PD-1 Expression on Circulating CD8⁺ T-Cells as a Prognostic Marker for Patients With Gastric Cancer

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Abstract. *Background: Programmed death-1 (PD1) is an immunoinhibitory receptor, and PD1 overexpression on T-cells is involved in immune evasion in cancer. This study investigated the prognostic significance of PD1 expression on T-cells in gastric cancer. Materials and Methods: PD1 expression on CD4⁺ and CD8⁺ T-cells obtained from peripheral blood mononuclear cells was evaluated by multicolor flow cytometry. Results: Based on cutoff values from receiver operating characteristic analysis, patients were sub-grouped according to PD1 positivity as having high or low PD1⁺CD4⁺ T-cell (cutoff $\geq 34.2\%$) and PD1⁺CD8⁺ T-cell (cutoff $\geq 28.7\%$) frequencies. Five-year overall survival rates differed significantly between the groups with low and high frequency of PD1⁺CD4⁺ T-cells (75.1% vs. 27.2%, respectively; $p=0.0008$). The 5-year overall survival rates were 78.3% and 37.2%, respectively, for the corresponding PD1⁺CD8⁺ T-cell subgroups ($p=0.0004$). Multivariate analysis revealed that the frequency of PD1⁺CD8⁺ T-cells was an independent prognostic indicator. Conclusion: The frequency of PD1⁺CD8⁺ T-cells may predict the prognosis of patients with gastric cancer.*

Although the prognosis of patients with gastric (GC) has improved because of better diagnostic techniques and intraoperative and postoperative care, this malignancy still ranks second among all cancer-related deaths worldwide (1). Thus, determining postoperative prognostic factors for patients with GC has a clinical importance. As such, serum tumor markers are both easy to measure and useful for diagnosis, predicting survival rates, and monitoring recurrence following surgery. Carcinoembryonic antigen

(CEA) and carbohydrate antigen (CA) 19-9 are the most frequently used tumor markers for diagnosing, treating, and predicting prognoses in GC (2-4).

Programmed death 1 receptor (PD1, also known as CD279) is a well-known immune-checkpoint molecule expressed by chronically stimulated CD4⁺ and CD8⁺ T-cells after T-cell activation (5-7). PD-L1 (8, 9) and PD-L2 (10, 11) are the ligands for PD1, and the PD1/PD-L1 interaction is strongly associated with T-cell dysfunction and contributes to maintaining peripheral tolerance to self-antigens (12). These molecules are now receiving considerable attention as targets of cancer immunotherapy. In fact, immune-checkpoint inhibitors including antibodies to programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte antigen-4 (CTL-4) have been shown to be effective treatments for various tumor types (13-15).

We previously demonstrated that the frequency of PD1⁺CD4⁺ and PD1⁺CD8⁺ T-cells was significantly increased in patients with GC compared to healthy controls. PD1⁺CD4⁺ and CD8⁺ T-cells produced significantly less interferon- γ than PD1⁻CD4⁺ and CD8⁺ T-cells, indicating that PD1⁺CD4⁺ and CD8⁺ T-cell function was impaired in patients with GC. Because CD4⁺ and CD8⁺ T-cells play critical roles in tumor immunity, we hypothesized that the frequency of PD1⁺CD4⁺ and PD1⁺CD8⁺ T-cells would be associated with the prognosis of patients with GC. Therefore, the aim of this study was to determine the prognostic significance of the frequency of PD1⁺CD4⁺ and PD1⁺CD8⁺ T-cells in patients with GC.

Materials and Methods

Patients and healthy donors. Seventy-two patients (53 male and 19 female) with gastric adenocarcinoma who underwent curative gastrectomy (R0 resection) at our Institution between April 2009 and April 2013 were enrolled in this study. The study protocol was approved by the Institutional Review Board of Tottori University Hospital (Yonago, Japan; Approval number 448). None of the patients received radiotherapy, chemotherapy, or other medical interventions before surgery. Clinicopathological findings were

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Key Words: Gastric cancer, PD1, PD-L1, prognosis, T-cells.

Table I. Programmed cell death 1 (PD1)+CD4⁺ and PD1+CD8⁺ T-cell frequencies according to patient characteristics.

