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[1]Department of Pathology, Kurume University School of Medicine, Kurume, Japan;
[2]Department of Dermatology, Kurume University School of Medicine, Kurume, Japan;
[3]Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Japan;
[4]Life Science Research Institute, Saga-ken Medical Centre Koseikan, Saga, Japan

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Programmed Death-Ligand 1 and Programmed Death-Ligand 2 Expression Can Affect Prognosis in Extramammary Paget's Disease

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[1]*Department of Pathology, Kurume University School of Medicine, Kurume, Japan;*
[2]*Department of Dermatology, Kurume University School of Medicine, Kurume, Japan;*
[3]*Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Japan;*
[4]*Life Science Research Institute, Saga-ken Medical Centre Koseikan, Saga, Japan*

Abstract. *Background: Extramammary Paget's disease (EMPD) is a type of carcinoma that usually progresses slowly but may cause metastasis and subsequent death of patients. We investigated the relationship between the expression of programmed death-ligand 1 (PD-L1)/programmed death-ligand 2 (PD-L2) and stromal CD8$^+$ tumor-infiltrating lymphocytes (TILs) in EMPD and clinicopathological findings, including prognosis. Materials and Methods: We examined 47 cases of EMPD and performed immunohistochemical staining of formalin-fixed paraffin-embedded full-face sections. Results: PD-L1 expression in tumor cells was observed in 13 cases (27.7%) while PD-L2 expression was observed in 21 cases (44.7%). The cumulative postoperative recurrence-free rate in the group with positivity for PD-L1 and/or PD-L2 with a low CD8$^+$ TIL count was significantly lower than that of the corresponding group with a high CD8$^+$ TIL count and of the PD-L1- and PD-L2-negative group (p=0.026). Conclusion: The expression of PD-L1/PD-L2 in tumor cells was shown to be a factor for poor prognosis.*

Extramammary Paget's disease (EMPD) is a rare cutaneous carcinoma that often manifests in the vulva, penis, scrotum, axillae, and perianal region in the elderly, and its etiology remains poorly understood (1-3). Histopathologically, the carcinoma tends to persist in the epidermis but infiltration into the dermis has also been observed. Local mass formation and regional lymph node metastases have also been noted, and mortality linked to the primary disease can also occur as a result of distant lymph node metastasis or hematogenous metastasis (4). The tumor-node-metastasis (TNM) classification for EMPD that is currently used in Japan is a system proposed by Ohara *et al*. (5) and is being implemented on a trial basis but there has been no international consensus regarding staging thus far. In addition, the current guidelines are based on histological features and do not yet define EMPD on a molecular basis that can guide curative therapies.

In recent years, immune checkpoint inhibitors (ICIs) such as nivolumab (a monoclonal antibody against human programmed death-1 (PD1) have been increasingly and frequently used as a new treatment for various malignant tumors such as malignant melanoma, non-small cell lung cancer and renal cell carcinoma (6, 7). ICIs are drugs that release suppression of the immune response to cancer, and by inhibiting binding to PD1 and its ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2), these drugs effectively maintain the immune response to tumor. Response rates with a single ICI have been reported to be approximately 10-30% (8). The expression of PD-L1 and PD1 in tumor and immune cells infiltrating the stroma has been reported to be useful as a predictor of therapeutic efficacy (8-10).

The immunological microenvironment is currently being studied in many types of malignant tumor such as malignant melanoma, head and neck cancer, and hepatocellular carcinoma (11-14). However, few reports have examined the immunological tumor microenvironment in the context of EMPD (15). In this study, we investigated the relationship between the expression of PD-L1/PD-L2, immune checkpoint ligands, and tumor stromal CD8$^+$ T-cells in EMPD, as well as the clinicopathological findings including prognosis.

Correspondence to: Jun Akiba, MD, Ph.D., Department of Diagnostic Pathology, Kurume University Hospital, 67 Asahi-machi, Kurume, Fukuoka, 830-0011, Japan. Tel.: +81 942317546, Fax: +81 942320905, e-mail: akiba@med.kurume-u.ac.jp

Key Words: Extramammary Paget's disease, prognosis, PD-L1, PD-L2, CD8+ TILs.

Materials and Methods

In this study, we examined 47 cases diagnosed with EMPD. All EMPDs were surgically resected at our institutional hospital between 2009 and 2017. Clinical follow-up data were available for all 47 cases. Specimens were fixed in 10% neutral buffered formalin, following paraffin embedding. Consecutive sections measuring 4 μm in thickness were cut and stained with hematoxylin and eosin. A medical history of colorectal carcinoma was assessed to distinguish between primary and secondary diseases. Two pathologists (A.K. and J.A.) independently conducted immunohistochemical evaluation. Disagreement between pathologists was resolved by a joint review to obtain a single consensus.

Recurrence was defined as clinically confirmed post-operative lymph node metastasis. The date of recurrence was defined as the date of pathological diagnosis of lymph node metastasis. This study complies with the institutional guidelines on human experimentation by the Ethical Committee of our institution [approval #413].

