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P53, hTERT, WT-1, and VEGFR2 are the most suitable targets for cancer vaccine therapy in HLA-A24 positive pancreatic adenocarcinoma

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Abbreviations: CTL, cytotoxic T lymphocyte; TAA, tumor-associated antigen; ELISPOT, enzyme-linked immunospot; MAGE, melanoma-associated antigen; hTERT, human telomerase reverse transcriptase; WT-1, Wilms tumor-1; VEGFR, vascular endothelial growth factor receptor; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction

Abstract

Cancer vaccine therapy is one of the most attractive therapies as a new treatment procedure for pancreatic adenocarcinoma. Recent technical advances have enabled the identification of cytotoxic T lymphocyte (CTL) epitopes in various tumor-associated antigens (TAAs). However, little is known about which TAA and its epitope are the most immunogenic and useful for a cancer vaccine for pancreatic adenocarcinoma. We examined the expression of 17 kinds of TAA in 9 pancreatic cancer cell lines and 12 pancreatic cancer tissues. CTL responses to 23 epitopes derived from these TAAs were analyzed using enzyme-linked immunospot (ELISPOT), CTL, and tetramer assays in 41 patients, and factors affecting the immune responses were investigated. All TAAs were frequently expressed in pancreatic adenocarcinoma cells, except for adenocarcinoma antigens recognized by T cells (ART)1, melanoma-associated antigen (MAGE)-A1, and MAGE-A3. Among the epitopes recognized by CTLs in more than two patients in the ELISPOT assay, 6 epitopes derived from 5 TAAs, namely, MAGE-A3, p53, human telomerase reverse transcriptase (hTERT), Wilms tumor (WT)-1, and vascular endothelial growth factor receptor (VEGFR)2, could induce specific CTLs that showed cytotoxicity against pancreatic cancer cell lines. The frequency of lymphocyte subsets correlated well with TAA-specific immune response. Overall survival was significantly

longer in patients with TAA-specific CTL responses than in those without. P53, hTERT, WT-1, and VEGFR2 were shown to be attractive targets for immunotherapy in patients with pancreatic adenocarcinoma and the induction of TAA-specific CTLs may improve the prognosis of these patients.

Key words: epitope; immunotherapy; CTL: cytotoxic T lymphocyte; ELISPOT: enzyme-linked immunospot

Précis: P53, hTERT, WT-1, and VEGFR2 were the most suitable epitopes for cancer

vaccine therapy in pancreatic adenocarcinoma. Outcomes were significantly better in

patients with CTL responses than in those without.

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death worldwide [1]. Despite recent advances in diagnostic techniques, pancreatic adenocarcinoma is diagnosed at an advanced stage in most patients and, consequently, the overall 5-year survival rate is less than 5% [2]. Thus, the development of a new treatment option is needed to improve the prognosis of pancreatic cancer patients without toxicity.

Immunotherapy is one of the most attractive therapies as a new treatment procedure for melanoma and other solid tumors [3]. Recent technical advances have enabled the identification of various tumor-associated antigens (TAAs) [4-21]; however, few of their epitopes are inducers of cytotoxic T lymphocyte (CTL) responses against tumors [22]. Several kinds of epitope have also been identified in patients with pancreatic adenocarcinoma [23,24]. However, previous studies focused on the identification and evaluation of a particular antigen, and different TAAs have not yet been compared simultaneously; therefore, little is known about which epitope is the most immunogenic and useful in eliciting clinical responses in pancreatic adenocarcinoma patients.

In the present study, we compared CTL responses to various TAA-derived epitopes in identical patients with pancreatic adenocarcinomas and examined the factors

that affect immune responses. This approach provided information that is useful for

selecting immunogenic TAAs and suitable patients, and developing a new

immunotherapy for pancreatic adenocarcinoma.

