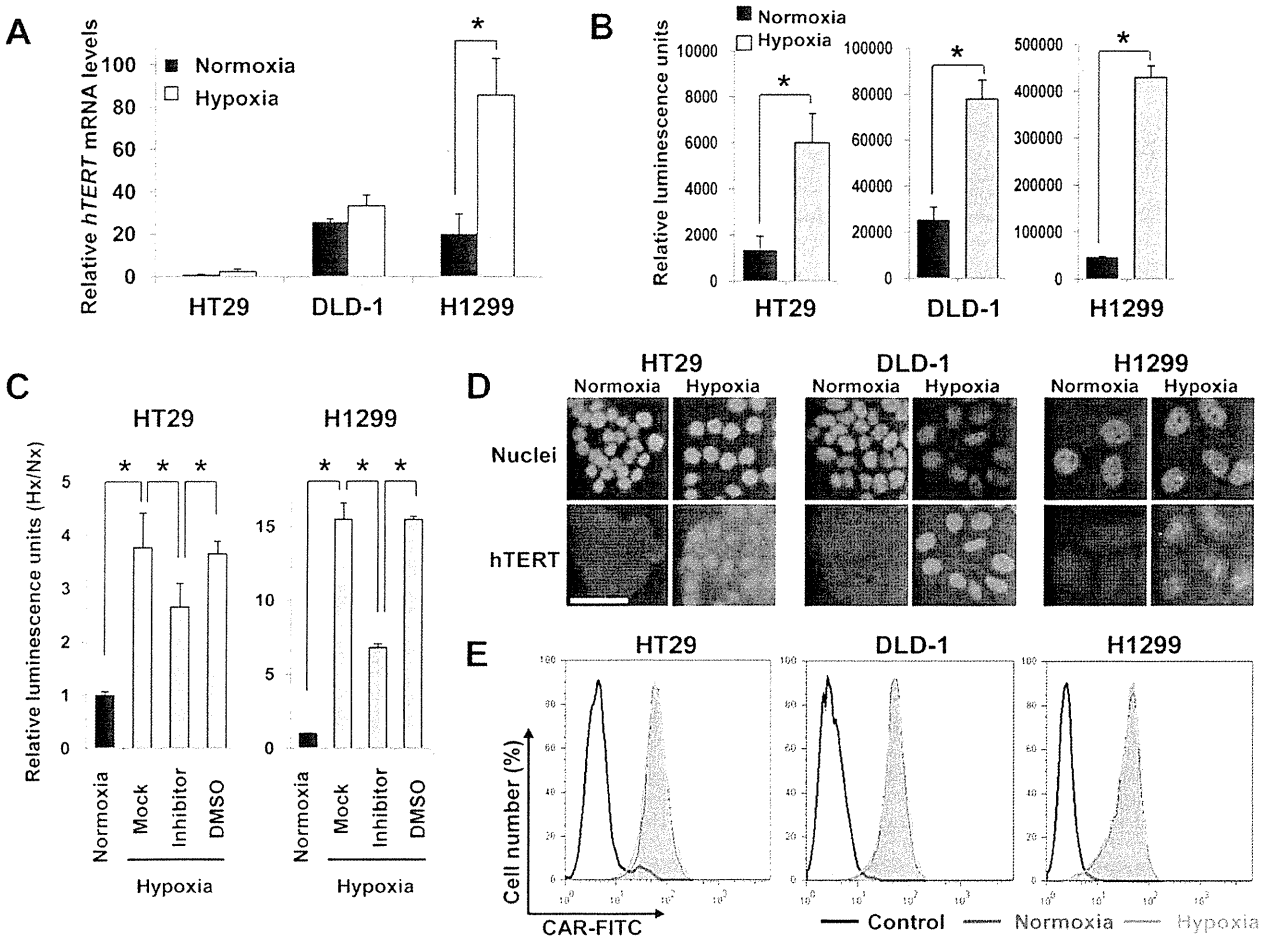

Figure 2. Effect of hypoxia on hTERT and CAR expression in human cancer cells. A, *hTERT* mRNA expression was assessed in human cancer cells that were maintained under normoxia (Nx) or hypoxia (Hx) for 18 h, using quantitative real-time RT- PCR analysis. The levels of *hTERT* mRNA were plotted as fold induction relative to the values of *hTERT* mRNA in HT29 cells incubated under normoxia, which was set at 1.0. Data are shown as mean values ± SD of triplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test). **B** and **C**, *hTERT* gene promoter activity was assessed in human cancer cells that were transfected with the hTERT reporter vector (pGL3-hTERT) and then cultured under normoxia or hypoxia for 24 h, using luciferase reporter assay. The GFP expression vector (pCMV-EGFP) was used as a reporter for transfection efficiency, and the activities of hTERT promoter were determined as ratio of luciferase activity to GFP expression. Data are shown as mean values ± SD of triplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test). **C**, HT29 and H1299 cells were treated with 30 μM HIF-1α inhibitor or DMSO solvent control in hypoxic condition. The levels of luminescence were plotted as fold induction relative to the values of luminescence in cancer cells incubated under normoxia, which were set at 1.0. **D**, Subcellular localization of hTERT protein expression in human cancer cells under normoxia or hypoxia was assessed using immunofluorescent staining. Cells cultured under a normoxic or hypoxic condition for 48 h were stained with anti-hTERT antibody (green). Nuclei were counterstained with DAPI (blue). Scale bars = 50 μm. **E,** Flow cytometric analysis of CAR expression in human cancer cells maintained under normoxia (green) or hypoxia (red) for 18 h. Cells were incubated with a mouse anti-CAR antibody followed by FITC-labeled rabbit anti-mouse IgG. An isotype-matched normal mouse IgG was used as a control (black).
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activation of the *hTERT* gene promoter was involved in the enhancement of virus replication in hypoxic tumor cells (Fig. 2 and 4). These results suggest that the *hTERT* gene promoter is useful for regulation of the replication of oncolytic adenoviruses in tumor cells in a hypoxic microenvironment.

The replication of OBP-301 depends on the activity of the *hTERT* gene promoter, which contains two HREs and is activated by HIF-1α under hypoxic conditions [23–25]. Hypoxic conditions that induced nuclear accumulation of HIF-1α (Fig. 1) upregulated *hTERT* gene promoter activity in human cancer cells (Fig. 2B and 2C). Consistent with this *hTERT* gene promoter activation, OBP-301 replication was significantly higher than that of Ad5 with the endogenous *E1* promoter (Fig. 4). These findings suggest that hypoxia enhances OBP-301 virus replication through HIF-1α-mediated activation of the *hTERT* gene promoter.

Recently, oncolytic virotherapy has garnered interest as potential therapeutic strategy for hypoxic tumors [29]. A hypoxia-responsive promoter that is upregulated by HIF-1 has been used for the tumor-specific replication of an oncolytic adenovirus [30–32]. Although an oncolytic adenovirus that is regulated by a hypoxia-responsive promoter will also be effective against hypoxic tumor cells following HIF-1 activation, non-hypoxic tumor cells in which HIF-1 is not activated may be less sensitive to these viruses. In contrast, the *hTERT* gene promoter-regulated oncolytic adenovirus OBP-301 would be effective

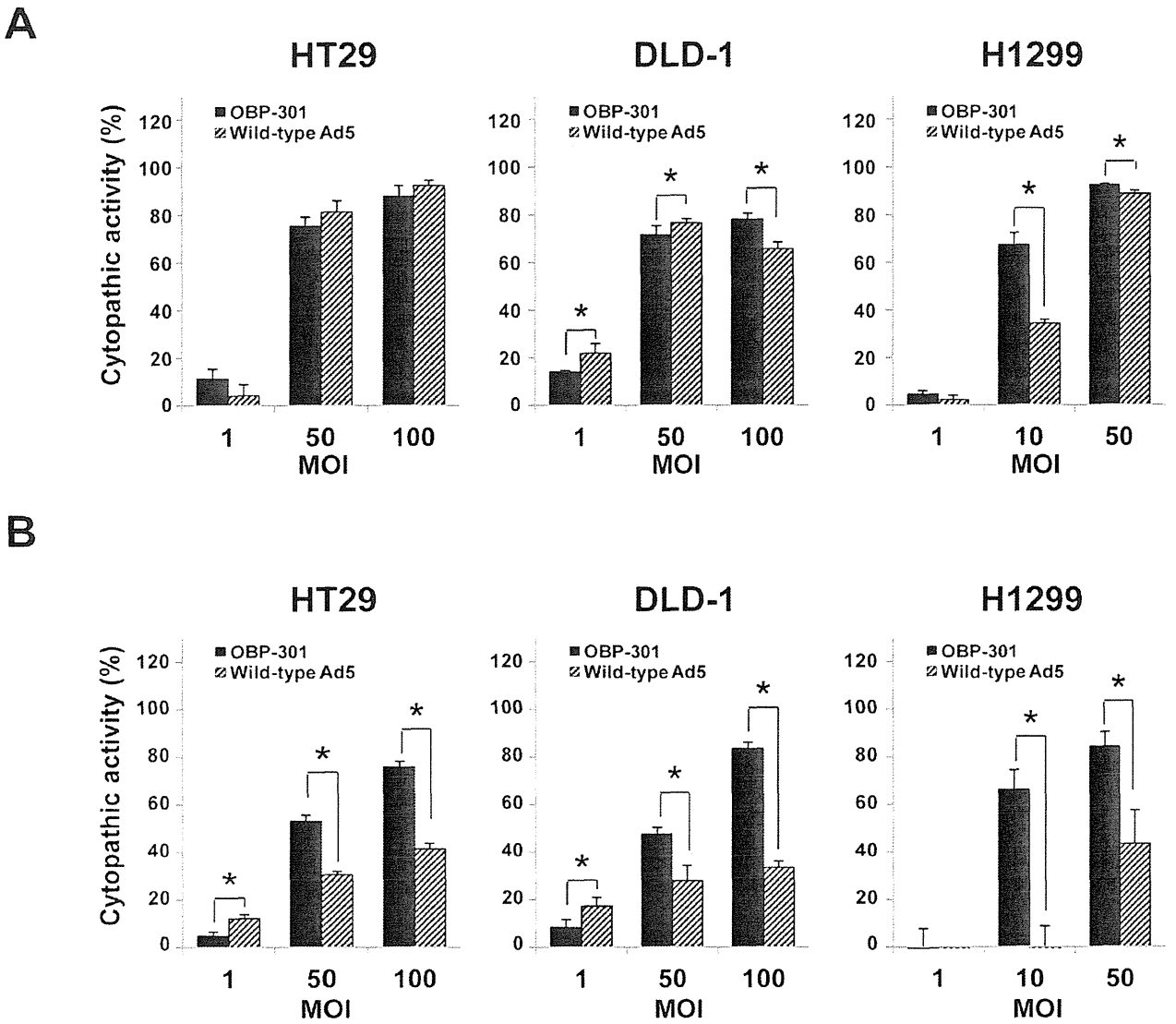

Figure 3. Cytopathic effect of OBP-301 and wild-type adenovirus serotype 5 (Ad5) under normoxic or hypoxic conditions. Cells were infected with OBP-301 (solid bars) or wild-type Ad5 (diagonal bars) at the indicated MOIs under normoxic (**A**) or hypoxic (**B**) conditions for 3 days. Cell viability was determined using an XTT assay. Cell viability was calculated relative to that of mock-treated cells, whose viability was set at 100%. Cytopathic activity was further calculated using the following formula; Cytopathic activity (%) = 100 (%) – cell viability (%). The results shown are the mean values ± SD of quadruplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's t test).
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against both hypoxic and normoxic tumor cells through hTERT activation.