Immunohistochemical (IHC) staining. We conducted IHC using paraffin-embedded sections. IHC was performed using antibodies as follows: anti-PD-L1 (1:200; clone E1L3N; Cell Signaling Technology, Denver, MA), anti-PD-L2 (1:200; clone 176611; R&D Systems, Minneapolis, MN), and anti-CD8 (1:200; clone 4B11; Leica Microsystems, Newcastle, UK). IHC was performed using a Leica Bond-III staining instrument (Leica Microsystems). Tonsil epithelium, alveolar macrophages, and lymph nodes were used as positive controls for PD-L1, PD-L2, and CD8, respectively. Vascular endothelium was used as a negative control. The expression of PD-L1 and PD-L2 in tumor cells was evaluated following previous reports, including our report (8, 12). Based on these reports, cut-off values for PD-L1 and PD-L2 were set as 1% and 50%, respectively. In brief, PD-L1 positivity was defined as PD-L1 expression in more than 1% of all tumor cells. PD-L2 positivity was defined as PD-L2 expression in more than 50% of all tumor cells. $CD8^+$ tumor-infiltrating lymphocytes (TILs) were evaluated based on previous reports (16). To investigate lymphocyte infiltration of the stroma, $CD8^+$ TILs were counted in five high-power fields of view (magnification: ×400) and the average number of cells per field of view was assessed. The median number of $CD8^+$ TILs was 14.8 per high-power field of view (range=1.5-27.7). Cases with more $CD8^+$ TILs than the median were collectively defined as having a high $CD8^+$ TIL count, whereas cases with fewer $CD8^+$ TILs than the median were defined having a low $CD8^+$ TIL count.

Statistical analyses. Correlations between the number of $CD8^+$ TILs in invasive and non-invasive regions in the same case were analyzed using Pearson's correlation coefficients. The t-test and paired t-test were used to compare the average of continuous variables, and chi-square test or the Fisher's exact probability test were used to compare the proportions of categorical variables in PD-L1 and PD-L2 expression, and $CD8^+$ TILs. The survival of patients was estimated based on the Kaplan-Meier method and their differences were evaluated using the log-rank test for disease-free survival. Clinical and pathological variables were subjected to univariate analysis using a Cox proportional hazard model. All tests were two-sided and a p-value of less than 0.05 indicated a statistically significant difference. Statistical analyses were performed using JMP software version 13 (SAS Institute, Cary, NC, USA) and R software version 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria).

Table I. *Patient characteristics*.

Clinicopathological factor	Value (n=47)
Age, years	
Mean±SD	73.4±10.5
Gender, n	
Male/female	22/25
Time to diagnosis, months	
Median (range)	12 (1-120)
Tumor site, n (%)	
Vulva/scrotum/penis	42 (89.4)
Anus	4 (8.5)
Axillary	1 (2.1)
Mass formation, n (%)	
Yes	5 (10.6)
Serum CEA, n (%)	
≥5 ng/ml	2 (4.3)
Serum CA19-9, n (%)	
≥37 ng/ml	2 (4.3)
Surgical margin, n (%)	
Positive	12 (25.5)
Lymphatic invasion, n (%)	
Yes	3 (6.4)
Preoperative lymph node metastasis, n (%)	
Yes	2 (4.3)
Invasion to dermis, n (%)	
Present	14 (29.8)
Absent	33 (70.2)
Recurrence, n (%)	
Yes	7 (14.9)
Follow-up period, days	
Median (range)	1,154 (202-2,603)
Death, n (%)	
Yes	2 (4.3)

Results

Patient characteristics. Patient characteristics are summarized in Table I. The mean age of patients at the time of first visit was 73.4±10.5 years, and the sex ratio was 22:25. The median period to diagnosis was 12 months (range=1-120 months). Lesions were located in the vulva/scrotum/penis in most cases (42/47, 89.4%). There were five cases (10.6%) with mass formation, and two cases (4.3%) with elevated serum carcinoembryonic antigen and cancer antigen-19-9 levels. Positive surgical margins were identified in 12 cases (25.5%), lymphatic invasion in 3 cases (6.4%), and preoperative lymph node metastasis in 2 cases (4.3%). Invasive carcinoma was present in only 14 cases (29.8%). Recurrence was found postoperatively in 7 cases (14.9%), and mortality from primary disease occurred in two cases (4.3%). The median follow-up period was 1,154 days (range=202-2,603 days).

IHC findings. The staining patterns of PD-L1/PD-L2 and $CD8^+$ TILs are shown in Figure 1. Cells expressing PD-L1 and PD-L2 in the cell membrane, cytoplasm, or both were

