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tance of posttranscriptional modification of IL-6 mRNA by Arid5a and regnase-1. The balance between Arid5a and regnase-1 may determine the pathological increase of IL-6 in various diseases including autoimmunity and even cytokine storm.

IL-6 RECEPTOR-MEDIATED SIGNALING SYSTEM

The IL-6 receptor–signaling system is made up of two receptor chains and downstream signaling molecules (Kishimoto et al. 1992). The IL-6 receptor (IL-6R) constitutes the IL-6-binding chain, which occurs in two forms, 80 kDa transmembrane and 50–55 kDa– soluble IL-6R (sIL-6R), whereas 130 kDa gp130 constitutes the signal-transducing chain. Both of these proteins belong to the cytokine receptor family with a Trp-Ser-X-Trp-Ser motif (Yamasaki et al. 1988; Hibi et al. 1990). sIL-6R without the cytoplasmic region is present in human serum and after IL-6 binding to sIL-6R; the resultant complex induces the IL-6 signal on gp130-expressing cells (Narazaki et al. 1993). The pleiotropic effect of IL-6 on various cells derives from the broad range of gp130 expression observed on cells (Taga and Kishimoto 1997). After binding

of IL-6 to IL-6R, the IL-6/IL-6R complex in turn induces homodimerization of gp130 and triggers a downstream signal cascade (Fig. 4) (Murakami et al. 1993). The activated IL-6 receptor complex is generated in the form of a hexameric structure comprising two molecules each of IL-6, IL-6R, and gp130 (Boulanger et al. 2003). Of these components, IL-6R is a unique binding-receptor for IL-6, whereas the signal-transducing chain gp130 is shared by members of the IL-6 family of cytokines, that is, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, IL-11, cardiotrophin 1, cardiotrophin-like cytokine, IL-27, and IL-35. Although all of these cytokines thus bind to their specific binding receptors, they use the same gp130 for their signals (Kishimoto et al. 1994, 1995). The only exception is virus-encoded IL-6, which is the product of KSHV (also known as human herpesvirus 8), and directly binds to and activates gp130 (Aoki et al. 2001). The mechanism that the IL-6 cytokine family members use to employ the common signal transducer makes it clear why members of the IL-6 family show functional redundancy. The molecular elucidation of the IL-6-signaling system finally solved the long-standing mystery of why cytokines featured pleiotropy and redundancy.

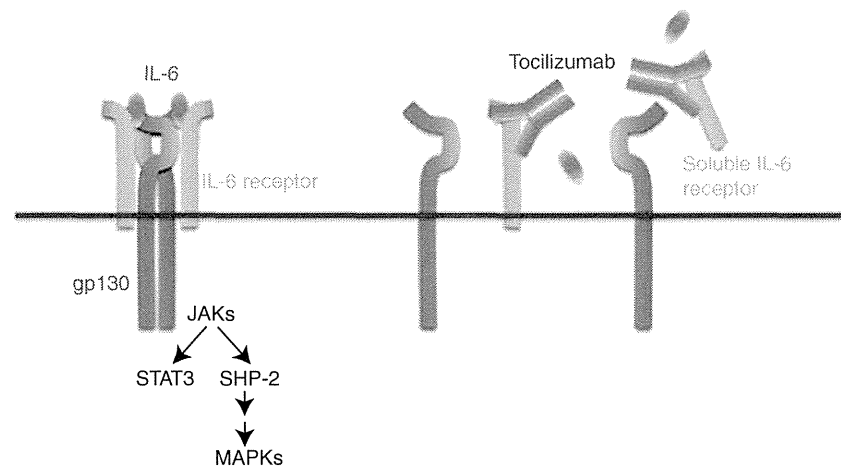


Figure 4. IL-6 receptor system and IL-6 blocker, a humanized anti-IL-6 receptor antibody tocilizumab. IL-6 binds to soluble and transmembrane IL-6R and the complex, then induces homodimerization of gp130, leading to activation of the signaling system. A humanized anti-IL-6R antibody, tocilizumab, blocks IL-6-mediated signaling pathway by its inhibition of IL-6 binding to both receptors. JAKs, Janus kinases; SHP-2, SH2-domain containing protein tyrosine phosphatase-2.