The infection efficacy of Ad5-based oncolytic adenoviruses has been suggested to depend mainly on the expression level of CAR on the target cell surface [28]. Hypoxia has been shown to downregulate CAR expression in tumor cells in a HIF-1α dependent manner [33]. However, in the present study, high CAR expression was maintained in all of the human cancer cells tested, even under hypoxic conditions (Fig. 2E). These results are consistent with a previous report [27], which demonstrated that hypoxia has no influence on adenoviral infectivity of target cancer cells. The expression levels of integrin αvβ3 and αvβ5 are also involved in the infection efficacy of adenoviruses [34]. Previous reports have shown that hypoxia upregulates the expression levels of integrin αvβ3 and αvβ5 in tumor cells [35,36]. These results suggest that hypoxic conditions would mainly suppress the replication of an adenovirus rather than the infection efficiency of the adenovirus.

Tumor tissues frequently contain hypoxic areas due to an immature vascular network. Various exogenous and endogenous hypoxia-related proteins have recently been developed as markers for identification of hypoxic regions of tumor tissues. Increased HIF-1 expression is a useful endogenous marker of hypoxic areas close to blood vessels. Expression of the exogenous hypoxia marker, Pimonidazole, is as effective a marker as HIF-1 for the detection of severely hypoxic regions [37]. In this study, OBP-301-mediated E1A expression was detected in pimonidazole-positive regions as well as normoxic regions (Fig. 5). These results indicate that the telomerase-specific oncolytic adenovirus OBP-301 could

Table 1. Comparison of the ID_{50} values of OBP-301 and Ad5 against human cancer cells under normoxia and hypoxia.

Cell lines	Viruses	ID_{50} value[a] (MOI)		Ratio[b] (Hx/Nx)
		Normoxia	Hypoxia	
HT29	OBP-301	20.2±1.5	51.7±7.3	2.5
	Ad5	25.6±4.3	212.8±52.0	9.4
DLD-1	OBP-301	8.2±2.7	26.3±10.3	3.0
	Ad5	6.6±1.9	2359.6±440.7	597.1
H1299	OBP-301	8.1±1.2	5.3±0.9	0.8
	Ad5	15.1±0.9	18.8±7.8	1.3

[a]The ID_{50} values of OBP-301 and Ad5 were calculated from the data of the XTT assay at day 3 after infection. Data are shown as the mean values ± SE of triplicate experiments.
[b]The ratio was calculated by division of the ID_{50} value under hypoxia (Hx) by the ID_{50} value under normoxia (Nx).
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infect and replicate in tumor cells under a hypoxic microenvironment including in tumor cells in which HIF-1 was active.

Recent advances in our knowledge of tumor microenvironments have provided evidence that hypoxic tumor cells contribute to cancer progression. For example, hypoxia activates the metastatic potential of tumor cells by inducing EMT [6,7] and facilitates the maintenance of cancer stem cells [8–11]. Therefore, the complete elimination of hypoxic tumor cells with metastatic and stemness properties is important for improvement of the clinical outcome of cancer patients. Recent reports have suggested that tumor cells undergoing EMT show reduced CAR expression [38,39], suggesting that tumor cells undergoing EMT are less sensitive to oncolytic adenovirus infection. Further study to investigate the cytopathic effect of OBP-301 in tumor cells undergoing EMT is warranted. In contrast, recent reports have shown that an oncolytic adenovirus induces oncolytic cell death in cancer stem cells [40–43]. Cancer stem cells have recently been shown to have increased hTERT expression compared to non-cancer stem cells [44,45]. Consistent with this high hTERT expression in cancer stem cells, Hemminki *et al.* has suggested that an oncolytic adenovirus that is regulated by specific promoters for hTERT, cyclooxygenase-2 or multidrug resistance, shows efficient cytopathic activity against human breast cancer stem cells [46]. Thus, the hTERT promoter-regulated oncolytic adenovirus OBP-301 may have the potential to eliminate highly progressive tumor cells in a hypoxic microenvironment, thereby contributing to the improvement of its therapeutic benefit against malignant tumors.

In conclusion, we have clearly demonstrated that the antitumor effect of the telomerase-specific oncolytic adenovirus OBP-301 against tumor cells in a hypoxic microenvironment is much stronger than that of a wild-type adenovirus. Regulation of virus replication by the *hTERT* gene promoter would be an effective antitumor strategy that would enhance the cytopathic activity of an oncolytic adenovirus against hypoxic tumor cells.

Materials and Methods

Cell lines

The human colorectal cancer (DLD-1 and HT29) and non-small cell lung cancer (H1299) cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Although cell lines were not authenticated by the authors, cells were immediately expanded after receipt and stored in liquid N_2. Cells were not cultured for more than 5 months following resuscitation. DLD-1 and H1299 cells were propagated as monolayer cultures in RPMI-1640 medium. HT29 was grown in McCoy's 5A medium. The transformed embryonic kidney cell line 293 obtained from the ATCC was maintained in Dulbecco's

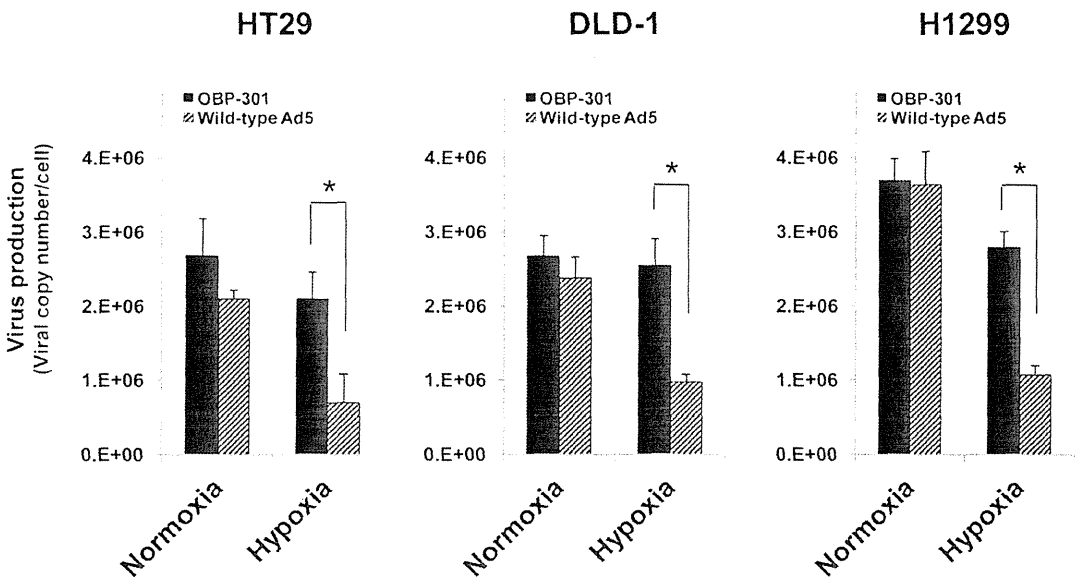

Figure 4. Quantification of viral DNA replication in human cancer cells under normoxia or hypoxia. The indicated human cancer cells were infected with OBP-301 or Ad5 at an MOI of 50 PFU/cell for 1 h, and were further incubated under normoxic (Nx) or hypoxic (Hx) conditions for 48 h. After incubation, cells were harvested and counted. *E1A* copy number in the cells at 48 h after incubation under normoxia or hypoxia was analyzed by quantitative PCR analysis. The amount of virus production was defined as the value of the *E1A* copy number relative to the number of cancer cells. Data are shown as the mean values ± SE of triplicate experiments. Statistical significance (*) was determined as $P<0.05$ (Student's *t* test).
doi:10.1371/journal.pone.0039292.g004

