The Role of Circulating Dendritic Cells in Patients with Unresectable Pancreatic Cancer

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Abstract. *Aim: Pancreatic cancer is a malignant neoplasm with a poor prognosis that might be associated with defective immune function. In this study, we aimed to clarify the role of circulating myeloid dendritic cells (cmDCs) and lymphoid (cl) DCs in patients with unresectable pancreatic cancer. Patients and Methods: This study covered the period from January 2001 to December 2009, and involved 104 patients with unresectable pancreatic cancer. We measured the number of cmDCs and clDCs using flow cytometry before and after chemotherapy, chemoradiotherapy and immuno-chemotherapy. Results: The percentage of the cmDC subset in the unresectable pancreatic cancer patients was significantly lower than in healthy volunteers (p=0.006). There was no difference in the cmDC subset between patients with distant organ metastasis and locally advanced pancreatic cancer. The patients with a high percentage (≥0.23%) of cmDC subset survived longer than patients with a low percentage (<0.23%) (p=0.0030). Multivariate analysis showed that cmDC was the only independent prognostic factor (p=0.0059). The percentage of cmDC subset was significantly increased after immuno-chemotherapy (p=0.0055). Conclusion: A high level of cmDCs is associated with better survival rate and is an independently favorable prognostic factor in patients with unresectable pancreatic cancer. It is likely that immuno-chemotherapy increases the number of cmDCs.*

Pancreatic cancer is a disease with one of the poorest prognoses. In Japan, it is the fifth leading cause of death from malignant neoplasms in men and the fourth in women (1). Despite improvements in diagnostic modalities, pancreatic cancer is still difficult to diagnose in the early stages. Surgical resection remains the most effective therapy against pancreatic cancer, and is the only one from which we can expect a radical recovery (2). However, even if pancreatectomy followed by adjuvant chemotherapy including gemcitabine is carried out, the prognosis for pancreatic cancer patients is still dismal. Furthermore, the majority of patients are classified with unresectable pancreatic cancer on diagnosis.

Dendritic cells (DCs) play a central role in the initiation and modulation of immune system responses (3). Techniques for isolating human DCs from peripheral blood have been established, and DCs are classified into two subsets: $CD11c^+$DCs (myeloid DCs); and $CD11c^-$DCs (lymphoid DCs) (4, 5). In particular, myeloid DCs play an essential role in antitumor immunity. Previously, we have reported that the number and function (allogeneic mixed lymphocyte reaction) of circulating myeloid (cm) DCs, were lower in patients with pancreatic cancer, and that chemoradiotherapy and surgical resection improved cmDC function (6). Moreover, we reported that the preoperative cmDC level in the peripheral blood mononuclear cells (PBMCs) of pancreatic cancer patients who underwent pancreatectomy might be a prognostic factor (7).

In this study, we investigated the number of cmDCs, circulating lymphoid (cl) DCs and other immune parameters in the peripheral blood of patients with unresectable pancreatic cancer due to locally advanced tumor or distant organ metastasis.

Patients and Methods

Between January 2001 and December 2009, 104 patients with unresectable pancreatic cancer due to locally advanced disease (n=23) or distant organ metastases (n=81) radiologically defined by the National Comprehensive Cancer Network (NCCN) guideline (8), were included in this study. The following cases were excluded: endocrine tumor, intraductal papillary mucinous carcinoma, acinar cell cancer, anaplastic cancer, duodenal cancer, distal common bile duct cancer, ampullary cancer, or double cancer. Among the 104 patients, who did not undergo pancreatectomy, 48 received single