Activation of gp130 in turn triggers activation of downstream signaling molecules, that is, the Janus kinase (JAK)-STAT3 pathway and the JAK-SHP-2-mitogen-activated protein (MAP) kinase pathway. The regulation of various sets of IL-6 responsive genes, including acute phase proteins, is accounted for by the transcription factor STAT3, which also induces the suppressor of cytokine signaling 1 (SOCS1) and SOCS3, which share the SH2-domain. In this context, SOCS1 binds to tyrosine-phosphorylated JAK (Naka et al. 1997), whereas SOCS3 binds to tyrosine-phosphorylated gp130 (Schmitz et al. 2000) to stop IL-6 signaling by means of a negative feedback loop.

IL-6 AND DISEASE

An immediate and transient expression of IL-6 is generated in response to environmental stress factors such as infections and tissue injuries. This expression triggers an alarm signal and activates host defense mechanisms against stress. Removal of the source of stress from the host is followed by cessation of IL-6-mediated activation of the signal-transduction cascade by negative regulatory systems such as ligand-induced internalization and degradation of gp130 and recruitment of SOCS (Naka et al. 1997), as well as degradation of IL-6 mRNA by regnase-1 leading to termination of IL-6 production. However, dysregulated and persistent IL-6 production of mostly unknown etiology, one of which may be the unbalance between Arid5a and regnase-1, in certain cell populations leads to the development of various diseases. This association of IL-6 with disease development was first shown in a case of cardiac myxoma. The culture of fluid obtained from the myxoma tissues of a patient who presented with fever, polyarthritis with positivity for antinuclear factor, elevated CRP level, and hypergammaglobulinemia, contained a large quantity of IL-6, which suggested that IL-6 may contribute to chronic inflammation and autoimmunity (Hirano et al. 1987). Subsequent studies have shown that dysregulation of IL-6 production occurs in the synovial cells of RA (Hirano et al. 1988), swollen lymph nodes of Castleman's disease

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(Yoshizaki et al. 1989), myeloma cells (Kawano et al. 1988), and peripheral blood cells or involved tissues in various other autoimmune and chronic inflammatory diseases and even malignant cells in cancers (Nishimoto et al. 1989, 2005).

Moreover, the pathological role of IL-6 in disease development has been shown in numerous animal models of diseases as well as the fact that IL-6 blockade by means of gene knockout or administration of anti-IL-6 or anti-IL-6R Ab can result in the preventive or therapeutic suppression of disease development. For example, IL-6 blockade resulted in a noticeable reduction in susceptibility to Castleman's disease-like symptoms in IL-6 transgenic mice (Katsume et al. 2002). Similar effects were observed in models of RA (Alonzi et al. 1998; Ohshima et al. 1998; Fujimoto et al. 2008), systemic lupus erythematosus (Mihara et al. 1998), systemic sclerosis (Kitaba et al. 2012), inflammatory myopathies (Okuyama et al. 2009), experimental autoimmune uveoretinitis (Haruta et al. 2011), experimental autoimmune encephalomyelitis (Serada et al. 2008), and many other diseases.

IL-6 TARGETING AS STRATEGY FOR TREATMENT OF IMMUNE-MEDIATED DISEASES

In view of the range of biological activities of IL-6 and its pathological role in various diseases described above, it was anticipated that IL-6 targeting would constitute a novel treatment strategy for various immune-mediated diseases. The development of tocilizumab was a direct result of this hypothesis. Tocilizumab is a humanized anti-IL-6R monoclonal Ab of the IgG1 class that was generated by grafting the complementarity determining regions of a mouse antihuman IL-6R Ab onto human IgG1 (Sato et al. 1993), and it blocks IL-6-mediated signal transduction by inhibiting IL-6 binding to transmembrane and soluble IL-6R (Fig. 4). The outstanding efficacy, tolerability, and safety of tocilizumab were verified in numerous worldwide clinical trials initiated in the late 1990s. This has resulted in the approval of this biologic for the treatment of RA in more than 100 countries (Tanaka et al. 2013),

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as well as for systemic and polyarticular juvenile idiopathic arthritis (Yokota et al. 2008, 2012; De Benedetti et al. 2012) and Castleman's disease (Nishimoto et al. 2005) in several countries. Although other biologics including TNF inhibitors, T-cell stimulator blocker, B-cell depletory, or IL-1R antagonist are currently used for RA, tocilizumab has proved its superior efficacy as monotherapy for moderate-to-severe active RA (Tanaka and Kishimoto 2011; Emery et al. 2013) and is recommended as a first-line biologic. Moreover, the outstanding efficacy of tocilizumab for systemic juvenile idiopathic arthritis has led to the recognition of the start of a new era in the treatment of this disease, which was long considered to be one of the most intractable juvenile diseases (Sandborg and Mellins 2012).

