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

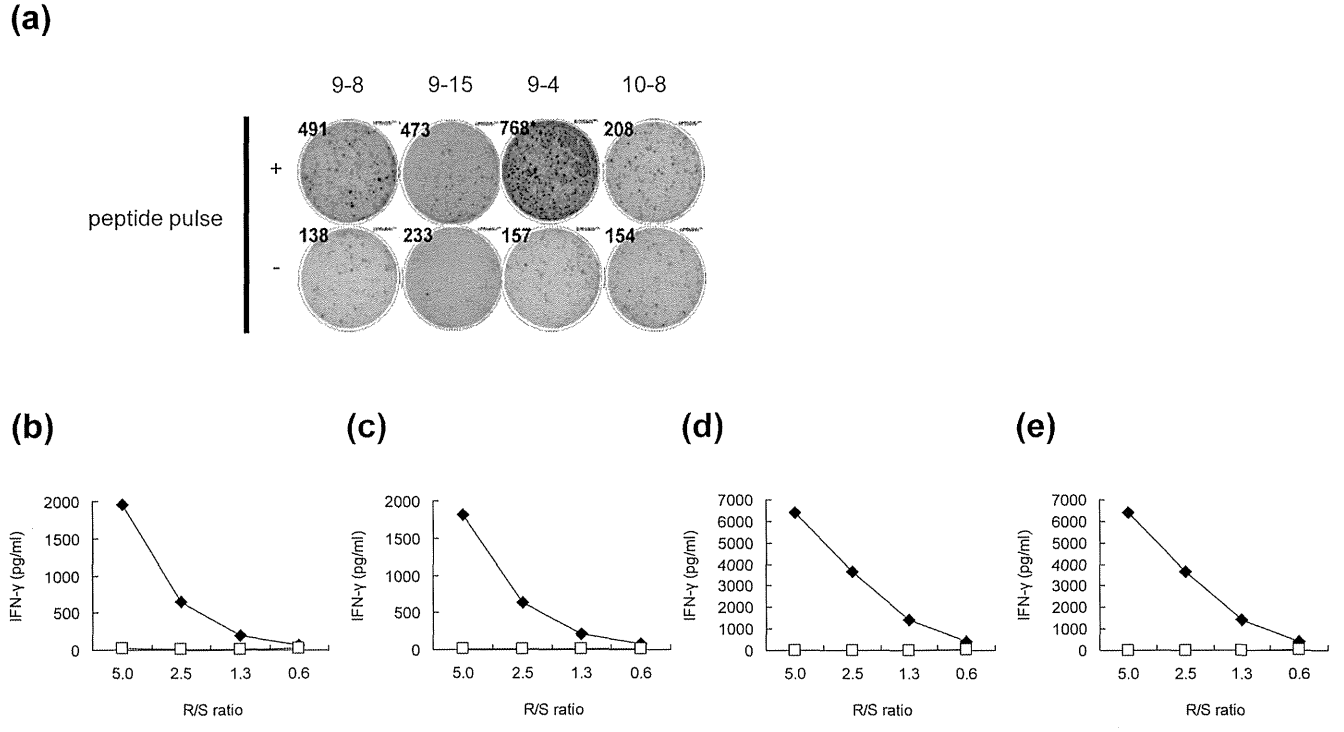

Figure 1. The IFN-γ production in response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide. (a) The IFN-γ production from cells induced by the indicated peptide-pulsed DCs was examined by an ELISPOT assay using T2 cells. "+" indicates the wells in which cells were stimulated with T2 cells pulsed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

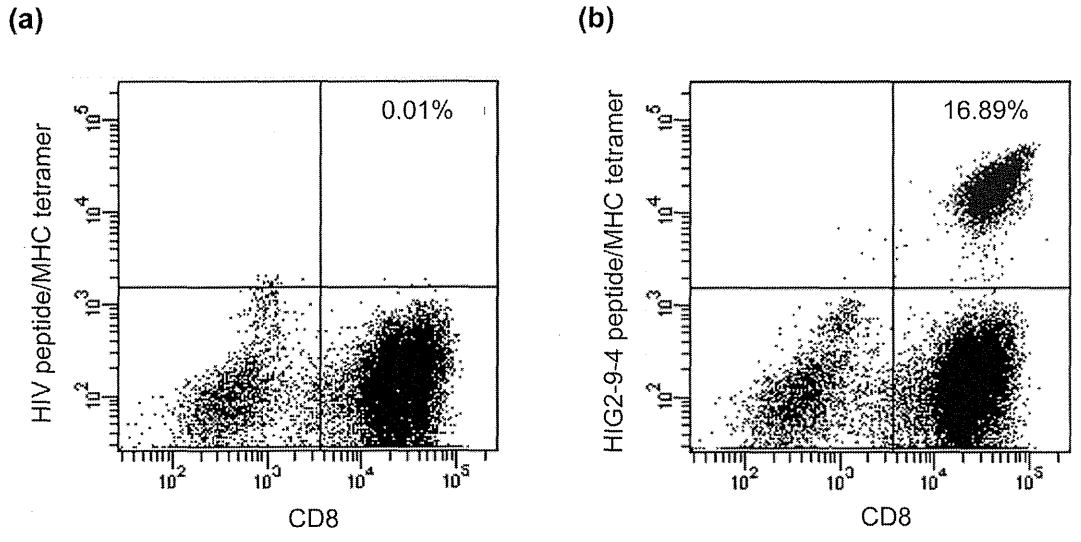

Figure 2. The expression of a HIG2-9-4 peptide-specific T cell receptor on CD8+ T cells. The expression of the HIG2-9-4 peptide-specific T cell receptor was examined on $CD3^+CD4^-$ cells following CTL expansion culture of HIG2-9-4 peptide-induced CTLs. (a) A quadrant gate was set based on the staining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

