

## Prognostic Implication of Programmed Death-1-Positive Tumor-infiltrating Lymphocytes in Diffuse Large B-Cell Lymphoma

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**Background:** Programmed death-1 (PD-1) is physiologically expressed by germinal center-associated helper T-cells and has an inhibitory effect on T-cell activity. **Methods:** We examined 63 cases of diffuse large B-cell lymphoma (DLBCL) and determined the number of PD-1-positive helper T-cells in a representative tumor area after immunohistochemical staining using a monoclonal antibody against PD-1. The PD-1-positive cells were counted in 3 high-power fields (HPFs; 400 $\times$ ). **Results:** Patients were divided into 2 groups: one with a high number of PD-1-positive cells (>20/HPF, n=33) and one with a low number of PD-1-positive cells ( $\leq$ 20/HPF, n=30). The former group showed decreased overall survival, but at a statistically non-significant level (p=0.073). A high number of PD-1-positive cells was more common in patients at an advanced clinical stage and with high international prognostic index score (p=0.025 and p=0.026, respectively). The number of extranodal sites also somewhat correlated with the PD-1 staining status (p=0.071). However, the number of PD-1-positive cells was not associated with patient age, serum lactate dehydrogenase level, and Eastern Cooperative Oncology Group performance score. **Conclusions:** The high number of PD-1-positive cells might be associated with an unfavorable outcome in DLBCL patients.

**Key Words:** Lymphoma, large B-cell, diffuse; Programmed death-1 (PD-1); Lymphoma; Prognostic factor

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma in Western populations, and it is the most common malignant lymphoma in East Asian populations, representing 60% of all B-cell lymphomas.<sup>1,2</sup> Morphologically, DLBCL is a tumor in which large B-cells, which originate from B-lymphocytes, increase their size; however, its histological appearance and clinical condition can vary considerably. DLBCL was classified as a heterogeneous disease entity with various morphological appearances which showed different degrees of heritability and clinical progress according to the World Health Organization's (WHO) classification of 2008.<sup>3</sup>

The treatment of DLBCL was greatly improved by the addition of rituximab to a merger therapy of cyclophosphamide, adriamycin, vincristine, and prednisone, and it is used as the

primary standard treatment in DLBCL patients who are positive for CD20.<sup>4-6</sup> However, we can expect a long-term survival rate of only 50% in patients receiving rituximab-CHOP, the standard treatment for DLBCL, and the remaining 50% of patients die from the progression or recurrence of lymphoma. Accordingly, to increase the long-term survival rate of DLBCL patients with a poor prognosis, more aggressive treatments are administered. To determine the prognosis in non-Hodgkin lymphoma patients, the International Non-Hodgkin Lymphoma Prognosis Factor Project created the International Prognostic Index (IPI) in 1993. Thereafter, the IPI has been used largely to evaluate the prognosis of DLBCL; however, many DLBCL patients with the same IPI score have a different clinical course and treatment response. From this background, there is a clear

need to reevaluate the clinical value of the IPI for DLBCL and to analyze and identify other prognostic factors.<sup>7</sup>

Efforts have been made to find pathological factors with which the prognosis of DLBCL can be predicted more accurately, including p53 and Bcl-2, which have roles in the cell cycle and apoptosis, respectively,<sup>8,9</sup> and Bcl-6, which is frequently rearranged in DLBCL;<sup>10-13</sup> however, there is still considerable debate because these factors do not coincide with the prognosis. Kim *et al.*<sup>14</sup> analyzed the relationship between DLBCL and the protein levels of p53 and Bcl-2.

Alizadeh *et al.*<sup>15</sup> classified DLBCL into 3 subgroups: germinal center B-cell-like (GCB), activated B-cell-like (ABC), and type 3, according to the results of gene expression profiling using cDNA microarrays. Patients in the GCB group showed a markedly better survival rate than those in the ABC group. They reported that type 3 did not segregate because it was heterogeneous, but it showed a similarly poor progression as that of the ABC group. Thereafter, various studies have examined the relationship between the DLBCL classification systems and prognostic factors using immunohistochemistry. Recently, DLBCL has been separated into two subgroups, GCB and non-GCB, according to the observation that there is a clear prognostic difference between the GCB and non-GCB groups.<sup>10,16-19</sup> According to Hans *et al.*,<sup>18</sup> DLBCL can be easily classified into the GCB and non-GCB subgroups by using antibodies of CD10, Bcl-6, and Mum-1.