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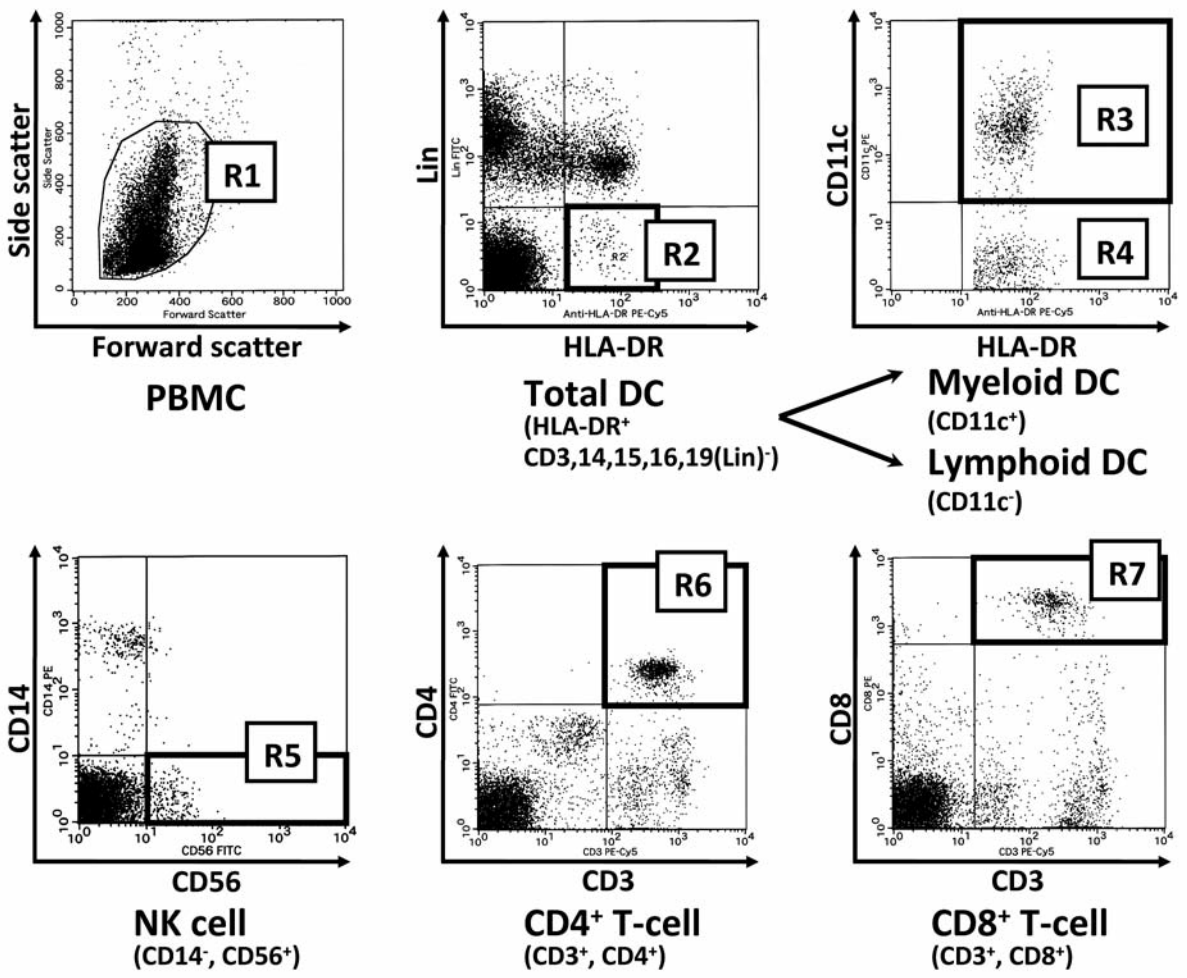

Figure 1. *Procedure of flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) by FACScan (BD Bioscience). The figures were chosen from typical cases of patients with pancreatic cancer. Dendritic cells (DCs) are detected in region R2 as the population of Lin$^-$/HLA-DR$^+$ and divided into two fractions by the expression of CD11c (region R3; CD11c$^+$DC (cmDCs) and region R4 CD11c$^-$DC (clDCs)). The natural killer (NK) cell fraction is gated in the CD14$^-$/CD56$^+$ population (region R5). CD3$^+$/CD4$^+$ T lymphocytes are detected in region R6 and CD3$^+$/CD8$^+$ T lymphocytes are detected in region R7.*

agent or combination chemotherapy selected from gemcitabine, tegafur-gimeracil-oteracil potassium (S-1), 5-fluorouracil (5-FU) and/or cisplatin (CDDP); 27 received chemoradiotherapy; 22 received immunochemotherapy as previously reported (9); and the remaining 7 received best supportive care.

