Abstract. Background/Aim: Treatment combining dendritic cells (DCs) pulsed with three types of major histocompatibility complex (MHC) class I and II (DC/WT1-I/II)-restricted Wilms’ tumor 1 (WT1) peptides with chemotherapy may stabilize disease in pancreatic cancer patients. Materials and Methods: Laboratory data from seven patients with pancreatic cancer who underwent combined DC/WT1-I/II vaccination and chemotherapy were analyzed. The DC phenotypes and plasma cytokine profiles were analyzed via flow cytometry. Results: The post-treatment neutrophil to lymphocyte (N/L) ratio was a treatment-related prognostic factor for better survival. Moreover, the mean fluorescence intensities (MFIs) of human leukocyte antigen (HLA)-DR and cluster of differentiation (CD)83 on DCs were significantly increased after chemoimmunotherapy. Interestingly, interleukin (IL)-6 level in plasma was significantly increased after chemoimmunotherapy in non-super-responders. Conclusion: An increased N/L ratio, as well as HLA-DR and CD83 MFI levels may be prognostic markers of longer survival in patients with advanced pancreatic cancer who undergo chemoimmunotherapy.
among patients who exhibited positive delayed-type hypersensitivity (DTH) reactions against the WT1 peptides (8). Moreover, patients with strongly-positive DTH reactions maintained WT1-specific memory CTLs throughout the entire treatment period. Therefore, we suggested that a combination treatment involving DC/WT1-I/II and chemotherapy could lead to disease stability in patients with advanced pancreatic cancer (8). In the present study, we analyzed the prognostic markers for the outcomes of patients with pancreatic cancer who underwent chemoimmunotherapy with DC/WT1-I/II vaccination and chemotherapy.

Materials and Methods

Study design. The ethics committee of the Jikei Institutional Review Board at the Jikei University School of Medicine and the clinical study committee of Jikei University Kashiwa Hospital (No. 14-60 (3209) and 21-204 (6082)) reviewed and approved this study. All 7 patients with pancreatic cancer provided written informed consent and all procedures were performed in accordance with the Helsinki Declaration. All patients underwent DC/WT1-I/II vaccination and chemotherapy. The laboratory data of patients who underwent chemoimmunotherapy (e.g., C-reactive protein level (CRP), lymphocyte number, neutrophil number, neutrophil to lymphocyte (N/L) ratio) were analyzed prior to and after the treatments. The N/L ratio was defined as the ratio of the number of neutrophils to the number of lymphocytes in the blood.

DC-WT1-I/II vaccines. DCs were generated from peripheral blood mononuclear cells (PBMCs) that had been prepared from leukapheresis products using Ficoll-Plaque Premium (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) density gradient solution as previously described (9). The DCs were pulsed with a mixture of three WT1 peptide types that were restricted to HLA-A*02:01, A*02:06 (126-134: RMFPNAPYL), A*24:02 (235-243: CYTWNQMNL) and MHC-class II (332-347: KRYFKLSLHQ MHSRKH; NeoMPS Inc., City, CA, USA) (8).

Chemioimmunotherapy. The chemotherapeutic agent gemcitabine was intravenously administered at a dose of 1,000 mg/m² on days 1, 8 and 15 of a 28-day cycle. After the first gemcitabine administration cycle, the pancreatic cancer patients were treated with a combination of DC/WT1-I/II and gemcitabine. The DC/WT1-I/II vaccine (approximately 1×10⁷ cells/dose) was intradermally administered biweekly. Nearly all vaccines overlapped with the standard chemotherapy (8).

Phenotype analysis. DCs generated from PBMCs that had been prepared from leukapheresis products were stained with the following monoclonal antibodies (mAb) for 30 min on ice: fluorescein isothiocyanate (FITC)-conjugated anti-human HLA-ABC (W6/32), CD80 (2D10), CD40 (5C3), phycoerythrin (PE)-conjugated anti-human CC chemokine receptor (CCR) 7 (150503; R&D Systems, Minneapolis, MN, USA), HLA-DR (L243), CD83 (HB 15c) and CD86 (IT2.2; BioLegend, San Diego, CA, USA). The cells were subsequently washed, fixed and analyzed using MACSQuant Analyzers (Miltenyi Biotec Inc., CA, USA) and the FlowJo analysis software (Tree Star, OR, USA).