Furthermore, there are strong indications based on favorable results detailed in numerous recent case reports, series, and pilot studies of the off-label application of tocilizumab that it can be used for the treatment of various immune-

mediated diseases (Tanaka and Kishimoto 2012; Tanaka et al. 2012). These comprise autoimmune, chronic inflammatory, autoinflammatory, and other diseases. The first group includes systemic sclerosis, inflammatory myopathies, large vessel vasculitis, systemic lupus erythematosus, relapsing polychondritis, autoimmune hemolytic anemia, acquired hemophilia A, neuromyelitis optica, and Cogan's syndrome. The second group includes adult-onset Still's disease, amyloid A amyloidosis, polymyalgia rheumatica, RS3PE, Behcet's disease, uveitis, Crohn's disease, graft-versus-host disease, pulmonary arterial hypertension, and IgG4-related diseases. The third group comprises such autoinflammatory diseases as TNF-receptor-associated periodic syndrome and chronic inflammatory neurological cutaneous articular syndrome, whereas other diseases include atherosclerosis, type 2 diabetes mellitus, atopic dermatitis, sciatica, and cancer-associated cachexia. Clinical trials are in progress to identify additional indications for tocilizumab (Table 1).

Table 1. Ongoing clinical trials of tocilizumab

Targeted diseases	Status	Identifier
Diabetes mellitus (type 2), obesity	Phase 2	NCT01073826
Graves' ophthalmopathy	Phase 3	NCT01297699
Cardiovascular disease in RA (vs. etanercept)	Phase 4	NCT01331837
Polymyalgia rheumatica	Phase 2	NCT01396317
	Phase 2	NCT01713842
Giant-cell arteritis	Phase 2	NCT01450137
	Phase 3	NCT01791153
Steroid refractory acute GVHD	Phase 1/2	NCT01475162
After HSCT	Phase 2	NCT01757197
Non-ST elevation myocardial infarction	Phase 2	NCT01491074
Noninfectious uveitis	Phase 1/2	NCT01717170
Systemic sclerosis	Phase 2/3	NCT01532869
Transplant rates awaiting kidney transplantation	Phase 1/2	NCT01594424
JIA-associated uveitis	Phase 1/2	NCT01603355
Recurrent ovarian cancer	Phase 1/2	NCT01637532
Behcet's syndrome	Phase 2	NCT01693653
Schizophrenia	Phase 1	NCT01696929
Erdheim–Chester disease	Phase 2	NCT01727206
Primary Sjogren's syndrome	Phase 2/3	NCT01782235
Fibrous dysplasia of bone	Phase 2	NCT01791842

Registered with ClinicalTrials.gov for diseases other than rheumatoid arthritis, juvenile idiopathic arthritis, and Castleman's disease.

GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplant; JIA, juvenile idiopathic arthritis.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The first report of the existence of soluble factors for the enhancement of IgG and IgE Ab responses was published by Kishimoto and Ishizaka in 1973, and it took 13 years until the actual cloning of the IL-6 gene (Hirano et al. 1986). After this success, the fundamental research progressed rapidly and the whole picture of the IL-6 signaling system was completed in the early 1990s (Kishimoto et al. 1994). In parallel with this development, the pathological involvement of IL-6 in various diseases was also established (Nishimoto et al. 1989, 2005; Yoshizaki et al. 1989). It was found that if free serum concentration of tocilizumab is maintained at more than

1 µg/ml, CRP remains negative (Nishimoto et al. 2008), indicating that IL-6 plays a major role in the induction of CRP expression and that IL-6 may be involved in the development of almost all chronic inflammatory diseases with CRP elevation. Clinical trials of tocilizumab started in the late 1990s and this humanized monoclonal Ab was approved for the treatment of Castleman's disease in 2005 in Japan, nearly 20 years after the successful molecular cloning of the IL-6 gene (Fig. 5) (Kishimoto 2005). During the following years, tocilizumab has become a first-line biologic for the treatment of moderate-to-severe active RA and the only approved first-line biologic for systemic juvenile idiopathic arthritis. It is anticipated that during the next decade this IL-6 blocker will be widely used for