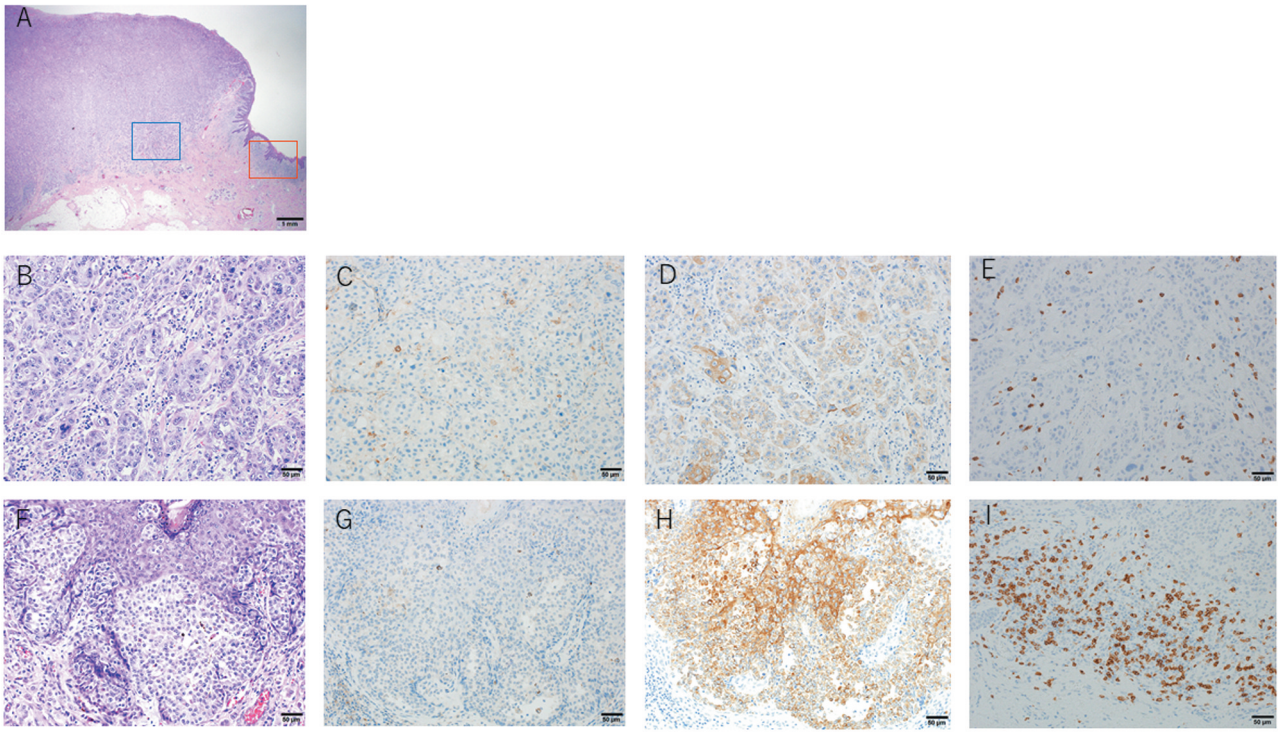

Figure 1. *Representative microphotographs of morphological findings and immunohistochemistry for programmed death-ligand 1 (PD-L1)/programmed death-ligand 2 (PD-L2) and CD8 in in situ and invasive extramammary Paget's disease. A: Low magnification view showing invasion (blue) and in situ (red) regions. Morphological findings (B, F) and immunohistochemical findings for PD-L1 (C, G), PD-L2 (D, H) and CD8 (E, I) in invasive (upper panel) and in situ (lower panel) region.*

defined as positive. While PD-L1 did not show any specific patterns of expression, a number of tumors demonstrated positive PD-L2 expression in deeper regions of the tumor.

PD-L1 was expressed in 13 cases (27.7%) in total and was observed in seven cases (14.5%) of carcinoma *in situ*. Of 14 invasive carcinoma cases, expression of PD-L1 was detected in both the non-invasive regions and invasive regions of six cases (42.9%). In the remaining eight cases, no expression was observed in either of these regions. The expression of PD-L1 was evenly distributed between the non-invasive and invasive areas in the cases with PD-L1-positive invasive carcinoma.

PD-L2 was expressed in 21 cases (44.7%). PD-L2 expression was observed in 12 cases (25.5%) of carcinoma *in situ*. Similar to PD-L1, PD-L2 was expressed in both the non-invasive and invasive regions of nine out of 14 invasive carcinoma cases (64.3%). In the remaining five cases, no expression was observed at all. PD-L2 expression was also evenly distributed between the non-invasive and invasive regions in PD-L2-positive invasive carcinoma cases. The number of $CD8^+$ TILs was significantly corelated in invasive and non-invasive regions of invasive carcinoma when compared within the same case (Figure 2A, r=0.671, p=0.009). Moreover, the number of $CD8^+$ TILs was significantly less in invasive than in non-invasive regions of invasive carcinoma (Figure 2B, p<0.001). The numbers of $CD8^+$ TILs were comparable between non-invasive regions of invasive carcinoma and carcinoma *in situ*. No significant difference was observed in the number of $CD8^+$ TILs between carcinoma *in situ* and non-invasive regions of invasive carcinoma cases.

PD-L1/PD-L2 in tumor cells and clinicopathological correlations. There were no significant differences between the PD-L1-positive and PD-L1-negative groups with respect to clinicopathological factors patient age, sex, period to diagnosis, preoperative lymph node metastasis, surgical margin positive, lymphatic invasion, and presence of invasion to the dermis. In addition, there was no significant difference in PD-L1 expression and number of $CD8^+$ TILs.

There were also no significant differences in clinicopathological factors between the PD-L2-positive and PD-L2-negative groups. In addition, there was no significant difference in PD-L2 and $CD8^+$ TIL count (Table II).

Correlations between PD-L1/PD-L2/CD8 TIL count and patient prognosis. The PD-L1-positive group had a shorter recurrence-free survival than the PD-L1-negative group (Figure 3A, log-rank test: p<0.001). The PD-L2-positive group also exhibited a shorter recurrence-free survival period compared

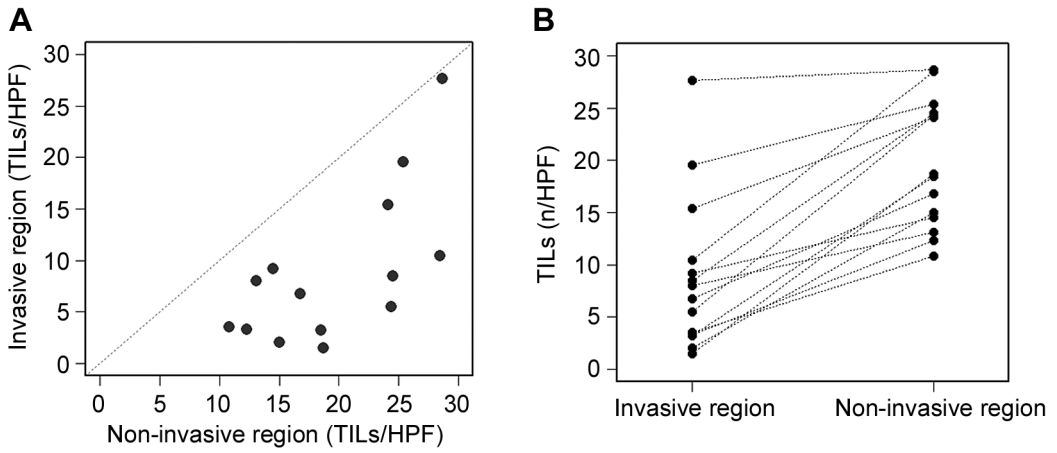

Figure 2. *Correlation of the number of CD8⁺ tumor-infiltrating lymphocytes (TILs) in invasive and non-invasive regions of invasive carcinoma. A: The number of CD8⁺ TILs in invasive and non-invasive regions was significantly positively correlated when compared within the same case (r=0.671, p=0.009). B: The number of CD8⁺ TILs was significantly less in invasive than in non-invasive regions of invasive carcinoma (p<0.001).*