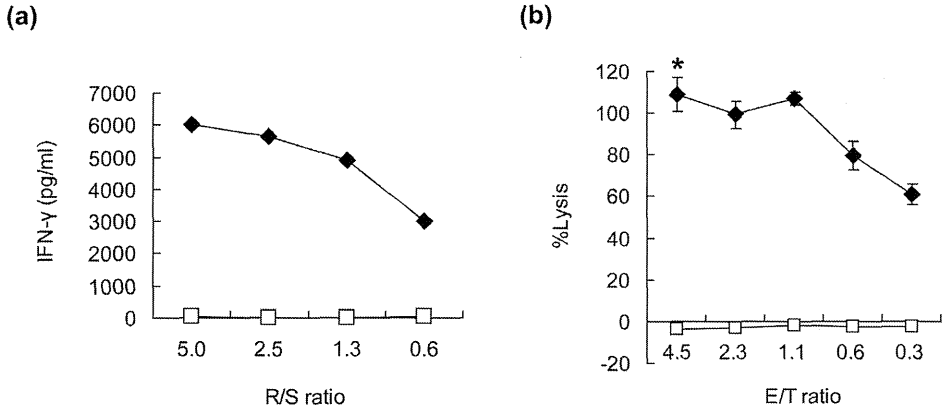

Figure 3. The IFN-γ production and cytotoxic activity of a HIG2-9-4 peptide-specific CTL clone. (a) An established CTL clone was stimulated with T2 cells pulsed with the HIG2-9-4 peptide (closed diamonds) or HIV peptide (open squares). The IFN-γ production in the culture supernatant was examined by ELISA.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

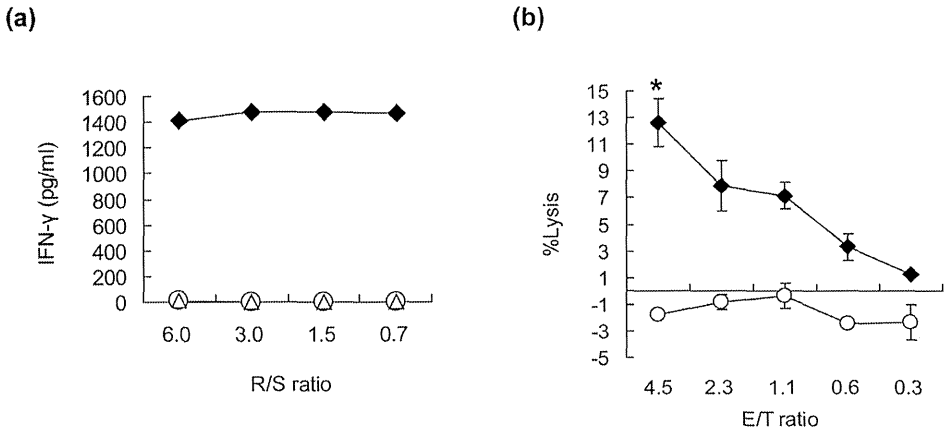

Figure 4. The recognition of HIG2 and HLA-A*02:01-expressing cells by a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:01 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:01 alone (open triangle),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

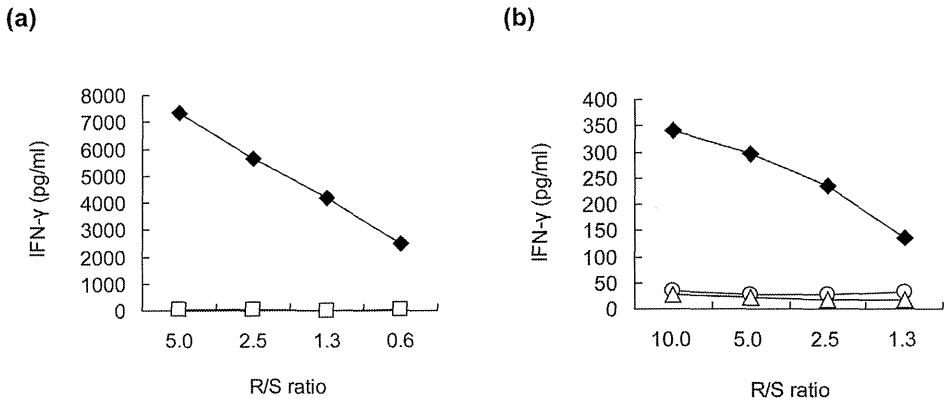

Figure 5. The HLA-A*02:06-restricted response of a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was induced from HLA-A*02:06-positive PBMCs, and stimulated with HLA-A*02:06-positive PSCCA0922 cells pulsed with the HIG2-9-4 peptide (close diamond) or HIV peptide (open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