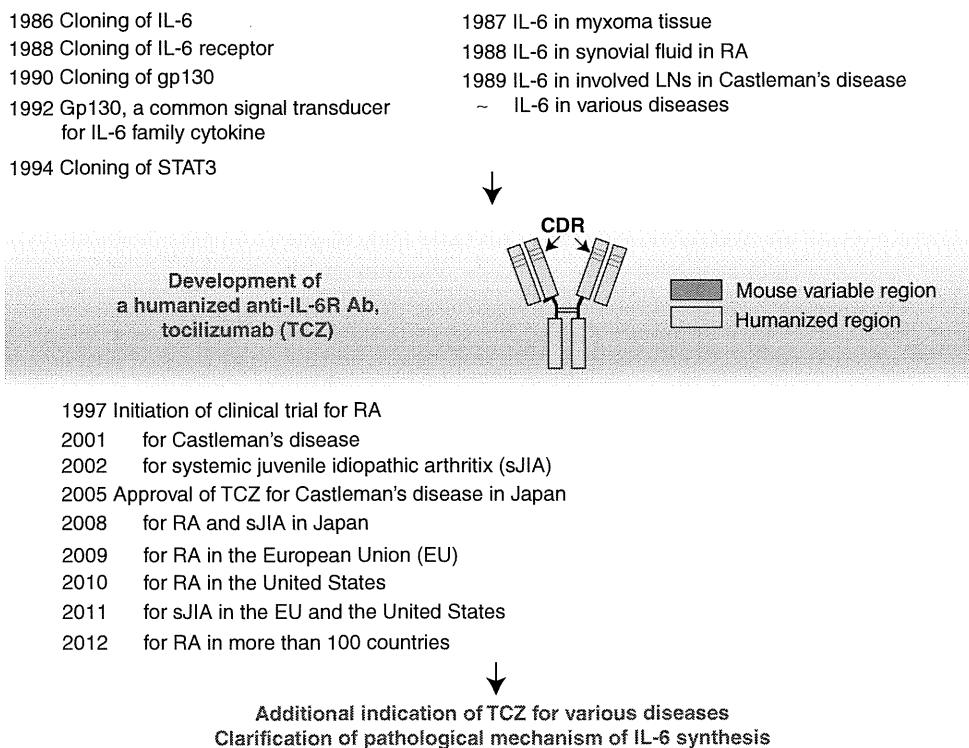


Figure 5. Major discoveries in IL-6-related research and establishment of IL-6 targeting strategy for immune-mediated diseases. Basic research regarding IL-6 clarified the molecular basis of the characteristics of cytokine, redundancy, and pleiotropy, whereas clinical research revealed its pathological significance in disease development. These findings led to concept that IL-6 targeting would constitute a novel therapeutic strategy for immune-mediated diseases and indeed, tocilizumab, a humanized anti-IL-6R antibody became an innovative biologic for the treatment of intractable diseases such as RA, systemic juvenile idiopathic arthritis (sJIA), and Castleman's disease. It will be expected that this strategy would be widely applicable for other immune-mediated diseases. LNs, lymph nodes; TCZ, tocilizumab; CDR, complementarity determining region.

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the treatment of various as-yet intractable diseases including cytokine storm and its application will overcome the refractoriness of such diseases.

To achieve this goal, however, there are several hurdles to overcome. First, additional clinical trials will be needed to evaluate the efficacy and safety of tocilizumab for various diseases. The second, and also important, hurdle is to clarify the mechanisms that render tocilizumab efficacious for phenotypically different diseases. In the case of amyloid A amyloidosis, the ameliorative effect of tocilizumab can be explained by its potent suppression of serum amyloid A protein synthesis (Tanaka et al. 2011). In the case of RA, it has been shown that tocilizumab treatment led to improvement in systemic and joint inflammatory markers (Garnero et al. 2010; Terpos et al. 2011; Kanbe et al. 2012), but it remained to be determined whether it can rectify fundamental immunological abnormalities, such as autoantibody production or imbalance of effector CD4⁺ T-cell subsets (Tanaka 2013). However, recent preliminary results indicate that tocilizumab treatment can correct the imbalance between Th17 and Treg in peripheral blood CD4⁺ T cells (Samson et al. 2012; Pesce et al. 2013). Moreover, it was shown that the treatment caused a reduction in the level of serum IgG4-class anticyclic citrullinated peptide Ab in RA (Carbone et al. 2013). Anti-aquaporin 4 (AQP4) Ab plays a pathological role in neuromyelitis optica, and tocilizumab treatment was found to improve clinical symptoms and reduce serum anti-AQP4 Ab titers, perhaps by inhibiting cell survival of the plasmablasts secreting this Ab (Chihara et al. 2011; Araki et al. 2013). If IL-6 blockade can actually correct these immunological abnormalities, it will in fact be possible to use tocilizumab for the treatment of a wide variety of immune-mediated diseases.

