

**Title:** Prognostic value of programmed death ligand 1 and programmed death 1 expression in thymic carcinoma

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## STATEMENT OF TRANSLATIONAL RELEVANCE

Programmed death 1/programmed death ligand 1 (PD-1/PD-L1) pathway contributes to evasion of host immunity through negative regulation of T cells in the tumor microenvironment. Although the prognostic implications of PD-1/PD-L1 expression have been reported in various malignancies, its role in thymic carcinoma remains unclear. Herein, for the first time, we provide evidence that high PD-L1 expression in tumor cells is associated with favorable prognosis, whereas an increase in the number of PD-1<sup>+</sup> tumor infiltrating lymphocytes is correlated with poor prognosis in thymic carcinoma. Significant proportional correlations were also observed between PD-L1 expression and cytotoxic T lymphocyte abundance. These indicators can be used to stratify patients for more effective clinical management. More importantly, our findings may also contribute to establishing a basis for the potential application of targeting the PD-1/PD-L1 pathway via immunotherapy as a novel treatment for thymic carcinoma.

## ABSTRACT

**Purpose:** The immune checkpoint of the programmed death 1/programmed death ligand 1 (PD-1/PD-L1) pathway is believed to play an important role in evasion of host antitumor immune surveillance in various malignancies; however, little is known about its role in thymic carcinoma. This study investigated PD-1/PD-L1 expression and its association with clinicopathological features, the expression of immune-related proteins in tumor infiltrating lymphocytes (TILs), and patient prognosis.

**Experimental Design:** PD-L1 and PD-1 expression was evaluated by immunohistochemistry in 25 thymic carcinoma tissue specimens. Copy number alterations of the *PD-L1* gene in 11 cases were assessed in formalin-fixed, paraffin-embedded material using quantitative real-time PCR.

**Results:** Compared to normal subjects, 3 thymic carcinoma patients showed an increase in *PD-L1* copy number, whereas 8 did not. PD-L1 was significantly overexpressed in cases with copy number gain as compared to normal cases. High PD-L1 expression was associated with higher disease-free and overall survival rates as compared to cases with low expression. Prognostic analysis revealed low PD-L1 expression and high number of PD-1<sup>+</sup> TILs as significant predictors of poor survival, together with Masaoka-Koga stage IVa/IVb disease and incomplete resection. In the quantitative analysis of TILs, PD-L1 expression correlated proportionally with the number of infiltrating cytotoxic T lymphocytes.

**Conclusions:** Here, for the first time, we report that PD-L1 and PD-1 expression might be useful

prognostic predictors in thymic carcinoma. Further studies are expected to substantiate the prognostic value of PD-L1 and PD-1 expression, and the potential efficacy of targeting the PD-1/PD-L1 pathway in thymic carcinoma via immunotherapy.

## INTRODUCTION

Thymic epithelial tumors are the most common type of malignancy arising in the anterior mediastinum and include thymoma and thymic carcinoma; the latter is a relatively rare, high grade malignancy that directly invades adjacent structures and also undergoes distant metastasis. The most effective therapeutic modality for treating thymic carcinoma is surgical complete resection,<sup>1,2</sup> which increases the 5-year overall survival from 33.3-65.0% to 53.0-65.7%.<sup>3-8</sup> Moreover, thymic carcinoma is widely recognized to respond poorly to many chemotherapeutic agents, with an objective response rate of 20-36%, although concomitant administration of carboplatin and paclitaxel yields longer survival.<sup>9, 10</sup> Second-line treatments have also met with varying degrees of success, whereas therapeutic efficacy of sunitinib, an oral tyrosine kinase inhibitor, was recently reported in refractory patients previously treated with platinum-based chemotherapy.<sup>11</sup> As such, there is a need for more effective therapeutic strategies, which requires detailed knowledge of underlying molecular oncogenic mechanisms.

Programmed death ligand 1 (PD-L1), which is expressed by antigen-presenting cells, is an immunomodulatory glycoprotein that acts as a negative regulator in immune responses.<sup>12</sup> Programmed death 1 (PD-1) receptor is expressed on T cells and binds PD-L1 to inhibit phosphatidylinositol 3-kinase/Akt, which in turn suppresses T cell interleukin-2 production and reduces T cell proliferation and survival.<sup>13</sup> The PD-1/PD-L1 pathway has recently been shown to play pivotal roles in normal immune system regulation.<sup>13</sup> Furthermore, PD-L1 is also upregulated in

various malignancies and facilitates tumor progression by inhibiting T cell responses, allowing tumors to evade host immune surveillance mechanisms.<sup>14</sup> Indeed, PD-L1 expression and PD-1<sup>+</sup> tumor infiltrating lymphocyte (TIL) abundance have been reported to be prognostic factors in various malignancies.<sup>15-21</sup> In previous reports on PD-L1 expression in thymic epithelial tumors, it was stated that high PD-L1 expression was associated with an advanced Masaoka-Koga stage and more aggressive histologies such as type B2/B3 thymoma or thymic carcinoma.<sup>22-24</sup> In contrast, survival analyses based on PD-L1 expression were inconsistent, as one study determined a significant association between high PD-L1 expression and poor overall survival (OS) in an age- and sex-adjusted analysis, whereas the others did not find any prognostic impact. Additionally, PD-1 expression in TILs has still not been examined in any detail. Finally, no study has evaluated PD-1/PD-L1 expression in thymic carcinoma as a subset of thymic epithelial tumors, in distinction from thymoma.

Blocking PD-1/PD-L1 signaling is a potential immunotherapeutic strategy for restoring antitumor T cell responses in various malignancies. Anti-PD-1 antibody has already been approved for clinical use in malignant melanoma, non-small cell lung cancer, and renal cell carcinoma, whereas anti-PD-L1 antibody has demonstrated therapeutic efficacy in patients resistant to conventional chemotherapeutic agents in clinical trials.<sup>25</sup> Immunotherapy targeting the PD-1/PD-L1 pathway is therefore expected to improve treatments of malignancies, particularly advanced, chemotherapy-resistant, or metastatic cases.

To this end, the present study investigated PD-L1 expression in thymic carcinoma and its correlation with clinicopathological features including PD-1<sup>+</sup> TIL numbers and patient prognosis. Copy number alterations of the *PD-L1* gene were also examined, and their relationship to PD-L1 expression was assessed.

