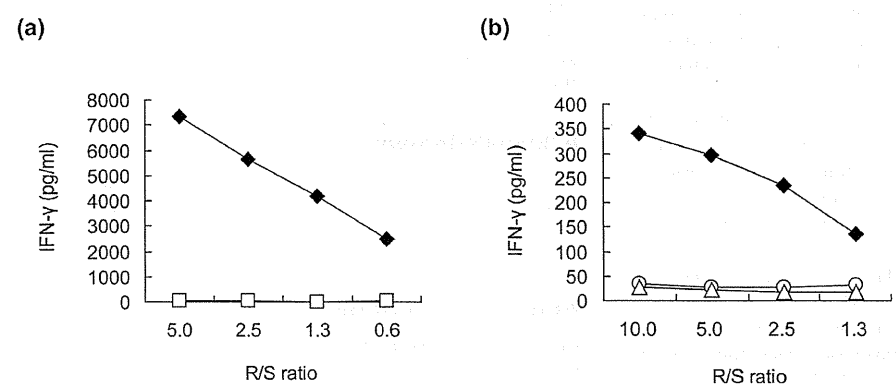

Figure 5. The HLA-A*02:06-restricted response of a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was induced from HLA-A*02:06-positive PBMCs, and stimulated with HLA-A*02:06-positive PSCCA0922 cells pulsed with the HIG2-9-4 peptide (close diamond) or HIV peptide (open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

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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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Phase I clinical trial of multiple-peptide vaccination for patients with advanced biliary tract cancer

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Abstract

Background: The prognosis of patients with advanced biliary tract cancer (BTC) is extremely poor and only a few standard treatments are available for this condition. We performed a phase I trial to investigate the safety, immune response and anti-tumor effect of vaccination with three peptides derived from cancer-testis antigens.

Methods: This study was conducted as a phase I trial. Nine patients with advanced BTC who had unresectable tumors and were refractory to standard chemotherapy were enrolled. Three HLA-A*2402 restricted epitope peptides-cell division cycle associated 1 (CDCA1), cadherin 3 (CDH3) and kinesin family member 20A (KIF20A)-were administered subcutaneously, and the adverse events and immune response were assessed. The clinical effects observed were the tumor response, progression-free survival (PFS) and overall survival (OS).

Results: The three-peptide vaccination was well-tolerated up to a dose of 3 mg per peptide (9 mg total). No grade 3 or 4 adverse events were observed after vaccination. Peptide-specific T cell immune responses were observed in all patients and stable disease was observed in 5 of 9 patients. The median PFS and OS were 3.4 and 9.7 months. The Grade 2 injection site reaction and continuous vaccination after PD judgment appeared to be prognostic of OS.

Conclusions: Multiple-peptide vaccination was well tolerated and induced peptide-specific T-cell responses.

Trial registration: This study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR000003229).

Keywords: Cancer vaccine, Peptide vaccine, Immunotherapy, Biliary tract cancer

Background

Biliary tract cancer (BTC) is not a common disease worldwide, but is prevalent in East Asia and Latin America. The occurrence rate is gradually increasing and there is a high mortality rate because most cases of BTC are not diagnosed until advanced and inoperable. At this time, very few standard treatments have been established for BTC [1,2], and thus development of new treatment modalities is urgently needed. Recently, cancer vaccines using synthetic peptides have been undergoing development throughout the world, and some of them have already been shown to be safe and effective [3-12]. We have previously reported that cancer peptide vaccines are capable of inducing antigen-specific cytotoxic T cells *in vivo* and providing some clinical benefit to some patients with advanced biliary tract cancer [13]. In this study, we selected three cancer-testis antigens that were identified by using cDNA microarray technology coupled with laser microdissection and were overexpressed in nearly 100% of BTC. We then performed a phase I study to assess the safety and antigen-specific immune response of a three-peptide vaccination using the selected antigens in patients with advanced biliary tract cancer. Patients were vaccinated on a continuous basis over the long-term even if their disease had progressed. We assessed the safety of the vaccination by CTCAE v3.0 as a primary endpoint and the antigen-specific immune response and clinical effects as secondary endpoints.

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Methods

Patient eligibility

Patients with unresectable BTC (intrahepatic bile duct cancer, extrahepatic bile duct cancer and gallbladder cancer) who were refractory to standard chemotherapy were eligible for this study. All patients were required to have an HLA-A type of A*2402. Additional inclusion criteria consisted of age between 20 and 80 years, no severe organ function impairment, white blood cell count between 2000 and 10000/mm^3, hemoglobin > 8 mg/dL, platelet count > 100,000/mm^3, AST and ALT < 100 IU/L, and total bilirubin < 2 mg/dl. Performance status measured by the ECOG scale was 0 to 2. It was required that there be an at least 4-week interval since the last chemotherapy. The exclusion criteria consisted of pregnancy, serious infections, severe underlying disease, severe allergic disease and a judgment of unsuitability by the principal investigator.

Study design and endpoints

This study was conducted as a phase I trial. Patients who received standard chemotherapy under a diagnosis of inoperable BTC between June 2009 and May 2010 were invited to participate after providing their informed consent. The HLA-A genotypes of these patients were examined, and the 9 patients with an HLA-A type of A*2402 were enrolled. Three peptides were used for the vaccine, CDCA1 (VYGIRLEHF) [14], CDH3 (DYLNEW GSRF) [15] and KIF-20A (KVYLRVRPLL) [16]. These peptides were chosen from among the antigens identified by using cDNA microarray technology coupled with laser microdissection and were the most overexpressed in BTCs. The purity (>97%) of the peptides was determined by analytical high-performance liquid chromatography (HPLC) and mass spectrometry analysis. The endotoxin levels and bioburden of these peptides were tested and determined to be within acceptable levels based on the GMP grade for the vaccines (PolyPeptide or NeoMPS Inc., San Diego, CA, USA). These peptides were mixed with incomplete Freund's adjuvant (IFA; Montanide ISA51, SEPPIC),which has been used in many clinical studies, and were injected subcutaneously into the inguinal or the axicilla site. Each of the three peptides at doses of 1 mg, 2 mg or 3 mg was injected subcutaneously into three patients once a week until the 8th vaccination and every two weeks after the 9th vaccination as a monotherapy as long as possible even if the patient was judged to exhibit disease progression. The primary endpoint in this study was the assessment of toxicities caused by the vaccination based on the Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v.3.0). The secondary endpoints were assessment of the immunological response, tumor response, progression-free survival (PFS) and overall survival (OS) from the 1st dose given. For the image analysis, CT scan or ultrasound was performed during the pre-vaccination period and at every 4th vaccination. This study was approved by the institutional review board at Tokyo Women's Medical University and was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000003229). Informed consent was obtained from all the patients and the procedures followed were in accordance with the Declaration of Helsinki.