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

Author Contributions

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

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

TAKESHI IIDA[1], MAKOTO IWAHASHI[1], MASAHIRO KATSUDA[1], KOICHIRO ISHIDA[1], MIKIHITO NAKAMORI[1], MASAKI NAKAMURA[1], TEIJI NAKA[1], TOSHIYASU OJIMA[1], KENTARO UEDA[1], KEIJI HAYATA[1], HIRONAO YASUOKA[2] and HIROKI YAMAUE[1]

[1]Second Department of Surgery, School of Medicine, and [2]Division of Pathology, Department of Clinical Laboratory Medicine, School of Medicine, Wakayama Medical University, Wakayama 641-8510, Japan

Received October 23, 2013; Accepted November 11, 2013

DOI: 10.3892/or.2013.2911

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

Correspondence to: Dr Makoto Iwahashi, Second Department of Surgery, School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8510, Japan
E-mail: makoto@wakayama-med.ac.jp

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

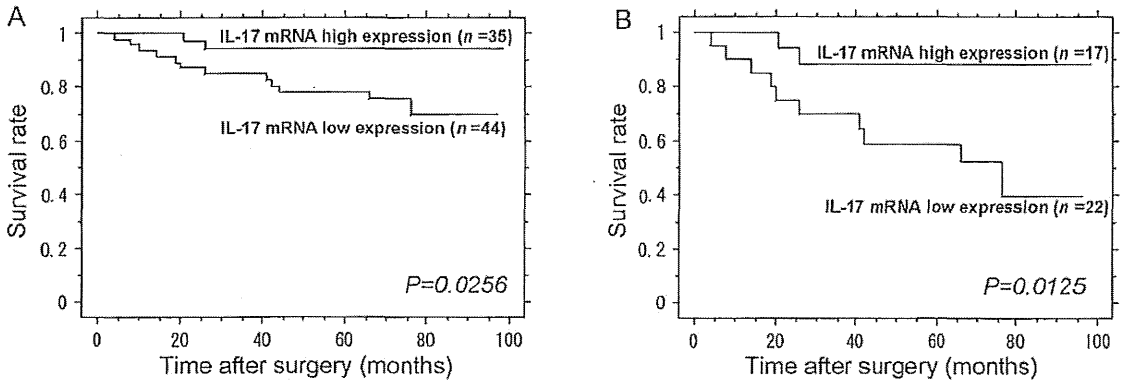

Figure 1. (A) Overall survival curves of the 79 gastric cancer patients who underwent curative R0 resection stratified according to IL-17 mRNA high or low expression in peritoneal lavage as determined by real-time RT-PCR. A significant difference in survival was noted between the IL-17 mRNA high expression 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

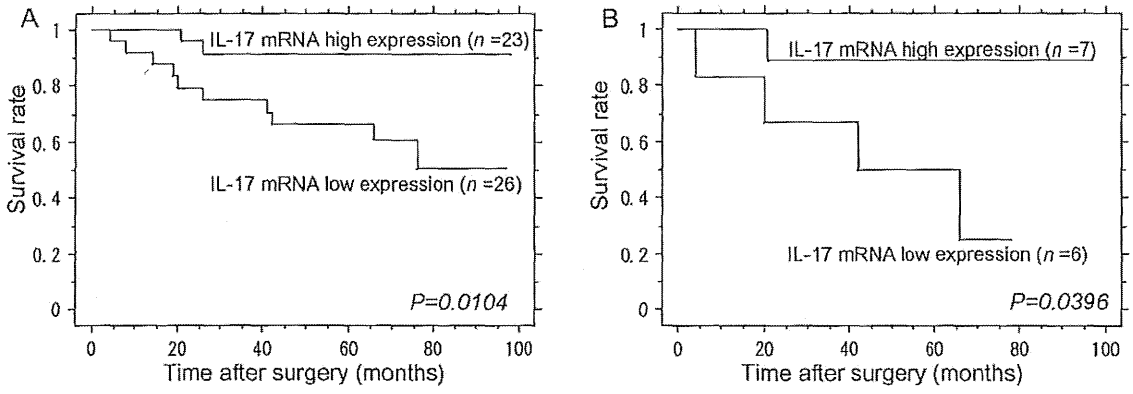

Figure 2. Overall survival curves of the 79 gastric cancer patients who underwent curative resection with pT2/3/4 stage tumors stratified according to IL-17 mRNA high or low expression in peritoneal lavage. (A) Overall survival of the 79 patients subdivided according to IL-17 mRNA high or low expression 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,