Finally, the mystery remains why IL-6 is persistently expressed in distinct cell populations in various diseases. Accurate and detailed analyses of proteins such as Arid5a and regnase-1 and of microRNAs that regulate IL-6 synthesis will be helpful for solving this mystery, whereas clarification of the mechanism(s) involved will facilitate the identification of more specific target

molecules and investigations into the pathogenesis of specific diseases.

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

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Summary: Wilms tumor gene (*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/m²) 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 reason, 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

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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 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 I 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 I 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 phenotype. 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.

WT1-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 μ L 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/m² 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 ($\geq 38^{\circ}\text{C}$) or infection (febrile 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

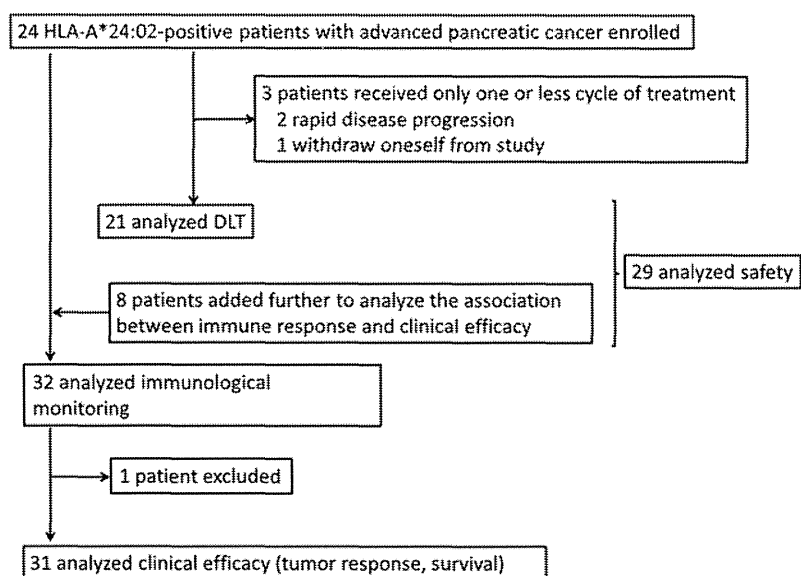


FIGURE 1. Study profile.

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 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 WT1₂₃₅ 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 WT1₂₃₅ tetramer⁺ lymphocytes were defined as WT1 tetramer⁺ CD3⁺ CD8⁺ T lymphocytes (WT1-CTLs). Data acquisition were performed on a FACS Aria instrument (BD Biosciences), and data analysis were 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% (one-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 Fig. 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.²⁷ 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

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)
$\geq 10,000$	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.

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 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 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 febrile 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)
Hypoalbuminemia	9	4	0	0	13 (44.8)	0 (0.0)	0 (0.0)
Alanine aminotransferase	9	4	0	0	13 (44.8)	0 (0.0)	0 (0.0)
Aspartate aminotransferase	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, Supplemental 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 months¹; (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

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 Not evaluable	1†	1	0
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 to 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.