Lymphocyte preparation for immunologic monitoring

The performance of the immunologic assay at the center laboratory was periodically standardized and validated by Clinical Laboratory Improvements Amendments (CLIA) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use (ICH) guidelines. PBLs were obtained from the patients at the pre-vaccination period and after every 4th vaccination. Peripheral blood was taken by venipuncture, collected in an EDTA tube and transferred to the center laboratory within 24 hrs at room temperature. Within 24 hrs of blood collection, PBLs were isolated with Ficoll-Paque Plus (GE Healthcare Bio-sciences, Piscataway, NJ) density gradient solution and were stored at -80°C in cell stock media (Juji Field) without serum at 5 × 10^6 cells/ml. After thawing, the cell viability was confirmed to be more than 90% by trypan-blue dye.

Enzyme-linked immunospot (ELISPOT) assay

The peptide-specific CTL response was estimated by ELI SPOT assay following *in vitro* sensitization. Frozen PBMCs derived from the same patient were thawed at the same time, and the viability was confirmed to be more than 90%. PBMCs (5 × 10^5/ml) were cultured with 10 μg/ml of the respective peptide and 100 IU/mL of IL-2 (Novartis, Emeryville, CA) at 37°C for two weeks. The peptide was added to the culture at day 0 and day 7. Following CD4+ cell depletion by a Dynal CD4 Positive Isolation Kit (Invitrogen, Carlsbad, CA), an IFN-γ ELISPOT assay was performed using a Human IFN-γ ELISpot PLUS kit (MabTech, Nacka Strand, Sweden) according to the manufacturer's instructions. Briefly, HLA-A*2402-positive B-lymphoblast TISI cells (IHWG Cell and Gene Bank, Seattle, WA) were incubated with 20 μg/ml of vaccinated peptides overnight, and then the residual peptide in the media was washed out to prepare peptide-pulsed TISI cells as the stimulation cells. Prepared CD4- cells were cultured with peptide-pulsed TISI cells (2 × 10^4 cells/well) at a 1/1, 1/2, 1/4 or 1/8 mixture ratio of responder cells to stimulator cells (R/S ratio) on a 96-well plate (Millipore, Bedford, MA) at 37°C overnight. Non-peptide-pulsed TISI cells were used as a negative control for the stimulator cells. To confirm IFN-γ productivity, the responder cells were stimulated with PMA and ionomycin (3 μg/ml) overnight, then applied to an IFN-γ ELISPOT assay (2.5 × 10^3 cells/well)

Table 1 Patient characteristics

Patients	Age/Sex	Tumor site*		Prior therapy**	Peptide (mg)
		Primary	Metastases		
1	38/F	IBD	Lung, bone	Ope, GEM, CBDCA, TS-1, DTX	1
2	69/M	GB	Liver, LN	Ope, GEM, TS-1	1
3	60/F	GB	Liver, LN	Ope, GEM, TS-1	1
4	66/F	IBD	Liver, lung, LN, bone	Ope, GEM, TS-1	2
5	75/M	IBD	Lung	Ope, GEM, TS-1	2
6	61/F	IBD	Liver, LN, peritoneum	GEM, TS-1, CDDP	2
7	46/M	EBD	Liver, LN	Ope, GEM, TS-1	3
8	76/M	EBD	Lung	Ope, GEM, TS-1	3
9	62/F	EBD	Lung	Ope, GEM, TS-1	3

*IBD: intrahepatic bile duct; GB: gallbladder; EBD: extrahepatic bile duct; LN: lymph node.
**Ope: operation; GEM: gemcitabine; CBDCA: carboplatin; TS-1: tegafur-gimeracil-oteracil potassium; DTX: docetaxcel; CDDP: cisplatin.

without stimulator cells. All ELISPOT assays were performed in triplicate wells. The plates were analyzed by an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology, Ltd., Shaker Heights, OH) and ImmunoSpot Professional Software Version 5.0 (Cellular Technology, Ltd.). The number of peptide-specific spots was calculated by subtracting the number of spots in the control well from the number of spots in each of the wells with peptide-pulsed TISI cells. The sensitivity of our ELISPOT assay was estimated as an approximately average level by an ELISPOT panel of the Cancer Immunotherapy Consortium [CIC (http://www.cancerresearch.org/cic/tools-initiatives/immune-assay-proficiency-harmonizationpanels)].

Flow cytometry assay

Conventional two-color analysis was performed with FITC-conjugated anti-human CXCR3 mAb plus PE-conjugated anti-human CCR4 mAb (R&D Systems, Minneapolis, MN) in order to assess the host immune condition of the type 1/type 2 subsets.

Statistical analysis

PFS and OS rates were analyzed using the Kaplan-Meier method. Statistical analyses of prognostic factors were done using the log-rank test. A p-value less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using IBM SPSS Statistics 21 (IBM, USA).