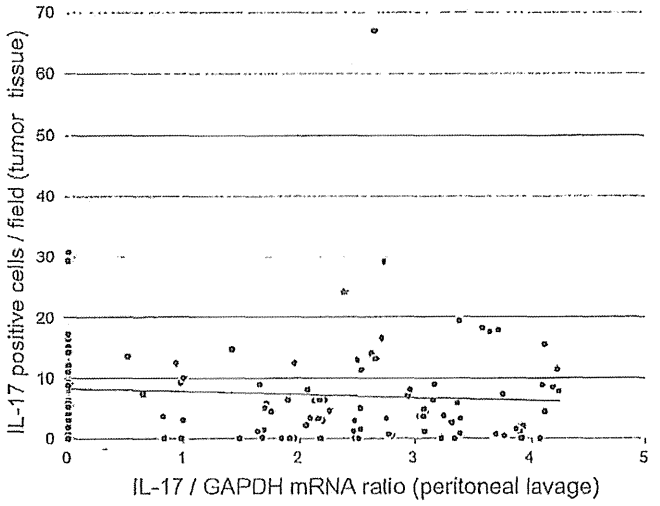

Figure 3. Correlation between IL-17-positive cells in tumor tissues and IL-17 mRNA expression in peritoneal wash. Immunohistochemical staining for IL-17 in primary tumor tissues was performed in the same patient samples whose peritoneal lavage was examined for IL-17 mRNA. The longitudinal axis shows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

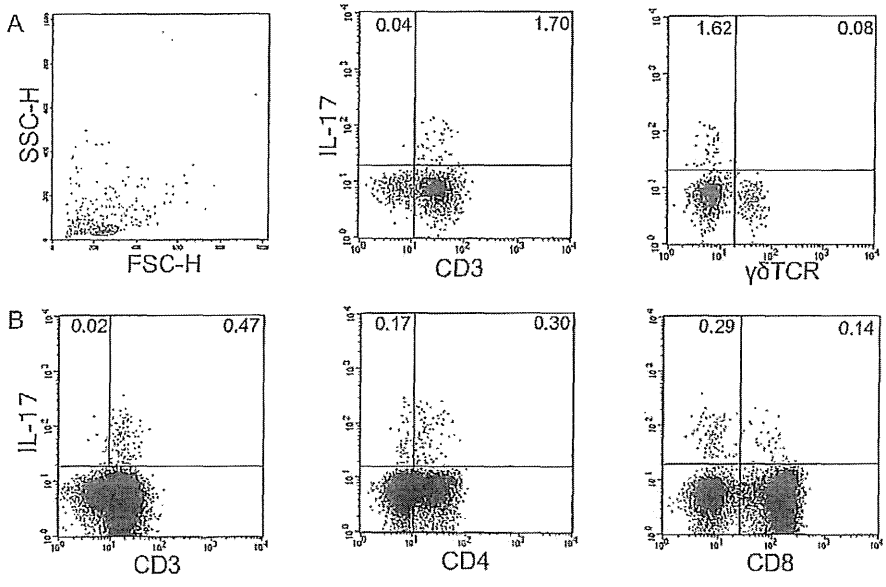

Figure 4. Flow cytometric analysis using anti-IL-17, -CD3, -CD4, -CD8 and -γδ TCR antibodies to identify IL-17-producing cells in peritoneal washes. (A) Cells were stained with PE-anti-IL-17 mAb, PerCP-anti-CD3 mAb and FITC-anti-γδ TCR mAb after stimulation with PMA and ionomycin for 5 h. (B) Cells were 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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Cancer Chemother Pharmacol (2014) 73:97–102
DOI 10.1007/s00280-013-2323-6

ORIGINAL ARTICLE

Phase II clinical study of alternate-day oral therapy with S-1 as first-line chemotherapy for locally advanced and metastatic pancreatic cancer

Hiroki Yamaue · Sohei Satoi · Takamasa Kanbe · Motoki Miyazawa · Masaji Tani · Manabu Kawai · Seiko Hirono · Kenichi Okada · Hiroaki Yanagimoto · A-Hon Kwon · Tomoyuki Mukouyama · Hiroaki Tsunoda · Kazuo Chijiiwa · Jiro Ohuchida · Jun Kato · Kazuki Ueda · Taketo Yamaguchi · Shinichi Egawa · Kazuhiko Hayashi · Tetsuhiko Shirasaka

Received: 26 May 2013 / Accepted: 10 October 2013 / Published online: 22 October 2013

Abstract
Purpose Based on the results of first-line chemotherapy for advanced pancreatic cancer, S-1 was confirmed to be non-inferior to gemcitabine. However, the recommended regimen of 4 weeks of administration followed by 2 weeks of drug withdrawal frequently causes adverse effects. On the other hand, we experienced in clinical practice that alternate-day administration of S-1 reduced adverse effects and were tolerable for advanced pancreatic cancer patients unwilling to continue the standard daily administration. We therefore conducted a multicenter cooperative prospective study to compare daily with alternate-day administration of S-1 for advanced pancreatic cancer.
Methods Patients with advanced pancreatic cancer were eligible for enrollment in this trial. S-1 was administered at a dose of 40–60 mg twice daily, calculated according to body surface area, on Monday, Wednesday, Friday, and Sunday. Each treatment cycle was 42 days. The primary end point was overall survival (OS). Secondary end points were safety, response rate (RR), progression-free survival (PFS), and time to treatment failure (TTF).
Results Forty-eight patients were evaluable for response. OS as the primary end point was 8.4 months (95 % CI 5.4–10.8), and the 1-year survival rate was 29.2 %. PFS was 5.5 months, and TTF was 3.9 months. RR was 10.4 %, and the disease control rate was 79.2 %. Grade 3/4 hematological and non-hematological toxicities were minor. All of these adverse reactions were tolerable and reversible.
Conclusions The current data demonstrate the mitigation of adverse effects with alternate-day administration of