Programmed death-1 (PD-1) belongs to the immunoglobulin CD28 receptor family, and it is expressed in germinal center-associated helper T-cells. PD-1 is known to weaken the immune response of T-cells by significantly affecting the pathway for the inhibition of the activating mechanism of T-cells.<sup>20,21</sup> How the expression of this receptor affects the immunity of tumor cells is not understood; however, it seems to lead to the immune evasion of cancer cells.<sup>22</sup> Carreras *et al.*<sup>23</sup> reported that overall survival increased when high numbers of PD-1-positive tumor-infiltrating lymphocytes were observed in follicular lymphoma patients. In contrast, Muenst *et al.*<sup>24</sup> reported that overall survival decreased as the number of PD-1-positive tumor-infiltrating lymphocytes increased in patients with classical Hodgkin lymphomas. The connection between the increase and decrease in the number of infiltrating PD-1-positive lymphocytes and the prognosis of lymphomas has been examined, but studies that examine the prognostic relationship between DLBCL and the infiltration of PD-1-positive lymphocytes have not yet been carried out.

The aim of this study was to examine whether there was a

correlation between the PD-1-positive tumor-infiltrating lymphocytes and clinicopathologic prognostic factors in DLBCL. We analyzed the overall survival rate according to the increase or decrease in the number of PD-1-positive tumor-infiltrating lymphocytes. We then examined the usefulness of the number of PD-1-positive tumor-infiltrating lymphocytes as a diagnostic factor in GCB and non-GCB patients, which has a close relationship with the number of PD-1-positive tumor-infiltrating lymphocytes in DLBCL. We also examined whether PD-1 is an informative prognostic factor for DLBCL using a comparative analysis of each clinical prognostic factor involved in DLBCL, e.g., the IPI.

## MATERIALS AND METHODS

### Patients

We initially assessed 84 cases, which included 33 surgically diagnosed cases of DLBCL at Konkuk University Hospital from May 2005 to August 2010, and 51 DLBCL cases who were diagnosed at Hanyang University Hospital from 1995-2002. However, the final cohort consisted of 63 cases since we excluded 13 cases that showed an inappropriate tissue condition and 8 cases with insufficient clinical information. We collected the patients' clinical information such as age, sex, clinical stage, whether or not the patient died, test results (Eastern Cooperative Oncology Group performance status [ECOG PS], extranodal site involvement, and serum lactate dehydrogenase [LDH] levels).

### Tissue microarray (TMA) production

Two pathologists collected tumor lesion tissue from paraffin blocks by re-examining hematoxylin-eosin stained slides and selecting appropriate samples. TMAs were generated by transplanting the tumor cores from the paraffin blocks to a manual tissue arrayer (Tissue Microarray TMA01, SeongKohn Trader's Co., Seoul, Korea). Single core tissue biopsy specimens of 3 mm diameter were taken from representative regions of paraffin-embedded blocks. Ten cores of tumor and 3 cores of control tissue (one from tonsil, one from spleen, one from follicular lymphoma) were transplanted in each TMA block.

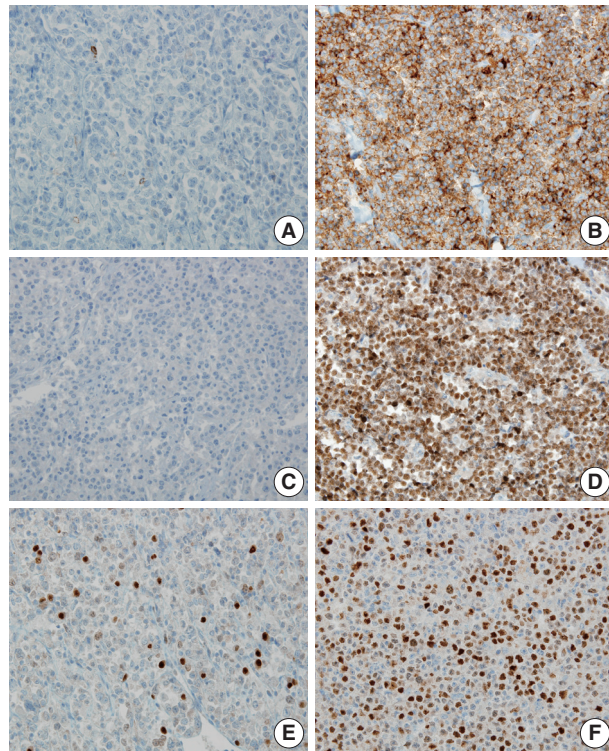
### Immunohistochemical stains and interpretation