Results

Patient characteristics

Nine patients (4 males and 5 females; median age: 61.4 years; range: 38-76) whose HLA type was A*2402 were enrolled in this study (Table 1). Their primary tumor site was the intrahepatic bile duct in 4 cases, the extrahepatic bile duct in 3 cases, and the gallbladder in 2 cases. They had several metastases to the liver, lungs, lymph nodes, peritoneum and bone. Previous therapies consisted of operation, gemcitabine (GEM), cisplatin (CDDP), tegafur-gimeracil-oteracil potassium (TS-1), carboplatin (CBDCA) or docetaxcel (DTX). One patient dropped out after the 1st follow-up study because of another disease and 4 patients elected to stop vaccination at the time of PD judgment. Four patients decided to continue the vaccination as long as possible after PD judgment.

Assessment of toxicity

Toxicity was assessed by CTCAE v3.0 (Table 2). Four of 9 patients developed grade 1 injection site reaction and 5 developed grade 2 injection site reaction. Low hemoglobin, WBC, lymphopenia, neutrophil and platelet counts were observed before the 1st vaccination and were not worsened throughout the vaccination term. No other adverse events were seen throughout the peptide vaccination. Therefore, the multiple-peptide vaccine therapy was well-tolerated up to a dose of 3 mg for each peptide, or 9 mg total.

Table 2 Summary of adverse events

Adverse events	Total (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)
Hemoglobin	5 (55.6)	3 (33.3)	2 (22.2)	0	0
WBC	2 (22.2)	0	2 (22.2)	0	0
Lymphopenia	3 (33.3)	2 (22.2)	0	1 (11.1)	0
Neutrophil	2 (22.2)	0	2 (22.2)	0	0
Platelet	3 (33.3)	3 (33.3)	0	0	0
Injection site reaction	9 (100)	4 (44.4)	5 (55.6)	0	0

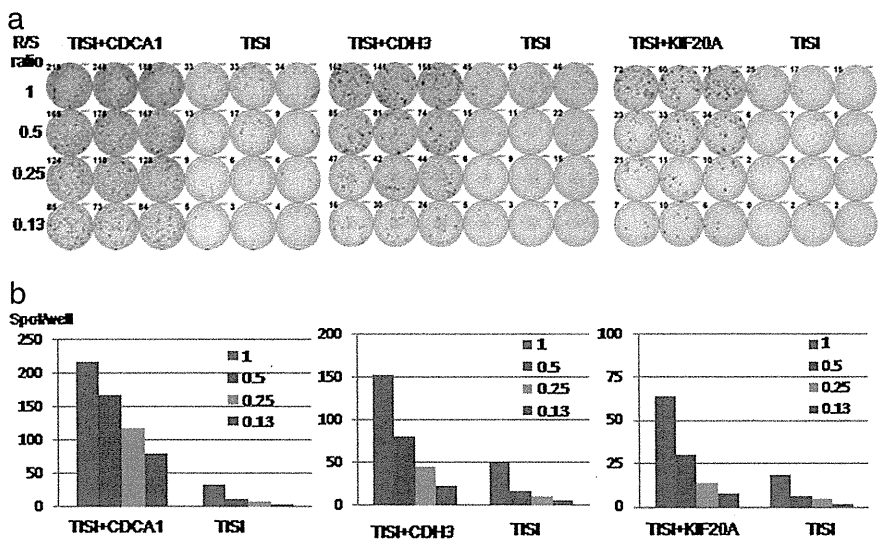

Figure 1 Immunological monitoring assay of T cell response to CDCA1, CDH3 and KIF20A in patient 8. (a) IFN-γ ELISPOT assay for CDCA1, CDH3 and KIF20A. **(b)** IFN-γ positive spots at several R/S ratio.

Antigen-specific immune response

Antigen-specific immune response was assessed by the ELISPOT assay. In the assay, CDCA1, CDH3 and KIF20A peptides-specific IFN-γ spots were observed in 9 of 9 patients (Figure 1). The response to every antigen in every patient was determined using our algorithm (Additional file 1: Figure S1) is summarized in Tables 3 and 4. The number of peptide-specific IFN-γ spots gradually increased with the number of vaccinations (Figure 2). These immune responses were not found for all antigens or for all patients after 8 vaccinations when the clinical assessments were done, but they were observed after the judgment of PD in some patients with continuous peptide vaccination. The group receiving 3 mg of each peptide tended to show stronger CTL induction throughout the course of this study.

Clinical response

As shown in Tables 3, 5 patients had stable disease (SD) and 4 had progressive disease (PD) as judged after the 8th vaccination. The 8 patients continued to be administered the vaccination and 4 of them continued to receive the vaccination for as long as possible, even if their disease developed PD. The vaccinations eventually achieved disease stability in the patients who received the long-term vaccination (Figure 3 shows the CT scan of patient 4), but in the end their diseases progressed, and they had all died within 2 years of the 1st vaccination. Their median progression-free survival (PFS) for all patients after the first vaccination was 3.4 months (95% CI: 0-7.0) and the 1 year PFS was 11.1% (Figure 4a). The median overall survival (OS) for all patients was 9.7 months (95% CI: 1.4-18.0) and the 1 year OS was 22.2% (Figure 4b).

Table 3 Summary of clinical outcome and immunological response

Patients	No. of vaccine	Clinical response*	PFS (days)	OS (days)	After vaccine	ISR** (Grade)	Peptide-specific CTL		
							CDCA1	CDH3	KIF20A
1	16	SD	212	310	No	2	0	1+	1+
2	8	PD	53	134	No	1	2+	0	1+
3	18	SD	127	205	No	1	1+	2+	1+
4	19	SD	505	659	Yes	2	1+	0	1+
5	27	SD	225	290	No	2	1+	1+	3+
6	10	SD	101	101		1	1+	1+	0
7	13	PD	64	164	Yes	1	2+	1+	2+
8	24	PD	64	353	Yes	2	3+	3+	3+
9	25	PD	57	380	Yes	2	2+	3+	2+

*SD: stable disease; PD: progressive disease.
**ISR: injection site reaction.