Plasma cytokine profiles. The patient plasma samples collected were stored at −80°C. The stored plasma cytokine profiles were determined using the Human MACSPlex Cytokine 12 Kit, which enables the simultaneous measurement of human granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, IFN-γ, interleukin (IL)-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A and tumor necrosis factor (TNF)-α in a single immunoassay (Miltenyi Biotec Inc.) via MACSQuant Analyzers (Miltenyi Biotec Inc., CA, USA). The cytokine concentrations were quantified according to the manufacturer’s instructions.

ELISA. To assess the production of IFN-γ and IL-10 in PBMCs upon stimulation with MHC-I/II-restricted WT1 peptides in vitro, PBMCs (1×10⁶ cells/ml per well) from 6 DC/WT1-I/II vaccination cycles were cultured for 6 days with 10 μg/ml WT1 class I and II peptides in the presence of 10 U/ml recombinant human (rh) IL-2 (Shionogi, Osaka, Japan) and 10 ng/ml IL-7 (Peprotech, Rocky Hill, NJ, USA). HIV env peptides were used as negative controls. The IFN-γ and IL-10 concentrations in the sample supernatants were analyzed using ELISA kits (BioLegend) according to the manufacturer’s instructions.

Statistical analysis. The statistical analyses of the overall survival (OS) and progression-free survival (PFS) prognostic factors were performed according to the Kaplan-Meier method and evaluated using the log-rank test. The immunological parameters of the pancreatic cancer patients were evaluated in a t-test analysis. Values were expressed as the mean±standard deviation (SD). A p-value of <0.05 was considered statistically significant.

Results

Patients’ characteristics. Patients with pathologically- or cytologically-confirmed, measurable, metastatic pancreatic adenocarcinoma or recurrent disease were enrolled in a non-comparative, open-label, phase I study (8). All 7 patients had stage IV disease and were treated with DC/WT1-I/II and chemotherapy. As shown in Table I, we identified 3 super-responders (OS>1 year) and 4 non-super-responders (OS≤1 year). After treatment, pancreatic cancer spread to the liver in one super-responder; however, this patient remains alive (760 days) with a 100% Karnofsky Performance Status (KPS) after receiving treatment and has received more than 51 vaccinations. The remaining 2 super-responders with stage IV pancreatic cancer died at 582 and 717 days after the first treatment.

Prognostic markers as indicated by laboratory data. There were no differences between the super-responders (OS>1 year) and non-super-responders (OS≤1 year) with regard to sex, age or tumor location (Table II). We analyzed the laboratory data prior to treatment, after the first course of gemcitabine (prior to the first vaccination) and after 6-8 rounds of DC/WT1-I/II vaccination combined with gemcitabine. There was no significant difference in the lymphocyte numbers between the super-responders and non-super-responders. Moreover, the pretreatment N/L ratio of

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each group was not significantly associated with OS. Interestingly, after the first course of gemcitabine (prior to the first vaccination) and after 6-8 DC/WT1-I/II vaccinations combined with gemcitabine, the N/L ratio (<4) decreased significantly in the super-responders ($p=0.025$ and $p=0.018$, respectively; Table II and Figure 1). These results suggested that an N/L ratio <4 was a prognostic marker that correlated with OS.

**DC phenotype.** The DCs from all 7 patients displayed a characteristic phenotype comprising of HLA-ABC, HLA-DR, CD40, CD80, CD86, CD83 and CCR7 expression (Figure 2, upper panel). There were no differences in the HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 mean fluorescence intensities (MFIs) on DCs from super-responders (OS>1 year) versus non-super-responders (OS≤1 year) (Figure 3). Interestingly, the HLA-DR and CD83 MFIs were significantly increased in the DCs of the super-responders following chemoimmunotherapy (Figure 2 and Figure 4). In contrast, the CD83 MFIs in the DCs of non-super-responders decreased after therapy, although this difference was not significant ($p=0.302$).