## **MATERIALS AND METHODS**

### **Patients**

Formalin-fixed, paraffin-embedded (FFPE) tissue samples acquired from 25 newly diagnosed consecutive thymic carcinoma patients at Kurume University Hospital between 1999 and 2015 were reviewed. All pathological diagnoses were made by two experienced pathologists (MH and OK) based on the 2015 World Health Organization classification with immunohistochemical detection of cluster of differentiation (CD)5 and CD117 as required.<sup>26</sup> None of the samples included immature lymphocytes positive for terminal deoxynucleotidyl transferase, and relapsed cases or cases of pathologically combined thymoma and thymic carcinoma were excluded. Each patient had undergone clinical follow-up at least every 6 months after diagnosis. The samples and medical records used in this study were approved by the Research Ethics Committee of Kurume University and conformed to the ethical guidelines of the Declaration of Helsinki. Informed consent to use materials and clinical information was obtained from all patients.

### **Tissue microarray construction**

Tissue microarrays (TMAs) were prepared using a standard tissue microarrayer (Azumaya Corp., Tokyo, Japan). A 3000- $\mu\text{m}$  core was obtained from the central area of each tumor to include the maximum number of tumor cells. This procedure was based on small biopsy specimens, which are often clinically obtained using image-guided techniques, where the central area of the tumor are likely to be extracted. The TMA blocks were sectioned at a thickness of 2.5  $\mu\text{m}$  and stained with hematoxylin and eosin or used for immunohistochemistry (IHC).

### **Immunohistochemistry**

The primary antibodies used for IHC were as follows: rabbit monoclonal anti-PD-L1 (EPR1161[2]; Abcam, Cambridge, UK) at 1:200; mouse monoclonal anti-PD-1 (NAT105; Abcam) at 1:50; mouse monoclonal anti-CD56 (1B6; Leica Biosystems, Wetzlar, Germany) at 1:100; mouse monoclonal anti-T cell intracellular antigen 1 (TIA-1) (2G9A10F5; Beckman Coulter, Brea, CA) at 1:200; and mouse monoclonal anti-granzyme B (GrB-7; Merck Millipore, Billerica, MA) at 1:500. Tissue sections were deparaffinized and then rehydrated with  $\text{H}_2\text{O}$ . Antigen retrieval was performed by heating in a microwave oven at 95°C for 20 minutes in ethylenediaminetetraacetic acid buffer (pH 8.0). Endogenous peroxidase activity was blocked by incubating in 3%  $\text{H}_2\text{O}_2$  for 5 minutes. TMA slides were incubated in primary antibodies as follows: 16 hours in a humidified chamber at 4°C for PD-L1 and 30 minutes at room temperature for PD-1/CD56/TIA-1/granzyme B. Samples were then incubated with horseradish peroxidase-conjugated anti-rabbit/mouse secondary antibody of the Dako REAL EnVision Detection System (Dako, Glostrup, Denmark) for 30 minutes at room temperature.



The immunoreaction was visualized by treatment with diaminobenzidine chromogen (Dako) for 5 minutes. Each slide was evaluated by two investigators (YS and MH) who were blinded to any clinical information, and discrepancies were debated until a consensus was reached.

### **Appraisal of PD-L1 expression**

#### *Quantification of PD-L1 expression*

PD-L1 expression was assessed under 400-fold magnification using an optical microscope in an entire field for each TMA spot and was quantified by the H-score approach, in which the proportion and intensity of tumor cells positive for PD-L1 is determined.<sup>23,27</sup> Specifically, the PD-L1 staining intensity of individual tumor cells was graded from 0 to 3 (0, negative; 1, weak; 2, moderate; 3, strong). The proportion of tumor cells exhibiting each intensity level with respect to the entire tumor cell population was calculated. The H-score was defined as the sum of the values obtained by multiplying the staining intensity and proportion, and ranged from 0 to 300 (Fig. 1).

#### *PD-L1 expression (H-score) cutoff value determination*

The optimal cutoff value for PD-L1 expression was defined based on the receiver operating characteristic curve and Youden's index.<sup>28,29</sup> Here, Masaoka-Koga stage and surgical curability were taken into account as variables that significantly affect prognosis.<sup>8, 30</sup> Ultimately, either Masaoka-Koga stage I/IIa/IIb/III disease who all performed complete resection or stage IVa/IVb who undergone diagnostic biopsy was applied as a dichotomous variable, with PD-L1 expression as a continuous variable. Youden's index revealed as optimal cutoff value an H-score of 20; cases with

PD-L1 expression higher than 20 and equal to or lower than 20 were defined as cases with high and low PD-L1 expression, respectively.

### **Immunohistochemical analysis of TILs expressing PD-1, TIA-1, and granzyme B**

The numbers of PD-1<sup>+</sup>, TIA-1<sup>+</sup>, and granzyme B<sup>+</sup> TILs were respectively enumerated in the area showing the strongest staining for each antibody. In this quantification, because PD-1<sup>+</sup> and granzyme B<sup>+</sup> TILs were not infiltrated in tumor nests or around vessels penetrating the tumor, they were counted in the stroma among tumor nests. In contrast, TIA-1<sup>+</sup> TILs were counted mainly in tumor nests or beside glandular structures within the tumor. However, in some cases they were counted in the stroma because of the absence of TIA-1<sup>+</sup> TILs in these sites. All TILs were counted in 5 high-power fields (HPFs) under 400-fold magnification. The mean number of TILs that demonstrated positive staining for each antibody per HPF was calculated.

### **Quantitative real-time polymerase chain reaction analysis of *PD-L1* copy number**

Copy number analyses of *PD-L1* in 11 FFPE tissue samples were performed by quantitative real-time polymerase chain reaction (PCR). Samples with adequate tumor regions were dissected and total DNA was extracted using a QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). *PD-L1* copy number was evaluated using the TaqMan DNA Copy Number Assay (Hs03704252\_cn; Applied Biosystems, Waltham, MA) and Taqman Copy Number Reference Assay *RNase P* (Applied Biosystems). Quantitative real-time PCR was performed using Taqman Universal Genotyping Master Mix (Applied Biosystems) according to the manufacturer's protocol on an Applied Biosystems 7500

real-time PCR system. Results were analyzed using CopyCaller Software (Applied Biosystems).