We reviewed the slides under a light microscope and reclassi-

**Table 1.** Antibodies used for immunohistochemical staining

Antibody	Clone	Source	Buffer	Antigen retrieval	Incubation time	Dilution	Cut-off value
PD-1	NAT	Abcam, Cambridge, UK	EDTA buffer pH 8.6	Mild	2 hr	1 : 200	20/HPF
CD10	56C6	Thermo, Fremont, CA, USA	EDTA buffer pH 8.6	Standard	60 min	1 : 20	30%
Bcl-6	Gl191E/A8	Cell Marque, Rocklin, CA, USA	EDTA buffer pH 8.6	Mild	100 min	1 : 10	30%
Mum1	MUM1p	Dako, Glostrup, Denmark	EDTA buffer pH 8.6	Standard	52 min	1 : 50	30%

PD-1, programmed death-1; HPF, high-power field.



**Fig. 1.** Immunohistochemical staining for subgrouping of diffuse large B-cell lymphoma. (A) CD10-negative. (B) CD10-positive. (C) Bcl-6-negative. (D) Bcl-6-positive. (E) Mum1-negative. (F) Mum1-positive.

fied the morphological and immunohistochemical subtypes of all cases according to the WHO classification index (revised in 2008).<sup>3,18</sup>

Immunohistochemistry for CD10, Bcl-6, Mum-1, and PD-1 was performed after cutting each TMA block to a thickness of 4  $\mu$ m. The details of the immune staining are summarized in Table 1.

We conducted all immunohistochemistry using avidin-biotin in an autoimmunostainer (Ventana, Tucson, AZ, USA). Especially, we used the mouse monoclonal antibody NAT (Abcam, Cambridge, UK) for PD-1 at a dilution of 1 : 200 for 2 hours in an EDTA buffer (pH 8.6). CD10, Bcl-6, and Mum-1 staining was defined as positive when it was observed in >30% of the tumor (Fig. 1). We examined each case of PD-1 at 3 dif-

ferent high power fields (HPF), counted the number of PD-1-positive tumor-infiltrating lymphocytes, and recorded the average value. The samples were classified as positive for >20/HPF and negative for  $\leq$ 20/HPF. We also examined the relationship between the two histological types and the number of PD-1-positive lymphocytes (Fig. 2).