Variable	PD1+CD4 ⁺ T-cells (%)	p-Value	PD1+CD8 ⁺ T-cells (%)	p-Value
Age				
<70 Years (n=31)	28.7±8.4	0.26	25.6±11.1	0.29
≥70 Years (n=41)	32.2±12.0		29.8±14.2	
Gender				
Male (n=53)	30.2±11.0	0.4	29.6±13.9	0.1
Female (n=19)	31.9±10.0		23.7±8.9	
Tumor size				
<4 cm (n=34)	26.6±7.9	0.0043	27.7±13.7	0.58
≥4 cm (n=38)	34.3±11.6		28.3±12.4	
Histology ^a				
Differentiated (n=35)	31.5±10.9	0.47	30.6±14.9	0.21
Undifferentiated (n=37)	29.8±10.6		25.6±10.5	
Depth of invasion ^b				
T1 (n=31)	27.5±10.1	0.016	25.1±11.7	0.042
T2/3/4 (n=41)	33.1±10.7		30.3±13.6	
Lymph node metastasis				
Absent (n=37)	28.4±10.8	0.039	25.5±11.6	0.037
Present (n=35)	33.0±10.2		30.7±14.0	
Lymphatic invasion				
Absent (n=22)	25.3±9.4	0.0011	23.8±11.6	0.027
Present (n=50)	33.0±10.5		29.9±13.2	
Vascular invasion				
Absent (n=25)	26.1±9.1	0.0035	24.1±11.0	0.053
Present (n=47)	33.1±10.8		30.1±13.6	
Stage of disease				
I (n=30)	28.1±11.2	0.036	25.1±11.8	0.038
II/III (n=42)	32.5±10.0		30.1±13.5	

^aDifferentiated: papillary, or tubular adenocarcinoma; undifferentiated: poorly differentiated, mucinous adenocarcinoma, and signet-ring cell carcinoma. ^bDepth of invasion: T1, tumor invasion of the *lamina propria* or submucosa; T2, tumor invasion of the muscularis propria; T3, tumor invasion of the subserosa; T4, tumor penetration of the serosa or tumor invasion of adjacent organs. All results are expressed as the mean±SD.

determined according to the Japanese Classification of Gastric Carcinoma (16).

Peripheral blood mononuclear cells (PBMC) preparation. A 30-ml peripheral blood sample was drawn from each of the controls or patients before surgery and centrifuged through a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). Informed consent was obtained from all participants before blood donation.

Flow cytometric analysis. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ, USA). The following antibodies were used to classify cells: anti-CD3-phycoerythrin (PE)-cyanine 5 (Biolegend, San Diego, CA, USA), anti-CD4–fluorescein isothiocyanate (FITC), anti-CD8-FITC, and anti-PD1-PE (all from BD Biosciences).

Statistical analysis. Mann–Whitney *U*-tests were used to determine the statistical significance of differences in the frequencies of PD1+CD4⁺ and PD1+CD8⁺ T-cells by clinicopathological characteristics. Correlations among the frequencies of T-cell subsets and serum CEA levels were analyzed by the Spearman rank correlation coefficient test. The Youden index was calculated using receiver operating characteristic (ROC) analysis to determine

optimal cutoffs for the frequencies of PD1+CD4⁺ and PD1+CD8⁺ T-cells in survival analyses. Survival curves were calculated according to the Kaplan–Meier method. Differences between curves were identified using the log-rank test. Multivariate analyses of factors considered prognostic of overall survival (OS) were based on Cox's proportional hazards model and a stepwise procedure. Differences with *p*<0.05 were considered significant. GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and Stat View (Abacus Concepts Inc., Berkeley, CA, USA) were used for statistical analyses.

Results

The mean frequencies of PD1+CD4⁺ and PD1+CD8⁺ T-cells were 30.7% (range=14.1-60.4%) and 28.0% (range=10.1-66.5%), respectively. Table I shows correlations between the frequencies of PD1+CD4⁺ and PD1+CD8⁺ T-cells and clinicopathological characteristics. Patients with tumors ≥4 cm, advanced-stage disease, lymph node metastasis, lymphatic and vascular invasion, and stage II/III tumors had significantly more PD1+CD4⁺ T-cells than did patients with tumors <4 cm (*p*=0.0043), early-stage disease (*p*=0.016),

Figure 1. Correlation between the frequency of programmed cell death 1 (PD1)⁺CD4⁺ T-cells and carcinoembryonic antigen (CEA) (A) and between the frequency of PD1⁺CD8⁺ T-cells and CEA (B).