Materials and Methods

Patients and clinical information

In this study, we examined 41 HLA-A24 positive patients with pancreatic adenocarcinoma and 14 healthy volunteers who were HLA-A24 positive but did not have any cancers, as negative controls. Fine-needle biopsy, a surgical specimen, or autopsy was used for the pathological diagnosis of pancreatic adenocarcinoma in 18 patients. Diagnosis of the remaining 23 patients was achieved using the radiological findings of computed tomography and/or magnetic resonance imaging. We investigated patient background, treatment procedures, and outcomes.

Clinical information was obtained from the medical records of patients. We evaluated the tumor stage using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (7th version) (UICC stage). The frequency of lymphocyte subsets was calculated by dividing the absolute lymphocyte count by the absolute leukocyte count. HLA typing of peripheral blood mononuclear cells (PBMCs) from patients and healthy volunteers was performed by the reverse sequence-specific oligonucleotide with polymerase chain reaction (PCR-RSSO). This study was approved by the Ethics Committees of Kanazawa University (No. 1237) and Kanazawa Medical Center (No. 17), and all patients gave written informed consent to participate in

accordance with the Helsinki Declaration.

Synthetic peptides and preparation of PBMCs

The 23 epitopes derived from 17 different TAAs used in the present study are listed in Table 1. We selected epitopes that had previously been identified as HLA-A24-restricted and suggested to have immunogenicity in various cancers not restricted to pancreatic cancer [4-21]. Epitopes derived from the HIV envelope protein (HIV env$_{584}$) [25] and cytomegalovirus (CMV) pp65 (CMVpp65$_{328}$) [26] were also used to assess T-cell responses. Peptides were synthesized at Mimotope (Melbourne, Australia), Sumitomo Pharmaceuticals (Osaka, Japan), COSMO BIO Co. (Tokyo, Japan), and Scrum Inc. (Tokyo, Japan). Purities were determined to be >80% by analytical high-performance liquid chromatography (HPLC). PBMCs were separated as described below; heparinized venous blood was diluted in phosphate-buffered saline (PBS) and loaded on Ficoll-Histopaque (Sigma, St. Louis, MO) in 50 ml tubes. After centrifugation at 2000 rpm for 20 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS, centrifuged at 1400 rpm for 10 min, and finally resuspended in complete culture medium consisting of RPMI (GibcoBRL, Grand Island, NY), 10% heat-inactivated FCS (Gibco BRL), 100 U/ml penicillin, and 100

µg/ml streptomycin (Gibco BRL).

Cell lines

The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% FCS and 500 µg/ml hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS [27]. MiaPaca2, AsPC1, BxPC3, Panc-1, CAPAN1, and CAPAN2 were purchased from the American Type Culture Collection (VA, USA). YPK-1 and YPK-2 were kind gifts from Prof. Oka and Dr. Yoshimura (Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan). PK-1 was provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Human pancreatic cancer cell lines were cultured in DMEM (GibcoBRL) or RPMI 1640 medium containing 10% fetal calf serum (FCS). All media contained 100 U/mL penicillin and 100 µg/mL streptomycin.

RNA preparation and real-time PCR

The expression of TAA messenger RNA (mRNA) in human pancreatic cancer cell lines and pancreatic adenocarcinoma tissues was analyzed by real-time polymerase chain reaction (PCR). Cell lines were harvested, centrifuged, and washed with PBS and

total RNA was then isolated using Quick-Gene (Fuji Film, Tokyo). Total RNA from

frozen pancreatic adenocarcinoma samples was isolated using a GenElute Mammalian

Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's protocol.

cDNA was synthesized from 150 ng of total RNA using a high-capacity cDNA reverse

transcription kit (PE Applied Biosystems, CA, USA), and was then mixed with TaqMan

Universal Master Mix (PE Applied Biosystems) and each TaqMan probe. Primer pairs

and probes for various TAAs and β-actin were obtained from the TaqMan assay reagents

library. Thermal cycling conditions were 25°C for 10 min, 37°C for 120 min, and 85°C

for 1 min. cDNA was subjected to quantitative real-time PCR analyses targeting various

TAAs and β-actin. Analyses were performed using the StepOne Real-Time PCR system

and StepOne v2.0 software. Relative gene expression values were determined. Data are

presented as fold differences in TAA expression normalized to the housekeeping gene

β-actin as an endogenous reference.