**Plasma cytokine level profiles.** To assess the T-helper 1 (Th1) and T-helper 2 (Th2) cell-related cytokine profiles following chemoimmunotherapy, the levels of GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A and TNF-α in plasma collected after 6-8 vaccinations were immediately and simultaneously analyzed. There were no differences between the super-responders and non-super-responders in terms of these plasma cytokine levels after vaccination (data not shown). The levels of immunosuppressive cytokines, such as IL-4, IL-10 and IL-6, were higher in samples from non-super-responders (OS≤1 year) than in those from super-responders (OS>1 year), although this difference was insignificant (Figure 5A). Moreover, the levels of the Th1-stimulating cytokines IFN-α, IFN-γ and TNF-α were also higher in non-responders; again, this difference was insignificant (Figure 5A). Interestingly, the IL-6 level was significantly increased after chemoimmunotherapy in non-super-responders ($p=0.049$) (Figure 5B).

**Cytokine production by PBMCs following WT1 peptide stimulation.** To assess the cytokine profiles upon WT1 peptide stimulation in vitro in greater detail, PBMCs were
cultured with MHC-I- and -II-restricted WT1 peptides after which the Th1 cytokine IFN-γ and Th2 cytokine IL-10 concentrations in the supernatants were determined. In this experimental setting, no differences in the IFN-γ and IL-10 concentrations were observed between the super-responders (OS>1 year) and non-super-responders (OS≤1 year) (Figure 6). Moreover, the PBMCs produced extremely high levels of IFN-γ relative to IL-10 (Figure 6).

**Discussion**

The data presented herein demonstrate that a decreased N/L ratio (<4) and increased HLA-DR and CD83 MFIIs may be prognostic markers of chemoimmunotherapeutic outcome. Results from a recent clinical trial suggest that chemotherapies, such as gemcitabine and S-1, an oral fluoropyridine, are effective chemotherapeutic agents for pancreatic cancer treatment in Japan (10). In that phase III study, the median OS was 8.8 months in the gemcitabine group, 9.7 months in the S-1 group and 10.1 months in the gemcitabine plus S-1 group. Therefore, an OS of >1 year generally indicates that the treatment was beneficial. In the present study, patients who received DC/WT1-I/II vaccinations combined with chemotherapy were classified into 2 groups: OS>1 year (super-responders) and OS≤1 year (non-super-responders). We first analyzed the pre-treatment laboratory data, including the albumin levels (data not shown), CRP levels, neutrophil numbers and lymphocyte numbers. There were no differences between the super-responders and non-super-responders in terms of these factors in our study. Our results support those from a recent report that indicated that the albumin level, CRP level, neutrophil number and lymphocyte number were not prognostic factors for the outcomes in 255 patients who had received standard

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**Figure 1.** Association of the neutrophil to lymphocyte ratio with overall survival. The neutrophil to lymphocyte (N/L) ratios of seven pancreatic cancer patients who received chemoimmunotherapy were analyzed prior to treatment (left panel), after the first gemcitabine course (middle panel) and after completing chemoimmunotherapy (right panel). NLR, N/L ratio.

**Figure 2.** Dendritic cell phenotypes. The indicated molecules expressed on the dendritic cells (DCs) from a super-responder (Patient no. 6) are shown prior to treatment and after chemoimmunotherapy.
Figure 3. The mean fluorescent intensities of surface molecules on dendritic cells. The pretreatment mean fluorescent intensities (MFIs) of HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 on dendritic cells (DCs) were compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means±SD.

Figure 4. The pre- and post-chemoinmunotherapy mean fluorescent intensities of surface molecules on dendritic cells. The pre- and post-chemoinmunotherapy mean fluorescent intensities (MFIs) of the indicated molecules on the surfaces of dendritic cells (DCs) were compared between super-responders (OS>1year) and non-super-responders (OS≤1year). Values are expressed as means. *p<0.05.
Figure 5. Plasma cytokine profiles. A. The levels of cytokines (IL-6, IL-4, IL-10, IFN-γ, TNF-α and IFN-α) shown in plasma samples from patients who received 6-8 vaccinations and chemotherapy are compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year). B. IL-6 levels in plasma samples (prior to treatment and after 6-8 vaccinations and chemotherapy) are compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year). Values are expressed as means±SD. *p<0.05.