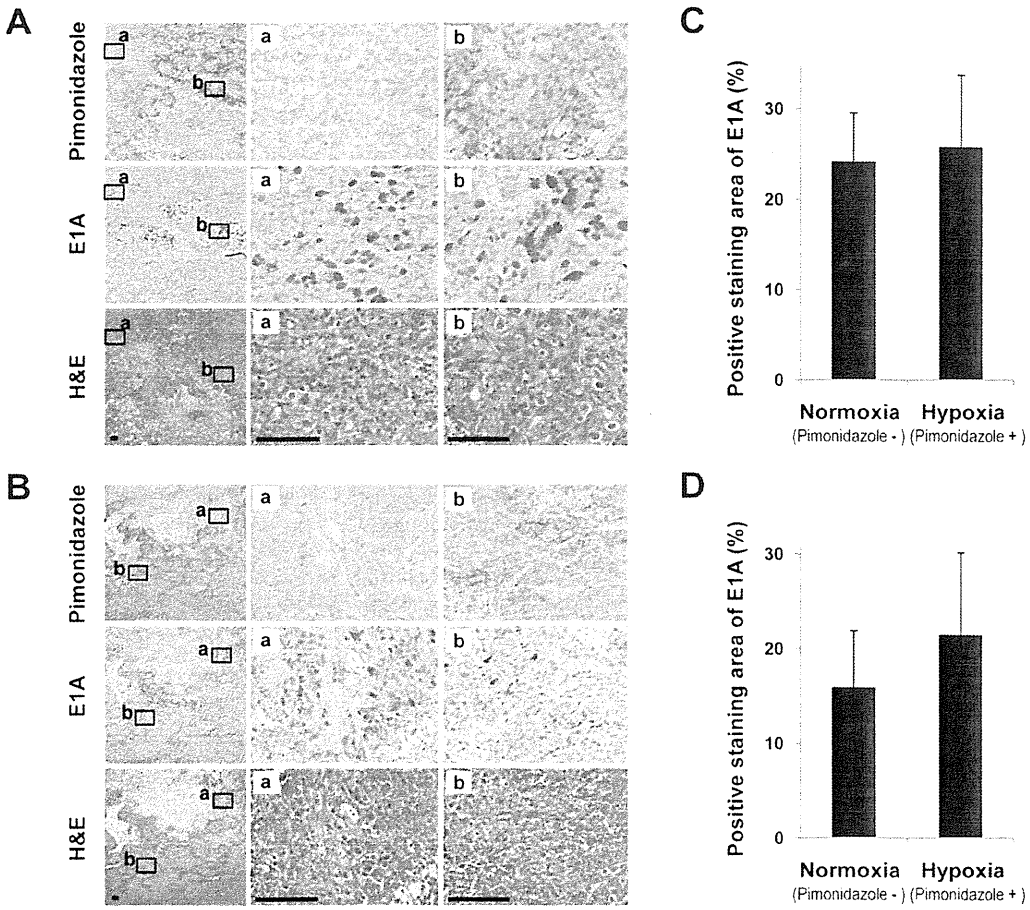

Figure 5. E1A expression in hypoxic areas of human xenograft tumors intratumorally injected with OBP-301. HT29 (**A** and **C**) and DLD-1 (**B** and **D**) tumor cells (5×10^6cells/mouse) were injected subcutaneously into the flank of athymic nude mice. Two weeks after inoculation, OBP-301 (1×10^8 PFU/tumor) was injected into the tumor for three cycles every 2 days. One day after final administration of OBP-301, the mice were intraperitoneally injected with the hypoxia marker pimonidazole hydrochloride (120 mg/kg). Thirty minutes after injection of pimonidazole hydrochloride, the mice were sacrificed and the tumors were harvested. Paraffin-embedded sections of HT29 and DLD-1 tumors were stained with hematoxylin and eosin (H&E). Tumor sections were also immunostained with an anti-pimonidazole antibody and an anti-adenovirus E1A antibody. **A** and **B**, Middle (**a**) and right (**b**) panels are higher magnifications of the boxed regions in the left panels. Original magnification: ×4 (left panels), ×40 (middle and right panels). Scale bars = 100 μm. **C** and **D**, Quantitative analysis of the E1A-positive areas in the normoxic and hypoxic regions of human xenografts tumor tissues. Data are shown as mean values ± SD of quadruplicate experiments.
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modified Eagle's medium containing high glucose (4.5 g/L). All media were supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin. To maintain human cancer cells under hypoxic conditions, the cells were incubated in a hypoxic chamber (Modular Incubator Chamber; Billups-Rothenberg, Del Mar, CA, USA) filled with a gas mixture of 1% O_2, 5% CO_2 and N_2. The cells were also incubated under normoxic conditions at 37°C in a humidified atmosphere with 5% CO_2 and 20% O_2. HIF-1α inhibitor LW6 was purchased from Calbiochem (San Diego, CA, USA) and used at the concentration of 30 μM.

Recombinant adenoviruses

The recombinant replication-selective, tumor-specific adenovirus OBP-301 (Telomelysin), in which elements within the *hTERT* gene promoter drive the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site, was previously constructed and characterized [14]. The wild-type Ad5 was used as a control vector. OBP-301 and Ad5 were generated in 293 cells and purified by cesium chloride step-gradient ultracentrifugation. Their infectious titers were determined by a plaque-forming assay using 293 cells. The ratios of viral particle/plaque-forming unit of OBP-301 and Ad5 are 26 and 27, respectively. Viruses were stored at −80°C.

Western blot analysis

Cells were maintained under a hypoxic or a normoxic condition for 18 h or 24 h. Whole cell lysates were then prepared in a lysis buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40) containing a protease inhibitor mixture (Complete Mini; Roche, Indianapolis, IN, USA). Lysates were electrophoresed on 4%–7% SDS polyacrylamide gels and proteins were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). The primary antibodies used for Western blotting were: mouse anti-HIF-1α monoclonal antibody (mAb) (BD Biosciences, San Diego, CA, USA) and mouse anti-β-actin mAb (Sigma, St. Louis, MO, USA). Horseradish peroxidase-conjugated antibody against mouse IgG

(GE Healthcare) was used as the secondary antibody. Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

Immunofluorescence staining

Cells grown in chamber slides were washed twice with ice-cold PBS, and then fixed with cold 4% paraformaldehyde in PBS for 15 min on ice. The cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min on ice and then blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. The slides were subsequently incubated with mouse anti-HIF-1α mAb (BD Biosciences) or mouse anti-hTERT mAb (KYOWA Medex, Tokyo, JP) for 1 h at room temperature. After two washes with PBS, the slides were incubated with Alexa Fluor 488- or Alexa Fluor 568-labeled goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. The slides were further stained with 10 mg/ml 4′,6-diamidino-2-phenylindole (DAPI), mounted using Fluorescence Mounting Medium (Dako, Glostrup, Denmark), and then photographed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Quantitative real-time RT-PCR analysis

Total RNA was extracted from cancer cells maintained under hypoxic or normoxic conditions for 18 h using the RNA-Bee regent (Tel-test; Friendswood, TX, USA). The *hTERT* mRNA copy number was determined by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) using a LightCycler instrument and a LightCycler Telo*TAGGG* hTERT Quantification Kit (Roche Diagnostics, Bagel, Switzerland). Data analysis was performed using LightCycler Software. The expression of *hTERT* mRNA was defined from the threshold cycle (Ct), and relative expression levels were calculated after normalization with reference to the expression of porphobilinogen deaminase (PBGD).

Transfection and luciferase reporter assay

Cells were seeded on 6-well plates at a density of 4×10^5 cells/well and incubated overnight. Each cell line was transfected with 3 μg of hTERT reporter plasmid (pGL3-hTERT) and 3 μg of GFP expression vector (pCMV-EGFP) as a reporter for transfection efficiency, using Lipofectamin LTX (Invitrogen) following the manufacturer's recommendations. Cells were then incubated under normoxic or hypoxic conditions. After 24 h incubation, luciferase activity was determined using a Bright-Glo reagent (Promega Corporation, Madison, WI, USA). Results presented are the ratios of luciferase activity to GFP fluorescent intensity and the means of three independent experiments.

Flow cytometric analysis

The cells that were maintained under hypoxic or normoxic conditions for 18 h were labeled with a mouse anti-CAR mAb (Upstate Biotechnology, Lake Placid, NY, USA) for 30 min at 4°C. An isotype-matched normal mouse IgG1 (Serotec, Oxfordshire, UK) was used as a negative control. The cells were then incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco, CA, USA) and were analyzed using flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA, USA).

Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^4 cells/well 20 h before viral infection. All cell lines were infected with OBP-301 or wild-type Ad5 at multiplicity of infections (MOI) of 0, 1, 5, 10, 50 or using 100 plaque-forming units (PFU)/cell. The cells were then incubated under normoxic or hypoxic conditions for 3 days. Cell viability was determined using a Cell Proliferation Kit II (Roche Diagnostics) that was based on a sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay according to the manufacturer's protocol. The cytotoxic activity and the ID_{50} value of each virus was calculated using cell viability data. Each experiment was performed in quadruplicate during the same day and repeated at least three times.

In vitro virus replication assay

Cells were seeded on 6-well plates at a density of 3×10^5 cells/well 20 h before viral infection and were infected with OBP-301 or wild-type Ad5 at an MOI of 50 for 1 h. Following removal of the viral inocula, the cells were further maintained under hypoxic or normoxic conditions and were then harvested at 48 h after virus infection. After cell counting, DNA was purified using the QIAmp DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. E1A copy numbers were determined by quantitative real-time PCR using the StepOnePlus Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and TaqMan Gene Expression Assays (Applied Biosystems). The sequences of the specific primers and probe used in this experiment were: E1A primers, 5′-CCT GAG ACG CCC GAC ATC-3′ and 5′-GGA CCG GAG TCA CAG CTA TCC-3′; E1A probe, 5′-FAM-CTG TGT CTA GAG AAT GC-MGB-3′. Data analysis was carried out using StepOne Software (Applied Biosystems).

In vivo human xenograft tumor models

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine (Approval ID: OKU-2009051). The HT29 and DLD-1 cells (5×10^6 cells per site) were inoculated subcutaneously into the flank of 5- to 6-week-old female BALB/c *nu/nu* mice (Japan SLC, Shizuoka, Japan). When the tumor size reached approximately 10 mm in diameter, OBP-301 was injected into the tumors at a dose of 1×10^8 PFU/tumor every 2 days for three cycles. To detect hypoxic areas within tumor tissues, pimonidazole hydrochloride (Hypoxyprobe -1; Hypoxyprobe Inc., Burlington, MA, USA) was injected intraperitoneally at a dose of 120 mg/kg body weight 24 h after the final treatment. The mice were then sacrificed and the tumors were harvested 30 min after pimonidazole injection. Four mice were used for each group.

Immunohistochemistry

Tumors were fixed in 10% neutralized formalin and embedded in paraffin blocks. Sections (4 μm) were prepared for hematoxylin/eosin staining and also for immunohistochemical examination. After deparaffinization and rehydration, antigen retrieval was performed by microwave irradiation in 10 mM citrate buffer (pH 6.0). After quenching of endogenous tissue peroxidase, tissue sections were incubated with mouse anti-adenovirus type 5 E1A mAb (BD Biosciences) and mouse anti-Hypoxyprobe-1 mAb (Hypoxyprobe Inc.). The sections were then incubated using the Histofine Mouse Stain Kit (Nichirei Biosciences, Tokyo, Japan). Immunoreactive signals were visualized by using 3,3′-diaminobenzidine tetrahydrochloride solution, and the nuclei were counterstained with hematoxylin. Signals were viewed under a microscope (BX50; Olympus). The percentage of the positive area in each field was analyzed using Image J software (version 1.45).

Statistical analysis

Determination of significant differences among groups was assessed by using the Student's *t* test. $P<0.05$ was considered significant.

Supporting Information

Figure S1 Suppression of HIF-1α expression in human cancer cells under hypoxic conditions by HIF-1 inhibitor. A, Western blot analysis of HIF-1α protein expression in human cancer cells (HT29 and H1299) under normoxic or hypoxic conditions. Cells were treated with 30 mM HIF-1α inhibitor or DMSO solvent control under hypoxic condition for 24 h. Cell lysates were subjected to Western blot analysis using an anti- HIF-1α antibody. β-actin was assayed as a loading control. **B,** Subcellular localization of HIF-1α expression in human cancer cells treated with 30 mM HIF-1α inhibitor or DMSO solvent control under hypoxia was assessed using immunofluorescent staining. Cells cultured under a hypoxic condition for 24 h were stained with anti-HIF-1α antibody (red). Nuclei were counter-stained with DAPI (blue). Scale bars = 50 μm.
(TIF)

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Author Contributions

Conceived and designed the experiments: YH HT FT TF. Performed the experiments: YH FT TK YW SY. Analyzed the data: YH HT FT FU SK TF. Contributed reagents/materials/analysis tools: YU. Wrote the paper: YH HT TF.