Enzyme-linked immunospot assay (ELISPOT assay)

Ninety-six-well plates (Millititer, Millipore, Bedford, MA) were coated with

anti-human interferon-γ (IFN-γ) (Mabtech, Nacka, Sweden) at 4°C overnight and then

washed 4 times with sterile PBS. The plates were then blocked with RPMI 1640

medium containing 5% FCS for 2 hr at room temperature. A total of 300,000 unfractionated PBMCs were added in duplicate cultures of RPMI 1640 containing 5% FCS together with the peptides at 10 µg/ml. After 24 hr, the plates were washed 8 times with PBS and incubated overnight with 100 µl of the biotin-conjugated anti-human IFN-γ antibody. After another 4 washes with PBS, streptavidin-AP was added for 2 hr. Finally, the plates were washed again 4 times with PBS and developed with freshly prepared NBT/BCIP solution (Biorad, Hercules, CA). The reaction was stopped by washing with distilled water and drying at room temperature. Colored spots with fuzzy borders, which indicated the presence of IFN-γ-secreting cells, were counted. The number of specific spots was determined by subtracting the number of spots in the absence of the antigen. Responses were considered positive if 10 or more specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence.

Peptide-specific CTL induction and cytotoxicity assay

Synthetic peptide-specific T cells were expanded from PBMCs in 96-well round-bottom plates (NUNC, Naperville, IL). Four hundred thousand cells/well were stimulated with synthetic peptides at 10 µg/ml, 10 ng/ml rIL-7, and 100 pg/ml rIL-12

(Sigma) in RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cultures were restimulated with 10 µg/ml peptide, 20 U/ml rIL-2 (Sigma), and 10^5 mitomycin C-treated autologous PBMCs as feeder cells on days 7 and 14. One hundred microliters of RPMI medium with 10% human Ab serum and rIL-2 at a final concentration of 10 U/ml was added to each well on days 4, 11, and 18. The cytotoxicity assay was conducted on day 22.

The C1R-A24 and human pancreatic cancer cell lines were used as target cells for the ^{51}Cr release assay. C1R-A24 cells were incubated overnight with 10 µg/ml synthetic peptides and labeled with 25 µCi of ^{51}Cr for 1 hour. Pancreatic cancer cell lines were also labeled with 25 µCi of ^{51}Cr for 1 hour without incubation with peptides. After three washes with PBS, target cells were plated at 3,000 cells/well in complete medium in round-bottom 96-well plates. Unlabeled K562 (120,000 cells/well) was added to reduce non-specific lysis. Peptide-stimulated PBMCs were added at various effector-to-target ratios as indicated. Maximum release was determined by the lysis of ^{51}Cr-labeled targets with 5% Triton X-100 (Sigma Chemical). Spontaneous release was <10% of maximum release for all experiments, except for when it was <15% when the target cells were human pancreatic cancer cell lines. Percent-specific cytotoxicity was determined using the following formula: 100×(experimental release − spontaneous

release) / (maximum release − spontaneous release), and specific cytotoxic activity was calculated as follows: (cytotoxic activity in the presence of the peptide) − (cytotoxic activity in the absence of the peptide). Specific cytotoxicity of more than 10% was considered to be positive.