Table 4 CTL response to CDCA1, CDH3 and KIF20A

Peptide dose	No.	Course	CTL response			
			CDCA1	CDH3	KIF20A	Positive control
1 mg	1	Pre	-	+	-	+++
		Post 1	-	-	+	+++
		Post 2	-	-	-	+++
	2	Pre	-	-	-	+++
		Post 1	++	-	+	+++
	3	Pre	+	-	-	+++
		Post 1	NA*	NA	NA	+++
		Post 2	-	+	-	+++
		Post 3	+	++	-	+++
		Post 4	+	-	+	+++
2 mg	4	Pre	+	-	+	+++
		Post 1	-	-	+	+++
		Post 2	+	-	+	+++
		Post 3	-	-	-	+++
	5	Pre	-	-	-	+++
		Post 1	-	-	-	+++
		Post 2	-	-	+	+++
		Post 3	+	-	+++	+++
		Post 4	-	-	++	+++
		Post 5	+	+	-	+++
		Post 6	-	-	-	+++
	6	Pre	-	+	-	+++
		Post 1	+	+	-	+++
		Post 2	+	+	-	+++
3 mg	7	Pre	NA	NA	NA	+++
		Post 1	-	-	-	+++
		Post 2	++	+	++	+++
	8	Pre	-	-	+	+++
		Post 1	-	+	-	+++
		Post 2	++	+	++	+++
		Post 3	+++	+++	+	+++
		Post 5	+++	+++	+++	+++
	9	Pre	+	-	+	+++
		Post 1	++	+	+	+++
		Post 2	-	+	-	+++
		Post 3	-	+++	+	+++
		Post 4	++	-	-	+++
		Post 6	++	+	++	+++

*NA: not analyzed.

Univariate analysis of the prognostic factors

The results of the univariate analysis of the prognostic factors are described in Table 5. The patients who developed grade 2 local skin reaction at the vaccination site showed a median OS of 11.8 months (95% CI: 8.7-14.9). This was better than the OS of patients with grade 1 local skin reaction of 4.5 months (95% CI: 2.4-6.5). There was a significant difference between these 2 groups ($p = 0.003$)

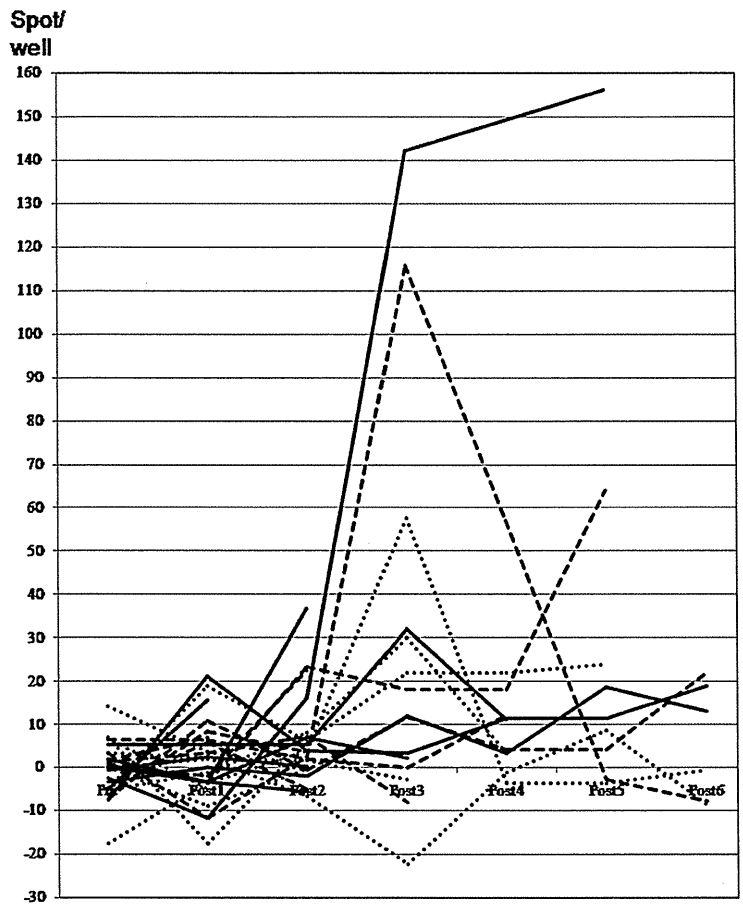

Figure 2 Kinetics of the peptide-specific T cell response after vaccination. Specific spots were counted as shown in Additional file 1: Figure S1 to CDCA1 (solid line), KIF20A (dotted line) and CDH3 (dashed line). The R/S ratio was 0.50.

Table 5 Prognostic factors of PFS or OS

Factors	PFS	OS
Sex (male/female)	0.426	0.302
Age (≧61/<61)	0.706	0.084
CRP (≧1.5/<1.5)	0.654	0.832
Hemoglobin (≧12/<12)	0.351	0.435
Lymphocyte (%) (≧27/<27)	0.145	0.132
Lymphocyte (number) (≧1500/<1500)	0.488	0.900
CDCA1 CTL spots (≧2+/<2+)	0.004	0.870
CHD3 CTL spots (≧2+/<2+)	0.235	0.611
KIF20A CTL spots (≧2+/<2+)	0.486	0.840
CXCR3 + CCR4- T cells (≧8%/<8%)	0.046	0.966
CXCR3 + CCR4- T cells (up/down)	0.007	0.604
Injection site reaction (≧Grade2/<Grade2)	0.145	0.003
Continuous vaccination after PD (Yes/No)	-	0.007

(Figure 4c). The OS of the patients with continuous vaccinations was also better than that of the patients who stopped the vaccination as their disease progressed. The OS of the patients with continuous vaccinations was 5.1 months (95% CI: 0-11.3) and that of the patients who stopped the vaccination was 2.6 months (95% CI: 2.1-3.1). There was a statistically significant difference between these 2 groups ($p = 0.007$) (Figure 4d).