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Wilms Tumor Gene 1 (WT1) Peptide–based Cancer Vaccine Combined With Gemcitabine for Patients With Advanced Pancreatic Cancer

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Summary: Wilms tumor gene 1 (WT1) protein is an attractive target for cancer immunotherapy. We aimed to investigate the feasibility of a combination therapy consisting of gemcitabine and WT1 peptide–based vaccine for patients with advanced pancreatic cancer and to make initial assessments of its clinical efficacy and immunologic response. Thirty-two HLA-A*24:02$^+$ patients with advanced pancreatic cancer were enrolled. Patients received HLA-A*24:02-restricted, modified 9-mer WT1 peptide (3 mg/body) emulsified with Montanide ISA51 adjuvant (WT1 vaccine) intradermally biweekly and gemcitabine (1000 mg/m^2) on days 1, 8, and 15 of a 28-day cycle. This combination therapy was well tolerated. The frequencies of grade 3–4 adverse events for this combination therapy were similar to those for gemcitabine alone. Objective response rate was 20.0% (6/30 evaluable patients). Median survival time and 1-year survival rate were 8.1 months and 29%, respectively. The association between longer survival and positive delayed-type hypersensitivity to WT1 peptide was statistically significant, and longer survivors featured a higher frequency of memory-phenotype WT1-specific cytotoxic T lymphocytes both before and after treatment. WT1 vaccine in combination with gemcitabine was well tolerated for patients with advanced pancreatic cancer. Delayed-type hypersensitivity-positivity to WT1 peptide and a higher frequency of memory-phenotype WT1-specific cytotoxic T lymphocytes could be useful prognostic markers for survival in the combination therapy with gemcitabine and WT1 vaccine. Further clinical investigation is warranted to determine the effectiveness of this combination therapy.

Key Words: Wilms tumor gene (WT1), WT1 peptide vaccine, cancer immunotherapy, pancreatic cancer, gemcitabine

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Pancreatic cancer remains a malignancy with high mortality.[1] Gemcitabine has been the standard first-line treatment for patients with advanced pancreatic cancer, but featured a median overall survival time (MST) of about 6 months and a 1-year overall survival (OS) rate of $\leq$20%.[2] Although many trials of gemcitabine-based combination therapies with cytotoxic or biological agents have been attempted, these therapies, with the exception of erlotinib,[3] have not achieved any survival results superior to those attained with gemcitabine alone.[1] Prognosis of patients with pancreatic cancer thus remains extremely poor, so that novel treatments are urgently needed to improve survival.

Among promising therapeutic strategies, active cancer immunotherapies, such as peptide-based cancer vaccines against tumor-associated antigens (TAAs), which elicit TAA-specific cytotoxic T lymphocytes (CTLs) that eventually eradicate cancer cells, have been and are being developed.[4] However, because their clinical efficacy has been limited,[5,6] several approaches have been tried to improve their efficacy. One approach is the use of combination therapies with certain chemotherapeutic agents, including gemcitabine, which can stimulate the immune system.[7–9] An additional benefit is that chemotherapy makes the tumor cells susceptible to CTL response,[10,11] whereas cancer immunotherapy can sensitize the tumor cells to subsequent chemotherapeutic agents. For this reasons, cancer vaccine in combination with certain chemotherapeutic agents can be expected to exert synergistic effects.

The Wilms tumor gene (*WT1*) is highly expressed in various kinds of malignancies and has been found to perform oncogenic rather than tumor-suppressor functions in tumorigenesis.[12,13] Moreover, both cellular and humoral immune responses against the WT1 protein are naturally elicited in cancer patients, indicating that the *WT1* gene product is actually immunogenic.[14–18] In view of these

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findings, we and others have been performing clinical studies of the efficacy of WT1 peptide–based immunotherapies for patients, including children, with various kinds of malignancies.[13,19–26]

This report describes a phase 1 clinical study of a WT1 peptide–based cancer vaccine combined with gemcitabine for patients with advanced pancreatic cancer. The main objective of this study was to investigate the feasibility of this combination therapy and to make initial assessments of its clinical efficacy and the immunologic response to WT1 peptide.

MATERIALS AND METHODS

Patient Characteristics

Patients with pathologically or cytologically confirmed, measurable, locally advanced, or metastatic pancreatic adenocarcinoma or with recurrent disease were recruited for this noncomparative, open-label, phase 1 study at 2 centers: Osaka University Hospital and Jikei University Kashiwa Hospital, in Japan. Another major eligibility criterion was HLA-A*24:02 positivity. We chose this phenotype because about 60% of Japanese population had this phonotype. Other eligibility criteria included age of 20 years and older, 75 years and younger, Karnofsky performance status 60%–100%, no previous history of treatment for locally advanced or metastatic disease, a minimum 6-month interval from completion of any previous treatment for recurrent disease, a life expectancy of ≥ 3 months, and adequate organ functions. This study was approved by the ethical review boards of the 2 centers and performed in accordance with the Helsinki Declaration. All patients provided written informed consent.

WT Peptide–based Cancer Vaccine (WT1 Vaccine)

A HLA-A*24:02-restricted, modified 9-mer WT1 peptide (mp235; CYTWNQMNL; Peptide Institute Inc., Osaka, Japan) was generated according to the Good Manufacturing Practice Guidelines. In our previous report about the first clinical use of WT1 peptide,[19] the dose-escalation of WT1 peptide from 0.3 to 3.0 mg was designed to decide the recommended dose in combination with the incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France), and 3 mg of WT1 peptide in combination with Montanide ISA51 was decided to be well tolerated. In our present study, we chose WT1 vaccine composed of 3 mg of WT1 peptide and Montanide ISA51 adjuvant. WT1 vaccine was prepared, according to our previous report.[19] WT1 peptide of 3 mg was dissolved in a small volume of dimethyl sulfoxide (DMSO; Sigma, St Louis, MO). The solution was then diluted to 400 μL with 5% glucose and finally emulsified with an equal weight of Montanide ISA51 adjuvant.

Treatment

Gemcitabine was intravenously administered at a dose of 1000 mg/m^2 on days 1, 8, and 15 of a 28-day cycle. WT1 vaccine was intradermally administered at 6 different sites (bilateral upper arms, lower abdomen, and femoral regions) on days 1 and 15 of a 28-day cycle. The initial treatment protocol was planned as 2 courses. Patients without early progressive disease upon the completion of protocol treatment could receive additional treatment until the occurrence of disease progression, unacceptable adverse events, or withdrawal of consent.

Study Assessment

Toxicity was graded using the National Cancer Institute's Common Toxicity Criteria of Adverse Events (CTCAE version 3.0). Dose-limiting toxicity (DLT) was defined as the following adverse events, during the first 2 courses, which were possibly, probably, or definitely related to treatment: grade 4 hematological toxicity lasting >7 days, grade 3 or worse neutropenia accompanied by high fever (≥38°C) or infection (febril neutropenia), and any nonhematological toxicity of grade 3 or worse in other organ systems, including vaccine-injection sites. Biliary tract infection secondary to biliary obstruction was not considered to be a DLT unless it occurred in conjunction with grade ≥ 3 neutropenia. Computed tomography was performed every 4 weeks during the protocol treatment and

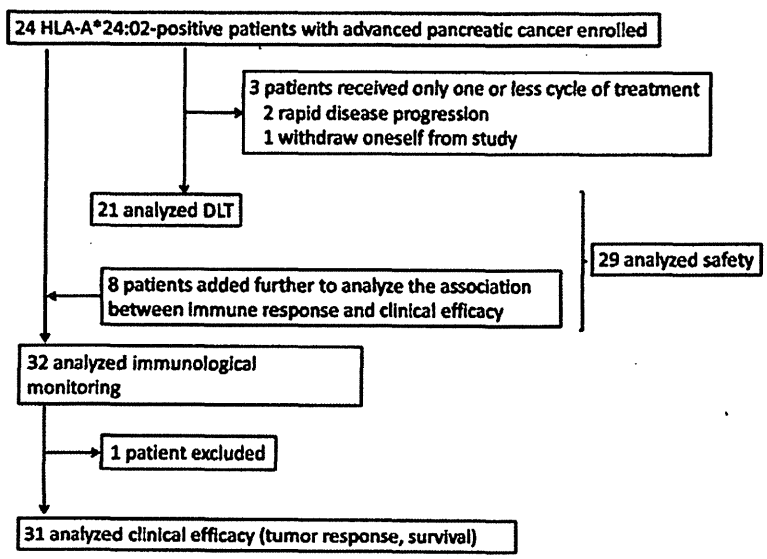

FIGURE 1. Study profile.

every 6–8 weeks during the additional treatment until disease progression, and tumor response was assessed by the investigators according to the Response Evaluation Criteria in Solid Tumors criteria. Stable disease (SD) was defined as a disease that was stable for >8 weeks after the beginning of treatment. The concentration of the tumor marker carbohydrate antigen 19-9 (CA19-9) was measured at baseline and each course.

WT1-specific Immunologic Assessment

As WT1-specific immunologic assessment, delayed-type hypersensitivity (DTH) to WT1 peptide and the WT1 peptide/HLA-A*24:02 tetramer assay was examined. DTH was examined on day 1 of each course during the protocol treatment and optionally at suitable time during the additional treatment. All DTH tests were performed and measured by the investigators. Briefly, 30 μg of WT1 peptide in saline and saline alone were intradermally injected in the forearm, and the maximum diameter of erythema and other skin reaction, including induration, were measured after 48 hours. DTH-positivity was defined as erythema >2 mm in diameter, which size was the minimum size measurable with a ruler at the clinical practice.

Peripheral blood (PB) mononuclear cells for WT1 peptide/HLA-A*24:02 tetramer assay were collected on day 1 of each course during the protocol treatment and appropriately during the additional treatment, and cryopreserved until use. The following tetramer and monoclonal antibodies were used: PE-conjugated $WT1_{235}$ tetramer [HLA-A*24:02-restricted natural 9-mer WT1 peptide (CMT WNQMNL)] (MBL, Nagoya, Japan), anti-CD4-FITC, anti-CD16-FITC, anti-CD45RA-APC (BioLegend, San Diego, CA), anti-CD19-FITC, anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA), anti-CD3-PerCP, anti-CD8-APC-Cy7, anti-CD14-FITC (BD Biosciences, San Jose, CA), and anti-CD56-FITC (eBioscience, San Diego, CA). Lineage antigen (CD4, CD14, CD16, CD19, and CD56)-negative, $CD3^-$, $CD8^-$, and $WT1_{235}$ tetramer$^+$ lymphocytes were defined as WT1 tetramer$^+$ $CD3^+$ $CD8^+$ T lymphocytes (WT1-CTLs). Data acquisition was performed on a FACS Aria instrument (BD Biosciences), and data analysis was performed with FACS Diva software (BD Biosciences).