Tetramer staining and flow cytometry

TAA-specific tetramers were purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). Tetramer staining was performed as described below. One million isolated PBMCs or peptide-specific CTLs pulsed with TAA-derived peptides were washed, resuspended in 200 μl of PBS without calcium or phosphate, and stained with 40 μg/ml tetrameric complexes and monoclonal antibodies against cell surface proteins for 30 min at room temperature. The following monoclonal antibodies were used: anti-CD8-APC (BD PharMingen, San Diego, CA), anti-CCR7-FITC, anti-CD45RA-PerCP, and tetramer-PE. Cells were washed, fixed with 0.5% paraformaldehyde/PBS, and analyzed on a Becton Dickinson FACSAria II system.

Statistical analysis

Fisher's exact test and unpaired Student's *t*-test were used to analyze the effect of

variables on immune responses in pancreatic cancer patients. Overall survival was

calculated from the day of pancreatic cancer diagnosis until the date of death or the last

day of the follow-up period. Cumulative survival proportions were calculated using the

Kaplan-Meier method, and any differences were evaluated using the log-rank test. A

p-value of less than 0.05 was considered to be significant, and all the tests were

two-sided. All statistical analyses were performed using the SPSS statistical software

program package (SPSS version 11.0 for Windows).

Results

Patients

Patient characteristics are summarized in the Supplementary Table. The median age of patients was 72 years, and patients included 24 males (59%). The main localization of the tumors was the pancreatic head in 39% of patients and the pancreatic body or tail in 61%. The majority of patients (93%) had advanced-stage cancer, namely, UICC stage III or IV. Therapeutic procedures mainly involved chemotherapy consisting of protocols such as gemcitabine monotherapy, S-1 monotherapy, or a combination of both drugs. Only 11 patients received the best supportive therapy to relieve physical and spiritual pain. A total of 61% of patients had died by the last day of the follow-up period and the median overall survival time of patients was 7.2 months.

TAA expression in pancreatic cancer cell lines and human cancer tissues

We evaluated the expression of 17 different TAAs in 9 human pancreatic cancer cell lines using real-time PCR. Although differences were observed from cell to cell, TAAs were expressed in more than 40% of pancreatic adenocarcinoma cell lines, except for adenocarcinoma antigens recognized by T cells (ART)1 (11%) and ART4 (33%) (Table 2). We then investigated TAA expression in 7 surgical and 5 autopsy specimens.

The expression of most TAAs in pancreatic adenocarcinoma specimens was similar to or more frequent than that in human pancreatic cancer cell lines, except for melanoma-associated antigen (MAGE)-A1 and MAGE-A3 (Table 2).

Detection of TAA-specific T cells by IFN-γ ELISPOT analysis

IFN-γ ELISPOT responses were evaluated with PBMCs to determine how frequently T cells respond to TAA-derived peptides and control peptides in patients with pancreatic adenocarcinoma (Figure 1A). Positive responses to at least one TAA-derived peptide were observed in 28 of 41 (68%) patients. On the other hand, 14 of 23 (61%) peptides were recognized by T cells obtained from at least one patient. $ART1_{188}$, $ART4_{161}$, $ART4_{899}$, lymphocyte-specific protein tyrosine kinase $(Lck)_{208}$, $MAGE-A3_{195}$, $p53_{161}$, human telomerase reverse transcriptase $(hTERT)_{461}$, $hTERT_{324}$, Wilms tumor $(WT)-1_{235}$, vascular endothelial growth factor receptor $(VEGFR)2_{169}$, and $VEGFR1_{1084}$ were recognized in more than two patients, which suggested that these peptides have the potential to be immunogenic. Peptides 24 $(HIVenv_{584})$ and 25 $(CMVpp65_{328})$ were recognized in 0% and 38% of patients, respectively.