Analysis of the relation between better PFS and the type 1 host immune condition

The type 1 host immune condition was analyzed based on the ratio of the CXCR3 + CCR4- T cell population (mean: 8.1%). The patients with a CXCR3 + CCR4- T cell population of more than 8% showed longer PFS and the patients whose CXCR3 + CCR4- T cell population increased after the 4^{th} vaccinations also showed longer PFS (Table 5). Most of the patients with stable disease (SD) showed an increase in the CXCR3 + CCR4- type 1 T cell population (Figure 5a) and a decrease in the CXCR3-

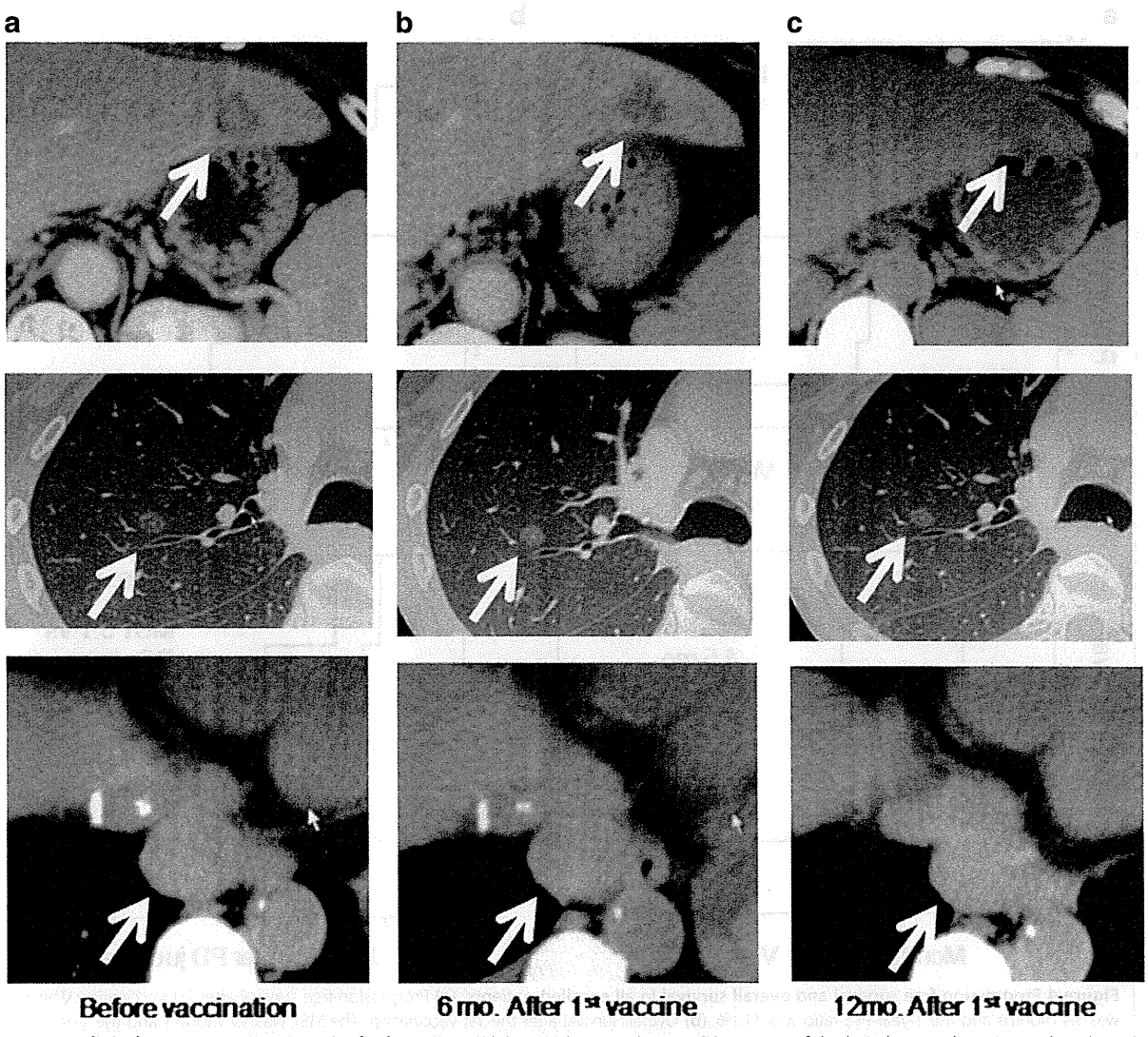

Figure 3 Clinical response assessment in the longest-surviving patient, patient 4. CT imaging of the liver, lung and para-aortic lymph node metastasis before vaccination **(a)**, 6 months after 1st vaccination **(b)** and 12 months after 1st vaccination **(c)**. The tumor sizes were stable across the 505 days after 1st vaccination.

CCR4+ type 2 T cell population after the 4th vaccination (Figure 5b). In contrast, all the patients with progressive disease (PD) showed a decrease in the CXCR3 + CCR4- T cell population and an increase in the CXCR3-CCR4+ T cell population after the 4th vaccination. Therefore, the type 1 host immune condition was suspected to be an important factor for achieving disease stability through the induction of peptide-specific CTLs *in vivo*.

Discussion

It is difficult to detect BTCs in the early stage, and this difficulty is partly responsible for the poor prognosis of the disease. Operation is the most effective treatment, but recurrence or metastasis occurs at a high rate following curative operation. Other than operation, chemotherapy is the only therapy currently available. Nevertheless, GEM plus CDDP, which is the best choice of chemotherapy for BTC, only yields a median overall survival of 11.7 months and progression-free survival of 8.0 months. However, a median overall survival of 9.7 months was realized using a three-peptide vaccination after the failure of standard chemotherapies, and thus this modality might have potential for improving the OS in patients with BTC.