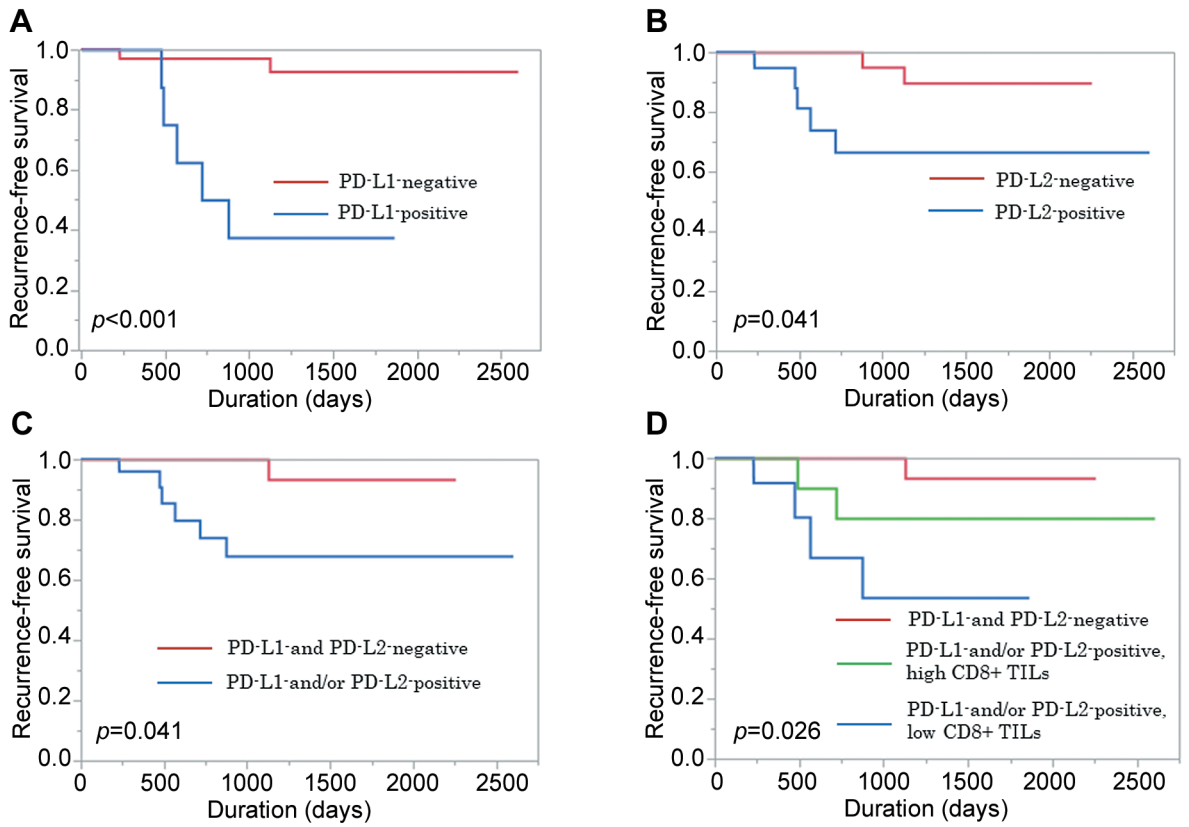

Figure 3. *Kaplan–Meier analysis of recurrence-free survival of patients with extramammary Paget's disease. A: Comparison of prognosis between cases with programmed death ligand 1 (PD-L1-)-positive and PD-L1-negative disease. The PD-L1-positive group had a shorter recurrence-free survival than the PD-L1-negative group (p<0.001). B: Comparison of prognosis between PD-L2-positive and PD-L2-negative cases. The PD-L2-positive group also exhibited a shorter recurrence-free survival period compared with the PD-L2-negative group (p=0.041). C: Comparison of prognosis between cases positive for PD-L1 and/or PD-L2 and cases negative for PD-L1 and PD-L2. The PD-L1/PD-L2-positive group had a shorter recurrence-free survival than the group negative for both PD-L1 and PD-L2 (p=0.041). D: Comparison of prognosis according to PD-L1 and PD-L2 status and the number of CD8⁺ tumor-infiltrating lymphocytes (TILs). Those positive for PD-L1 and/or PD-L2 with a low CD8⁺ TIL count exhibited shorter recurrence-free survival than those with a high CD8⁺ TIL count, and those negative for both PD-L1 and PD-L2 (p=0.026).*

Table II. *Relationship between the expression programed death ligand-1 (PD-L1)/programmed death ligand-2 (PD-L2) and various factors.*