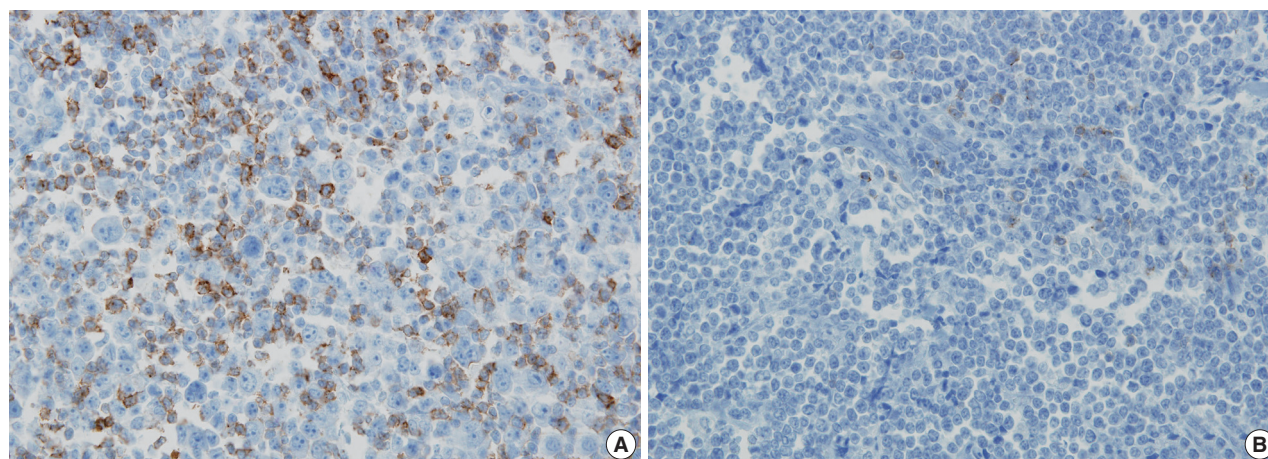
### Immunohistochemical subtyping

We classified the 63 DLBCL patients into 2 subtypes, as shown in Table 2, from the results of CD10, Bcl-6, and Mum-1 immunohistochemistry using the method suggested by Hans *et al.*<sup>18</sup> Accordingly, the patients were classified into the GCB group regardless of the results for Bcl-6 and Mum-1 if CD10 was positive. When CD10 was negative, the patients were classified into the GCB group if Bcl-6 was positive and Mum-1 was negative, and were classified into the non-GCB group if Bcl-6 and Mum-1 were negative. If CD10 and Bcl-6 were negative, the patients were classified into the non-GCB type regardless of the result for Mum-1.

### Statistical analysis

We used the chi-square test to analyze the statistical relationships between each clinical prognosis factor derived from the clinical records and the patients' PD-1 status and the relationship between the GCB or non-GCB groups and the patients' PD-1 status. The overall survival distribution was analyzed by the Kaplan-Meier method. The follow-up period was defined from the first day of diagnosis until death or to the most recent treatment day using months as the unit of time and the difference in overall survival between the PD-1-positive and -negative groups were comparatively analyzed with the log-rank test. All analyses were performed with SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA), and the differences were considered significant when the p-value was smaller than 0.05.





**Fig. 2.** Immunohistochemical staining for programmed death-1 (PD-1). High numbers of PD-1-positive tumor-infiltrating lymphocytes (A, positive: >20/high-power field [HPF]); low numbers of PD-1-positive tumor-infiltrating lymphocytes (B, negative: ≤20/HPF).

**Table 2.** Clinical characteristics of 63 patients with DLBCLs and statistical comparison between PD-1-negative and PD-1-positive groups

	Total (%)	PD-1 N (%)	PD-1 P (%)	p-value
Total No.	63	30 (47.6)	33 (52.4)	
Age (yr)				
≤60	31 (49.2)	13 (43.3)	18 (54.5)	0.374
>60	32 (50.8)	17 (56.7)	15 (45.5)	
LDH				
Normal	30 (55.6)	15 (60.0)	15 (51.7)	0.542
High	24 (44.4)	10 (40.0)	14 (48.3)	
Stage				
I/II	37 (58.7)	22 (73.3)	15 (45.5)	0.025
III/IV	26 (41.3)	8 (26.7)	18 (54.5)	
ECOG PS				
Low (0-2)	50 (82.0)	25 (86.2)	25 (78.1)	0.412
High (3-4)	11 (18.0)	4 (13.8)	7 (21.9)	
Extranodal sites				
<2	48 (80.0)	26 (89.7)	22 (71.0)	0.071
≥2	12 (20.0)	3 (10.3)	9 (29.0)	
IPI risk group				
Low (0-2)	49 (77.8)	27 (90.0)	22 (66.7)	0.026
High (3-5)	14 (22.2)	3 (10.0)	11 (33.3)	
Subtype				
GCB	21 (33.3)	10 (33.3)	11 (33.3)	0.174
Non-GCB	42 (66.7)	20 (66.7)	22 (66.7)	

DLBCL, diffuse large B-cell lymphoma; PD-1, programmed death-1; N, negative (PD-1-positive lymphocytes ≤20/HPF); P, positive (PD-1-positive lymphocytes >20/HPF); LDH, lactate dehydrogenase; ECOG PS, Eastern Cooperative Oncology Group performance status; IPI, International Prognostic Index; GCB, germinal center B-cell.

## RESULTS

### Clinical information

We analyzed 36 females and 27 males with an age distribution of 19-85 years (mean, 57.3 years). We classified those who

were older than 60 years as the high-risk group. The follow-up period was 1-110 months (average, 32.7 months). We observed 19 deaths during the follow-up period, of which 1 was from suicide that was classified as a follow-up loss; consequently, the mortality rate for DLBCL was 28.6% (18 of 63 cases). Serum LDH levels ranged from 116-5,559 IU/dL, while serum LDH levels >400 IU/dL were classified as the high-risk group. According to the clinical stage, 20 cases were stage I (31.7%), 17 cases were stage II (27.0%), 15 cases were stage III (23.8%), and 11 cases were stage IV (17.5%). ECOG scores of 0-2 were classified as the low-risk group (50 cases, 82.0%) and scores of 3-4 were classified as the high-risk group (11 cases, 18.0%). The involvement of 2 or more extranodal sites was classified as the high-risk group, where 12 cases (20.0%) were classified as high risk and 48 cases (80.0%) were classified as low risk.