Staging was performed in accordance with TNM Classification of Malignant Tumors, Sixth Edition (10). Unresectable patients were followed up for at least 24 months. Only one patient still survives after initiating chemotherapy; the remaining 103 patients have died. Twenty three age-matched healthy volunteers with no history of malignant or severe disease also participated in this study. We obtained written informed consent from all patients and healthy volunteers in accordance with the provisions of the Declaration of Helsinki. This study received approval from the Ethics Committee of our hospital.

Heparinised peripheral blood samples were obtained in the early morning from all patients before starting medical treatments against cancer, and also from all healthy volunteers using the same method. Further blood samples were taken 2 months after starting cancer treatment from 39 patients; 13 who received single agent gemcitabine; 13 who received combination therapy of gemcitabine and irradiation; and 13 who received combination therapy of gemcitabine and immunotherapy using personalized peptide vaccine (9). No patients or volunteers had any inflammatory disease at the time of blood sampling.

Flow cytometry. The numbers of peripheral blood mononuclear cells (PBMCs), CD4$^+$/CD3$^+$ T lymphocytes, CD8$^+$/CD3$^+$ T lymphocytes, natural killer (NK) cells, c-m-DCs and c-l-DCs were measured using Flow Cytometry. The phenotypes of PBMCs were measured by two- or three-color flow cytometric analysis using monoclonal antibodies (mAbs), which were directly conjugated with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or PE cyanin 5.1 (PE-Cy5). PBMCs were prepared by Lymphoprep (Nycomed Pharma,

Table I. *Comparison of clinical characteristics and immunological parameters in unresectable pancreatic cancer patients and healthy volunteers.*

	Healthy volunteers (n=23)	Unresectable pancreatic cancer patients (n=104)	*p*-Value
Age (years)	62.0 (56-82)	65.3 (38-85)	0.35
Gender (M:F)	11:12	44:60	0.36
WBC ($\times 10^2$/ul)	5000 (3600-6800)	5600 (3000-13100)	0.16
Hb (g/dl)	13.2 (12.1-15.3)	12.1 (8.5-14.9)	0.003
Albumin (g/dl)	4.25 (3.8-4.5)	3.8 (2.3-5)	$p<0.001$
T-Bil (mg/dl)	0.45 (0.2-0.9)	0.8 (0.3-10.0)	0.052
PBMC ($\times 10^6$ cell/ml)	2.4 (1.2-4.8)	2.63 (0.97-7.57)	0.148
NK (%)	6.57 (0.16-25.26)	7.5 (0.53-21.58)	0.791
CD4 (%)	24.57 (2.56-49.63)	19.21 (2.79-53.10)	0.062
CD8 (%)	8.27 (0.54-29.98)	6.9 (1.04-23.63)	0.524
cmDCs (%)	0.35 (0.09-0.82)	0.23 (0.03-0.82)	0.006
clDCs (%)	0.13 (0.02-0.67)	0.17 (0.05-0.80)	0.142

The data are expressed as median and range. M:F, Male:female; WBC, white blood cell; Hb, hemoglobin; T-Bil, total bilirubin; PBMCs, peripheral blood mononuclear cell; NK, natural killer cell; CD4, CD4-positive T lymphocyte; CD8, CD8-positive T lymphocyte; cmDCs, circulating myeloid dendritic cell; clDC, circulating lymphoid dendritic cell.