### **Statistical analysis**

The relationship between PD-L1 positivity and clinicopathological features was evaluated using Fisher's exact test for categorical variables. The Mann-Whitney U test was used to compare the median value of continuous variables according to PD-L1 positivity as well as PD-L1 expression based on *PD-L1* copy number status. Pearson's product-moment correlation coefficient was used to determine the correlation between PD-L1 expression and the number of TIA-1<sup>+</sup> or granzyme B<sup>+</sup> TILs. In the analysis of recurrence and survival, the starting point was defined as the day of surgical resection or biopsy. The end of the disease-free survival (DFS) period was defined as the day of recurrence, and that of the OS period was defined as the day of death or the date of last follow-up, respectively. Survival curves to estimate DFS and OS were determined by the Kaplan-Meier method, with comparison of each curve using a log-rank test. Cox proportional hazards regression models were used to assess the prognostic values of each factor. *P*-values declared in this study were all based on two-sided tests, and those less than 0.05 were interpreted as statistically significant. JMP version 11 software (SAS Institute Inc., Cary, NC) was used to perform all statistical analyses.

## **RESULTS**

### **Clinicopathological findings**

The baseline clinicopathological features of patients are summarized in Supplementary

Table S1. The median follow-up period was 33 months (range, 1-124). The 25 cases of thymic carcinoma included 17 males (68%) and 8 females (32%), with a median age of 63 years (range, 32-81). Surgical complete resection was achieved in 16 cases (64%), which was attained in all cases representing Masaoka-Koga stage I/IIa/IIb/III disease with microscopically confirmed negative surgical margin. In 9 cases (36%) of Masaoka-Koga stage IVa/IVb disease, only diagnostic biopsies were performed and specific nonsurgical treatments were administered. Four cases (25%) received neoadjuvant therapies, including 2 cases of radiotherapy (13%) and 2 of chemoradiotherapy (13%); subsequent surgical complete resections were successfully performed for all cases.

#### **Immunohistochemical analysis of PD-L1 expression**

PD-L1 was mainly expressed on the cell membrane with or without cytoplasm of tumor cells, exhibiting a near-uniform pattern in tumor regions (Fig. 1). The median value of PD-L1 expression was 100, with a range of 0-300 (95% confidence interval [CI], 77.8-154.4). According to our predefined cutoff value, 20/25 cases (80%) were classified as having high PD-L1 expression.

#### ***PD-L1* copy number analysis and association with PD-L1 expression**

The validity of the *PD-L1* copy number assay was confirmed using 5 normal control cases of reactive tonsils, which had a mean copy number of 2.07 (95% CI, 1.83-2.31). The mean copy number in each case was determined by triplicate PCRs with adjustment by control cases, which revealed *PD-L1* gene copy number gains in 3 cases (27%), while 8 cases (73%) showed a normal *PD-L1* status (Fig. 2A). Cases with *PD-L1* copy number gain showed significantly higher PD-L1

expression than normal cases ( $P = 0.040$ , Fig. 2B).

### **Relationship between PD-L1 positivity and clinicopathological features**

Table 1 depicts the distributions of clinicopathological characteristics according to positivity for PD-L1. PD-L1 expression status was not associated with age ( $P = 0.586$ ), sex ( $P = 1.000$ ), Masaoka-Koga stage ( $P = 0.312$ ), histology ( $P = 1.000$ ), tumor size ( $P = 0.838$ ), or surgical curability ( $P = 0.312$ ). Of note, the number of PD-1<sup>+</sup> TILs was also not associated with PD-L1 expression status ( $P = 0.837$ ).

### **Clinical outcome according to PD-L1 expression**

On Kaplan-Meier analysis, OS of all patients was significantly longer in cases with high as compared to low PD-L1 expression ( $P = 0.010$ , Fig. 3A). Furthermore, significant associations were also encountered in the subgroup of patients with completely resected tumor ( $P = 0.0009$ , Fig. 3B) and the subgroup of patients with thymic squamous cell carcinomas ( $P = 0.005$ , Fig. 3C). Patients with high PD-L1 expression also had significantly better DFS than those with low PD-L1 expression ( $P = 0.004$ , Fig. 3D).

### **Potential prognostic factors affecting overall survival**

Each clinicopathological factor, including PD-L1 and PD-1 expression status, was evaluated for its prognostic value (Table 2). In all patients, Masaoka-Koga stage IVa/IVb disease (hazard ratio [HR], 6.578;  $P = 0.004$ ), incomplete resection (HR, 6.578;  $P = 0.004$ ), low PD-L1 expression (HR, 5.837;  $P = 0.025$ ), and increased number of PD-1<sup>+</sup> TILs (HR, 1.496;  $P = 0.037$ ) were identified as

significant predictors of poor survival in the univariate analysis. Furthermore, the statistical significance of both PD-L1 expression and PD-1<sup>+</sup> TILs as prognostic factors was observed even in the squamous cell carcinoma subset, in which the HR for low PD-L1 expression was even higher (HR, 8.073;  $P = 0.014$ ) than that in the entire cohort of thymic carcinoma patients.

### **Multivariate analysis of prognostic factors affecting overall survival**

Although a larger multivariate model was used to identify prognostic factors, the results had limited value because of the small number of samples (Supplementary Table S2). For reference, all potential prognostic factors identified in the univariate analysis retained their significance in the multivariate analysis, represented as Masaoka-Koga stage (HR, 7.000;  $P = 0.005$ ), surgical curability (HR, 7.000;  $P = 0.005$ ), PD-L1 expression (HR, 6.854;  $P = 0.028$ ), and the number of PD-1<sup>+</sup> TILs (HR, 1.546;  $P = 0.026$ ). In addition, these factors were prognostically significant in patients with squamous cell carcinomas.

### **Correlation between PD-L1 expression and cytotoxic T lymphocyte abundance**

To further characterize TILs and assess the association between high PD-L1 expression and improved survival, we evaluated TIA-1 expression in TILs which is a representative marker of cytotoxic cells. Based on morphological findings, the acquired knowledge of antitumor immunity principled by T cells,<sup>14</sup> and the absence of CD56<sup>+</sup> lymphocytes confirmed by our immunohistochemical surveillance, the TIA-1<sup>+</sup> cells were validated to be cytotoxic T lymphocytes (CTLs). CTLs were subsequently counted in each case and statistical analysis represented that

PD-L1 expression was significantly correlated with the number of CTLs ( $r = 0.566$ ,  $P = 0.003$ , Fig. 4). Additionally, the number of granzyme B<sup>+</sup> lymphocytes was also investigated to determine the correlation between PD-L1 expression and the number of activated CTLs. However, there was little correlation between PD-L1 expression and activated CTL abundance ( $r = 0.051$ ,  $P = 0.808$ ).

## DISCUSSION

This study demonstrated for the first time that high PD-L1 expression is associated with favorable OS in thymic carcinoma. This result was supported by the finding that high PD-L1 expression was correlated with an increase in CTL infiltration. Furthermore, we also found that an increase in the number of PD-1<sup>+</sup> TILs predicted poor patient survival. These results suggest that the PD-1/PD-L1 pathway is a potential immunotherapeutic target in thymic carcinoma.