These 5 types of clinical information, i.e., age, serum LDH level, clinical stage, ECOG PS, and number of extranodal sites, are all important prognostic factors, and they are also the 5 articles that are included in the IPI. The IPI is sorted into 4 categories according to the prognosis with a range of 0-5 points by calculating 1 point each when the age is >60 years at the first diagnosis, when the clinical stage is III/IV, when serum LDH is high, when ECOG PS is >2, and when there are ≥2 extranodal sites. The resulting IPI scores are classified as follows: 0-1, low risk; 2, low-intermediate risk; 3, high-intermediate risk; and 4-5, high risk. In this study, the patients with a score of 0-2 points (14 patients, 22%) were classified into the low-risk group and those with a score of 3-5 points were classified into the high-risk group.<sup>3</sup>

There was no significant relationship between the patients' overall survival and the 5 types of clinical information ( $p > 0.05$ ).

### Immunohistochemistry

There were 47 CD10-negative cases (74.6%), 16 CD10-positive cases (25.4%), 34 Bcl-6-negative cases (54.0%), 29 Bcl-6-positive cases (46.0%), 26 Mum-1-negative cases (41.3%), and 37 Mum-1-positive cases (58.7%). They were sorted into the GCB (21 cases, 33.3%) and non-GCB (42 cases, 66.6%) groups when the patients were classified according to the guidelines of Hans *et al.*<sup>18</sup> The distribution of PD-1-positive tumor-infiltrating lymphocytes ranged from 0/HPF to 201/HPF (mean, 21/HPF).

There was no significant relationship between the immunohistochemical subtypes and the patients' overall survival ( $p > 0.05$ ).

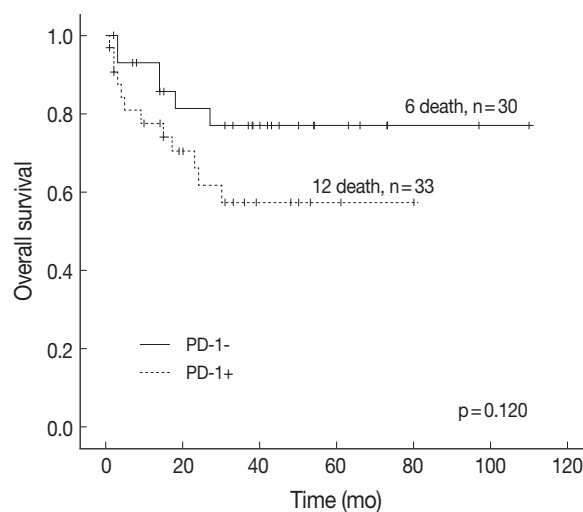
### Relationship between the number of PD-1-positive tumor-infiltrating lymphocytes and the clinical prognoses and histological subtypes

We classified the 63 DLBCL patients into 2 groups according to their prognosis with the information derived from their clinical records, i.e., age, serum LDH level, stage, ECOG PS, number of extranodal sites, and IPI score. The percentage of patients (54.5%) with a higher clinical stage was higher in the patient group with PD-1-positive lymphocyte infiltration  $> 20$ /HPF (PD-1 P) and the percentage of patients (73.3%) with a lower stage was higher in the patient group with PD-1-positive lymphocyte infiltration  $\leq 20$ /HPF (PD-1 N). In addition, the percentage of low-risk (0-2) patients (90.0%) according to the IPI was significantly higher in the patient group with PD-1 N. These 2 prognostic factors, i.e., clinical stage and IPI, showed significant statistical relationships with PD-1 ( $p = 0.025$ ,  $p = 0.026$ , respectively). As the PD-1 N group had a closer relationship with the lower clinical stage and low-risk IPI groups, reduced levels of lymphocyte infiltration were shown to have a significant relationship with a better prognosis (Table 2).