Statistical Analysis

The safety profile constituted the primary end point. A treatment schedule was considered to be acceptable if the probability of developing DLT was estimated to be <20%. If the estimated probability of DLT occurrence was 10%, the upper limit of the 90% (1-sided) confidence interval (CI) of DLT probability was <20%, based on the projected sample size of 20 patients. For a more accurate determination of the associations with clinical efficacy and immunologic parameters, in total 32 patients were enrolled (8 patients were further enrolled after the completion of safety assessment with the initial 24 patients as shown in Figure 1). The secondary end points included objective response, CA19-9 response, defined as a decrease in CA19-9 concentration of at least 50% in the patients with >100 U/mL of CA19-9 at baseline, progression-free survival defined as time from date of beginning of the treatment to date of disease progression as confirmed by the investigators or death without progression, OS, immunologic responses to WT1 peptide, and correlations between clinical benefit response (CBR)[2] and quality of life (QOL) assessed using by the Functional Assessment of Cancer Therapy-General (FACT-G) measurement system.[39] The nonparametric, Wilcoxon signed-rank test or Mann-Whitney U test was used to calculate P values for change in immune cells because the data were skewed. We judged P values of <0.01 to be significant. χ^2 test was used to calculate P values for associations between DTH and clinical efficacy. The statistical analyses were performed with SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC). Correlations between CBR and the physical and functional scores based on replies to the FACT-G QOL questionnaire were analyzed with a linear mixed-effects model, for which SAS for Windows release 9.1 (SAS Institute Inc.) was used.

RESULTS

Patient Characteristics

A total of 63 patients with advanced pancreatic cancer, whose median age was 63.0 years old, were screened and checked a phenotype in HLA-A locus. Twenty-two patients failed to enroll in this trial because of lack of HLA-A*24:02 phenotype. A total of 32 HLA-A*24:02$^+$ patients with advanced pancreatic cancer were finally enrolled in this trial between 2008 and 2010. Of 32 patients, 28 had inoperable advanced pancreatic cancer (6 locally advanced and 22 metastatic diseases), and the remaining 4 had recurrent

TABLE 1. Patients Characteristics at Baseline

Characteristics	N (%)
Age (y)	
Median	60.0
Range	41–75
Sex	
Male	17 (53.1)
Female	15 (46.9)
Karnofsky performance status (%)	
~70	7 (21.9)
80	10 (31.3)
90	12 (37.5)
100	3 (9.4)
Disease extent	
Inoperable advanced disease	28 (87.5)
Locally advanced	6 (18.8)
Metastatic	22 (68.8)
Recurrent disease	4 (12.5)
Local relapse	1 (3.1)
Distant metastasis	3 (9.4)
Primary tumor site	
Head	15 (46.9)
Body/tail	17 (53.1)
Metastatic sites	
Liver	17 (53.1)
Distant lymph node	16 (50.0)
Lung	7 (21.9)
Peritoneum	6 (18.8)
Others*	4 (12.5)
CA19-9 concentration at baseline (U/mL)	
Median	248
Range (U/mL)	<5–75,050
<5	3 (9.4)†
6–99	10 (31.3)
100–999	7 (21.9)
1000–9999	5 (15.6)
>10,000	7 (21.9)

*Other metastatic sites included bone, ovary, or adrenal gland.
†All patients had the Lewis blood group-negative phenotype.
CA19-9 indicates carbohydrate antigen 19-9.

disease. Table 1 summarizes the patient baseline characteristics. Three patients did not complete the first 2 courses of treatment: 2 patients showed rapid disease progression, and 1 refused to continue the treatment. It was determined by the supervising Data Safety and Monitoring Board that the elimination of these cases was unlikely to be or was not related to the protocol treatment. Of the initial 24 patients, 21 could thus to be used for assessment of DLT, 29 of all 32 patients for assessment of adverse events (Fig. 1).

Safety

Administration of WT1 vaccine in combination with gemcitabine was well tolerated. All adverse events are listed in Table 2. The initial assessment of safety for 21 patients found that a grade 4 central nervous system cerebrovascular ischemia considered to be a DLT had occurred in 1 patient. The most commonly reported adverse event was skin toxicity related to WT1 vaccine. All patients developed grade 1 or 2 skin reactions with swelling, redness, erythema, and induration with or without involvement of small vesicles at the local vaccine-injection sites. Hematological abnormalities were similar to those observed with the administration of gemcitabine alone, and none of the patients developed DLTs associated with hematological abnormalities or febril neutropenia. Eight grade 3 nonhematological adverse events (1 instance of hyponatremia and 7 hepatobiliary/pancreas infections) were detected and attributed to complications associated with disease progression or biliary obstruction. Other major nonhematological adverse events included grade 1 or 2 skin rash, anorexia, nausea, and fever, all of which were previously reported as major adverse events associated with gemcitabine. Hepatic transaminase elevation was principally related to disease progression and/or hepatobiliary infection. Except for local skin reactions, none of the patients experienced adverse events considered to be related to WT1 vaccination.

Clinical Response and Survival Analysis

The clinical efficacy results for all 32 patients are summarized in Table 3. Two patients were excluded from some of these analyses. One patient, who had followed a satisfactory and interesting treatment course and finally undergone a surgical resection (Supplementary Figure 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A317 and Table 3), was excluded from the evaluations of response and survival because the diagnosis of pancreatic cancer could not be pathologically confirmed due to the lack of viable tumor cells in the resected specimens. The other patient was excluded from the evaluation of response because of withdrawal of consent before the first evaluation. Thus, of the total of 32 patients, 30 could be used to evaluate response to treatment and 31 to assess survival. Six of 30 patients (20.0%) reached partial response (PR), and 16 of them (53.3%) showed SD at least for >8 weeks (Table 3). Median progression-free survival was 4.2 months (95% CI, 3.6–4.6) (Fig. 2A) and MST was 8.1 months (95% CI, 6.3–10.0) (Fig. 2B). Six-month and 1-year OS rates were 71.0% (95% CI, 54.9–87.1) and 29.0% (95% CI, 12.9–45.1), respectively (Fig. 2B).

Ten of 19 patients with >100 U/mL of CA19-9 at baseline (52.6%) showed a decrease in CA19-9 serum concentration of at least 50% (Table 3).

TABLE 2. Adverse Events Reported in 29 Patients who Completed the First 2 Courses of Treatment

	Grades				N (%)		
	1	2	3	4	Any Grade (N = 29)	Grade 3 or 4 (N = 29)	DLT (N = 21)
Hematological abnormalities							
Neutropenia	3	6	13	0	22 (75.9)	13 (44.8)	0 (0.0)
Leukocytopenia	4	12	8	0	24 (82.8)	8 (27.6)	0 (0.0)
Lymphopenia	3	12	8	0	23 (79.3)	8 (27.6)	0 (0.0)
Anemia	6	15	2	0	23 (79.3)	2 (6.9)	0 (0.0)
Thrombocytopenia	15	6	1	0	22 (75.9)	1 (3.4)	0 (0.0)
Nonhematological events							
CNS ischemia	0	0	1	0	1 (3.4)	1 (3.4)	1 (4.8)
Hepatobiliary tract infection with normal ANC	0	1	7	0	8 (27.6)	7 (24.1)	0 (0.0)
Hyponatremia	3	0	1	0	4 (13.8)	1 (3.4)	0 (0.0)
Hypoalbminemia	9	4	0	0	13 (44.8)	0 (0.0)	0 (0.0)
ALT	9	4	0	0	13 (44.8)	0 (0.0)	0 (0.0)
AST	10	1	0	0	11 (37.9)	0 (0.0)	0 (0.0)
Bilirubin	2	4	0	0	6 (20.7)	0 (0.0)	0 (0.0)
Hyperkalemia	3	0	0	0	3 (10.3)	0 (0.0)	0 (0.0)
Hemorrhage in urinary tracts	2	1	0	0	3 (10.3)	0 (0.0)	0 (0.0)
Proteinuria	2	0	0	0	2 (6.9)	0 (0.0)	0 (0.0)
Hypokalemia	1	0	0	0	1 (3.4)	0 (0.0)	0 (0.0)
Anorexia	9	0	0	0	9 (31.0)	0 (0.0)	0 (0.0)
Rush*	5	3	0	0	8 (27.6)	0 (0.0)	0 (0.0)
Fever	6	1	0	0	7 (24.1)	0 (0.0)	0 (0.0)
Nausea	7	0	0	0	7 (24.1)	0 (0.0)	0 (0.0)
Diarrhea	2	1	0	0	3 (10.3)	0 (0.0)	0 (0.0)

Adverse events were graded using the National Cancer Institute Common Toxicity Criteria of Adverse Events (CTCAE version 3.0).
*Exclude skin reaction at WT1 vaccine-injection sites.
ANC indicates absolute neutrophil count; CNS, central nervous system; DLT, dose-limiting toxicity.

Correlations between CBR and either physical or functional scores assessed with the FACT-G QOL questionnaire were analyzed. For assessment of CBR, 16 of the initial 24 patients (66.7%) could be used. Nine (56.3%) of these patients (3 with PR, 5 with SD, and 1 with progressive disease) were classified as CBR responders (data not shown). CBR responders showed improvement in physical and functional scores during the first 2 courses, whereas both scores for CBR nonresponders tended to become worse (Supplementary Figure 2, Supplmental Digital Content 2, http://links.lww.com/JIT/A318).