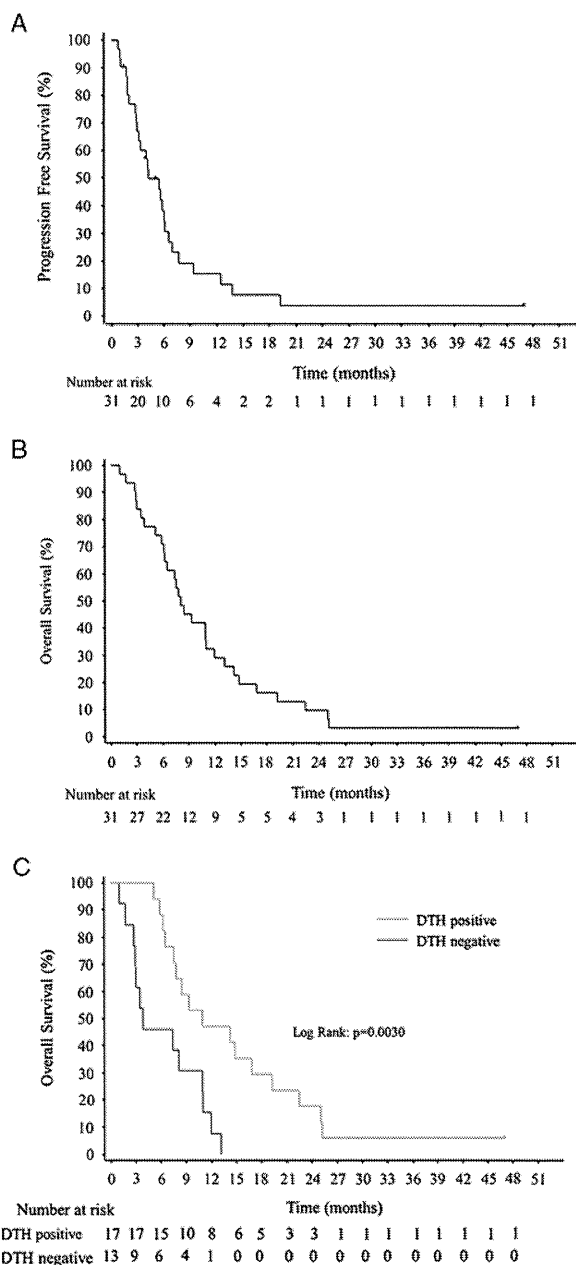


FIGURE 2. Kaplan-Meier survival curves. A, Progression-free survival (N = 31). B, Overall survival (N = 31). C, Overall survival in DTH-positive (gray line) or DTH-negative patients (black line). DTH indicates delayed-type hypersensitivity.

($\chi^2 = 15.908$, $P = 0.0012$) (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) 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.^{28,29} 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,³⁰ 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-specific 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-targeting immunotherapy gave damage to tumor cells, but not WT1-expressing normal tissue, including hematopoietic cells.^{31,32} 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 of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer (GEST study) conducted in Japan and Taiwan between 2007 and 2009 has been reported.³³ Median OS and OS rate at 12

TABLE 4. Association Between DTH and Survival

	Overall Survival				Total
	> 12 mo (Superior)	≤ 12, > 8 mo (Good)	≤ 8, > 4 mo (Moderate)	≤ 4 mo (Poor)	
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.

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 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