Figure 2. Overall (A) and disease-specific (B) survival curves based on the frequency of programmed cell death 1 (PD1)⁺CD4⁺ T-cells.

without lymph node metastasis ($p=0.039$), without lymphatic ($p=0.0011$) and vascular invasion ($p=0.0035$), and with stage I tumors ($p=0.036$). Patients with advanced-stage disease, lymph node metastasis, lymphatic invasion, and stage II/III tumors also had significantly more PD1⁺CD8⁺ T-cells than did patients with early-stage cancer ($p=0.042$), without lymph node metastasis ($p=0.039$), without lymphatic invasion ($p=0.027$), and with stage I tumors ($p=0.038$).

Because CEA is the most frequently used tumor marker in GC, we next determined correlations among the frequencies of PD1⁺CD4⁺ and PD1⁺CD8⁺ T-cells and serum CEA level and found that there were no significant correlations between the frequencies of PD1⁺CD4⁺ or PD1⁺CD8⁺ T-cells and serum CEA level (Figure 1).

ROC analysis showed that the optimal cut-off values for the frequencies of PD1⁺CD4⁺ and PD1⁺CD8⁺ T-cells were 34.2% and 28.7%, respectively. Based on these results,

patients were subdivided into groups with low and high frequency of PD1⁺CD4⁺ T-cells ($n=20$ vs. 52 , respectively); and PD1⁺CD8⁺ T-cells ($n=30$ and $n=42$, respectively). The 5-year OS rates was significantly higher for those with a low PD1⁺CD4⁺ T-cell frequency (75.1% vs. 27.2%, respectively, $p=0.0008$; Figure 2A). The pattern was similar for the 5-year disease-specific survival rate for those with a low PD1⁺CD4⁺ T-cell frequency (85.6% vs. 45.4%, $p=0.0052$; Figure 2B). The 5-year OS rates were 78.3% and 37.2% in those with low and high frequency of PD1⁺CD8⁺ T-cells, respectively, which was statistically significantly different ($p=0.0004$, Figure 3A), as was 5-year disease-specific survival (91.5% and 51.0%, respectively, $p=0.0004$; Figure 3B). Finally, multivariate analysis of clinicopathological factors considered prognostic of OS was performed. The covariates included in the analysis were age, gender, tumor size, histology, depth of invasion, lymph node metastasis, lymphatic invasion, vascular

Figure 3. Overall (A) and disease-specific (B) survival curves based on the frequency of programmed cell death 1 (PD1)+CD8+ T-cells.

invasion, adjuvant chemotherapy, the extent of lymph node dissection, serum CEA level, and the frequencies of PD1+CD4+ and PD1+CD8+ T-cells. Multivariate analysis revealed that the frequency of PD1+CD8+ T-cells, but not PD1+CD4+ T-cells, was an independent prognostic indicator, along with tumor size (Table II).

Discussion

Co-signaling molecules are cell-surface glycoproteins that can direct, modulate, and fine-tune T-cell receptor (TCR) signaling. On the basis of their functional outcomes, co-signaling molecules can be divided into co-stimulators and co-inhibitors, which promote and suppress T-cell activation, respectively. By expressing them at the appropriate time and location, co-signaling molecules positively or negatively control the priming, growth, differentiation, and functional maturation of T-cell responses (5). PD1 and its ligand PD-L1 are co-signaling molecules that are co-inhibitors. In close proximity to the TCR signaling complex, PD1 delivers a co-inhibitory signal upon binding to either of its ligands. It is interesting that tumors may have exploited the PD1/PD-L1 pathway to evade eradication by the immune system. In fact, mounting evidence suggests that PD-L1 expression on solid tumors dampens antitumor T-cell responses (9, 12, 17-20). Additionally, blocking PD-L1 inhibited tumor growth and delayed tumor progression in multiple murine models (19-22); furthermore, the adoptive transfer of tumor-specific PD1-/- TCR transgenic T-cells rejected tumors even when cytotoxic T-lymphocyte-associated antigen (CTLA)-4-/- transgenic T-cells were unable to (17). Moreover, PD-L1 tumor expression has been shown to correlate with worse clinical outcomes in various solid tumors (23-27). With regard to the correlation between PD1 expression on T-cells and immune evasion, Matsuzaki *et al.* recently demonstrated that tumor-infiltrating New York esophageal squamous cell carcinoma 1 (NY-ESO-1)-specific CD8+ T-cells up-regulated PD1

Table II. Multivariate analysis of gastric cancer patients using Cox proportional hazard model and a stepwise procedure.