Peptides $ART4_{161}$, $ART4_{899}$, Cyclophilin B $(Cyp-B)_{315}$, Lck_{208}, $hTERT_{324}$, and $VEGFR1_{1084}$ were recognized in more than one healthy volunteer and/or the percentage

of positive responses was higher in healthy volunteers than in pancreatic adenocarcinoma patients, which indicated that the responses to these peptides were not specific to T cells from patients with pancreatic adenocarcinoma (Figure 1B). In other words, peptides $ART1_{188}$, $MAGE-A3_{195}$, $p53_{161}$, $hTERT_{461}$, $WT-1_{235}$, and $VEGFR2_{169}$ have specific immunogenic potential in patients with pancreatic adenocarcinoma.

The number of peptide-specific IFN-γ-producing T cells was counted to examine the frequency of T cells responsive to TAA-derived peptides. A range of 10 to 46 T cells per 300,000 PBMCs in patients with pancreatic adenocarcinoma produced IFN-γ (Figure 1C).

TAA-specific CTL induction and cytotoxic activity

We attempted to induce peptides specific to CTLs from the PBMCs of pancreatic adenocarcinoma patients. Cytotoxicity assays were performed in more than five patients for each peptide. Of the 11 peptides recognized in more than two patients in the IFN-γ ELISPOT assay, 6 peptides ($MAGE-A3_{195}$, $p53_{161}$, $hTERT_{461}$, $hTERT_{324}$, $WT-1_{235}$, and $VEGFR2_{169}$) could induce their specific CTLs, which were confirmed to be able to respond to C1RA24 cells pulsed with corresponding peptides by the cytotoxicity assay, as shown in Figure 2A.

We conducted a cytotoxicity assay to determine whether peptide-specific CTLs from healthy volunteers could show their cytotoxic activity against pancreatic carcinoma cell lines. $P53_{161}$-, $hTERT_{461}$-, and $hTERT_{324}$-specific CTLs showed cytotoxicity against YPK-2 (HLA-A24-, p53-, and hTERT-positive), but not against Panc-1 (HLA-A24-negative, p53- and hTERT-positive). $MAGE-A3_{195}$-, $WT-1_{235}$-, and $VEGFR2_{169}$-specific CTLs also showed cytotoxic activity against YPK-2 (HLA-A24-, MAGE-A3-, WT-1-, and VEGFR2-positive), but not against YPK-1 (HLA-A24 positive, MAGE-A3-, WT-1-, and VEGFR2-negative). Representative data are shown in Figure 2B.

Phenotypic analysis of TAA-derived peptides specific to T cells

To analyze the characteristics of TAA-derived peptides specific to T cells and select the appropriate epitope for immunotherapy in patients with pancreatic adenocarcinoma, we performed phenotypic analysis by tetramer staining and FACS analysis. We first attempted to detect $MAGE-A3_{195}$-, $hTERT_{461}$-, and $WT-1_{235}$-specific tetramer-positive T cells in PBMCs and CTLs induced by the corresponding peptides in healthy volunteers. The ratio of tetramer-positive T cells was increased in CTLs and their frequencies were 1.481-2.930% of $CD8^+$ T cells, suggesting that these tetramers

work well (Figure 3A). We also conducted similar assays in pancreatic adenocarcinoma

patients and detected tetramer-positive T cells in CTLs (Figure 3B).

We then examined the naïve/effector/memory phenotype of tetramer-positive

cells in the PBMCs of patients. The memory phenotype was investigated by the

criterion of CD45RA/CCR7 expression [28]. In tetramer analysis, the frequencies of

MAGE-A3$_{195}$-, hTERT$_{461}$-, and WT-1$_{235}$-specific tetramer-positive T cells were

0.003-0.044%, 0.006-0.053%, and 0.030-0.191% of CD8$^+$ T cells, respectively. The

frequency of CD45RA$^-$/CCR7$^+$ (central memory), CD45RA$^-$/CCR7$^-$ (effector memory),

and CD45RA$^+$/CCR7$^-$ (effector) T cells in tetramer-positive cells depended on the

patient and all phenotypes were observed in all patients, except for patients 1, 8, 28, 29,

and 4 (Supplementary Figure 1).