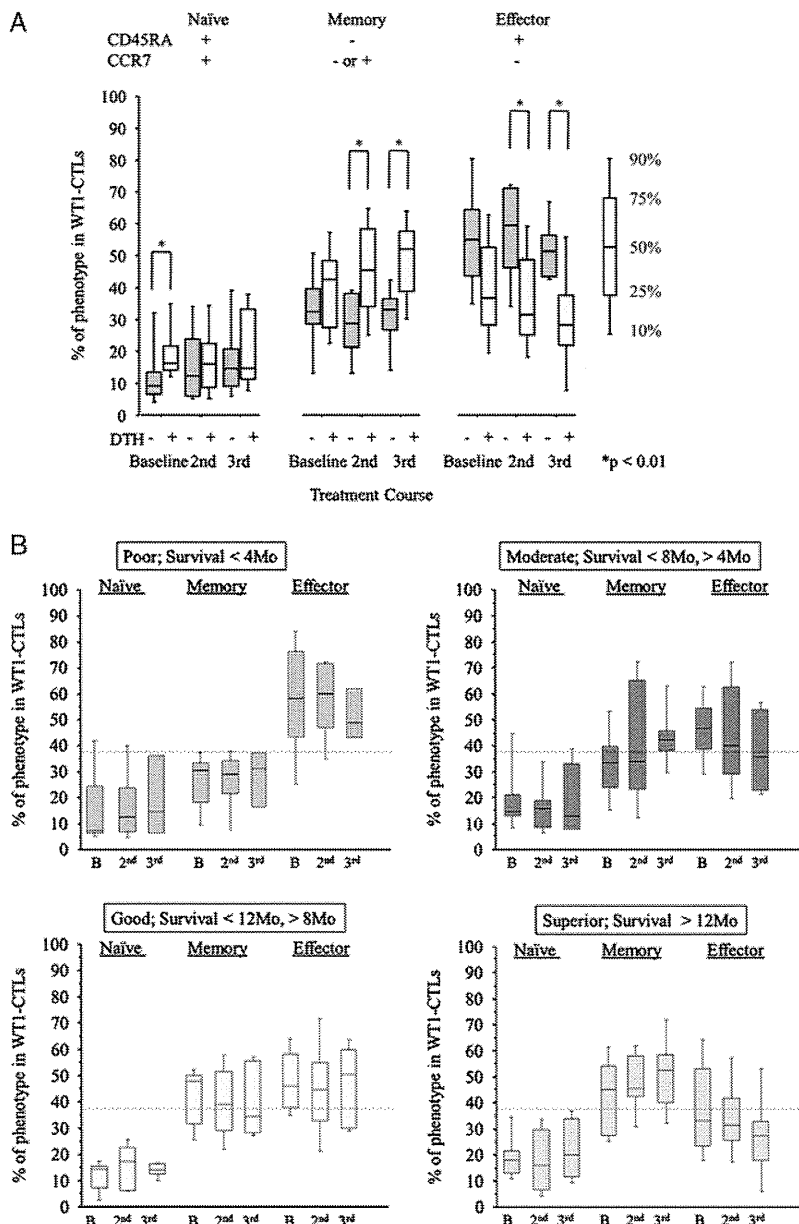


FIGURE 3. Analysis of WT1-specific immune response. A, Immunologic monitoring of the phenotype analysis of WT1 tetramer+ CD3+CD8+ T lymphocytes (WT1-CTLs) in DTH-positive (light gray columns) and DTH-negative patients (dark gray columns). B, Immunologic monitoring of the phenotype analysis of WT1 tetramer+ 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 WT1₂₃₅ tetramer [HLA-A*24:02-restricted natural 9-mer WT1 peptide (CMTWNQMNL)], naïve (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.