Oslo, Norway) gradient centrifugation of heparinized peripheral blood, mixed with each of the mAbs and incubated for 30 min at 4˚C. Cells were stained with the following combination of mAbs: PE-conjugated anti-CD14 and FITC-conjugated anti-CD56 mAbs for NK cells; PE-Cy5-conjugated anti-CD3, FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 for T lymphocytes; PE-Cy5-conjugated anti-HLA-DR, a mixture of FITC-conjugated anti-CD3, CD14, CD15, CD16, CD19 so-called "lineage cocktail (Lin)" and PE-conjugated anti-CD11c for DCs. Each phenotype and the proportions of stained cells were analyzed using FACScan (Becton Dickinson, Sunnyvale, CA, USA) and Cell Quest software (Becton Dickinson). At least 100,000 events were counted for each mononuclear fraction by FACScan. Each cell population determined by flow cytometry is shown in Figure 1. Region R1 includes lymphocytes and monocytes, but excludes debris. DCs were detected in region R2 as the population of Lin-/HLA$^-$DR$^+$ cells. Two subsets of DCs were identified within the Lin-/HLA$^-$DR$^+$ population, which were based on differential expression of CD11c: cmDCs (CD11c$^+$ population; region R3) and clDCs (CD11c$^-$ population; R4). The absolute number (per ml) in each subset of DCs was calculated by multiplying the percentage of each region of the DCs by the PBMC count. The numbers of NK cells (CD14$^-$/CD56+ population; region R5), CD4$^+$/CD3$^+$ T lymphocytes (region R6), and CD8$^+$/CD3$^+$ T lymphocytes (region R7) were also calculated in the same manner.

Statistical analysis. The data were expressed as the median and range of values. Mann-Whitney *U*-test and Wilcoxon signed-rank test were used for statistical analysis using JMP software (statistical program ver.5.5; SAS Inc., Cary, NC, USA). The Kaplan-Meier method was used to generate survival curves. Survival time was taken from the day of blood sampling to death or the end of observation. According to the median of the number and percentage of cmDCs, the survival curve was compared using the log-rank test. Cox proportional hazards regression analysis was used to calculate hazard ratios for overall survival. The differences were considered significant when the *p*-values were less than 0.05.

Results

Clinical characteristics and immunological parameters of unresectable pancreatic cancer patients and healthy volunteers are shown in Table I. Serum albumin and hemoglobin levels in the patients were significantly lower than in the healthy volunteers ($p<0.001$ and $p=0.003$). Most immunological parameters including the number of PBMCs, the percentage of NK, CD4 T cells, CD8 T cells, and clDCs did not show significant differences, but the percentage of the cmDC subset in the PBMCs in unresectable pancreatic cancer was significantly lower than in healthy volunteers ($p=0.006$). In the subgroup analysis, there was no significant difference in the percentage of cmDCs (cmDCs (%)) in patients with locally advanced disease and those with distant metastases.

The median value of cmDCs (%) in the patients with unresectable pancreatic cancer was 0.23% in this study. We divided the 104 unresectable patients into two groups according to the median value of cmDCs (%): 51 patients were classified into the high cmDC group (*i.e.*, % of cmDCs in PBMCs was greater than 0.23; and 53 were classified into the low cmDC group). As shown in Table II, there was a significant difference in gender ratio between the high and low cmDC groups ($p=0.042$). However, there were no significant differences between them with respect to any other clinical or immunological parameters. When it came to the overall survival rate, this was significantly longer in the high cmDC group compared with the low cmDC group ($p=0.0030$, cmDCs (%) ≥0.23, n=51, median survival time: 7.6 months, 1 year survival: 29.4%; cmDCs (%) <0.23, n=53, median survival time: 6.1 months, 1 year survival: 17.3%, Figure 2). As shown in Table III, we discovered that the percentage of cmDCs and

Table II. *Comparison of clinical characteristics and immunological parameters in high and low cmDC patients with unresectable pancreatic cancer.*

	High cmDC (n=51)	Low cmDC (n=53)	*p*-Value
Age (years)	64.8 (38-81)	65.8 (41-85)	0.434
Gender (M:F)	35:16	26:27	0.042
WBC ($\times 10^2$/ul)	5600 (3500-9400)	5600 (3000-13100)	0.75
Hb (g/dl)	12.3 (8.5-14.7)	11.8 (8.1-14.9)	0.203
Albumin (g/dl)	3.9 (2.3-5.0)	3.7 (2.4-4.7)	0.087
T-Bil (mg/dl)	0.7 (0.4-8.0)	0.9 (0.3-10.0)	0.741
CA19-9 (U/ml)	722.0 (5.0-25468)	468.1 (2.0-80714)	0.292
Stage (IIA:IIB:III:IV)	1:0:10:40	2:1:8:42	0.581
Locally : Metastasis	12:39	11:42	0.806
Tumor location (Ph:Pbt)	29:22	29:24	0.758
Type of therapy (chemo:Im-chemo:CRT:BSC)	25:8:16:2	22:13:13:5	0.425
PBMC ($\times 10^6$ cells/ml)	2.48 (0.97-7.57)	2.85 (1.25-6.88)	0.239
NK (%)	7.56 (1.31-21.58)	7.31 (0.53-20.22)	0.272
CD4 (%)	21.14 (5.17-38.00)	17.92 (2.79-53.10)	0.086
CD8 (%)	7.13 (1.04-23.63)	5.87 (1.12-22.79)	0.241
clDCs (%)	0.19 (0.07-0.67)	0.17 (0.05-0.80)	0.998