In this study, we selected three new peptides for BTC. CDCA1 is a molecular linker between the kinetochore attachment site and the tubulin subunits. CDH3 is a

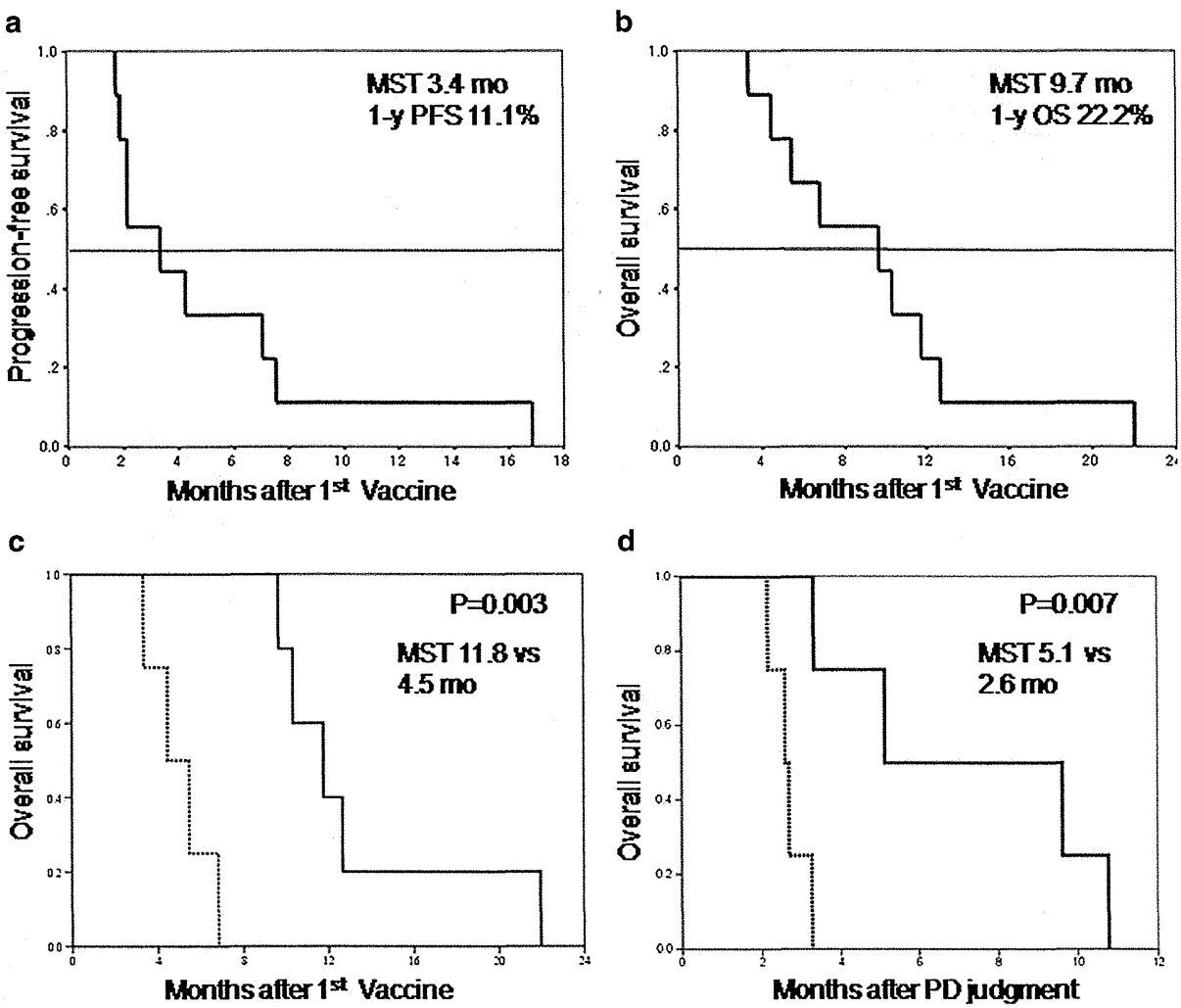

Figure 4 Progression-free survival and overall survival in all enrolled patients. (a) Progression-free survival after 1st vaccination. The MST was 3.4 months and the 1-year PFS ratio was 11.1%. **(b)** Overall survival after the 1st vaccination. The MST was 9.7 months and the 1-year OS ratio was 22.2%. **(c)** Overall survival of the patients with Grade 2 ISR (solid line) compared to that of the patients with Grade 1 ISR (dotted line). There was a statistically significant difference between these 2 groups ($p = 0.003$). **(d)** Overall survival of the patients receiving continuous vaccination (solid line) compared to that of the patients who stopped the vaccination at the determination of PD (dotted line). There was a statistically significant difference between these 2 groups ($p = 0.007$).

cell-cell adhesion molecule and takes part in the signal transduction for cell growth and differentiation. KIF20A is a conserved motor domain that binds to microtubules. We previously reported on other combinations of peptides for vaccination in patients with BTC. We found that DEPDC1 and LY6K strongly induced antigen-specific CTLs after the 4th vaccination. In contrast, IMP3 and TTK induced CTLs only weakly and late after the vaccination. The three-peptide vaccination in this study also showed a delayed induction of peptide-specific CTLs. We speculate that this might have been due to differences in the abilities of the peptides to induce a host immune response. In these cases, most of the patients were judged as having PD before the strong CTLs were induced *in vivo*. The protocol of this study permitted the continuation of vaccination after the diagnosis of PD, and the patients who continued vaccination after the start of PD had a strong CTL induction and showed better prognosis compared with other patients who stopped the vaccination at the time of PD judgment. Therapeutic cancer vaccination seems to show a delayed clinical effect [17] and the early discontinuance of vaccination might cause a misappraisal of the true capacity of the cancer vaccine. Therefore, there may be need of a further clinical study in which