WT1-specific Immune Response

Exploratory analyses of the immune response consisted of assessment of DTH to WT1 peptide and WT1 tetramer$^+$ CD3$^+$ CD8$^+$ T lymphocytes (WT1-CTLs) in PB of all 32 patients. All patients were DTH-negative at baseline, but 31 were at least once assessed as DTH after WT1 vaccination and 18 patients (58.1%) showed DTH-positivity, all of which conversion was detected during the protocol treatment. All of the DTH-positive patients showed at least ≥ 4 mm diameter of erythema, which was a length that was easy enough to measure. Next, for evaluation of associations between survival and DTH, the patients were classified into 4 groups according to survival time: Superior (> 12 mo), good (8–12 mo), moderate (4–8 mo), and poor (≤ 4 mo) responders. These categories were based on the following findings: (i) MST for best supportive care only is no more than 3–4 months[1]; (ii) MST of our patients was 8.1 months; and (iii) survival time of > 12 months generally indicates that the treatment has been beneficial. DTH-positivity of superior and good responders was 68.7% (11/16), whereas that of poor responders was 0% (0/7). The association between DTH-positivity and longer survival time was statistically significant ($\chi^2 = 15.908$, $P = 0.0012$) (Table 4). Therefore, survival was retrospectively reanalyzed in terms of DTH-positivity or DTH-negativity. MST was 3.9 and 10.9 months for DTH-negative (N = 13) and DTH-positive (n = 17)

TABLE 3. Summary of Clinical Efficacy Results

	All Patients	DTH Positive	DTH Negative
Best overall response [N (%)]			
Complete response	0 (0.0)	0 (0.0)	0 (0.0)
Partial response	6 (20.0)	3 (17.6)	3 (23.1)
Stable disease*	16 (53.3)	12 (70.6)	4 (30.8)
Progressive disease	8 (26.7)	2 (11.8)	6 (46.2)
Excluded	1†	1	0
Not evaluable	1		
CA19-9 response (> 100 U/mL at baseline)	N = 19	N = 11	N = 7
Positive‡ [N (%)]	10 (52.6)	7 (63.6)	3 (42.9)
PFS	N = 31	N = 17	N = 13
Range (d)	21–1504 +	55–1504 +	21–373
Median PFS (mo)	4.2 (1.1–7.4)	5.4 (2.6–8.2)	2.9 (−1.6 to 7.1)
3-mo PFS (%)	67 (50–84)	82 (64–100)	46 (9–73)
OS	N = 31	N = 17	N = 13
Range (d)	30–1504 +	154–1504 +	30–443
Median OS (mo)	8.1 (6.3–10.0)	10.9 (1.2–20.7)	3.9 (−3.0–10.7)
6-mo OS (%)	71 (55–87)	88 (73–104)	46 (19–73)
12-mo OS (%)	29 (13–45)	47 (18–65)	7.7 (−6.8 to 22)

(): 95% CI.
*Stable disease conformation is determined at least for > 8 weeks.
†This patient was reached partial response after 3 courses of treatment, and finally underwent the surgical resection. This patient was excluded the analysis of clinical response, PFS, and OS.
‡"Positive" CA19-9 response is defined as a ≥ 50% decrease in CA19-9 concentration after treatment.
CA19-9 indicates carbohydrate antigen 19-9; CI, confidence interval; DTH, delayed-type hypersensitivity; OS, overall survival; PFS, progression-free survival.

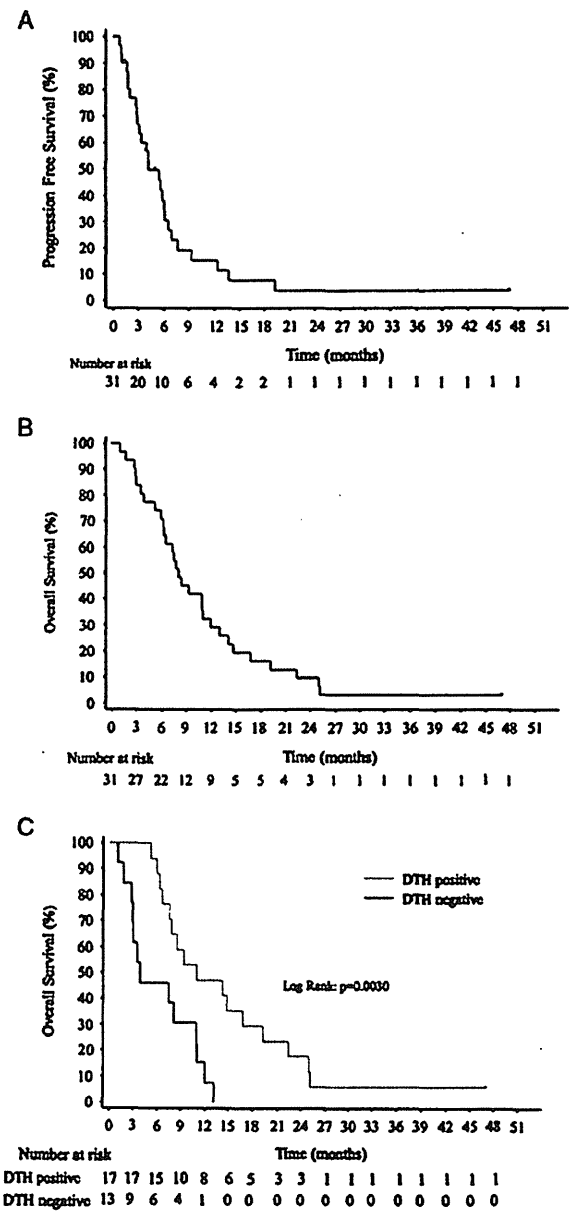

FIGURE 2. Kaplan-Meier survival curves. A, Progression-free survival (N = 31). B, Overall survival (N = 31). C, Overall survival in DTH-positive or DTH-negative patients. DTH indicates delayed-type hypersensitivity.

patients, respectively, with a statistically significant difference ($P = 0.0030$) (Fig. 2C and Table 3).

The number of WT1-CTLs and the percentages of naive ($CD45RA^+CCR7^+$), memory ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$), and effector ($CD45RA^+CCR7^-$) phenotypes in WT1-CTLs did not show any significant changes during the protocol treatment by the analysis using all patients (Supplementary Table 1, Supplemental Digital Content 3, http://links.lww.com/JIT/A319 and Supplementary Table 2, Supplemental Digital Content 4, http://links.lww.com/JIT/A320). Next, these immunologic parameters were compared between patients showing DTH-positivity and DTH-negativity. The difference in the number of WT1-CTLs was not statistically significant (Supplementary Table 1, Supplemental Digital Content 3, http://links.lww.com/JIT/A319). Phenotype analysis of WT1-CTLs showed that the percentage of naive-phenotype was higher in DTH-positive than in DTH-negative patients at baseline (Fig. 3A). After treatment, DTH-positive patients showed a significantly higher percentage of memory-phenotype and consequently a lower percentage of effector-phenotype WT1-CTLs than did their DTH-negative counterparts (Fig. 3A and Supplementary Table 2, Supplemental Digital Content 4, http://links.lww.com/JIT/A320). Furthermore, the percentage of memory-phenotype WT1-CTLs for the superior responders seemed to be relatively higher than that of effector-phenotype WT1-CTLs (Fig. 3B), whereas this tendency was quite the opposite for the poor responders (Fig. 3B and Supplementary Table 3, Supplemental Digital Content 5, http://links.lww.com/JIT/A321).

Case Report

A 44-year-old male with a locally advanced pancreatic head cancer (T4N1M0; stage III) received WT1 vaccine in combination with gemcitabine, and achieved PR (Fig. 4A). Five months after the beginning of the treatment, this patient underwent a complete surgical resection. Histopathologic examination of the resected specimen showed an invasive ductal adenocarcinoma with mononuclear cell infiltration around the cancer region and moderate to severe fibrotic change (Fig. 4B). This patient proved to be positive for DTH to WT1 peptide after 1 treatment course (Fig. 4C). The number of WT1-CTLs transiently decreased during the first 2–3 treatment courses but subsequently increased again, while the percentage of memory-phenotype WT1-CTLs remained high during the treatment courses (Fig. 4C). Of note, the percentage of WT1-CTLs in the tumor-infiltrating $CD3^+CD8^+$ T lymphocytes was 2.48%, which was about 6 times higher than that in PB (0.39%) (Fig. 4D). This patient had been receiving monthly administration of WT1 vaccine in combination with gemcitabine for 3 years and has maintained a Karnofsky performance status of 100% with no evidence of disease recurrence.

DISCUSSION

This study was designed with a DLT target rate of 10% during the first 2 treatment courses, but only one of the 21 initial evaluable patients (4.8%) actually experienced DLT. These results confirmed that WT1 vaccine in combination with gemcitabine is acceptable for patients with advanced pancreatic cancer. Cerebrovascular ischemia, reported here as a DLT, could be also caused by pancreatic cancer itself and/or the administration of gemcitabine, both of which are sometimes associated with a high risk of developing thrombotic disease.[27,28] Therefore, this adverse event was considered to be multifactorial and judged to be "possibly" related to treatment.

Except for skin reactions at the local injection sites, the toxicity profiles of WT1 vaccine in combination with gemcitabine were consistently similar to those of gemcitabine alone. As the *WT1* gene is physiologically expressed in hematopoietic progenitor cells,[13] damage to hematopoiesis is one of the major concerns in WT1 peptide–based immunotherapy. The incidence of hematological adverse events in our study, however, was similar to that observed for treatment with gemcitabine alone,[29] and these events were easily managed and reversible. These findings suggest that WT1 vaccine does not synergistically intensify hematological adverse events associated with gemcitabine. It seems unlikely that WT1-specific CTLs elicited by WT1 vaccine might damage normal WT1-expressing hematopoietic progenitor cells as well as WT-expressing tumor cells, as following reasons. First, in the previous clinical studies, we and others reported that WT1-specifc CTLs elicited by WT1 vaccine decreased WT1-expressing leukemia cells and suppressed the disease progression of WT-expression cancer cells, but not significantly damaged normal hematopoiesis.[19,23–26] Second, it was demonstrated that, using mice in vivo experiments, WT1-tagetting immunotherapy gave damage to tumor cells, but not WT1-expressing normal tissue, including hematopoietic cells.[40,41] The reason why the normal WT1-expressing hematopoietic cells are able to escape from the attack by WT1-specific CTLs is not well known. Further investigations should be required to address this issue.

The clinical efficacy of treatment with WT1 vaccine in combination with gemcitabine, especially in terms of survival, seemed to be better than of that with gemcitabine alone.[1,2] About half of patients who had been induced WT1-specific immunity after vaccination showed better clinical outcome with 12 months or longer survival time, suggesting additional or synergistic effects of WT1 vaccine in combination with gemcitabine. Furthermore, the former contributed to pain relief and thus to improvement of QOL. Recently, the result of the phase III study; GEST study conducted in Japan and Taiwan between 2007 and 2009 has been reported.[40] Median OS and OS rate at 12 months in the gemcitabine alone group were 8.8 months and 35.4%, respectively. These results seemed a little better than those in our study. One reason for this may be the difference in the proportion of the patients with the locally advanced pancreatic cancer, in which survival data were apparently

TABLE 4. Association Between DTH and Survival

	Overall Survival				
	>12 mo (Superior)	≤12, >8 mo (Good)	≤8, >4 mo (Moderate)	≤4 mo (Poor)	Total
DTH positive	8*	3	6	0	17*
DTH negative	1	4	1	7	13
Total	9	7	7	7	30

$\chi^2 = 15.908$, $P = 0.0012$.
*One patient was excluded from this analysis.
DTH indicates delayed-type hypersensitivity.

much better than those in metastatic ones. In our study, this proportion was 18.8%, which was lower than that in GEST study (23.8%). The other reason may be PS at baseline, which was also one of the important prognostic factors. The proportion of the patients with ECOG-PS 0, 1, and 2 at baseline in our study were 46.9%, 31.3%, and 21.9%, respectively, whereas those in GEST study were 65.3%, 34.7%, and 0.0%, respectively. It is apparent that our patients are predicted to worse prognosis than those in GEST study. Despite lower proportion of locally advanced stage and worse PS, however, the survival data gained from the patients with DTH-positivity seemed to be better than those in GEST study. These results suggested additional or synergistic effects of WT1 vaccine. Although the number of patients in our present study was too small to reach any definitive conclusions about clinical efficacy, these findings have been sufficiently encouraging to prompt us to conduct a further clinical study to determine the potency of this combination therapy. No combination chemotherapy, with the exception of FOLFIRINOX,[30] has resulted in a