Factor	Hazard ratio	95% CI	p-Value
Tumor size			
≥ 4 vs. <4 cm	3.676	1.069-10.09	0.0064
PD1+CD8+ T-cells			
High vs. low frequency	4.403	1.821-10.68	0.001

PD1: Programmed cell death 1; CI: confidence interval.

expression, which resulted in suppression of the CD8+ T-cells (28). This result suggested that PD1 and PD-L1 may be associated with tumor immune evasion. Considering the close correlation between PD-L1 expression on cancer cells and prognosis in various cancer types, it is likely that PD1 expression on T-cells is also associated with prognosis in cancer patients. In this regard, it has been demonstrated that higher frequencies of PD1+CD8+ tumor-infiltrating lymphocytes were significantly associated with poor prognosis in head and neck cancer, and pancreatic cancer. A recent meta-analysis of 29 studies investigating PD1 expression and OS in patients with epithelial malignancies showed that PD1 expression in tumor-infiltrating lymphocytes was associated with a shorter OS (36). Furthermore, Shen *et al.* showed that higher frequencies of PD1+ peripheral CD8+ T-cells, but not CD4+ T-cells, were closely associated with poor prognosis in pancreatic cancer (29).

In this study, we demonstrated, for the first time, that the frequencies of PD1+CD4+ and PD1+CD8+ T-cells were closely associated with the prognosis of patients with GC. Furthermore, multivariate analysis revealed that the frequency of PD1+CD8+ T-cells was an independent prognostic indicator for patients with GC. CEA is the most frequently used tumor marker in GC and has been shown to

be useful for predicting the prognosis of patients with GC. In this study, there was no statistically significant correlation between the frequency of PD1⁺CD8⁺ T-cells and serum CEA levels, indicating that the frequency of PD1⁺CD8⁺ T-cells can be used as a prognostic indicator independently of serum CEA levels in patients with GC.

The mechanisms responsible for PD1 and PD-L1 up-regulation in GC remain unclear. In this regard, previous reports have demonstrated that PD1 up-regulation on CD8⁺ T-cells is associated with interleukin (IL)-10 and IL-6, but not transforming growth factor β . We previously reported that serum IL-6 concentrations in GC were significantly higher than in healthy controls (30). Additionally, we demonstrated that GC cells produce IL10 (31). Further investigations to clarify the mechanisms responsible for PD1 and PD-L1 up-regulation in GC are urgently required.

Immune checkpoint inhibitors, such as antibodies to PD1 and CTLA-4, have been shown to be effective treatments for various tumor types. Under these circumstances, it is extremely important to develop biomarkers that predict the efficacy of immune checkpoint inhibitors. In this regard, Kansy *et al.* recently demonstrated that the PD1 status of CD8⁺ T-cells was associated with anti-PD1 therapeutic outcomes in a murine model of head and neck cancer (32). Because no patients received immune checkpoint inhibitors in this study, the usefulness of PD1 status on CD8⁺ T-cells as a biomarker to predict the efficacy of immune checkpoint inhibitors remains unclear. Further investigations to clarify the usefulness of PD1 status on CD8⁺ T-cells as a biomarker to predict the efficacy of immune checkpoint inhibitors in GC patients are urgently required.

Our study had certain limitations. Firstly, because it was retrospective it was subject to bias. Secondly, the number of patients included in our study was small and the results must, therefore, be confirmed in a large-scale trial.

In conclusion, the frequency of PD1⁺CD8⁺ T-cells was useful for predicting the prognosis of patients with GC. Because it can be measured quickly, easily, and non-invasively, the frequency of PD1⁺CD8⁺ T-cells may be a useful biological marker of patients with GC in routine clinical settings.

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Human Rights Statement and Informed Consent

All procedures undertaken in this study were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1964 and later versions. Informed consent was obtained from all patients prior to inclusion in the study.

Conflicts of Interest

The Authors have no conflicting financial interests.

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