H. Yamaue (✉) · M. Miyazawa · M. Tani · M. Kawai · S. Hirono · K. Okada · J. Kato
Second Department of Surgery, Wakayama Medical University, 10-31 Kimiidera, Wakayama City, Wakayama 641-8510, Japan
e-mail: yamaue-h@wakayama-med.ac.jp

S. Satoi · H. Yanagimoto · A.-H. Kwon
Department of Surgery, Kansai Medical University, Moriguchi, Japan

T. Kanbe · T. Mukouyama · H. Tsunoda
Department of Internal Medicine, San-in Rosai Hospital, Yonago, Japan

K. Chijiiwa · J. Ohuchida
Department of Surgical Oncology and Regulation of Organ Function, Miyazaki University School of Medicine, Miyazaki, Japan

K. Ueda
Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan

T. Yamaguchi
Department of Gastroenterology, Chiba Cancer Center, Chiba, Japan

S. Egawa
Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan

K. Hayashi
Department of Chemotherapy and Palliative Care, Tokyo Women's Medical University, Tokyo, Japan

T. Shirasaka
Kitasato Institute for Life Science, Kitasato University, Tokyo, Japan

S-1, and this appears to be a more sustainable option for advanced pancreatic cancer.

Keywords Alternate-day · Oral therapy · S-1 · Chemotherapy · Pancreatic cancer

Introduction

Pancreatic cancer is known for its most unfavorable prognosis, with a 5-year survival rate of approximately 9 % [1]. Surgery is the only treatment expected to completely eradicate the condition, but 80 % of patients are diagnosed with the cancer when they have already reached an inoperable status. For unresectable patients, chemotherapy is commonly used, and since Burris et al. [2] demonstrated the significant efficacy of gemcitabine (GEM) in prolonging life expectancy over 5-fluorouracil (5-FU) in a comparative study conducted in 1997, GEM has become the major chemotherapeutic agent; yet the median survival time (MST) of unresectable patients treated with GEM remains 5–7 months, suggesting that its effect on survival is inadequate. In Japan, a tegafur/gimeracil/oteracil combination capsule (S-1) is used to treat various types of cancers, and a domestic late phase II trial to evaluate S-1 in patients with pancreatic cancer showed that the response rate (RR) was 37.5 %, and the median progression-free survival time (PFS) was 3.7 months [3]. Furthermore, phase III studies (GEST study) of GEM + S-1 combination therapy (GS therapy), GEM, and S-1 were conducted in Japan and Taiwan in patients with unresectable advanced pancreatic cancer, and a controlled trial of the effects of GEM versus S-1 on survival showed that the hazard ratio (HR) was 0.96 (97.5 % confidence interval [CI] 0.78–1.18), demonstrating the non-inferiority of S-1 to GEM [4]. The standard regimen of S-1 treatment used in the GEST study, a 4-week daily administration followed by a 2-week rest period, has frequently been associated with digestive symptoms such as anorexia, diarrhea, and stomatitis, which can result in a need to discontinue treatment altogether. It is still unclear, however, whether the therapeutic efficacy of modified regimens with reduced overall dosage or of shortened treatment cycles is as effective as the standard dosage regimens in patients reporting adverse events with S-1. In recent years, an alternate-day administration of S-1 has been reported to alleviate adverse reactions without reducing the efficacy of treatment. Arai et al. [5] started treatment for 92 patients with advanced recurrent gastric cancer with a schedule of administration for 4 consecutive weeks followed by a 2-week rest period, but later switched to an alternate-day regimen for 72 patients, upon their own request, in whom the therapy had to be interrupted due to grade 1 or higher non-hematological toxicities (31.5 %). As a result, the number of patients with grade 2 or higher non-hematological toxicities dropped remarkably to 2 (2.8 %), and the average duration of therapy for the alternate-day regimen was extended to 272 days, as opposed to 47 days with daily administration. In the study, time to progression (TTP) was 170 days, MST was 11 months, and the disease control rate in the evaluable patients was reported to be 53 % (31/58). Since we have observed the reduction in adverse events and the long-term administration rendered possible by replacing the S-1 regimen with an alternate-day administration, we also conducted a clinical phase II study of alternate-day S-1 administration for the treatment of advanced recurrent pancreatic cancer in an attempt to alleviate adverse reactions and to achieve long-term administration.