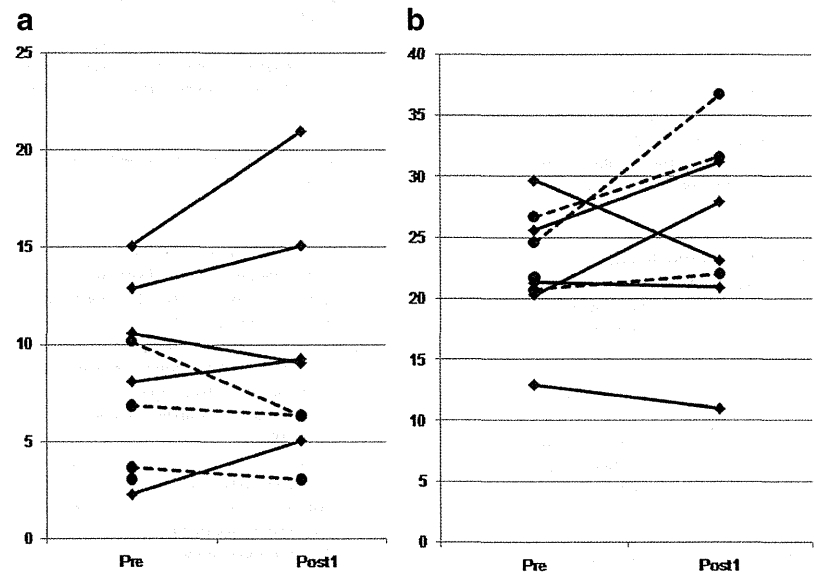

Figure 5 Kinetics of type 1 T cell population and type 2 T cell population after vaccination. (a) The population of CXCR3 + CCR4- type 1 T cells increased in most of the patients with stable disease (SD) (solid line) and decreased all the patients with progressive disease (PD) (dashed line) after 4 vaccinations. **(b)** The population of CXCR3-CCR4+ type 2 T cells was decreased in most of the patients with SD (solid line) and increased in all the patients with PD (dashed line) after 4 vaccinations.

the vaccination is continued for the long term even after a diagnosis of PD in the early stage of study, because the induction of CTLs was often fairly slow.

Several phase III randomized studies of cancer vaccines have been performed [18], but very few of them were successful [19]. The clinical efficacy of cancer vaccines is currently limited because of the immune checkpoint. Anti-CTLA-4 mAb (ipilimimab) [20], anti-PD-1 [21,22] and PD-L1 [23] have shown promising results in some clinical studies. Although the blockage of the immune checkpoint itself is an effective therapy, it also seems to be necessary to administer the cancer antigen-specific CTLs. Cancer peptide vaccines could induce antigen-specific CTLs *in vivo*, so the combination of a cancer vaccine and immune checkpoint blockade would be a more successful anti-cancer strategy in the future. Another approach might be to improve the immune condition of the host. A proper number of lymphocytes, especially type 1 T cells, seems to be needed to acquire a good immune response, which in turn has been associated with a better prognosis [24,25]. In order to ensure the success of clinical trials, a new classification method or biomarker is needed to stratify patients according to their immune condition [26-30].

In this report, a new three-peptide vaccine was shown to be safe and to elicit an effective immune response in patients with advanced biliary tract cancer. No patients exhibited a CR or PR, but it was suggested that the OS could be extended by continuous administration of this vaccination. In order to establish this immunotherapy as the standard therapy for biliary tract cancer, it will be necessary to assess the survival improvement in a phase II/III randomized trial with an appropriate number of subjects. We have reported 4 peptides previously and 3 new peptides in this study. All 7 of these peptides could be used simultaneously for patients with advanced BTC, or one or more of them could be selected for patients in an adjuvant setting after operation and examination of the antigen expression profile in their tumor cells.

Conclusions

We have shown that a cancer peptide vaccine therapy using a mixture of three peptides was well tolerated and could induce peptide-specific CTLs in patients with advanced BTC. The peptide vaccine was found to have a sufficient effect on survival to merit further evaluation in clinical trials.

Additional file

Additional file 1: Algorithm of the assessment of CTL response to antigen.

Abbreviations

BTC: Biliary tract cancer; CBDCA: Carboplatin; CDCA1: Cell division cycle associated 1; CDDP: Cisplatin; CDH3: Cadherin 3; CTCAE: Common terminology criteria for adverse events; CTL: Cytotoxic T lymphocyte; DTX: docetaxcel; ELISPOT: Enzyme-linked immunospot; GEM: Gemcitabine; HPLC: High-performance liquid chromatography; IFA: Incomplete Freund's adjuvant; KIF20A: Kinesin family member 20A; OS: Overall survival; PBL: Peripheral blood lymphocyte; PFS: Progression-free survival; SD: Stable disease; TS-1: Tegafur-gimeracil-oteracil potassium.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AA participated as the principle investigator of the study and drafted the manuscript. NT and NM participated the acquisition of data. KT coordinated the analysis of immunological data. YK and RO participated in the design and coordination of the study. TO and MY participated in the study supervision. All authors read and approved the final manuscript.

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JOURNAL OF TRANSLATIONAL MEDICINE

RESEARCH Open Access

A phase I study of combination vaccine treatment of five therapeutic epitope-peptides for metastatic colorectal cancer; safety, immunological response, and clinical outcome

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Abstract

Background: To evaluate the safety of combination vaccine treatment of multiple peptides, phase I clinical trial was conducted for patients with advanced colorectal cancer using five novel HLA-A*2402-restricted peptides, three peptides derived from oncoantigens, ring finger protein 43 (RNF43), 34 kDa-translocase of the outer mitochondrial membrane (TOMM34), and insulin-like growth factor–II mRNA binding protein 3 (KOC1), and the remaining two from angiogenesis factors, vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2.