The data are expressed as median and range. M:F, Male:female; WBC, white blood cell; Hb, hemoglobin; T-Bil, total bilirubin; Locally, locally advanced cancer; Metastasis, distant metastasis; Ph/Pbt, pancreas head/ pancreas body and tail; Chemo, chemotherapy; Imchemo, immunochemotherapy; CRT, chemoradiotherapy; BSC, best supportive care; PBMCs, peripheral blood mononuclear cell; NK, natural killer cell; CD4, CD4-positive T lymphocyte; CD8, CD8-positive T lymphocyte; cmDCs, circulating myeloid dendritic cell; clDCs, circulating lymphoid dendritic cell. Clinical and pathological staging was based on the TNM Classification of Malignant Tumors, sixth edition.

the hemoglobin level were risk factors for prognosis by using the univariate Cox proportional hazard regression model. Multivariate analysis showed that the percentage of cmDCs was the only independent prognostic factor (p=0.0059, hazard ratio=0.750, 95% confidence interval: 0.610-0.920).

We analyzed the change in the cmDC level before and after treatment in the 39 patients who provided blood samples two months after starting their cancer therapies. The immunochemotherapy group showed significant improvement in the level of cmDCs two months after starting treatment (p=0.0055); in contrast, the cmDC level in the chemotherapy and chemoradiation groups did not differ before and after treatment (Figure 3).

Discussion

Surgical resection for pancreatic cancer is the only curative treatment option (2); however, because in the majority of patients with pancreatic cancer, the tumor is classified as unresectable a mere 20% of patients will actually undergo surgical resection (11). Although many antitumor drugs have been developed, including the newer immunotherapies introduced in the last few decades, the treatment of unresectable pancreatic cancer with beneficial effects remains to be investigated. Patients with unresectable pancreatic cancer are classified into two groups: those with locally advanced disease and those with distant metastasis. According to previous reports, the proportion of the patients with pancreatic cancer classified into stage III was at least 13% and stage IV was at least 55% (12, 13). Furthermore, the median survival time by stage III or IV was 7.2 months or 2.5 months, respectively and 1-year survival rate was 27% or 8%, respectively (12). As shown in Figure 2, the overall survival rate in the high c-m-DCs group was significantly longer than that in the low c-m-DCs group. The median survival time and 1 year survival rate in the high cmDC group, in which patients with stage IV comprise 80%, was 7.6 months and 29.4%, respectively, which is similar to the stage III patients from other studies described above.

For patients with unresectable pancreatic cancer, it is important to identify risk factors in order to predict their prognosis or the effect of some treatments. Stocken reported that prognostic factors in advanced pancreatic cancer included serum albumin, lactic dehydrogenase (LDH), white blood cell count, CA19-9 level and metastasis (14). According to other reports, higher serum LDH levels were an independent factor for poor prognosis in pancreatic cancer patients with distant metastasis, and hemoglobin level and Karnofsky performance scale status were prognostic factors in patients with unresectable locally advanced pancreatic cancer treated with chemoradiotherapy (15, 16). In this current study, results of univariate analysis showed that cmDCs and hemoglobin levels were identified as prognostic factors in patients with unresectable pancreatic cancer. Furthermore, as a result of multivariate analysis, we clearly revealed cmDCs as the only independent risk factor of prognosis in patients with