The *PD-L1* copy number analysis demonstrated that PD-L1 expression was significantly higher in cases with *PD-L1* copy number gain than in normal cases, implying that *PD-L1* gene alteration is an important mechanism of PD-L1 overexpression. Previous studies that focused on B cell lymphomas have also reported that *PD-L1* transcripts are more highly expressed in tissue samples and cell lines with increased *PD-L1* copy number than in normal cases, although it was not possible to make a direct comparison.<sup>31, 32</sup> To clarify the exact biological mechanisms underlying PD-L1 overexpression in thymic carcinoma, additional studies and data on transcriptional levels are required. Furthermore, *PD-L1* was found to be amplified in 27% of thymic carcinomas. Although

few studies have evaluated genetic copy number aberrations in thymic carcinoma, relationships between poor OS and *BCL2* copy number gain or *CDKN2A/B* copy number loss have been observed, the frequency of which was 42% and 14%, respectively.<sup>33</sup> Further studies on genetic alterations in the tumor are necessary to improve therapeutic strategies and survival for thymic carcinoma patients.

Thus far, few studies have examined PD-L1 expression in thymic epithelial tumors.<sup>22-24</sup> Confined to thymic carcinoma as a subset of thymic epithelial tumors, only one of these reports mentioned the prognostic role of PD-L1, finding that there was no difference in OS according to PD-L1 positivity.<sup>23</sup> However, our results indicated a significant association between high PD-L1 expression and better prognosis. This discrepancy may be attributable to differences in patient selection; the previous study evaluated thymoma and thymic carcinoma collectively, whereas thymomas and heterogeneous cases of combined thymoma and thymic carcinoma were excluded from the present study. We consider that thymoma and thymic carcinoma should be analyzed individually because they are widely known to vary in terms of prognosis and molecular biological profiles.<sup>26</sup> In fact, when the 25 thymic carcinoma samples evaluated here were analyzed along with 82 thymoma samples in our previous study,<sup>24</sup> OS was comparable between cases with high and low PD-L1 expression in both individual histology types; thymic carcinoma, however, demonstrated slightly higher PD-L1 positivity than thymoma, consistent with the previous report. Nevertheless, our study analyzed thymic carcinoma independently and found that high PD-L1 expression was associated with favorable survival. Thus, thymic carcinoma and thymoma should be investigated



independently to clarify the role of PD-L1 expression in each tumor type.

The prognostic value of PD-L1 expression has been investigated for various malignancies, including malignant melanoma, non-small cell lung cancer, and renal cell carcinoma.<sup>34-36</sup> Most studies have emphasized an association between high PD-L1 expression and poor survival based on the ability of PD-L1 to negatively regulate antitumor T cell responses. In contrast, high PD-L1 expression has been linked to better prognosis in some malignancies.<sup>17, 35, 37</sup> In these reports, a significant association between high PD-L1 expression and increased TILs was identified.<sup>17, 34, 35, 37</sup> Moreover, it was also shown that expression of the immunosuppressive molecules PD-L1, indoleamine-2, 3-dioxygenase, and FoxP3 by tumor cells was induced following CD8<sup>+</sup> T cell infiltration or interferon-gamma production in murine *in vivo* models of melanoma.<sup>38</sup> This suggests that PD-L1 expression by tumor cells is upregulated as a result of a feedback mechanism against activation of host antitumor immunity. In addition, increases in TIA-1<sup>+</sup> TILs have been reported as a favorable prognostic factor in some malignancies, although the activated status of CTLs was not taken into account.<sup>39-41</sup> Based on these considerations, the association between high PD-L1 expression and improved prognosis in thymic carcinoma may be explained by high PD-L1 expression following the activation of host antitumor immune responses initiated by infiltrated CTLs. In order to substantiate the determined prognostic value of PD-L1 expression in thymic carcinoma, investigation of the interactions between immune-related molecules including PD-L1 within the tumor microenvironment is required.

This is the first report to investigate PD-1<sup>+</sup> TILs in thymic carcinoma, demonstrating that an increased number of these cells predicts poor patient prognosis. Similarly, abundant PD-1<sup>+</sup> TILs were previously reported to be a poor prognostic factor in some malignancies.<sup>19,20</sup> These outcomes may be attributed to activation of T cell-mediated antitumor immune suppression via PD-1/PD-L1 signaling. Some studies have reported a proportional correlation between PD-L1 expression in tumor cells and the number of PD-1<sup>+</sup> TILs, which may reflect PD-1/PD-L1 pathway activation; however, others have found an inverse correlation.<sup>20,42,43</sup> Although we could not provide definitive considerations in this argument because of the inability to directly compare studies, the ability to predict patient survival based on the number of PD-1<sup>+</sup> TILs may be useful for stratifying patients in clinical settings.

We found that PD-L1 was frequently overexpressed by tumor cells and PD-1<sup>+</sup> TILs infiltrated in thymic carcinoma. In recent years, PD-1/PD-L1-targeted immunotherapy has been shown to be clinically effective and thus represents a promising therapeutic alternative in selected patients with malignant tumors. Such efficacy is also expected for thymic carcinoma, which responds poorly to various chemotherapeutic agents; however, this has not been examined yet. In some other malignancies, immunotherapy using PD-L1- and PD-1-based antibodies appeared to show enhanced activity, favoring PD-L1-positive tumors over PD-L1-negative tumors.<sup>25,44</sup> Although the practical potency is still unknown, these findings support our statement that the clinical use of immunotherapeutic antibodies targeting the PD-1/PD-L1 pathway may be effective for treating thymic carcinoma based on high PD-L1 positivity.

It is still controversial which anti-PD-L1 antibody is the best to determine PD-L1 expression by IHC in tumor tissue samples. This is a critical issue because immunohistochemical PD-L1 positivity has been reported to predict therapeutic efficacy in anti-PD-1/PD-L1 antibody immunotherapy in several clinical trials, as mentioned previously. In the present study, the PD-L1 clone EPR1161[2], which recognizes the C-terminus extracellular domains of PD-L1, was employed. By contrast, previous reports studying PD-L1 in thymic epithelial tumors used clone 5H1, which targets extracellular domains of PD-L1, or E1L3N, which targets intracellular domains, respectively.<sup>22, 23</sup> These three studies (including ours) demonstrated slightly different populations of cases with high PD-L1 expression in thymic carcinoma (58.7-80%), which may have resulted from the use of distinct antibodies and PD-L1 cutoff values. Although the specificity of each antibody in IHC has certainly been confirmed, it remains unknown whether or not a particular antibody can predict the therapeutic efficacy of anti-PD-1/PD-L1-antibody immunotherapy. To establish a valid eligibility criterion for the clinical use of these agents, further studies evaluating the optimal methods (including the determination of the anti-PD-L1 antibody used for IHC and measures other than immunohistochemical PD-L1 expression) to accurately predict therapeutic efficacy are necessary in these treatments.