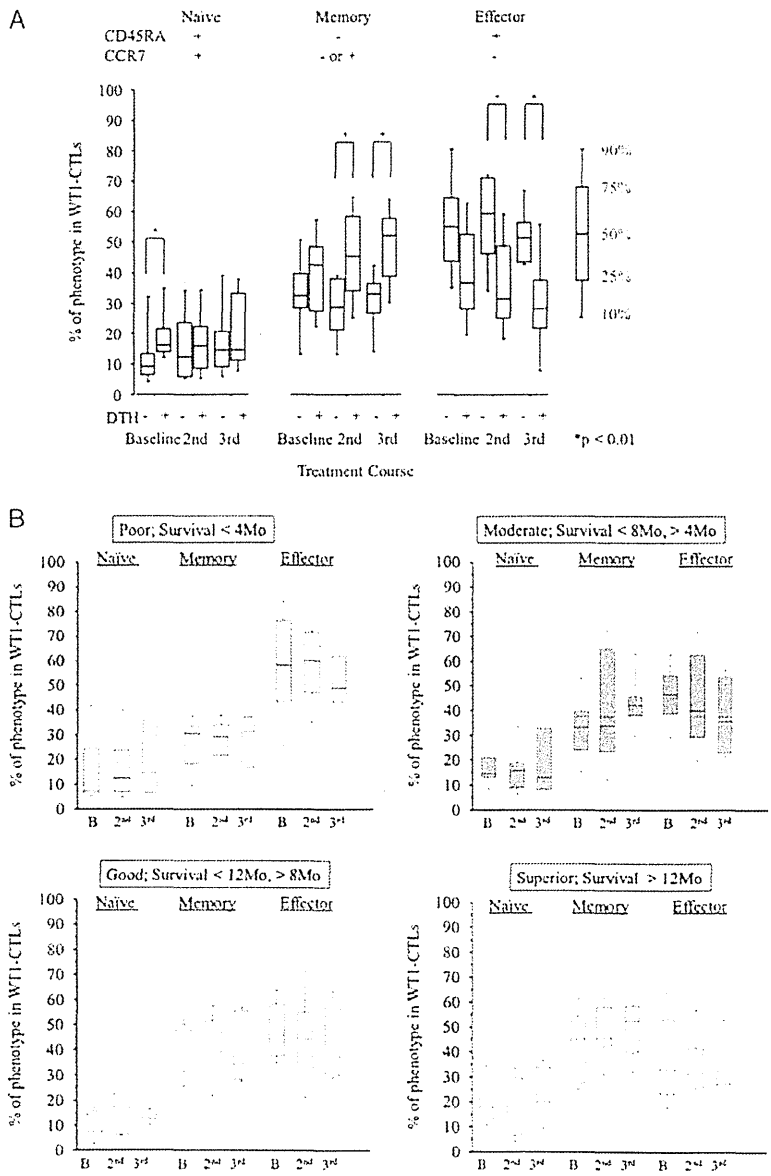

FIGURE 3. Analysis of WT1-specific immune response. A, Immunologic monitoring of the phenotype analysis of WT1 tetramer$^+$ CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs) in DTH-positive (red columns) and DTH-negative patients (blue columns). B, Immunologic monitoring of the phenotype analysis of WT1 tetramer$^+$CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs) in the patients of 4 groups classified according to overall survival time. The broken line represents the median percentage of memory-phenotype WT1-CTLs at baseline for all patients. WT1 tetramer = PE-conjugated $WT1_{235}$ tetramer [HLA-A*24:02-restricted natural 9-mer WT1 peptide (CMTWNQMNL)], naive (CD45RA$^-$CCR7$^+$), memory (CD45RA-CCR7$^+$ or CD45RA-CCR7$^-$), and effector (CD45RA$^-$CCR7$^-$). 2nd indicates day 1 in the second course; 3rd, day 1 in the third course; B, baseline; DTH, delayed-type hypersensitivity.

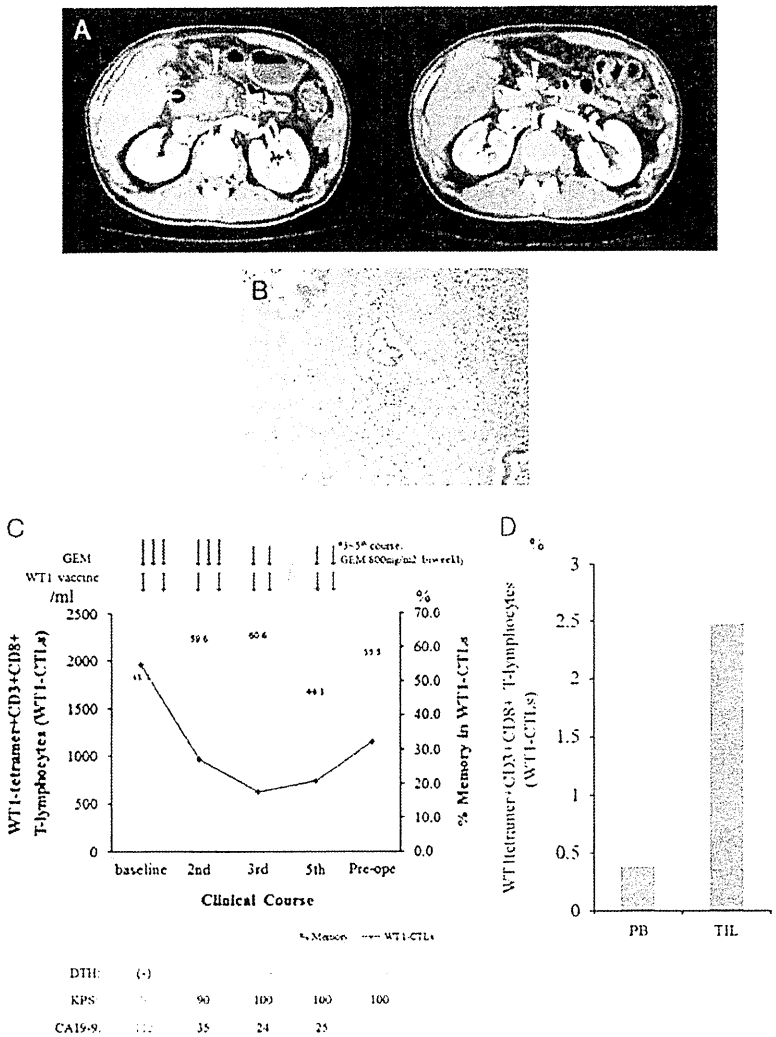

FIGURE 4. Clinical course and immunologic monitoring of 1 patient. A, Abdominal computed tomography (CT) scan before and after treatment. Left: CT scan at baseline showed a large hypodense lesion in the head of the pancreas, which had also invaded the supramesenteric artery and portal vein. Right: 5 months after treatment (before operation), a follow-up CT scan showed >80% regression of the primary lesion. Yellow arrows shows primary lesion of pancreas. B, Microscopic findings of the resected specimen (hematoxylin-eosin stain). C, Clinical course and immunologic monitoring. The blue line represents the absolute number of WT1 tetramer$^+$CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs), and the yellow column represents the percentage of memory-phenotype WT1-CTLs. D, Percentages of WT1-CTLs in the peripheral blood (PB) and tumor-infiltrating lymphocytes (TIL). CA19-9 indicates carbohydrate antigen 19-9; CTLs, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; GEM, gemcitabine; KPS, Karnofsky performance status.

significant improvement in survival of patients with pancreatic cancer although some combination therapies are thought to be more effective for several cancers than single-agent treatments.[1] The use of FOLFIRINOX, however, may have to be limited to patients with good performance status as this regimen has much higher toxicity that sometimes can impair QOL.[31,32] In contrast, as toxicities associated with cancer vaccines are generally mild and acceptable, combination therapies using chemotherapy and cancer vaccine can be expected to exert their clinical benefits without worsening of QOL, which is often impaired by combination chemotherapies using several kinds of cytotoxic agents.

Immunologic monitoring is an important step in the development of evidence-based immunotherapy. Our data provided 2 useful prognostic markers of better clinical outcomes for the combination therapy used in our study. One is DTH to WT1 peptide and the other the frequency of memory-phenotype WT1-CTLs in PB although we did not find the correlation between clinical effects, including survival, and the frequency or absolute numbers of nonphenotypically divided WT1-specific CTLs statistically (data not shown). DTH-positive patients had a notably better prognosis than DTH-negative patients, and the OS curve for DTH-positive patients showed a late separation beyond the median. As DTH has long been used for evaluation of antigen memory for bacterial, viral, and cancer antigens,[32] the occurrence of DTH to WT1 peptide may reflect the development and persistence of memory-phenotype WT1-CTLs. This can be

inferred from our observation that DTH-positive patients showed a significantly higher frequency of memory-phenotype WT1-CTLs than did DTH-negative patients after WT1 vaccination. Furthermore, patients who survived 12 months or longer (superior responders) seemed to have the highest frequency of memory-phenotype WT1-CTLs in their PB although the number of patients in each subgroup was too small to make a statistically valid comparison. It was reported that long-term survivors who had been treated with mutant K-ras vaccine against pancreatic cancer showed the persistence of vaccinated peptide-recognizing T cells (long-term T-cell memory response) for many years after the last vaccination.[33] This report and our results suggest that the development and persistence of TAA-specific CTLs with memory-phenotype resulting from treatment with cancer vaccine contributed to the longer survival. Further investigations are needed to validate these findings in the larger-scale clinical trial.

Despite its potent cytotoxicity, gemcitabine reportedly has immune-modulating functions, such as increase in antigen cross-presentation,[34] and inhibition of B-cells,[35] myeloid-derived suppressive cells,[36] and regulatory T cells,[37] resulting in enhancement of the antigen-specific CTL function. Recently, we reported that gemcitabine enhanced the WT1 expression on human pancreatic cancer cells thus sensitizing the cancer cells to WT1-specific CTL.[11] Furthermore, it was reported that lymphopenia-induced memory-phenotype WT1-CTLs from naive-phenotype WT1-CTLs without self-antigen-induced tolerance.[38] Transient mild to moderate lymphopenia induced by gemcitabine and immediate recovery of T cells could thus promote both the differentiation of naive-phenotype WT1-CTLs into memory-phenotype WT1-CTLs and their proliferation in the clinical application of the combination therapy of gemcitabine and WT1 vaccine. In view of these immunostimulatory properties of gemcitabine, this combination therapy can be expected to generate additional or synergistic effects.