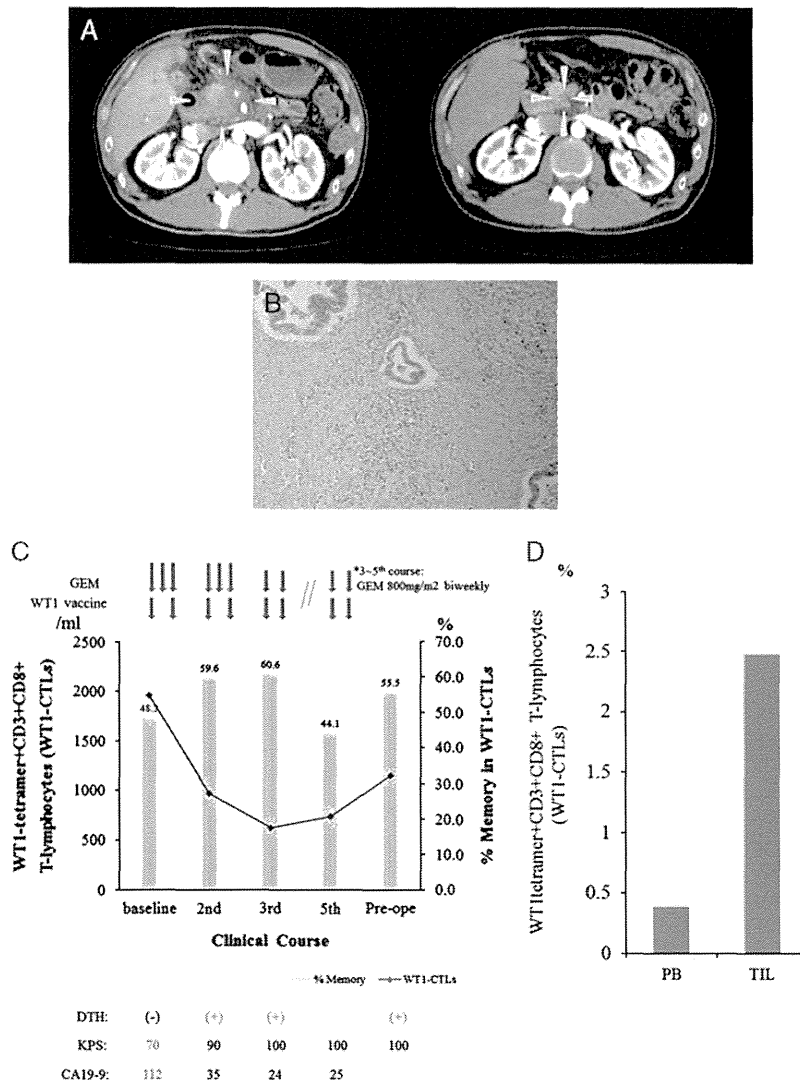


FIGURE 4. Clinical course and immunologic monitoring of 1 patient. A, Abdominal computed tomography (CT) scan before and after treatment. Left: CT scan at baseline showed a large hypodense lesion in the head of the pancreas, which had also invaded the supramesenteric artery and portal vein. Right: 5 months after treatment (before operation), a follow-up CT scan showed >80% regression of the primary lesion. Gray arrows shows primary lesion of pancreas. B, Microscopic findings of the resected specimen (hematoxylin-eosin stain). C, Clinical course and immunologic monitoring. The black line represents the absolute number of WT1 tetramer+CD3+CD8+ T lymphocytes (WT1-CTLs), and the gray 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.

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,³⁴ has resulted in a 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.^{34,35} 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,³⁶ 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.³⁷ 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,³⁸ and inhibition of B-cells,³⁹ myeloid-derived suppressive cells,⁴⁰ and regulatory T cells,⁴¹ 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.⁴² 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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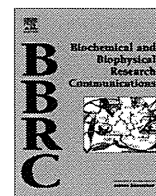
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Generation and characterization of a bispecific diabody targeting both EPH receptor A10 and CD3



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ABSTRACT

The EPH receptor A10 (EphA10) is up-regulated in breast cancer but is not normally expressed in healthy tissue, thus it has been suggested that EphA10 may be a useful target for cancer therapy. This study reports a diabody, an antibody derivative binding two different target molecules, EphA10 expressed in tumor cells and CD3 expressed in T cells, which showed T cell dependent-cytotoxicity. The diabody, which has His-tagged and FLAG-tagged chains, was expressed in *Escherichia coli* and purified in both heterodimer (Db-1) and homodimer (Db-2) formulations by liquid chromatography. Flow cytometry analysis using EphA10-expressing cells showed that binding activity of heterodimers was stronger than that of homodimers. Addition of diabodies to PBMC cultures resulted in T-cell mediated redirected lysis, and the bioactivity was consistent with the stronger binding activity of heterodimeric diabody formulations. Our results indicate that diabodies recognizing both EphA10 and CD3 could have a range of potential applications in cancer therapy, such as breast cancers that express the EPH receptor A10, especially triple negative breast cancer.

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1. Introduction

The EPH receptor A10 (EphA10) [1], known as an ephrin receptor family protein, is known to be involved in cancer progression. The roles of EphA10 in cancer have not yet been fully elucidated [2], although it has been shown to be a contributing factor in tumor progression and invasion and has been associated with enhanced tumorigenic properties and reduced survival times in breast carcinoma. Its expression in normal human tissue seems to be confined to the testis [1] and it is up-regulated in several cancers including prostate cancer [3], ovarian cancer and breast cancer [4,5]. EphA10 transcripts are absent in normal prostate and breast cells but are present in cancer cells of prostate and breast, respectively. Interestingly, high levels of EphA10 are found in the context of triple

negative breast cancers (TNBCs) [5]. Targeting EphA10 by blocking EphA10-dependent activation of the MAPK pathway has resulted in tumor growth inhibition *in vivo*. Therefore, EphA10 has emerged as a promising target for antibody therapies, while the exact functions and mechanism of action of EphA10 in normal physiology or in pathological conditions remain to be determined.

Creating bispecific antibodies (BsAbs), which are capable of simultaneous binding to two different targets, could overcome many defects of monoclonal antibody therapies [6]. Such molecules would be able to retarget not only a large variety of cancer cells but other cell types as well, such as lymphocytes [7–9]. The potential of this approach has been demonstrated by several studies and large amounts of heterogeneous BsAbs have been produced using techniques of molecular biology. In particular, a diabody, which is a kind of BsAb, is constructed from non-covalently associated bivalent molecules, created from scFvs by shortening the polypeptide linker between the VH and VL domains [10–13]. These antibody derivatives may be used as therapeutic drugs to treat cancer and blood coagulation diseases.

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