There are some limitations to our results. First, although a certain level of significance was observed in the multivariate analysis, this study included a small sample size, and the statistical power may have been insufficient. Additional studies with larger samples are necessary to confirm

the prognostic value of PD-L1 and PD-1 in thymic carcinoma. Second, our results were based on a retrospective analysis, which may have resulted in selection bias because of the various therapeutic protocols that were used. Therefore, prospective studies are also needed to validate our findings.

In conclusion, the present study demonstrated that high PD-L1 expression by tumor cells was associated with favorable prognosis, whereas an increased number of PD-1<sup>+</sup> TILs was correlated with poor prognosis in thymic carcinoma. These indicators can be used to stratify patients for more effective clinical management. Importantly, our findings provide a basis for the use of immunotherapy targeting the PD-1/PD-L1 pathway as a potential novel treatment in thymic carcinoma.

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### Figure legends

**Figure 1.** Immunohistochemical analysis of PD-L1 expression in thymic carcinoma. Hematoxylin and eosin staining (upper row, original magnification 40×) and PD-L1 immunostaining (lower row, original magnification 40× and lower row inset, original magnification 200×) are shown. PD-L1 expression (H-score) in each case represents (A,D) 300 (proportions of each intensity score: 0 = 0%; 1 = 0%; 2 = 0%; 3 = 100%), (B,E) 120 (0 = 30%; 1 = 30%; 2 = 30%; 3 = 10%), and (C,F) 3 (0 = 97%; 1 = 3%; 2 = 0%; 3 = 0%)

**Figure 2.** *PD-L1* copy number status and PD-L1 expression in thymic carcinoma. A, quantitative real-time PCR-based *PD-L1* copy number analysis in 11 thymic carcinoma tissue samples. Each bar represents the results showing the mean ( $\pm$  SD) of triplicate PCRs. Cases 9, 10, and 11 showed copy number gain (gray bar). B, cases with copy number gain showed significantly higher PD-L1 expression than normal cases.

**Figure 3.** Kaplan-Meier survival curves based on PD-L1 expression in thymic carcinoma (black and orange lines, high and low PD-L1 expression, respectively). A, survival curves in all patients. B and C, survival curves in the subgroup of patients who had undergone complete resection (B) and those with squamous cell carcinoma (C). D, survival curves for DFS in patients who had undergone complete resection. In all analyses, cases with high PD-L1 expression experienced significantly better OS or DFS than cases with low PD-L1 expression.

**Figure 4.** Correlation between PD-L1 expression and the number of infiltrated CTLs. PD-L1

expression was significantly correlated with CTL abundance.

Table 1. Comparison between PD-L1 expression status and clinicopathological features

Characteristic	PD-L1 high	PD-L1 low	<i>P</i>
	n = 20 (80%)	n = 5 (20%)	
Age, years old (median, range)	66.5 [32-74]	62 [49-81]	0.586
Sex, male	13 (65%)	4 (80%)	1.000
Masaoka-Koga stage, IVa/IVb	6 (30%)	3 (60%)	0.312
Histological type, SCC	15 (75%)	4 (80%)	1.000
Tumor size, mm (median, range)	55.5 [13-90]	60 [25-80]	0.838
Curability, complete resection	14 (70%)	2 (40%)	0.312
PD-1 positive TILs / HPF (median, range)	1.5 [0-81.4]	1.6 [0-35.4]	0.837

Abbreviations: HPF, high power field; PD-1, programmed death 1; PD-L1, programmed death ligand 1; SCC, squamous cell carcinoma; TIL, tumor infiltrating lymphocyte.

Table 2. Univariate analysis for prognostic factors affecting overall survival

Characteristic, risk factor	All cases (n = 25)			SCC (n = 19)		
	HR	[95% CI]	P	HR	[95% CI]	P
Age, < 63 years old	1.359	[0.408-5.217]	0.622	1.311	[0.370-5.175]	0.675
Sex, female	1.067	[0.279-3.541]	0.918	1.024	[0.260-3.612]	0.971
Masaoka-Koga stage, IVa/IVb	6.578	[1.789-30.971]	0.004	5.014	[1.304-24.008]	0.019
Histological type, SCC	3.195	[0.592-59.531]	0.205	-	-	-
Tumor size, > 56 mm	1.265	[0.359-4.279]	0.704	1.219	[0.341-4.844]	0.762
Curability, incomplete resection	6.578	[1.789-30.971]	0.004	5.014	[1.304-24.008]	0.019
PD-L1 expression, low expression	5.837	[1.265-30.167]	0.025	8.073	[1.538-59.367]	0.014
PD-1 positive TILs, per increase by 10 cells / HPF	1.496	[1.029-2.162]	0.037	1.476	[1.019-2.188]	0.041

Abbreviations: CI, confidence interval; HPF, high power field; HR, hazard ratio; PD-1, programmed death 1; PD-L1, programmed death ligand 1; SCC, squamous cell carcinoma; TIL, tumor infiltrating lymphocyte.