TAA-specific T-cell responses and clinical features of pancreatic cancer patients

In the present study, we analyzed the clinical features that can affect TAA-specific

immune responses. When we divided patients into two groups based on their

frequencies of lymphocyte subsets in peripheral leukocytes (less than 24%, the median

value among all patients, or equal to or more than 24%) and the strength of

TAA-specific immune responses into three groups according to the frequency of

TAA-specific T cells (less than 10 specific spots on ELISPOT assays, no response; 10-19 specific spots, weak response; equal to or more than 20 specific spots, strong response), the patients with more lymphocyte subsets in peripheral leukocytes showed stronger TAA-specific T-cell responses (Supplementary Figure 2). On the other hand, we could not find any relationship between TAA-specific immune responses and other clinical characteristics such as age, sex, tumor marker levels, UICC stage, or metastasis status.

We also analyzed the correlation between T-cell responses and the prognosis of pancreatic cancer patients. The median overall survival time of patients with T-cell responses to at least one TAA-derived peptide evaluated by the ELISPOT assay was 12.2 months, which was significantly longer than that without T-cell responses (4.3 months) ($p = 0.013$) (Figure 4A). On the other hand, no correlation was observed between positive T-cell responses and CMV-derived peptides and clinical outcomes (Figure 4B), suggesting that TAA-specific T-cell responses, but not the general immune response, is a prognostic factor in patients with pancreatic adenocarcinoma. The frequencies of regulatory T cells or the ratio of regulatory T cells to CD8$^+$ T cells had no impact on the outcomes of patients in this study.

Discussion

Immunotherapy is considered to be a fourth treatment procedure for cancer following surgical resection, radiotherapy, and chemotherapy [29]. Cancer vaccine therapy was previously shown to convey survival benefits to prostate cancer patients in a clinical phase III trial [30], and some candidates of other cancers have been identified and separately evaluated to determine whether a CTL response can be elicited, with the subsequent elimination of cancer cells and improvement in outcomes. Although a successful clinical response depends on how much tumor antigens elicit their specific CTLs, which are the most important effector cells for antitumor immune responses, to the best of our knowledge, no studies have attempted to identify which epitopes are optimal for peptide vaccine therapy in patients with pancreatic adenocarcinoma. Therefore, we simultaneously compared peptide-specific T-cell responses among various TAAs in 41 identical patients with pancreatic adenocarcinoma under the same experimental conditions.

Therapeutic function is the most important factor to consider when determining the usefulness of cancer antigens for peptide vaccine therapy. However, it is very difficult to compare the efficacy of more than one epitope, especially in patients with pancreatic adenocarcinoma whose survival time is very short. Under such circumstances, immunogenicity, specificity, oncogenicity, expression levels, % positive cells, and the

number of patients with antigen-positive cancer are considered to be alternative criteria [31]. On the basis of our results, $p53_{161}$, $hTERT_{461}$, $WT-1_{235}$, and $VEGFR2_{169}$ are considered the most optimal epitopes that satisfy all of the above criteria for peptide vaccine therapy in pancreatic adenocarcinoma patients. Although $MAGE-A3_{195}$ showed immunogenicity, its expression did not appear to be high in pancreatic adenocarcinoma tissue [32]. Therefore, it may be a candidate for cancer vaccine therapy when MAGE-A3 is confirmed to be overexpressed in pancreatic cancer tissue.