Factor	Subgroup	PD-L1			PD-L2		
		Positive (n=13)	Negative (n=34)	*p*-Value	Positive (n=21)	Negative (n=26)	*p*-Value
Age, years	Mean±SD	72.3±12.8	73.8±9.7	0.325	75.1±2.3	75.1±2.1	0.844
Gender	Male	6	16	>0.999	8	14	0.381
	Female	7	18		13	12	
Time to diagnosis (n=46)*	≥12 months	7	11	0.504	7	11	0.543
Tumor site, n	Vulva/scrotum/penis	13	29	0.303	18	24	0.644
	Anus	0	4	0.564	2	2	>0.999
	Axillary	0	1	>0.999	1	0	0.447
Mass formation, n	Yes	3	2	0.121	3	2	0.644
Serum CEA, n	≥5 ng/ml	0	2	>0.999	1	1	>0.999
Serum CA19-9, n	≥37 ng/ml	0	2	>0.999	1	1	>0.999
Surgical margin, n	Positive	3	9	>0.999	7	5	0.326
Lymphatic invasion, n	Yes	2	1	0.181	1	2	>0.999
Preoperative lymph node metastasis, n	Yes	1	1	0.481	0	2	0.495
Invasion to dermis, n	Present	6	8	0.163	9	5	0.112
	Absent	7	26		12	21	
PD-L1, n	Positive				12	14	0.520
	Negative				9	12	
PD-L2, n	Positive	7	14	0.520			
	Negative	6	20				
$CD8^+$ TIL count, n	High	4	22	0.052	12	14	>0.999
	Low	9	12		9	12	

CEA: Carcinoembryonic antigen; CA19-9: cancer antigen-19-9; TIL: tumor-infiltrating lymphocyte. *Data not available in one case.

Table III. *Univariate analysis of clinicopathological factors associated with recurrence-free survival.*

Characteristic		HR (95% CI)	*p*-Value
Age	≥75 *vs.* 75 Years	2.054 (0.456 to 9.250)	0.349
Gender	Male *vs.* female	0.554 (0.107 to 2.861)	0.481
Time to diagnosis	≥12 *vs.* <12 Months	0.662 (0.128 to 3.418)	0.623
Mass formation	Yes *vs.* no	4.551 (0.861 to 24.055)	0.075
Surgical margin positive	Yes *vs.* no	5.772 (0.672 to 49.593)	0.110
Presence of lymphatic invasion	Yes *vs.* no	6.290 (1.214 to 32.587)	0.028
Preoperative lymph node metastasis	Yes *vs.* no	5.772 (0.672 to 49.593)	0.110
Presence of invasion to dermis	Yes *vs.* no	20.964 (2.503 to 175.558)	0.005
PD-L1	Positive *vs.* negative	3.982 (0.884 to 17.938)	0.072
PD-L2	Positive *vs.* negative	4.773 (0.918 to 24.816)	0.063
PD-L1/PD-L2 expression	One or both positive *vs.* both negative	6.756 (0.809 to 56.397)	0.078

CI: Confidence intervaI; HR: hazard ratio; PD-L1/PD-L2: programmed death ligand-1/-2.

with the PD-L2-negative group (Figure 3B, log-rank test: $p=0.041$). Together, the group positive for PD-L1 and/or PD-L2 had a shorter recurrence-free survival than the PD-L1- and PD-L2-negative group (Figure 3C, log-rank test: $p=0.041$). The group positive for PD-L1 and/or PD-L2 with a low $CD8^+$ TIL count exhibited shorter recurrence-free survival periods than the corresponding group with a high $CD8^+$ TIL count, and the PD-L1- and PD-L2-negative group (Figure 3D, log-rank test: $p=0.026$). In the univariate analysis of recurrence-free survival, the presence of invasion to the dermis and lymphatic invasion were extracted as significant factors; although there was no statistically significant difference in recurrence, recurrence tended to be associated with PD-L1-positive, PD-L2 positive, and PD-L1- and/or PD-L2-positive status (Table III).

Discussion

We evaluated the expression of PD-L1, PD-L2, and $CD8^+$ TILs in patients with EMPD using IHC. Our data revealed that the expression of PD-L1 and PD-L2 in tumor cells increased with

invasion, serving as a poor prognostic factor. $CD8^+$ TILs were further shown to significantly decrease with infiltration. PD-L1 and PD-L2 status, as well as a low $CD8^+$ TIL count were found to be associated with shorter recurrence-free survival.

The expression of PD-L1/PD-L2 in tumor cells differs depending on the type of carcinoma. PD-L1 and PD-L2 are also expressed in non-small cell lung cancer, malignant melanoma, and renal cell carcinoma, and it has been reported that PD-L2 is more widely expressed in gastric cancer and head and neck squamous cell carcinoma than PD-L1 (17). In this study, we observed expression of both PD-L1 and PD-L2. Umezu *et al*. reported that blocking both PD-L1 and PD-L2 may enhance the activity of antitumor immunity compared with blocking PD-L1 alone. The reason for this is that inhibition of PD-L1 induces PD-L2 expression on tumor-associated macrophages, and the function of PD-L2 is enhanced (18). It has been suggested that simultaneous inhibition of PD-L1 and PD-L2 is necessary to activate antitumor immunity, which would be particularly important in cases of EMPD expressing both PD-L1 and PD-L2.

There have been several reports regarding PD-L1 and PD-L2 expression in EMPD. Karpathiou *et al*. (19) reported that no PD-L1 expression was observed in any of the cases described in their study, while Duverger *et al*. (20) and Mauzo *et al*. (21) reported expression in 57.1% and 14.2% of EMPD cases, respectively. Although there are fewer reports of PD-L2 expression, Pourmaleki *et al*. (22) reported no expression in all of their cases. The difference in the positivity rate may be related to the cut-off value provided, differences in antibody clones, and the ratio of invasive carcinoma among the cases. Pourmaleki *et al*. also compared the expression levels of PD-L1/PD-L2 in invasive and non-invasive regions in the same case and reported a strong correlation between them (22). Our findings were largely consistent with these results. Stated differently, in the context of EMPD, it may be possible to extract cases at high risk of developing invasive carcinoma by examining PD-L1 expression in biopsy samples obtained from non-invasive portions. However, an additional study using a large cohort should be conducted to further accumulate case data. In various cancer types, the expression of PD-L1 in tumor cells and the number of $CD8^+$ TILs were shown to be positively correlated (20, 23-27). $CD8^+$ TILs that infiltrate the stroma are believed to be one factor associated with a favorable prognosis (28-31). In this study, cases with PD-L1 and/or PD-L2, and a low $CD8^+$ TIL count had poor prognoses. Additionally, up-regulation of PD-L1/PD-L2 expression was observed along with invasion of tumor cells in this study. We believe that a dramatic change in the immune microenvironment occurred in parallel. As PD-L1/PD-L2 expression acts to suppress tumor immunity, tumor cells that express PD-L1/PD-L2 thereby evade the immune system, and we hypothesize that the tumor progresses due to suppression of the activation of $CD8^+$ TILs.