Figure 1

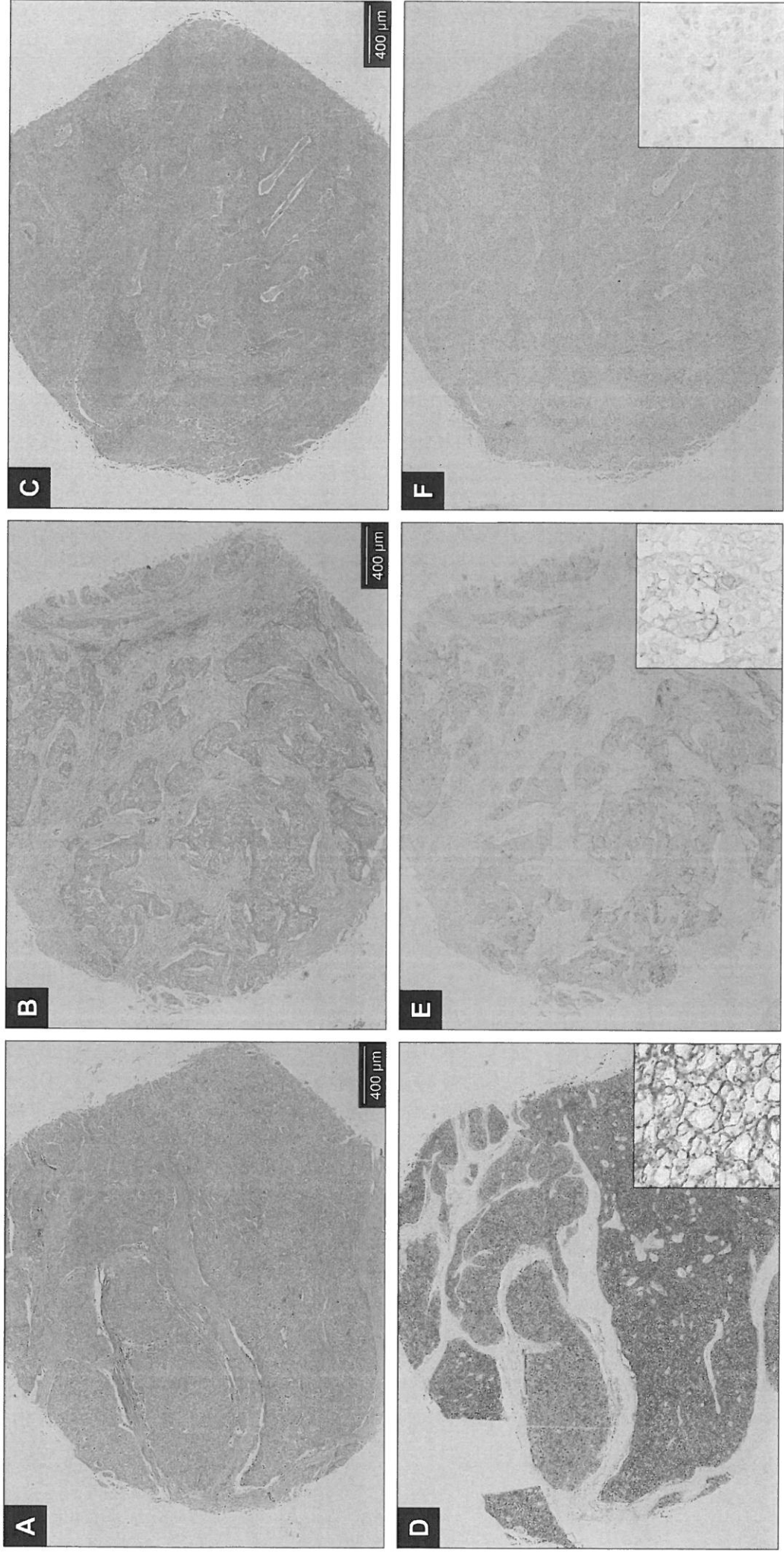


Figure 2

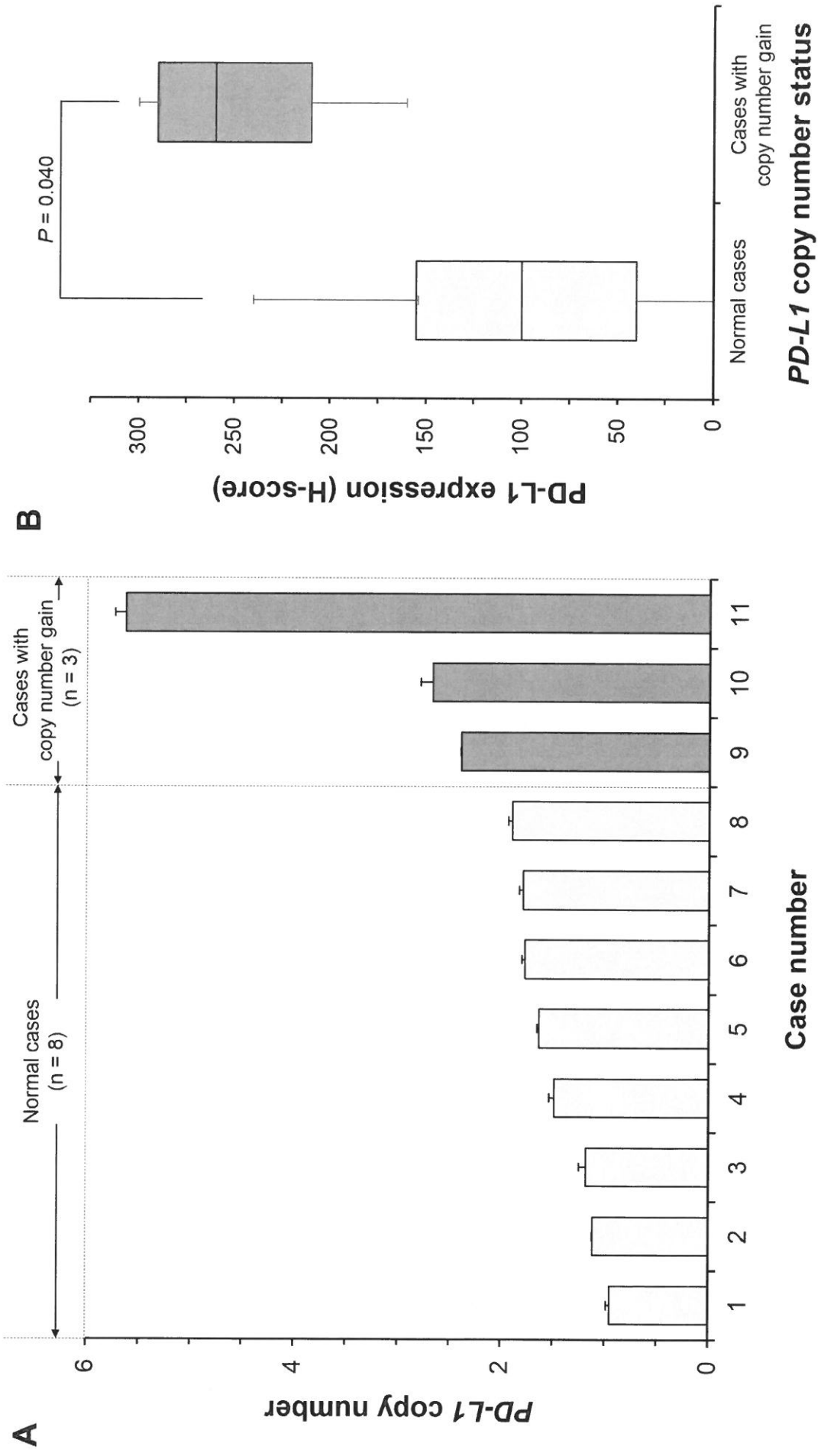


Figure 3

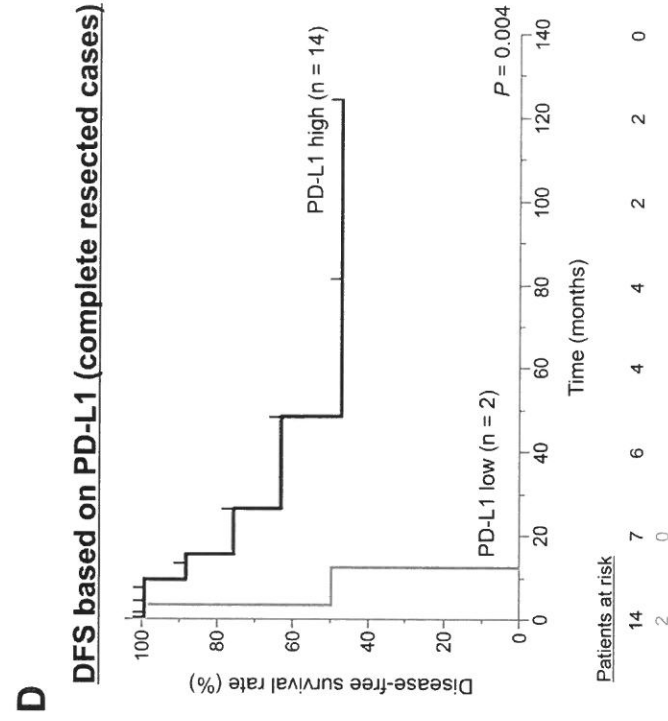