Patients and methods

Eligibility

The eligibility criteria for patients were as follows: pancreatic cancer with adenocarcinoma or adenosquamous cancer confirmed by histological testing; locally advanced and metastatic pancreatic cancer; a measurable lesion; ultrasonography examination taken 28 days prior to enrollment; no prior treatments (radiotherapy, chemotherapy, or immune therapy) other than resection of pancreatic cancer; patients in whom pre- and postoperative adjuvant chemotherapy had been administered were eligible if recurrence was confirmed 24 weeks after the final administration (or after day 169 counting from the day following the termination of treatment); patient age between 20 and 80 years; 0 or 1 ECOG performance status (PS); patients with the principal organ functions sufficiently maintained (see criteria below); orally administrable; no abnormal findings leading to clinical complications confirmed by electrocardiogram (ECG) taken within 28 days (4 weeks) of enrollment; and cases in which a patient's written consent had been obtained. The following criteria were used to define whether principal organ functions were sufficiently maintained, from laboratory data taken within 14 days of enrollment (tests conducted on the same day as the enrollment day 2 weeks prior were acceptable): white cell count >3,500/mm^3; neutrophil count >2,000/mm^3; hemoglobin >9.0 g/dL; blood platelet count >100,000/mm^3; total bilirubin <2.0 mg/dL; AST/ALT <150 IU/L; serum creatinine <1.2 mg/dL; and creatinine clearance >60 mL/min.

Treatment

The appropriate dose of S-1 was calculated as follows: patients with a body surface area of <1.25, 1.25–1.50, and >1.5 m^2 received daily doses of 80, 100, and 120 mg/day,

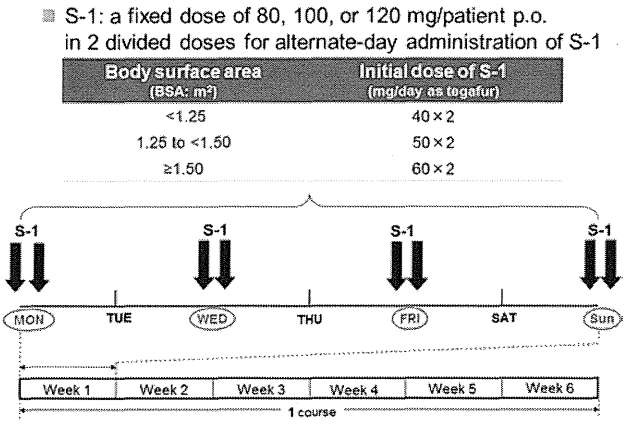

Fig. 1 Treatment schedule for alternate-day with S-1

respectively, administered orally in two equal amounts, after breakfast and after the evening meal. The initial dose of S-1 was administered either on a Monday, Wednesday, Friday, or Sunday (the specified days). S-1 was then administered according to the schedule for the alternate-day regimen for a cycle of 6 weeks. The first day of S-1 treatment was defined as day 1. The first dose of S-1 was taken after the evening meal if it could not be taken after breakfast. The day on which the first dose was administered was designated as day 1 even if the initial dose was taken after the evening meal. The second dose was then taken on the following specified day (e.g., if the initial dose was administered on Friday evening, the next dose would be taken on the following Sunday morning). S-1 administration was continued on Mondays, Wednesdays, Fridays, and Sundays until any one of the criteria for terminating the regimen was satisfied (Fig. 1). The days specified for administering S-1 could not be altered. No missed doses could be taken on days other than those initially prescribed. A dose reduction of 20 mg/day was recommended if grade 3 or higher hematological or non-hematological toxicity occurred in the previous cycle; dose re-escalation was not allowed. Patients who required more than 4 weeks of rest for recovery from any toxicity other than nausea, vomiting, or anemia, or who required a dose reduction of >20 mg/day, were withdrawn from the study.

Evaluation

Assessment of the response rate (RR) was carried out using the sum of complete (CR) and partial response (PR) rates. The antitumor efficacy was interpreted in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST v1.1) and evaluated according to the following criteria: The maximum response rate obtained for each patient by the final course was designated as the response rate of the patient, thereby making the confirmation of 4-week sustained efficacy unnecessary. The efficacy evaluation was carried out for all eligible cases. The number of non-evaluable cases was added only to the denominator of the efficacy evaluation. Stable disease (SD) referred to a stable condition in which none of the other conditions, that is, progressive disease (PD) confirmed CR, and confirmed PR, applied throughout the 6-week cycle. Adverse event nomenclature, grades, and dates of onset were recorded in the follow-up report forms by the participating physicians. The evaluation of adverse event grades and nomenclature were recorded according to the CTCAE v4.0. Overall survival (OS) and the secondary end points, progression-free survival (PFS), and time to treatment failure (TTF) were calculated using the Kaplan–Meier method.

Statistics

The primary end point was OS, and the secondary end points were PFS, TTF, RR, and the frequency and severity of adverse events. Forty-five patients were required, based on the assumption of an expected OS of 6 months and a threshold of 4 months, with an α-error of 0.05 and a β-error of 0.2. In order to allow for patients who were ineligible or who subsequently dropped out, it was planned that 50 patients would be included in this study.

Results

Patients

During the period from August 2009 to May 2011, a total of 50 patients were enrolled from 13 different institutions. Two of these patients did not meet the eligibility criteria: One was excluded due to the patient's refusal and the other on the grounds of inadequate renal function. The baseline characteristics of the patients are shown in Table 1.