By contrast, Iga *et al*. reported that in EMPD, $CD8^+$ TILs in the tumor stroma were predominant in cases with poor prognosis (15). Duverger *et al*. reported that $CD8^+$ TILs had no prognostic association with respect to cutaneous adnexal tumors, including EMPD (20). Thus, findings regarding $CD8^+$ TILs in cases of EMPD are inconsistent. The report by Iga *et al*. offered a detailed phenotype analysis of $CD8^+$ TILs. Infiltrate $CD8^+$ TILs exhibit an exhausted phenotype, and although these cells are present in the tumor stroma, they are hypothesized to have no effective effect on antitumor immunity. Additionally, the proportion of cases with PD-L1-positive tumor cells in their study was similar to ours, despite the inclusion of more invasive carcinoma cases than our study. In our study, since the expression of PD-L1 was more evident in invasive carcinoma cases, it is possible that changes in the immune microenvironment due to PD-L1-positive carcinoma cells are involved in the degree of $CD8^+$ TIL infiltration.

The use of ICIs may be limited to cases where surgery is not curative and the disease is progressive. In our study, while expression of PD-L1/PD-L2 was observed primarily in the tumor cells located in the invasive regions, almost no $CD8^+$ TILs were detected in the tumor stroma. The presence of stromal immune cells as well as PD-L1/PD-L2 expression on tumor cells is essential in determining the proper application of ICIs (32). In our study, the immune environment in the invasive portions of EMPD cases corresponds to so-called 'Desert or cold' tumors, on which ICIs can be expected to have a negligible effect. The effective use of ICIs requires activation of the immune microenvironment to a 'hot' or inflamed state (33). EMPD has also been described as having an immunophenotype similar to that of breast cancer (34). In breast cancer, it has been reported that interleukin-17A and matrix metalloproteinase-9 antibodies alter the tumor microenvironment to a 'hot' state, and even in cases of EMPD, it may be necessary to employ these immune-activators together with ICIs for more effective therapeutic activity (35, 36).

In addition, tumors with PD-L1 expression have been reported in various types of carcinomas with highly malignant potential, which may be one of the reasons for poor prognosis in PD-L1/PD-L2-positive cases (37). Although there is currently no histological classification for EMPD, it may be possible that cases with high malignant potential may be identified using PD-L1/PD-L2 expression.

Our study has several limitations. Firstly, we used a retrospective cohort involving a relatively small number of patients from a single facility. Secondly, it was not possible to conduct a multivariate analysis due to the rarity of EMPD. Finally, with only two patients succumbing to their disease, it is difficult to correlate the current findings with precise outcomes. Therefore, future investigations and validations using prospective studies with a larger patient sample size are required.

In conclusion, the expression of PD-L1/PD-L2 was shown to be a prognostic factor in EMPD. Furthermore, the invasion of tumor cells appears to correlate with a dramatic change in the immune microenvironment, such as a reduction in the number of $CD8^+$ TILs, which may result in the formation of a suppressive immune microenvironment and a corresponding poor prognosis. The expression of PD-L1/PD-L2 can also vary by case, and consideration of $CD8^+$ TIL infiltration as well as PD-L1/PD-L2 expression when using an ICI may be valuable for the selection of cases in which treatment can be expected to be most effective.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

Authors' Contributions

AK, JA, RK, SO, YN, HK, SS, TN and HY designed this study. AK drafted the article, and JA, TN and HY edited the article. AK and IM acquired and collected the data. ES performed the statistical analysis and drew the figures.

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References

1 Patterson JW and Weedon D: Weedon's Skin Pathology. Fourth Edition. Churchill Livingstone/Elsevier, 2016.

2 Chang K, Li GX, Kong YY, Shen XX, Qu YY, Jia ZW, Wang Y, Dai B and Ye DW: Chemokine receptors CXCR4 and CXCR7 are associated with tumor aggressiveness and prognosis in extramammary Paget disease. J Cancer *8(13)*: 2471-2477, 2017. PMID: 28900484. DOI: 10.7150/jca.19127

3 Wagner G and Sachse MM: Extramammary paget disease - clinical appearance, pathogenesis, management. J Dtsch Dermatol Ges *9(6)*: 448-454, 2011. PMID: 21205169. DOI: 10.1111/j.1610-0387.2010.07581.x

4 Ito T, Kaku Y, Nagae K, Nakano-Nakamura M, Nakahara T, Oda Y, Hagihara A, Furue M and Uchi H: Tumor thickness as a prognostic factor in extramammary Paget's disease. J Dermatol *42(3)*: 269-275, 2015. PMID: 557434. DOI: 10.1111/1346-8138.12764