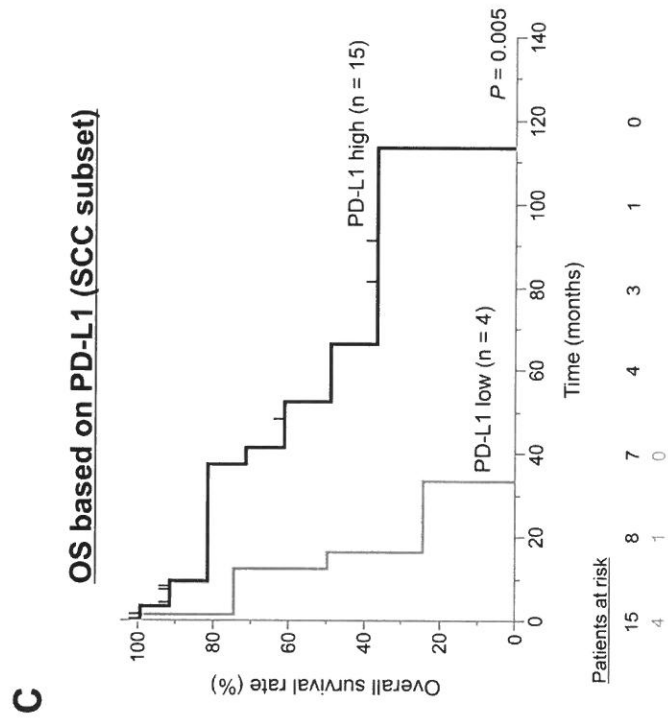
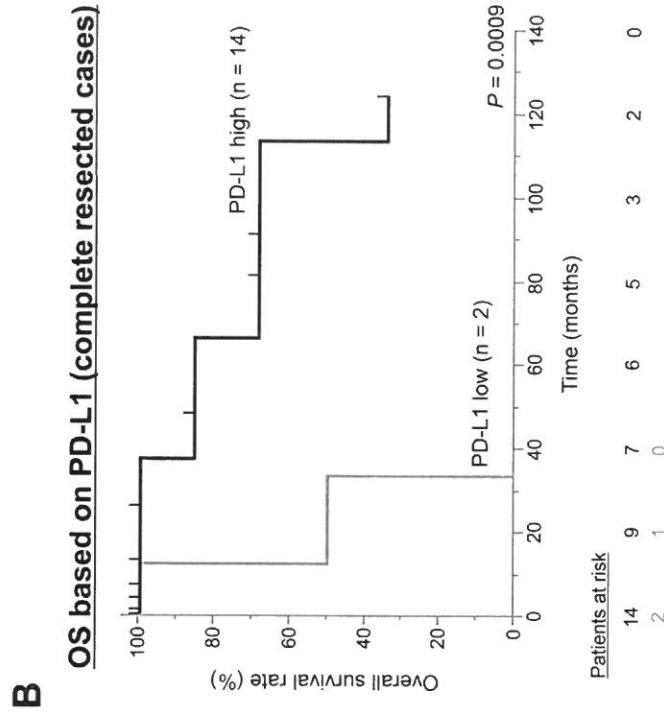
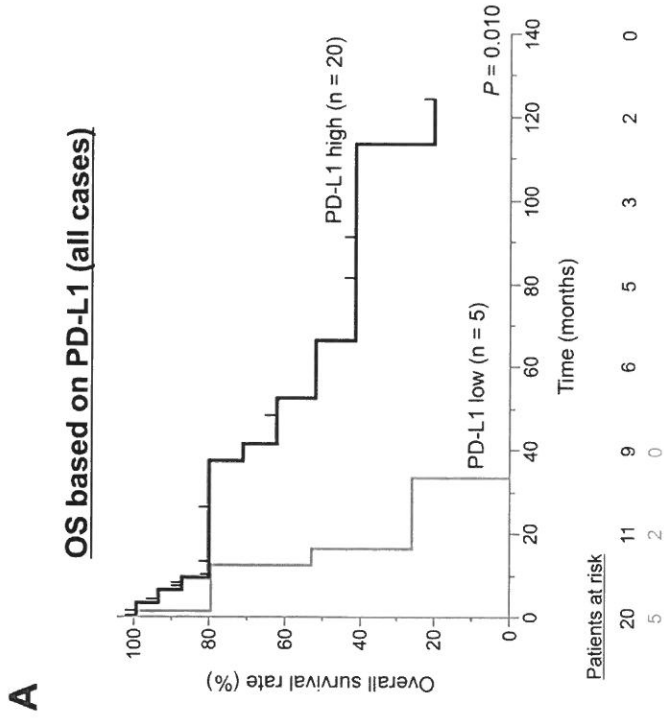
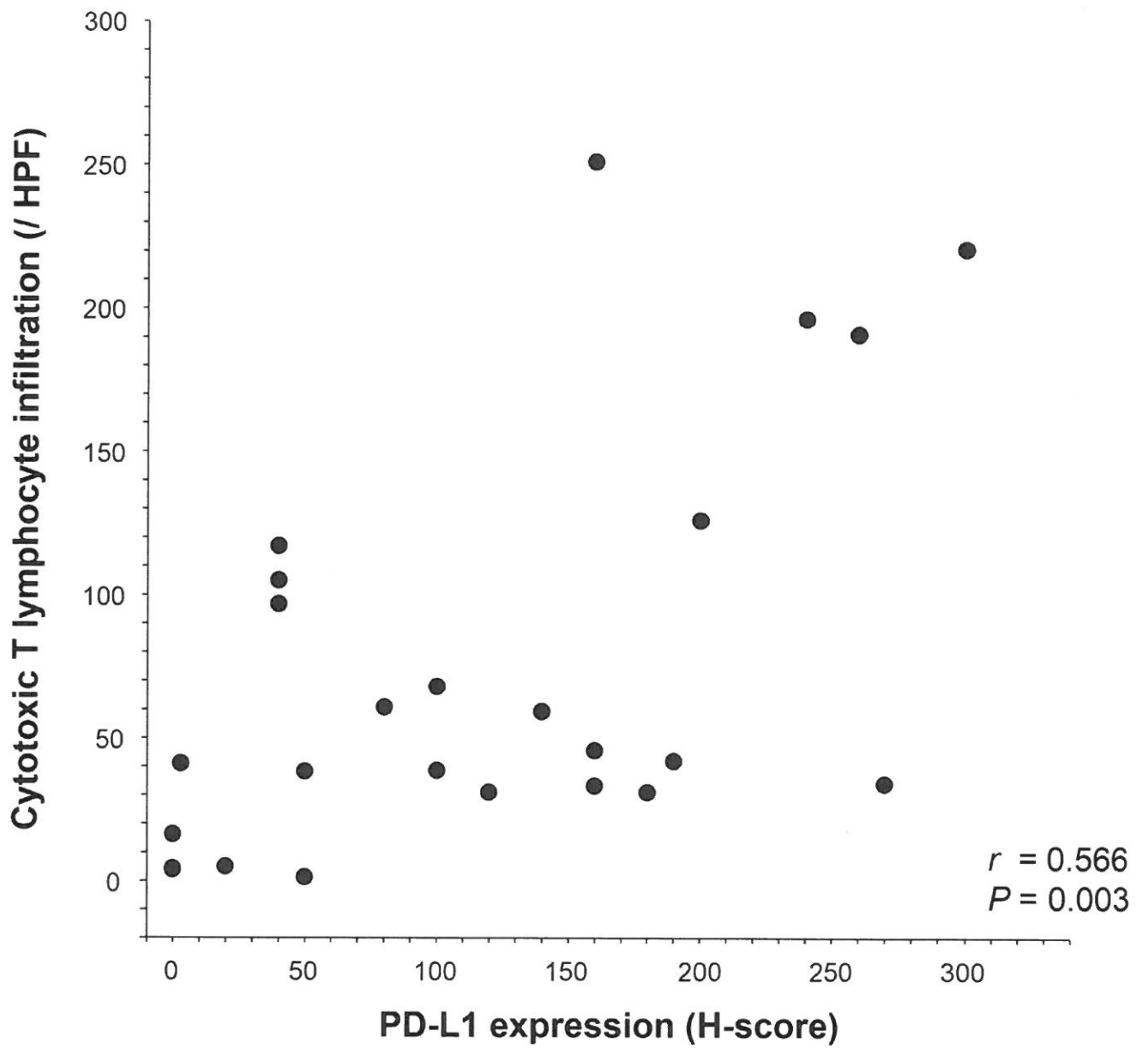