Treatment

The 48 patients received a total of 99 cycles of chemotherapy, with a median number of cycles of 2.6 (range 1–12). The dose of S-1 was reduced in one patient because of grade 3 anorexia and fatigue. The median relative dose intensity for the population was 98.9 %, indicating that patient compliance with S-1 chemotherapy was good. Reasons for withdrawal of treatment were progressive disease (79.2 %), patient's refusal (10.4 %), and adverse events (8.3 %). After discontinuation of alternate-day therapy, 14 patients (29.2 %) received GEM-based chemotherapy, 3 patients (6.2 %) received S-1-based chemotherapy, 3 patients (6.2 %) received GEM + S-1 chemotherapy, and 28 patients (58.3 %) received supportive care.

Table 1 Patient characteristics

Characteristic	Number (%)
Age (year)	
Median	67
Range	34–75
Sex	
Male	21 (44)
Female	27 (56)
Performance status	
0	40 (83)
1	8 (17)
Extent disease	
Locally advanced	11 (23)
Metastatic	37 (77)
Metastatic sites	
Liver	21 (44)
Peritoneum	10 (21)
Distant lymph nodes	6 (13)
Lung	1 (2)

Table 4 Patient characteristics in relation to the response

Variable	Number (%)
Complete response	0 (0.0)
Partial response	5 (10.4)
Stable disease	33 (68.8)
Progressive disease	10 (16.6)
Objective response rate (%)	5 (10.4)
95 % CI	(3.5–22.7)
Disease control rate (%)	38 (79.2)
95 % CI	(65.0–89.5)

Toxicity

The most common adverse events are listed in Tables 2 and 3. The only grade 3 or higher hematotoxicities reported were neutropenia (4.2 %) and cholecystitis (2.0 %), and most other instances remained below grade 2 (<30 %). Furthermore, the only grade 3 or higher non-hematotoxicities reported were anorexia and general malaise (2.0 %), and most of these adverse events were also below grade 2 (<20 %). Although gastrointestinal toxicities and myelosuppression were frequently observed with standard treatment, alternate-day treatment was manageable with appropriate medical care. There was no incidence of treatment-related death.

Response and survival

The antitumor effect is shown in Table 4. The objective response rate was 10.4 % (95 % CI 3.5–22.7 %), and the disease control rate was 79.2 % (95 % CI 65.0–89.5 %). At the median follow-up interval of 24 months, 3 patients were still alive and censored. The median overall survival time was calculated for all 48 patients: OS was 8.4 months (95 % CI 5.4–10.8), the one-year survival rate was 29.2 %, and PFS was 5.5 months. Time to treatment failure (TTF) was 3.9 months (95 % CI 2.6–7.3). The Kaplan–Meier survival curve is shown in Figs. 2, 3, and 4.

Discussion

While the results obtained in GEST showed that S-1 monotherapy was one of the standard therapeutic modalities

Table 2 Hematological toxicities

Event	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	≧Grade 3 (%)
Leukopenia	7 (14.6)	5 (10.4)	0 (0.0)	0 (0.0)	0 (0.0)
Neutropenia	4 (8.3)	1 (2.0)	2 (4.2)	0 (0.0)	2 (4.2)
Thrombocytopenia	6 (12.5)	8 (16.6)	0 (0.0)	0 (0.0)	0 (0.0)
Anemia	5 (10.5)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total bilirubin increased	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
AST increased	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Table 3 Non-hematological toxicities

Event	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)	≧Grade 3 (%)
Anorexia/fatigue	4 (8.3)	5 (10.4)	1 (2.0)	0 (0.0)	1 (2.0)
Mucositis	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
Vomiting	2 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Diarrhea	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Infection	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cholecystitis	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)	1 (2.0)

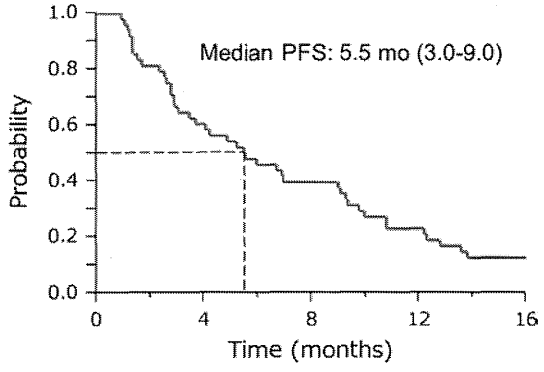

Fig. 2 Kaplan–Meier estimate of progression-free survival according to treatment of S-1

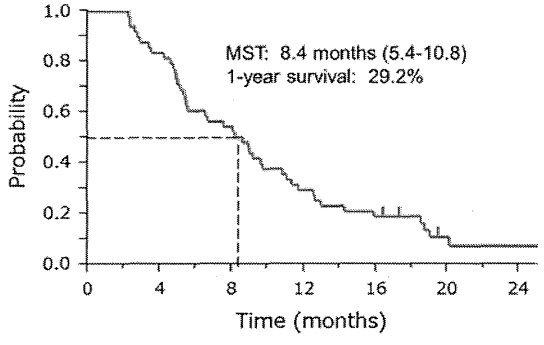

Fig. 3 Kaplan–Meier estimate of overall survival according to treatment of S-1

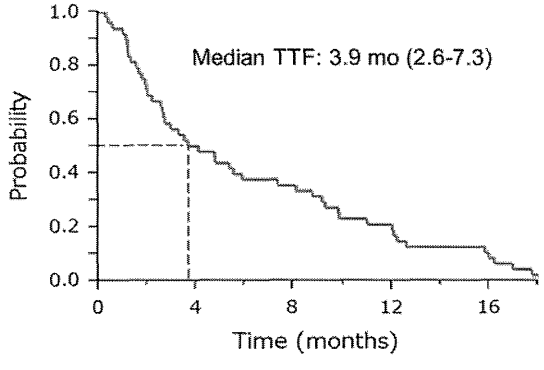

Fig. 4 Kaplan–Meier estimate of time to treatment failure according to treatment of S-1