A mutation in the p53 gene and overexpression of the p53 protein have been reported previously in pancreatic adenocarcinoma [33], and all pancreatic cancer cell lines and specimens used in our study expressed p53. Some strategies targeting p53 have been proposed over the last decade [34]. As peptide vaccine therapy, the wild-type p53 peptide is well preserved in mutant p53 because most mutations in the p53 gene are missense mutations, and are considered to be one of the attractive targets as a cancer antigen. The frequencies of the CTL response against HLA-A24-restricted $p53_{161}$ investigated by the ELISPOT assay in head and neck carcinoma and hepatocellular carcinoma were shown to be 35% and 10%, respectively [35,36]. Although the frequency of 7% in our study is lower, given the difference according to the primary tumor site or balance between sensitivity and specificity, induced CTLs showed

cytotoxic activity against pancreatic adenocarcinoma cell lines, which suggested that p53 may be an attractive target in patients with pancreatic cancer.

hTERT is widely overexpressed in various cancer cells including pancreatic cancer [37], which is consistent with our results. A clinical trial demonstrated that GV1001, a HLA class II epitope corresponding to the hTERT (611-626) fragment, was immunogenic in pancreatic cancer patients [38]. Another previous study evaluating T-cell responses to several hTERT epitopes in patients with hepatocellular carcinoma [39] demonstrated that $hTERT_{461}$- and $hTERT_{324}$-specific CTLs were induced in 5 (6.9%) and 9 (12.5%) of 72 patients, respectively. In the current study, these frequencies were equivalent and the killing of pancreatic cancer cell lines was demonstrated, which suggested that these epitopes also had immunogenicity in pancreatic cancer patients.

Peptide vaccine therapies using $WT-1_{235}$ and $VEGFR2_{169}$ combined with gemcitabine have already been conducted in pancreatic adenocarcinoma patients [23,24]. We clarified that $WT-1_{235}$- and $VEGFR2_{169}$-specific CTLs induced from PBMCs showed cytotoxicity for human pancreatic cancer cell lines, and the results of further investigations are anticipated.

We performed phenotypic analysis of TAA-derived epitope-specific T cells to determine the most appropriate epitope for immunotherapy in patients with pancreatic

adenocarcinoma. Epitope-specific tetramer^{+} cells in PBMCs were also found in patients without IFN-γ ELISPOT responses, which was consistent with the findings of previous studies [39,40] and suggested the existence of dysfunctional epitope-specific T cells. Epitope-specific tetramer^{+} cells were also identified at a very low frequency in PBMCs from healthy volunteers and increased in CTLs induced with TAA-derived peptides, which was also consistent with previous studies in which TAA-specific tetramer^{+} T cells were detectable in samples from healthy donors [41] or the *in vitro* stimulation of PBMCs with the epitopes derived from TAA could induce TAA-specific CTLs in healthy volunteers [42], even though the precise mechanism has not yet been clarified. Phenotypic analysis showed that the frequency of T cells with each memory and effector phenotype depended on the patient and also that peptide-specific memory T cells existed in PBMCs of patients with pancreatic adenocarcinoma. Because T cells with the memory phenotype exert stronger antitumor effects by secondary stimulation with the antigen, our results suggest that an additional immunological approach such as that consisting of a TAA-derived protein or peptide, recombinant virus, and engineered tumor cells to boost T-cell function may be useful to enhance host antitumor immune responses.

Another purpose of this study was to identify the factors influencing immune

responses. Our results suggested that the frequencies of the lymphocyte subsets in peripheral leukocytes were very important in the induction of TAA-specific CTLs. Although the relationship between cancer, inflammation, and immunity has already been documented [43], the precise mechanism has yet to be fully understood. One of the speculated reasons why PBMC from patients with lymphocytopenia could not induce a good immune response in our study is that the release of inhibitory immunological cytokines such as transforming growth factor β or IL-10 from pancreatic adenocarcinoma tissue decreases lymphocyte counts and impairs the function of lymphocytes both systemically and in the microenvironment [44]. It was also reported that lymphocyte counts and CTL responses were prognostic markers in advanced cancer cases receiving peptide vaccine therapy [45, 46]. Our results showing a correlation between the T-cell response and outcomes in pancreatic adenocarcinoma patients corresponded to these previous findings, which indicates that restricting the objective to those with an adequate lymphocyte subset could lead to a clinical trial with favorable outcomes.