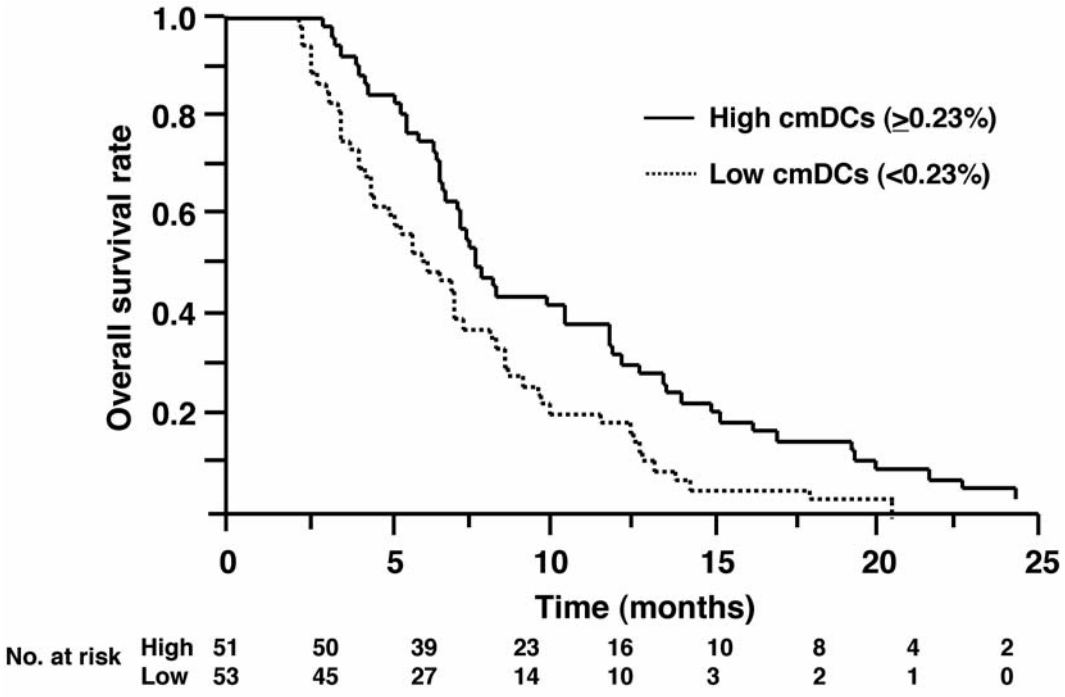

Figure 2. *Comparison of survival curves according to the proportion of circulating myeloid DCs subset in the peripheral blood mononuclear cells (PBMCs) in unresected pancreatic cancer patients. The solid line represents the overall survival curve in the high cmDC group that consisted of patients with cmDCs of at least 0.23%. The broken line represents the overall survival curve in the low cmDC group that consisted of patients with cmDCs less than 0.23%. The overall survival rate in the high cmDC group was significantly longer than in the low cmDC group (p=0.0107, cmDCs >0.23%, n=51, median survival time: 7.6 months, 1-/2- year survival: 29.4%/2.3%. cmDCs <0.23%, n=53, median survival time: 6.1 months, 1-/2- year survival: 18.9%/0%).*

unresectable pancreatic cancer. Thus, of the immunoparameters evaluated as prognostic factors in unresectable pancreatic cancer patients, cmDCs are good candidates, while PBMCs, NK, $CD4^+$ or $CD8^+$ cells are not.

Pancreatic cancer is a malignant neoplasm with dismal prognosis that might be associated with a defective immune function (6, 17, 18). DCs play a central role in antigen presentation to T lymphocytes. DCs express high levels of HLA-DR and lack the lineage markers CD3, CD14, CD15, CD16 and CD19. While the both myeloid and lymphoid DC subsets initiate antigen presentation to T lymphocytes, they play contrary roles. Myeloid DCs stimulate naïve T-cells and induce differentiation into type 1 helper T-cells (Th1 cells). Th1 cells induce cell-mediated immunity, which is the immune system that plays the most important role in the antitumor immunity. Lymphoid DCs also stimulate naïve T-cells, but induce their differentiation into type 2 helper T-cells (Th2 cells) or the generation of regulatory T-cells. Th2 cells induce humoral immunity (4, 19, 20). Generally, DCs exist in tissue of various organs including lymphoid tissue. When malignant cancer cells grow, cmDC migrate into these tissues and capture the cancer antigen. However, cancer cells often have immune escape mechanisms, and in such situations DCs are unable to recognize tumor cells as a target.