against advanced pancreatic cancer, there still was room for improvement with respect to the administration schedule, in order to reduce adverse events. At the same time, Lipkin et al. and Clarkson and Ota et al. [6, 7] demonstrated a significant difference between the cell cycles of a host's normal cells and cancer cells. The cell cycle of the normal cells was determined to be half to 1 day, and the length of S phase in which 5-FU was activated was 12 h. In contrast, the cell cycle of cancer cells was 4–5 days, and their S phase lasted for more than 24 h. Thus, by taking advantage of the difference in the cell cycles, a clinically optimum dosage regimen for 5-FU could be ascertained. If a reasonable number of normal cells were to avoid exposure to 5-FU (by means of a 1 day cessation of 5-FU treatment), it could be possible to avoid some of the toxic effects of 5-FU on the normal cells of the intestinal mucosa. In addition, because not only was the cell cycle of cancer cells longer (4–5 days), but also their S phase lasted for more than 24 h, Shirakawa et al. [8] argued that the alternate-day regimen for S-1 would not diminish the cytotoxic effects against cancer cells even if 5-FU were repeatedly activated every other day with a drug cessation period in between. Moreover, Arai et al. [9] treated gastric cancer cell lines with the same total dose of S-1 on alternate or consecutive days to compare these regimens. Although it was a basic study, the results demonstrated that alternate-day treatment with S-1 was equivalent to consecutive-day treatment in terms of the relative inhibition of tumor growth, but with lower toxicity. Furthermore, Sakuma et al. conducted a retrospective examination of the alternate-day regimen in 266 patients with gastric cancer (including advanced recurrent cancer and postoperative adjuvant chemotherapy). The results obtained in the study showed that the efficacy of the regimen was by no means inferior to that of the standard regimen, and with respect to the incidence of adverse events for each grade, extremely favorable results were obtained as follows: 0 % grade 3 or higher, 6 % grade 2, and 7.5 % grade 1 [10].

In this phase II study, the MST was 8.4 months, 1-year overall survival rate was 29.2 %, PFS was 5.5 months, and TTF was 3.9 months. Seven cases of grade 2 or higher non-hematotoxicities (14.6 %), and two cases of grade 3 or higher hematotoxicities (4.2 %) were reported; therefore, the efficacy and safety of the regimen have been confirmed. Although a high response rate was not obtained with S-1 alternate-day administration in this study, the disease control rate was approximately 80 %, and the frequency of adverse events was noticeably less compared to that of the 4-week S-1 regimen followed by a 2-week rest period [3, 4]. The transition to a second-line therapy was not specified in this study; however, these data were recorded as follows. The percentage of patients who underwent transition to GEM, S-1, or GEM + S-1 therapy, or to no further treatment was 29.2, 6.2, 6.2, and 58.3 %, respectively; in total, the percentage of patients undergoing transition to second-line treatment in this study was lower than the percentage undergoing transition to second-line treatment with GEM or GEM + S-1 in the GEST study (approximately 70 %). The fact that in 60 % of patients given the S-1 alternate-day regimen, the second-line treatment could not be

administered due to worsening of the overall health status induced by the first-line treatment has suggested that there is still room for improvement in the treatment efficacy/route of administration of first-line treatment for pancreatic cancer.

Therefore, by comparison with the standard regimen, the S-1 alternate-day regimen may have superior tolerability as well as continuity in the treatment for advanced recurrent gastric cancer or unresectable advanced pancreatic cancer. Compared to other types of carcinoma, unresectable advanced pancreatic cancer has been associated with a higher frequency of serious adverse events when treated with S-1; the alternate-day administration schedule of S-1 therefore has promising potential for not only making treatment more patient-friendly by alleviating side effects, but also achieving improvements in compliance and treatment outcomes [4, 11, 12].

In conclusion, from the results obtained in this study, we have designed and are conducting a randomized phase II study confirming non-inferiority, in terms of overall survival, of the alternate-day regimen for S-1, which has been suggested to result in superior safety and continuity and comparing safety and health-related quality of life in the standard and alternate-day regimens (PAN-01, UMIN000008604). The objective is to determine a standard treatment method necessary to conduct a superiority analysis for developing novel treatment approaches in the future. Furthermore, this research will facilitate the much-awaited development of combination chemotherapy maintaining the efficacy of each individual drug, by applying the alternate-day regimen, which promises fewer side effects, as a basic treatment.

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