5 Ohara K, Fujisawa Y, Yoshino K, Kiyohara Y, Kadono T, Murata Y, Uhara H, Hatta N, Uchi H, Matsushita S, Takenouchi T, Hayashi T, Yoshimura K and Fujimoto M: A proposal for a TNM staging system for extramammary Paget disease: Retrospective analysis of 301 patients with invasive primary tumors. J Dermatol Sci *83(3)*: 234-239, 2016. PMID: 27329007. DOI: 10.1016/j.jdermsci.2016.06.004

6 Marmarelis ME, Davis MR, Sethi NS, Krajewksi KM, McKay RR, Choueiri TK and Ott PA: Tumor control with PD1 inhibition in a patient with concurrent metastatic melanoma and renal cell carcinoma. J Immunother Cancer *4:* 26, 2016. PMID: 27099755. DOI: 10.1186/s40425-016-0129-x

7 Queirolo P and Spagnolo F: Atypical responses in patients with advanced melanoma, lung cancer, renal-cell carcinoma and other solid tumors treated with anti-PD1 drugs: A systematic review. Cancer Treat Rev *59:* 71-78, 2017. PMID: 28756306. DOI: 10.1016/j.ctrv.2017.07.002

8 Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, Patnaik A, Aggarwal C, Gubens M, Horn L, Carcereny E, Ahn MJ, Felip E, Lee JS, Hellmann MD, Hamid O, Goldman JW, Soria JC, Dolled-Filhart M, Rutledge RZ, Zhang J, Lunceford JK, Rangwala R, Lubiniecki GM, Roach C, Emancipator K and Gandhi L: Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med *372(21)*: 2018-2028, 2015. PMID: 25891174. DOI: 10.1056/NEJMoa1501824

9 Herbst RS, Baas P, Kim DW, Felip E, Perez-Gracia JL, Han JY, Molina J, Kim JH, Arvis CD, Ahn MJ, Majem M, Fidler MJ, de Castro G, Jr., Garrido M, Lubiniecki GM, Shentu Y, Im E, Dolled-Filhart M and Garon EB: Pembrolizumab *versus* docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): A randomised controlled trial. Lancet *387(10027)*: 1540-1550, 2016. PMID: 26712084. DOI: 10.1016/s0140-6736(15)01281-7

10 Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, Gottfried M, Peled N, Tafreshi A, Cuffe S, O'Brien M, Rao S, Hotta K, Leiby MA, Lubiniecki GM, Shentu Y, Rangwala R and Brahmer JR: Pembrolizumab *versus* chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med *375(19)*: 1823-1833, 2016. PMID: 27718847. DOI: 10.1056/NEJMoa1606774

11 Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, Okazaki T and Tokura Y: Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. Cancer *116(7)*: 1757-1766, 2010. PMID: 20143437. DOI: 10.1002/cncr.24899

12 Sato F, Akiba J, Kawahara A, Naito Y, Ono T, Takase Y, Murata K, Abe H, Yamaguchi T, Miyoshi H, Abe Y, Mihara Y, Tanikawa M, Akashi M, Kurose H, Umeno H and Yano H: The expression of programed death ligand-1 could be related with unfavorable prognosis in salivary duct carcinoma. J Oral Pathol Med *47(7)*: 683-690, 2018. PMID: 29719073. DOI: 10.1111/jop.12722

13 Kondo R, Akiba J, Ogasawara S, Nakashima O, Naito Y, Kusano H, Mihara Y, Tanigawa M and Yano H: Programmed death-ligand 1 expression is an unfavorable prognostic factor of hepatocellular carcinoma after archiving sustained virologic response for hepatitis C virus infection. Oncol Lett *18(2)*: 1458-1466, 2019. PMID: 31423211. DOI: 10.3892/ol.2019.10448

14 Sato F, Ono T, Kawahara A, Kawaguchi T, Tanaka H, Shimamatsu K, Kakuma T, Akiba J, Umeno H and Yano H: Prognostic impact of p16 and PD-L1 expression in patients with oropharyngeal squamous cell carcinoma receiving a definitive treatment. J Clin Pathol *72(8)*: 542-549, 2019. PMID: 31113825. DOI: 10.1136/jclinpath-2019-205818

15 Iga N, Otsuka A, Yamamoto Y, Nakashima C, Honda T, Kitoh A, Nakajima S, Egawa G, Nomura T, Dainichi T, Matsushita S, Tanizaki H, Yamamoto Y, Funakoshi T, Fujisawa Y, Fujimura T, Hata H, Ishida Y and Kabashima K: Accumulation of exhausted $CD8^+$ T cells in extramammary Paget's disease. PLoS One *14(1)*: e0211135, 2019. PMID: 30682105. DOI: 10.1371/journal.pone.0211135

16 Dieci MV, Radosevic-Robin N, Fineberg S, van den Eynden G, Ternes N, Penault-Llorca F, Pruneri G, D'Alfonso TM, Demaria

S, Castaneda C, Sanchez J, Badve S, Michiels S, Bossuyt V, Rojo F, Singh B, Nielsen T, Viale G, Kim SR, Hewitt S, Wienert S, Loibl S, Rimm D, Symmans F, Denkert C, Adams S, Loi S and Salgado R: Update on tumor-infiltrating lymphocytes (TILs) in breast cancer, including recommendations to assess tils in residual disease after neoadjuvant therapy and in carcinoma *in situ*: A report of the international immuno-oncology biomarker working group on breast cancer. Semin Cancer Biol *52(Pt 2)*: 16-25, 2018. PMID: 29024776. DOI: 10.1016/j.semcancer.2017.10.003

17 Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, Lunceford J, Cheng J, Chow LQM, Seiwert TY, Handa M, Tomassini JE and McClanahan T: PD-L2 expression in human tumors: Relevance to anti-PD1 therapy in cancer. Clin Cancer Res *23(12)*: 3158-3167, 2017. PMID: 28619999. DOI: 10.1158/1078-0432.Ccr-16-1761