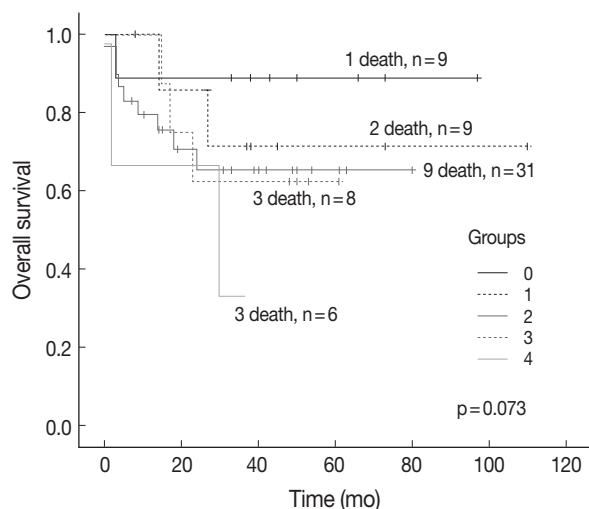
The trend between the level of extranodal site infiltration and the infiltration of PD-1-positive lymphocytes ( $p = 0.071$ ), provided additional support for the hypothesis that the reduced infiltration of PD-1-positive lymphocytes was related with a better prognosis. We clearly show the relationship between the patients sorted into the GCB and non-GCB groups and with the PD-1-positive infiltration rate in Table 2. There was no statistically significant relationship between the GCB or non-GCB groups and the infiltration of PD-1-positive lymphocytes (Table 2).

### Distribution of the overall survival rate according to the number of PD-1-positive tumor-infiltrating lymphocytes

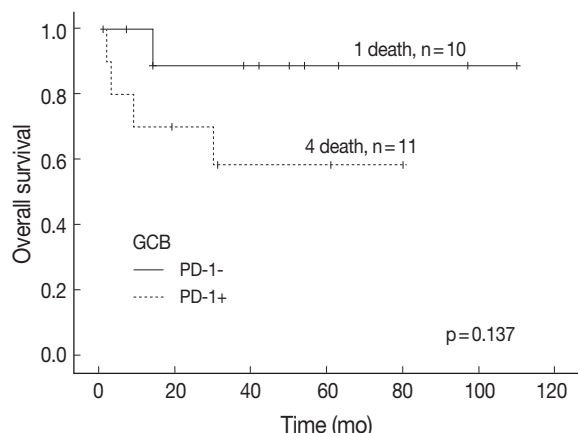
We analyzed the difference in the survival rate between the PD-1-positive and -negative patient groups according to the number of deaths during the follow-up period. When patients with an average number of PD-1-positive lymphocytes of 20/HPF were divided into the PD-1-positive and -negative groups, the PD-1-positive patients ( $n = 33$ ; number of deaths, 12) tended to show a lower overall survival rate ( $p = 0.120$ ) than the PD-1-negative patient group ( $n = 30$ ; number of deaths, 6) (Fig. 3); however, this was not a statistically significant difference. The survival rate was also analyzed when the patients were divided into the 5 following groups: 0/HPF ( $n = 9$ ; number of deaths, 1); 1-10/HPF ( $n = 9$ ; number of deaths, 2); 11-50/HPF ( $n = 31$ ; number of deaths, 9); 51-100 /HPF ( $n = 8$ ; number of deaths, 3); and  $> 100$ /HPF ( $n = 6$ ; number of deaths, 3) according to the number of PD-1-positive lymphocytes that infiltrated the tumor; the cumulative survival rate tended to decrease as the number of PD-1-positive lymphocytes increased ( $p = 0.073$ ), but it was not statistically significant (Fig. 4). Accordingly, Kaplan-Meier survival analysis confirmed that the cumulative survival rate decreased as the number of PD-1-positive tumor-infiltrating lymphocytes increased and the cumulative survival rate increased as the number of PD-1-positive tumor-infiltrating lymphocytes decreased. Such a tendency also appeared when the patient groups were divided into the GCB and non-GCB



**Fig. 3.** Kaplan-Meier survival analysis in diffuse large B-cell lymphoma according to the 2 groups of programmed death-1 (PD-1)-positive or PD-1-negative tumor-infiltrating lymphocytes. Patients with PD-1 expression above 20/high-power field tend to demonstrate poor but non-significant survival.