Table III. *Univariate and multivariate Cox proportional hazard regression analysis for overall survival.*

	Univariate analysis *p*-Value HR(95%CI)*	Multivariate analysis *p*-Value HR(95%CI)
cmDCs (%)	0.0037	0.0059
≥0.23 (n=51)	0.741 (0.605-0.907)	0.750 (0.610-0.920)
<0.23 (n=53)	1	1
Hb (g/dl)	0.0489	0.1203
≥12.1 (n=51)	0.815 (0.664-0.990)	0.850 (0.690-1.044)
<12.1 (n=53)	1	1

*Hazard ratio (95% confidence interval) of univariate and multivariate analysis.

Several reports showed that the number of circulating DCs was decreased in pancreatic cancer, hepatocellular carcinoma, breast cancer and squamous cell carcinomas of the head and neck (21-26). Furthermore, Bond A *et al*. reported the number and function of circulating DCs were diminished in patients with more advanced head and neck squamous cell carcinomas (25). Previously, we reported that the number and percentage of the cmDCs subset was

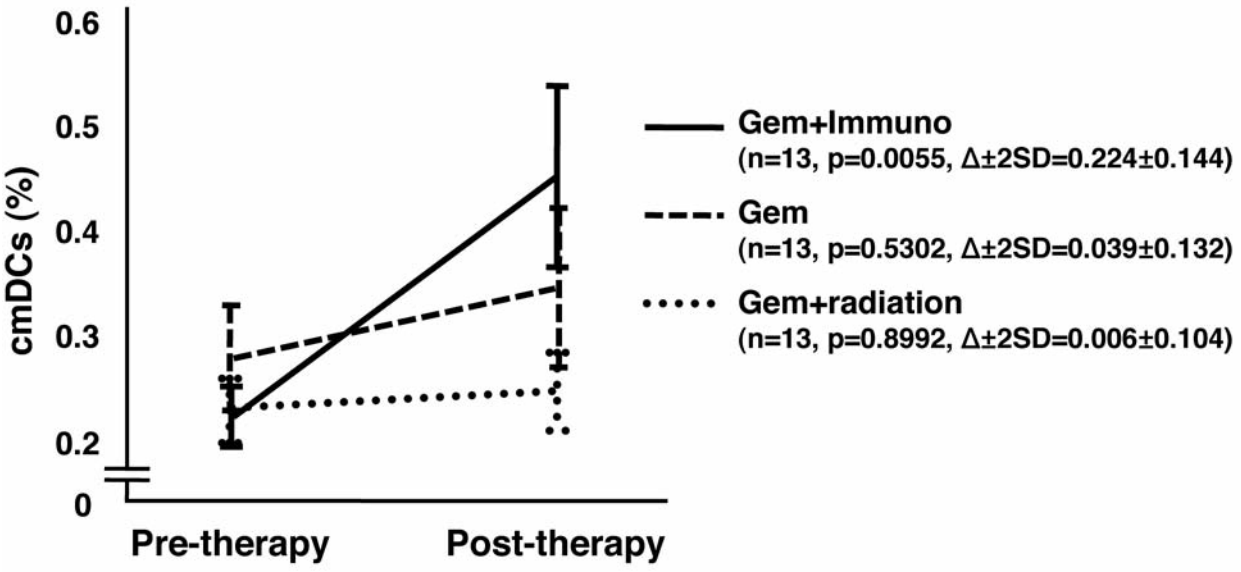

Figure 3. *Comparison of time course (before and 2 months after starting treatment) by the percentage of the cmDC subset in the peripheral blood mononuclear cells (PBMCs). The solid line represents the percentage of cmDCs in patients who underwent immnochemotherapy (Gem+Immuno). The broken line represents the percentage of cmDCs in patients who underwent chemotherapy (Gem). The dotted line represents the percentage in patients who underwent chemoradiotherapy (Gem+radiation). This figure indicates that cmDCs (%) were significantly increased after receiving immunochemotherapy (*p=0.0055), but similar findings were not found in patients who underwent chemotherapy or chemoradiotherapy.*