Figure 4



Supplementary Table S1. Patient characteristics

Characteristic	n = 25 (%)	
Age, years old		
Median [range]	63	[32-81]
Sex		
Male	17	68%
Female	8	32%
Masaoka-Koga stage		
I	4	16%
IIa	2	8%
IIb	1	4%
III	9	36%
IVa	3	12%
IVb	6	24%
Histological type		
Squamous cell carcinoma	19	76%
Adenocarcinoma	4	16%
Papillary adenocarcinoma	1	4%
Thymic carcinoma with adenoid cystic carcinoma-like features	1	4%
Mucinous adenocarcinoma	1	4%
Adenocarcinoma, NOS	1	4%
Small cell carcinoma	1	4%
Sarcomatoid carcinoma	1	4%
Tumor size, mm		
Median [range]	56	[13-90]
≤ 56 mm	13	52%
> 56 mm	12	48%
Curability		
Complete resection	16	64%
Incomplete resection (biopsy)	9	36%
Neoadjuvant therapy (complete resection cases)		
Chemotherapy	0	0%
Radiotherapy	2	13%
Chemoradiotherapy	2	13%
None	12	75%
Adjuvant therapy (complete resection cases)		
Chemotherapy	1	6%
Radiotherapy	4	25%
Chemoradiotherapy	0	0%
None	11	69%
Treatments after recurrence (complete resection cases)		
Chemotherapy	3	50%
Radiotherapy	1	17%
Chemoradiotherapy	0	0%
Best supportive care	2	33%
Treatments (incomplete resection cases)		
Chemotherapy	3	33%
Radiotherapy	0	0%
Chemoradiotherapy	4	44%
Best supportive care	2	22%

Abbreviation: NOS, not otherwise specified.

Supplementary Table S2. Multivariate analysis for prognostic factors affecting overall survival

Characteristic, risk factor	All cases (n = 25)			SCC (n = 19)		
	HR	[95% CI]	<i>P</i>	HR	[95% CI]	<i>P</i>
Masaoka-Koga stage, IVa/IVb	7.000	[1.805-34.979]	0.005	7.000	[1.304-24.008]	0.010
Curability, incomplete resection	7.000	[1.805-34.979]	0.005	7.000	[1.304-24.008]	0.010
PD-L1 expression, low expression	6.854	[1.236-54.411]	0.028	17.091	[1.538-59.367]	0.006
PD-1 positive TILs, per increase by 10 cells / HPF	1.546	[1.063-2.293]	0.026	1.607	[1.077-2.552]	0.023

Abbreviations: CI, confidence interval; HPF, high power field; HR, hazard ratio; PD-1, programmed death 1; PD-L1, programmed death ligand 1; SCC, squamous cell carcinoma; TIL, tumor infiltrating lymphocyte.