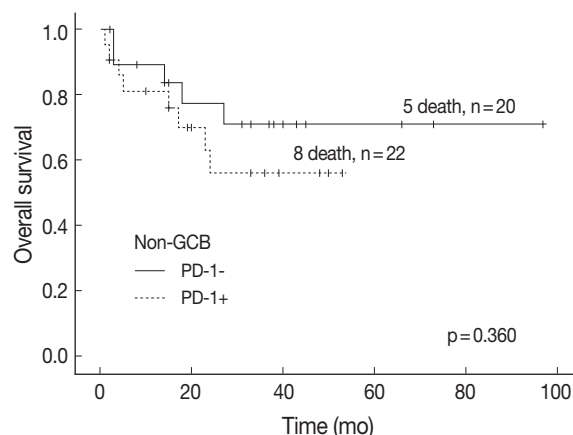


**Fig. 4.** Kaplan-Meier survival analysis in diffuse large B-cell lymphoma according to the numbers of programmed death-1 (PD-1)-positive tumor-infiltrating lymphocytes. Patients with high numbers of PD-1-positive tumor-infiltrating lymphocytes tend to demonstrate poor but non-significant survival (5 groups; 0, 0/high-power field [HPF]; 1, 1-10/HPF; 2, 11-50/HPF; 3, 51-100/HPF; 4, > 100/HPF).



**Fig. 5.** Kaplan-Meier survival analysis in germinal center B-cell subtype of diffuse large B-cell lymphoma according to the numbers of programmed death-1 (PD-1)-positive tumor-infiltrating lymphocytes. Patients with PD-1 expression above 20/high-power field (HPF) tend to demonstrate poor survival, but at a non-significant level (PD1-,  $\leq 20$ /HPF; PD1+, > 20/HPF).

groups. There was no significant relationship between the immunohistochemical subtypes and the patients' PD-1-positive status; however, the PD-1-positive group showed a lower cumulative survival rate than the PD-1-negative group. Unfortunately, this difference was not statistically significant, but we did observe a trend of  $p=0.137$  in the GCB group and  $p=0.360$  in the non-GCB group (Figs. 5, 6, respectively).



**Fig. 6.** Kaplan-Meier survival analysis in non-germinal center B-cell (non-GCB) subtype of diffuse large B-cell lymphoma according to the numbers of programmed death-1 (PD-1)-positive tumor-infiltrating lymphocytes. Patients with PD-1 expression above 20/high-power field (HPF) tend to demonstrate poor survival, but at a non-significant level (PD1-,  $\leq 20$ /HPF; PD1+, > 20/HPF).

## DISCUSSION

The aim of this study was to examine whether the levels of PD-1-positive tumor-infiltrating lymphocytes in immunohistochemical staining could be used as a prognostic factor for DLBCL patients. To find a prognostic factor closely connected with PD-1 staining we compared the already known clinical prognostic factors, i.e., age, serum LDH level, clinical stage, ECOG PS, extranodal sites, IPI, and the GCB and non-GCB immunohistochemical subtypes with the patients' PD-1 status. We found that clinical stage and the IPI had a statistically significant relationship with PD-1 positivity, and while extranodal site involvement was not significantly associated with PD-1 staining, it did demonstrate a close relationship.

The second aim of this study was to identify a histological type that was closely related to PD-1 immunostaining. Therefore, we classified the patients according to subtypes that are related to the prognosis of DLBCL patients because DLBCL was defined by WHO as a heterogeneous disease entity with diverse histological, molecular biological, and immunohistochemical characteristics. We then compared whether there was a difference in the number of PD-1-positive tumor-infiltrating lymphocytes between these subtypes. We classified the patients into the GCB and non-GCB groups according to the type classification system of Hans *et al.*<sup>18</sup> There was no significant correlation in the number of tumor-infiltrating PD-1-positive lymphocytes between these two groups; however, both groups showed a consistent reduction in the overall survival rate as the

number of PD-1-positive tumor-infiltrating lymphocytes increased.

The third aim of this study was to examine whether the infiltration of PD-1-positive lymphocytes was an accurate prognostic factor for DLBCL. However, while we did not observe a significant correlation, we demonstrated that the patient group with PD-1-positive tumor-infiltrating lymphocytes tended to show a lower overall survival rate than the PD-1-negative patient group, according to Kaplan-Meier survival curves for the follow-up period and the death rate. Therefore, we considered that an increase in the number of PD-1-positive tumor-infiltrating lymphocytes was correlated with a poor prognosis for DLBCL patients.