significantly higher in patients with pancreatic cancer than in the healthy volunteers, and that the function of cmDC in patients with unresectable pancreatic cancer significantly improved after chemo-radiation therapy that could induce apoptosis of the tumor (6). The cmDC level improved significantly in patients who underwent pancreatectomy and remained disease free after 12 months, but in patients with recurrence or metastasis it did not (18). Moreover, cmDC had potential as a prognostic factor for patients with resectable pancreatic cancer (7); namely, there were significantly longer survival rates in the resected pancreatic cancer patients whose cmDCs (%) was over 0.27% than in the lower cmDCs (%) patients. Soeda *et al*. reported the number of $CD11c^+$ DCs gradually increased over time in advanced pancreatic cancer patients who received a regular dose of gemcitabine monotherapy (27). In this study, we could not show similar findings in patients with unresectable pancreatic cancer who received chemotherapy or chemoradiotherapy. However, there was a significant improvement of the cmDC level in patients who received immunochemotherapy using gemcitabine and personalized peptide vaccine. In our previous report, the function of myeloid DCs was significantly improved after chemoradiotherapy (6). Based on these reports, we suggest that immunochemotherapy using gemcitabine may not only have the effect of inducing apoptosis of the cancer cells, as previously reported by Bold *et al*. (28), but might also have the effect of strengthening the antitumor immune response by inducing proliferation and activation of myeloid DCs.

The theoretical rationale of immunotherapy against pancreatic cancer is tumor-antigen presentation from antigen-presenting cells (APCs) including DCs to T lymphocytes, and the acquisition of aggressiveness against cancer tissue by the T lymphocytes. In particular, DCs are the most potent APCs which express higher MHC and co-stimulatory molecules. We previously reported a multicenter phase I/II study of gemcitabine and personalized peptide vaccination combination therapy in metastatic pancreatic cancer patients, in which we observed an overall response rate of 25%, a disease control rate of 55%, and median survival time of 8.5 months (29). Previous reports showed that several agents, such as interferon and 5-FU, can up-regulate the expression of MHC class I and tumor-associated antigens on colon cancer cells (30, 31). Moreover, anticancer drugs docetaxel, cyclophosphamide, doxorubicin and paclitaxel enhance immune response in anticancer vaccination therapy (32,33). These results may indicate that immunochemotherapy using gemcitabine induces the synergistic effect of apoptosis due to chemotherapy with the antitumor immune response due to immunotherapy. Immuno-chemotherapy, that can maintain or improve the number and function of DCs, might be the breakthrough that is so badly needed to obtain a better survival rate for patients with unresectable pancreatic cancer.

In this study, we evaluated only the circulating DCs of the PBMCs in patients with unresectable pancreatic cancer. It is difficult to evaluate the DCs accumulated in the cancer tissue of these patients. Several previous reports showed a correlation between the degree of tumor-infiltrating DCs and clinical outcome (34-36). Thus, these reports showed that, in colorectal and breast cancers, a better clinical outcome was significantly correlated with higher infiltration of immune cells into the tumor tissue (34-36). We previously performed further investigation into whether the degree of tumor-infiltration with mature DCs correlated with the prognosis in pancreatic cancer patients, who underwent pancreatectomy, and with the number or percentage of cmDCs (7). As a result, we found that there was a close positive correlation between the percentage of cmDCs subset in the PBMCs and the DCs number per field in the tissue of pancreatic cancer. Furthermore, it was found that the higher number of infiltrating DCs (≥3.5 per field) was significantly correlated with favorable outcome in resectable pancreatic cancer. The number of cmDCs in the PBMC is a reflection of the number in the tissue of pancreatic cancer. It is likely that the control of the biologic distribution and function of cmDC in the human body may improve prognosis in patients with pancreatic cancer, which at present is so dismal.

In conclusion, a high level of cmDCs was associated with a better survival rate, and was an independently favorable prognostic factor in patients with unresectable pancreatic cancer. It is likely that immunochemotherapy increases the number of cmDCs.

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