A limitation of this study was the lack of data for the clinical response. Tumor shrinkage or survival benefits are not always observed in all patients who exhibit immune responses. Further clinical studies using peptides that could induce

TAA-specific CTLs are needed to confirm our findings.

In conclusion, we simultaneously compared T-cell responses to various TAA-derived epitopes in patients with pancreatic adenocarcinomas; our results suggested that $p53_{161}$, $hTERT_{461}$, $WT-1_{235}$, and $VEGFR2_{169}$ were the most suitable epitopes for cancer vaccine therapy.

Conflict of interest

The authors do not have any conflict of interest.

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Figure legends

Figure 1: T-cell responses to TAA-derived peptides and control peptides in pancreatic adenocarcinoma patients **A)** and healthy volunteers **B)**. T-cell responses were evaluated by the IFN-γ ELISPOT assay. Responses were considered positive if 10 or more specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Black boxes indicate positive responses. **C)** The frequency of TAA-specific IFN-γ-producing T cells evaluated by the ELISPOT assay. Black bars indicate the response of one patient.

Figure 2: A) T-cell responses to peptides evaluated by the cytotoxicity assay. Peptide-specific CTL induction and cytotoxicity assays were performed on the PBMCs from at least five patients and representative data are shown when peptide-specific CTLs were induced in one or more patients. A percent-specific cytotoxicity of more than 10% was considered to be positive. Six peptides: 9, 14, 18, 19, 20, and 21, could induce their specific CTLs, and these could respond to C1RA24 cells pulsed with the corresponding peptides in the cytotoxicity assay. **B)** Cytotoxic activity against the pancreatic carcinoma cell lines of TAA-specific CTLs from healthy volunteers

evaluated by the cytotoxicity assay. Cytotoxicity was stronger against pancreatic carcinoma cells that were HLA-A24-restricted and expressed corresponding TAAs than against those not HLA-A24-restricted or not expressing corresponding TAAs.

Figure 3: Detection of TAA-specific, HLA-A24-tetramer^{+}, and CD8^{+} lymphocytes in PBMCs from healthy volunteers and pancreatic adenocarcinoma patients. **A)** Tetramer analyses were performed on eight healthy volunteers for each peptide (MAGE-A3$_{195}$, hTERT$_{461}$, and WT-1$_{235}$). Tetramer^{+} and CD8^{+} T cells were detectable in both PBMCs and CTLs induced by their corresponding peptides in at least one healthy volunteer, and representative data are shown in cases in which the ratio of tetramer^{+} and CD8^{+} T cells to CD8^{+} T cells was higher in CTLs induced with each TAA-derived peptide than in PBMCs. **B)** Tetramer analyses were performed on pancreatic adenocarcinoma patients using PBMCs and CTLs which was induced with TAA-derived peptides and showed cytotoxicity against pancreatic cancer cell lines in cytotoxicity assay. Levels of tetramer^{+} and CD8^{+} T cells were higher in CTLs induced with TAA-derived peptides than in PBMCs. Representative data are shown in cases in which the ratio of tetramer^{+} and CD8^{+} T cells to CD8^{+} was 0.017% in PBMCs and 1.145% in MAGE-A3$_{195}$-specific CTLs.

Figure 4: Kaplan-Meier plot of the overall survival of pancreatic cancer patients according to **A)** TAA-specific T-cell responses and **B)** T-cell responses to CMV-derived peptides. **A)** TAA-specific T-cell responses were defined as positive if 10 or more specific spots to at least one TAA-derived peptide were detected on the ELISPOT assay. The overall survival time of patients with TAA-specific T-cell responses was significantly longer than that of patients without TAA-specific T-cell responses. **B)** T-cell responses to CMV-derived peptides were defined as positive if 10 or more specific spots to CMV-derived peptides were detected on ELISPOT assays. No correlation was observed between positive T-cell responses to CMV-derived peptides and the clinical outcomes of patients.