Hsu *et al.*<sup>25</sup> recently reported that an increase in the number of PD-1-expressing intratumoral CD8 T-cells predicted a poor prognosis in nasopharyngeal cancer. They explained that cytotoxic T-cells had an important role in the inhibition of tumor recurrence, but PD-1 had a negative impact on the treatment course while being recognized by the marker T-cell and hindering the anti-tumor activity of the T-cells. They presented data suggesting significant reductions in overall survival, disease-free survival, and locoregional recurrence-free survival according to an increase in the proportion of CD8 T-cells expressing CD-1 in the tumor. Thomson *et al.*<sup>26</sup> presented the poor histological features of 77 cases in which PD-1-positive lymphocyte infiltration appeared; this was related with a poor outcome by decreasing cancer-specific survival in PD-1-positive patients compared to 136 cases of renal cell carcinoma. Therefore, these two previous studies have suggested that the infiltration of PD-1-positive lymphocytes hinders the immune response against tumor cells and leads to a poor prognosis.

On the other hand, Carreras *et al.*<sup>23</sup> analyzed the relationship between the numbers of PD-1-positive tumor-infiltrating lymphocytes in 100 cases of follicular lymphoma with disease-free survival. They observed that the disease-free survival rate of follicular lymphoma patients increased as the numbers of PD-1-positive lymphocytes increased. They suggested that regulatory T-cells (Tregs) in follicular lymphoma modulated the immune control effect of germinal center-associated T-cells and that an increase of PD-1-positive tumor-infiltrating lymphocytes acted as an indirect protective factor. They suggested that the role of Tregs differed on account of the differences in the microenvironments of lymphoma and solid cancer by comparing their research to that of Thomson *et al.*<sup>26</sup>

Meanwhile, Muenst *et al.*<sup>24</sup> showed a similar tendency to that reported in the present study when they observed a decrease in

overall survival as the number of PD-1-positive tumor-infiltrating cells increased in 280 cases of classical Hodgkin lymphoma. They suggested that FOXP3-positive Tregs hindered and controlled the expression of PD-1 in T-cells by showing an inverse correlation in the increase of FOXP3-positive Treg expression and the increase of PD-1-positive T-cells.

The above studies are difficult to interpret merely by the number of PD-1-positive cells, and it is necessary to consider the role of FOXP3-positive Tregs in the tumor microenvironment. In this respect, analysis of the microenvironment and the distribution of FOXP3-positive Tregs in DLBCL patients are well worth pursuing as a future study.

We can find effective test markers to predict the prognosis of DLBCL patients by conducting similar analysis of other immune antibodies besides PD-1. For example, Oh and Park<sup>27</sup> reported that the expression of CD138 was a meaningful indicator of poor prognosis in DLBCL patients, and Tzankov *et al.*<sup>28</sup> reported that high numbers of intratumoral FOXP3-positive Tregs were correlated with an improvement in the survival of DLBCL patients of the GCB type.

Our cases had comparatively short follow-up periods (range, 1 to 110 months; average, 32.7 months) and a small number of deaths (18/63 cases, 28.6%), indicating the tendency for the overall survival rate to decrease as the number of PD-1-positive cells increased. We expect that statistically significant results would be observed if the number of samples and the trace-observation period are increased.

The results from this research are as follows. Firstly, among the known prognostic factors for DLBCL, clinical stage and the IPI were significantly associated with the infiltration of PD-1-positive lymphocytes, while extranodal site involvement was weakly correlated with the infiltration of PD-1-positive lymphocytes. Secondly, when DLBCL is classified into two subgroups (GCB and non-GCB groups), there was no clear association between each subtype and PD-1-positive lymphocyte infiltration; however, there was a consistent decrease in the overall survival rate as the number of PD-1-positive lymphocytes increased in each subtype. Lastly, the PD-1-positive patient group showed a tendency for a lower survival rate compared to the PD-1-negative group, and the overall survival rate decreased as the number of PD-1-positive lymphocytes increased when the patients were divided into 5 groups according to their number of PD-1-positive cells.

In conclusion, we demonstrated that an increase in the number of PD-1-positive tumor-infiltrating lymphocytes tends to lead to poor prognosis of DLBCL patients.



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