Methods: Eighteen HLA- A*2402-positive colorectal cancer patients who had failed to standard therapy were enrolled in this study. 0.5 mg, 1.0 mg or 3.0 mg each of the peptides was mixed with incomplete Freund's adjuvant and then subcutaneously injected at five separated sites once a week. We also examined possible effect of a single site injection of "the cocktail of 5 peptides" on the immunological responses. ELISPOT assay was performed before and after vaccinations in the schedule of every 4 weeks.

Results: The vaccine treatment using multiple peptides was well tolerated without any severe treatment-associated systemic adverse events. Dose-dependent induction of peptide-specific cytotoxic T lymphocytes was observed. The single injection of "peptides cocktail" did not diminish the immunological responses. Regarding the clinical outcome, one patient achieved complete response and 6 patients revealed stable disease for 4 to 7 months. The median overall survival time (MST) was 13.5 months. Patients, in which we detected induction of cytotoxic T lymphocytes specific to 3 or more peptides, revealed significantly better prognosis (MST; 27.8 months) than those with poorer immune responses (MST; 3.7 months) ($p = 0.032$).

Conclusion: Our cancer vaccine treatment using multiple peptides is a promising approach for advanced colorectal cancer with the minimum risk of systemic adverse reactions.

Clinical trial registration: UMIN-CTR number UMIN000004948.

Keywords: Peptide vaccine, Peptide cocktail, Colorectal cancer, Phase I study

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Background

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death in industrialized countries [1]. In the last decade, the combined regimens of multiple anticancer drugs have been applied and markedly improved the survival of patients with CRC at stages III and IV [2]. However, many patients often face to progression of the diseases due to chemo-resistance.

Recent development in genome-based technologies has enabled us to obtain comprehensive gene expression profiles of malignant cells compared with normal cells [3]. By applying cDNA microarray technology coupled with laser micro-dissection, we had identified three oncoantigens, ring finger protein 43 (RNF43) [4], 34 kDa-translocase of the outer mitochondrial membrane (TOMM34) [5], and KOC1 (IMP-3; IGF-II mRNA binding protein 3) [6,7], as targets for development of cancer peptide vaccines for CRC. An oncoantigen was defined as a molecule with high immunogenicity in our immune system and with oncogenic function that plays a critical role in the growth of tumor cells. Since the oncoantigen is essential for the cell growth, the probability of immune escape of cancer cells by reducing or lacking these proteins is expected to be low [8,9]. In addition, these three molecules are specifically expressed in cancer cells, the risk of autoimmune reactions by vaccine treatment using the peptides derived from these proteins is expected to be minimum [4-6].

Although immunotherapy using tumor infiltrating cells (TIL) or vaccine treatment have been expected as a promising modality to treat cancer, recent reports have indicated several mechanisms in tumor tissues to protect cancer cells from immune attacks [10]. For example, the limitation of antitumor effects of cytotoxic T lymphocytes (CTL) was explained by tumor cell heterogeneity; a subset of tumor cells revealed downregulation or loss of expressions of human leukocyte antigen (HLA) or targeted antigen proteins [11,12]. The growth of solid neoplasms always accompanies with neovascularization [13] which is associated with the expression of vascular endothelial growth factor receptor 1 (VEGFR1) [14] and VEGFR2 [15]. These two molecules were highly expressed in tumor vascular endothelial cells. Hence, our vaccine treatment including the peptides derived from VEGFR1 and VEGFR2 is also able to target neovascular endothelial cells, suppress neovascurization, and then reduce the energy and oxygen supply into the tumor tissues.

In this study, since an HLA-A*2402 allele is the most common HLA-A allele in the Japanese population with the allelic frequency of approximately 60% [16], we selected five HLA-A*2402-restricted peptides derived from RNF43, TOMM34, KOC1, VEGFR1, and VEGFR2 for the clinical trial. The purpose of this study was to evaluate the safety and biological responses of these five peptides. Additionally, we compared the immunological responses of the separate injections of each of five peptides with those of the single injection of a cocktail of five peptides. We here demonstrate the safety of these peptides and a promising result of our cocktail treatment of five peptides for the improvement of prognosis of advanced CRC.

Patients and methods

Patients and eligibility criteria

The study protocol was approved by the Institutional Ethics Review Boards of Yamaguchi University (H18-82), was carried out in accordance with the Helsinki declaration on experimentation on human subjects, and was registered in the UMIN Clinical Trials Registry as UMIN000004948. Written informed consent was obtained from the patients at the time of enrollment. Patients were eligible for enrollment (1) when they had histologically confirmed CRC without indication of surgical resection, (2) when they had failed to respond to prior standard chemotherapy or were intolerable to the standard therapy, and (3) when they were HLA-A*2402-positive by DNA typing. We monitored for at least 4 weeks from the termination of the prior treatment to the beginning of the vaccine treatment, in order to wait patients' full recovery from adverse events with grade 3 or higher according to the Common Terminology Criteria for Adverse Events version3.0 (CTCAE). The patients were required to have an Eastern Cooperative Oncology Group performance status (PS) of 0 to 2, to be older than 20 years of age and to have a life expectancy of at least 3 months. Adequate bone marrow function (white blood cell count $\geq$2,000/mm^3 and platelet count $\geq$75,000/mm^3), renal function (serum creatinine $\leq$2.0 mg/dl) and liver function (transaminase within 3.0 times the institution's upper limit of normal) were required. Patients were excluded if they were pregnant, had severe ischemic heart disease, had active infectious disease, had any steroid-dependent autoimmune diseases, or had any prior peptide vaccination therapies.

Peptides

The RNF43-721 (NSQPVWLCL) [17], TOMM34-299 (KLRQEVKQNL) [5], KOC1(IMP-3)-508 (KTVNELQNL) [18], VEGFR1-1084 (SYGVLLWEI) [19] and VEGFR2-169 (RFVPDGNRI) [20] peptides restricted with HLA-A*2402 were synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA) according to a standard solid-phase synthesis method and purified by reverse-phase high performance liquid chromatography (HPLC). The purity (>95%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. Endotoxin levels and the bio-burden of these peptides were tested and determined to be within