In conclusion, the combination of WT1 vaccine with the standard gemcitabine therapy was well tolerated for patients with advanced pancreatic cancer. WT1 vaccine might have additional effects on gemcitabine to improve survival benefit. An increase in memory-phenotype WT1-CTLs could be a useful predictive marker for a favorable clinical outcome. To determine the clinical efficacy of this combination therapy, we have started a phase 2 randomized clinical study (UMIN000005248).

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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LETTER TO THE EDITOR

Maintenance of complete remission after allogeneic stem cell transplantation in leukemia patients treated with Wilms tumor 1 peptide vaccine

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The prognosis of patients after allogeneic hematopoietic stem cell transplantation (HSCT) is still not satisfactory because, while treatment-related mortalities have decreased, relapse after HSCT remains a major concern. The effectiveness of allogeneic HSCT for hematological malignancies is the result of immunologic rejection of recipient leukemia cells by donor T cells, known as the graft-versus-leukemia (GVL) effect.[1] It is thus obviously important to be able to exploit the GVL effect while minimizing graft-versus-host disease (GVHD). A targeted anti-leukemic immunotherapy, such as use of a leukemia vaccine,[2] is a promising strategy to boost the GVL effect.

Wilms tumor 1 (WT1) protein is one of the best targets for leukemia vaccines. Overexpression of the wild-type *WT1* gene has been detected in all types of human leukemia.[3–5] We performed a phase I clinical study of immunotherapy targeting the WT1 protein in patients with leukemia, and were able to show that WT1 vaccination was safe and could induce WT1-specific cytotoxic T lymphocyte (CTL).[6] Furthermore, reduction of minimal residual disease and long-lasting complete remission (CR) was observed in some leukemia patients who were given the WT1 vaccine.[7]

This report presents the results of phase I clinical study of WT1 vaccination for HLA-A*2402-positivie post-HSCT patients who were at high risk of relapse (HSCT in non-CR and 2nd HSCT for post-transplant relapse) or had already relapsed. The HLA-A*2402-restricted modified 9-mer WT1 peptide (amino acids 235–243 CYTWNQMNL)[8] was emulsified with Montanide ISA51 adjuvant. Patients were intradermally injected with 1.0 mg (three patients: UPNs 1, 4 and 6) or 3.0 mg (other six patients) of WT1 peptide four times weekly. When no adverse effects and no obvious disease progression were observed after the fourth injection, further WT1 vaccinations at 2-week intervals were administered.

Nine patients (five with acute myeloid leukemia (AML), one each with acute lymphoblastic leukemia, chronic myelomonocytic leukemia, multiple myeloma and T-cell lymphoblastic lymphoma) were enrolled in this study (Supplementary Tables 1 and 2). Local inflammatory response was observed at the vaccine injection sites of all patients. One patient (UPN5) suffered mild hypoxia (PaO_2 65 mm Hg at room air) and restrictive pulmonary dysfunction ($FEV_{1.0}$ 40%) 65 days after the start of WT1 vaccination (day 199 after HSCT; Figure 1a). He was diagnosed with bronchioleitis obliterans (BO), which was a symptom of chronic GVHD. The patient recovered soon after administration of inhaled steroids. While early and sudden discontinuation of prednisolone and tacrolimus (day 103 after HSCT) were considered to be the reason for development of BO, the possibility of an association between BO and WT1 vaccination cannot be entirely ruled out. In other eight patients, no severe toxicities related to WT1 vaccine were observed (Table1).

Three AML patients (UPN1–3), who had undergone HSCT in non-CR, started WT1 vaccine in CR (Supplementary Tables 1 and 2). They started WT1 vaccination on post-HSCT days 141, 76 and 93 and have remained in CR for 1038, 973 and 662 days, respectively (as of 8 April 2013; Table1), suggesting the potential of WT1 vaccination as a maintenance therapy after HSCT.

Six patients started WT1 vaccination in non-CR and two of them became CR after WT1 vaccination. One B-ALL patient (UPN4) with MLL-AF4 underwent bone marrow transplantation from an HLA-matched unrelated donor during the first CR. On post-HSCT day 111, MLL-AF4 and WT1 mRNA in peripheral blood (PB) had increased to 16 000 and 15 000 copies/μg RNA, indicating that the disease had relapsed. Tacrolimus and prednisolone doses were tapered off to induce GVL effects. The expression levels of MLL-AF4 and WT1 mRNA in PB had decreased to 2700 and 190

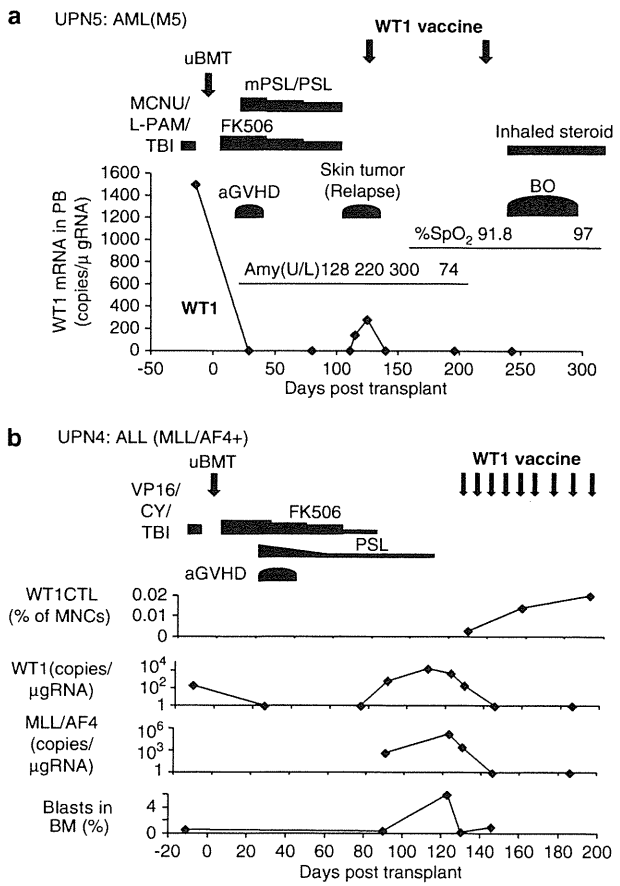

Figure 1. Clinical course of patients who attained CR after the start of WT1 peptide vaccination. (**a**) Clinical course of UPN5 who achieved CR after administration of WT1 vaccine but stopped vaccination because of the development of bronchioleitis obliterans. (**b**) Clinical course of UPN4. Residual leukemia cells that were detected by MLL/AF4 expression disappeared after the start of WT1 vaccination. In both cases, rapid tapering of immune-suppressive drugs preceded WT1 peptide vaccination.

Table 1. Patient outcomes

UPN	Disease	Status before vaccination	Adverse events	Number of vaccine doses	Outcome	Additional therapy	Survival	
							Post-HSCT	After start of vaccination
1	AML (M4)	CR	None	54	CR	–	1179+	1038+
2	AML(M4, DEK/CAN+)	CR	PLT↓	52	CR	–	1049+	973+
3	AML	CR	None	38	CR	–	759+	662+
4	B-ALL (MLL/AF4+)	Molecular relapse	None	71	CR	–	1312+	1179+
5	AML (M4)	Relapse	Amylase↑, bronchileitis obliterans (cGVHD)[a]	2	CR	–	972+	842+[b]
6	CMMoL	Relapse	None	25	PD[c]	Chemo	2265	381
7	MM	PD	None	19	PD	Chemo	1301+	804+
8	T-LBL	Relapse	None	4	PD	Second transplant	955	656
9	AML (M2)	Relapse	None	17	PD	Second transplant	1544+	749+

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CMMoL, chronic myelomonocytic leukemia; CR, complete remission; cGVHD, chronic graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; MM, multiple myeloma; PD, progressive disease; T-LBL, T-cell lymphoblastic lymphoma. (8 April 2013). [a]A causal relationship between vaccination and this event was not strongly suspected, but could not be ruled out. [b]Vaccination was discontinued. (The last injection was on post-HSCT day 60). [c]Size of the subcutaneous tumor decreased, but the disease relapsed in axial lymph nodes and stomach.

copies/μg RNA by day 132, and WT1 vaccination was started on day 133. MLL-AF4 mRNA had become undetectable by day 146, and had never appeared until post-HSCT day 1312 (day 1179 after the start of WT1 vaccination as of 8 April 2013; Figure 1b).

Skin tumors appeared in UPN5 (AML-M5) on post-HSCT day 103 and was diagnosed by biopsy as leukemia relapse. Tacrolimus was discontinued on day103, and WT1 vaccination was started on day 130. Cutaneous tumors had regressed 2 weeks after the start of WT1 vaccination, but vaccination was terminated after the second injection because of the development of BO as described earlier (Figure 1a). This patient has been remained in CR until post-HSCT day 972 (day 842 after the start of WT1 vaccination at 8 April 2013). While the exact contribution of the vaccination effect to the disease remission in addition to the GVL effect was unclear, the fact that both of these two patients still have remained in CR until now is encouraging to continue this trial. In the following phase II trials, the enumeration of WT1-specific CTLs should be performed more frequently after the start of vaccination to clarify the relationship between the effect of WT1 peptide vaccination and leukemia regression.

WT1 (a natural 9-mer WT1 peptide) HLA-A*2402 tetramer assays could be performed with peripheral blood mononuclear cell in seven of the nine patients to determine whether $WT1_{235}$ peptide-specific $CD8^+$ T cells had increased after WT1 vaccination. The gates for WT1 $tetramer^+$ cells were drawn as $<0.1\%$ of $CD8^+$ T cells were included in the tetramer-positive gate in multiple healthy individuals (Supplementary Figure 1A). $WT1_{235}$ $tetramer^+$ cells increased after the start of vaccination in three (UPNs1, 2 and 4) of the four patients who have remained in CR (Figure 1b and Supplementary Figure 1B). In the cases with progressive disease, continuous increase in the frequencies of $WT1_{235}$ $tetramer^+$ cells was not observed (Supplementary Figure 1B).

Our results suggest that WT1 vaccination should be started when the leukemia burden is minimal. The timing of the start of WT1 vaccination may be also important. For the cases with good outcomes, WT1 vaccination was started 76–140 days after transplantation (UPNs1–5), and at later times (days 299–1815) for PD cases (UPNs 6–9). A lymphopenic environment a few months after transplantation may be favorable for rapid and extensive expansion of tumor antigen-specific CTLs.

In summary, this report suggests that WT1 vaccine can be safely administrated for post-HSCT patients with hematological malignancies and has potential as a maintenance therapy. Clinical benefit of WT1 vaccination for post-HSCT patients will be evaluated in the subsequent phase II trials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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