Figure 6. IFN-γ and IL-10 production by peripheral blood mononuclear cells. IFN-γ and IL-10 production by peripheral blood mononuclear cells after 6 vaccinations is compared between super-responders (OS>1 year) and non-super-responders (OS≤1 year).
chemotherapy combined with MHC-I-restricted peptide-pulsed DCs (11). Interestingly, a low post-treatment N/R ratio (<4) was associated with a good prognosis (OS>1 year) for pancreatic cancer patients in this study. Previously, an early reduction in the N/L ratio after effective treatment was reported to be associated with improved survival in cancer patients (12). Gemcitabine has been shown to up-regulate antigenic peptides on the HLA molecules of tumor cells (13), increase antigen cross-presentation (14) and decrease the immunosuppressive myeloid-derived suppressive cell (MDSC) (15) and regulatory T-cell (Treg) populations (16), resulting in the augmentation of antitumor immunity. The rapid decrease in the N/R ratio immediately following the initial gemcitabine course, as shown above, may have been induced by the reduced tumor-associated inflammatory and immunosuppressive responses. Moreover, the combination of DC/WT1-I/II vaccination and gemcitabine administration was also associated with additional reductions in the blood N/L ratio. A low N/L ratio was predictive of longer survival in patients with advanced pancreatic cancer who received gemcitabine (17).

The treatment of patients with advanced pancreatic cancer using DC/WT1-I/II vaccination plus gemcitabine-based chemotherapy has been associated with disease stability (8). In a clinical phase I trial, WT1-specific DTH-positive patients exhibited significant improvements in OS and PFS compared to negative controls. Moreover, all patients with strong DTH reactions were super-responders. In DC-based vaccines, autologous DCs are generated from GM-CSF- and IL-4-treated monocytes and subsequently mature through incubation with penicillin-killed and lyophilized preparations of a low-virulence Streptococcus pyogenes (OK-432) strain (Su) and prostaglandin E2 (PGE2). The expression levels of HLA-ABC, HLA-DR, CD80, CD86, CD83 and CCR7 on the DCs derived prior to treatment did not differ between patients, thus indicating a uniform DC quality. Interestingly, in the super-responders, the HLA-DR and CD83 expression levels increased significantly after treatment relative to pre-treatment levels. Vaccination with fusion products generated from DCs and whole tumor cells has been reported to result in DC maturation (18, 19). Our results were also consistent with reports in which the phenotypic features of DCs were found to differentiate in vitro following vaccination. These results suggested that the increased surface expression of DC markers after treatment indicates improved antigen-presentation function in these cells (18). Patients with advanced pancreatic cancer exhibited impaired DC function; however, gemcitabine improved DC function (20). The significantly increased levels of HLA-DR and CD83 expression on the DCs derived from patients who received DC/WT1-I/II and gemcitabine suggest that chemoimmunotherapy may restore DC function. In the super-responders, the improved DC phenotype (HLA-DR and CD83) may be associated with longer survival.

The plasma cytokine profile may be important when assessing the prognostic markers associated with chemoimmunotherapy. In this study, the pancreatic cancer patients received DC/WT1-I/II vaccines combined with chemotherapy. Therefore, we analyzed the Th1 and Th2 cytokine profiles after vaccinations. Our results revealed no differences between the cytokine profiles of super-responders and non-super-responders. The levels of the immunosuppressive cytokines IL-4, IL-10 and IL-6 were higher in non-super-responders than in super-responders, although this difference was not significant. Unexpectedly, the levels of the Th1 cytokines IFN-γ, TNF-α and IFN-α were also higher in non-super-responders; however, this difference was not significant. We also analyzed in greater detail the Th1 and Th2 responses of PBMCs upon WT1 peptide stimulation in vitro. Similarly, there were also no differences between the super-responders and non-super-responders in terms of IFN-γ or IL-10 production by PBMCs after 6-8 vaccinations. Interestingly, IL-6 plasma levels in non-super-responders were significantly increased after chemoimmunotherapy relative to the pretreatment levels. IL-6 is one of the major immunosuppressive cytokines, which can induce tumor progression by manipulating immune responses. Therefore, an increased IL-6 level in plasma may be associated with poor prognosis. The immune checkpoint blockade targeted-agents, such as programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) have been used to successfully treat patients with advanced melanoma (21). Therefore, it may be more important to inhibit immunosuppressive responses than to stimulate immunity in patients with advanced pancreatic cancer. The primary limitation of our study is the relatively small size of the evaluated sample. Further studies are required to evaluate the prognostic markers of chemoimmunotherapy with DC/WT1-I/II.

Conflicts of Interest

The Authors declare that they have no competing interests.

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