18 Umezu D, Okada N, Sakoda Y, Adachi K, Ojima T, Yamaue H, Eto M and Tamada K: Inhibitory functions of PD-L1 and PD-L2 in the regulation of anti-tumor immunity in murine tumor microenvironment. Cancer Immunol Immunother *68(2)*: 201-211, 2019. PMID: 30357491. DOI: 10.1007/s00262-018-2263-4

19 Karpathiou G, Chauleur C, Hathroubi S, Habougit C and Peoc'h M: Expression of CD3, PD-L1 and CTLA-4 in mammary and extra-mammary Paget disease. Cancer Immunol Immunother *67(8)*: 1297-1303, 2018. PMID: 29943071. DOI: 10.1007/s00262-018-2189-x

20 Duverger L, Osio A, Cribier B, Mortier L, De Masson A, Basset-Seguin N, Lebbe C and Battistella M: Heterogeneity of PD-L1 expression and CD8 tumor-infiltrating lymphocytes among subtypes of cutaneous adnexal carcinomas. Cancer Immunol Immunother *68(6)*: 951-960, 2019. PMID: 30953116. DOI: 10.1007/s00262-019-02334-8

21 Mauzo SH, Tetzlaff MT, Milton DR, Siroy AE, Nagarajan P, Torres-Cabala CA, Ivan D, Curry JL, Hudgens CW, Wargo JA, Sahin AA, Pettaway CA, Prieto VG and Aung PP: Expression of PD1 and PD-L1 in extramammary Paget disease: Implications for immune-targeted therapy. Cancers *11(6)*, 2019. PMID: 31146499. DOI: 10.3390/cancers11060754

22 Pourmaleki M, Young JH, Socci ND, Chiang S, Edelweiss M, Li Y, Zhang M, Roshal L, Chi DS, Busam KJ, Mellinghoff IK and Hollmann TJ: Extramammary Paget disease shows differential expression of B7 family members B7-H3, B7-H4, PD-L1, PD-L2 and cancer/testis antigens NY-ESO-1 and MAGE-A. Oncotarget *10(58)*: 6152-6167, 2019. PMID: 31692889. DOI: 10.18632/oncotarget.27247

23 Knol AC, Nguyen JM, Pandolfino MC, Denis MG, Khammari A and Dreno B: Pd-l1 expression by tumor cell lines: A predictive marker in melanoma. Exp Dermatol *27(6)*: 647-655, 2018. PMID: 29505109. DOI: 10.1111/exd.13526

24 Kim H, Kwon HJ, Park SY, Park Y, Park E and Chung JH: Clinicopathological analysis and prognostic significance of programmed cell death-ligand 1 protein and mRNA expression in non-small cell lung cancer. PLoS One *13(6)*: e0198634, 2018. PMID: 29856861. DOI: 10.1371/journal.pone.0198634

25 Li X, Li M, Lian Z, Zhu H, Kong L, Wang P and Yu J: Prognostic role of programmed death ligand-1 expression in breast cancer: A systematic review and meta-analysis. Target Oncol *11(6)*: 753-761, 2016. PMID: 27422273. DOI: 10.1007/s11523-016-0451-8

26 Garcia-Diez I, Hernandez-Ruiz E, Andrades E, Gimeno J, Ferrandiz-Pulido C, Yebenes M, Garcia-Patos V, Pujol RM, Hernandez-Munoz I and Toll A: Pd-l1 expression is increased in metastasizing squamous cell carcinomas and their metastases. Am J Dermatopathol *40(9)*: 647-654, 2018. PMID: 29742559. DOI: 10.1097/dad.0000000000001164

27 Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Luber BS, Wang H, Xu H, Nayar SK, Wang TS, Sidransky D, Anders RA, Topalian SL and Taube JM: Pd-l1 expression in the Merkel cell carcinoma microenvironment: Association with inflammation, Merkel cell polyomavirus and overall survival. Cancer Immunol Res *1(1)*: 54-63, 2013. PMID: 24416729. DOI: 10.1158/2326-6066.Cir-13-0034

28 Stanton SE, Adams S and Disis ML: Variation in the incidence and magnitude of tumor-infiltrating lymphocytes in breast cancer subtypes: A systematic review. JAMA Oncol *2(10)*: 1354-1360, 2016. PMID: 27355489. DOI: 10.1001/jamaoncol.2016.1061

29 Santoiemma PP and Powell DJ, Jr.: Tumor infiltrating lymphocytes in ovarian cancer. Cancer Biol Ther *16(6)*: 807-820, 2015. PMID: 25894333. DOI: 10.1080/15384047.2015.1040960

30 Behr DS, Peitsch WK, Hametner C, Lasitschka F, Houben R, Schonhaar K, Michel J, Dollt C, Goebeler M, Marx A, Goerdt S and Schmieder A: Prognostic value of immune cell infiltration, tertiary lymphoid structures and PD-L1 expression in Merkel cell carcinomas. Int J Clin Exp Pathol *7(11)*: 7610-7621, 2014. PMID: 25550797.

31 El Sissy C, Marliot F, Haicheur N, Kirilovsky A, Scripcariu D, Lagorce-Pages C, Galon J and Pages F: Focus on the immunoscore and its potential clinical implications. Ann Pathol *37(1)*: 29-38, 2017. PMID: 28161000. DOI: 10.1016/j.annpat.2016.12.010

32 Ribas A and Hu-Lieskovan S: What does PD-L1 positive or negative mean? J Exp Med *213(13)*: 2835-2840, 2016. PMID: 27903604. DOI: 10.1084/jem.20161462

33 Mullard A: Can innate immune system targets turn up the heat on 'cold' tumours? Nat Rev Drug Discov *17(1)*: 3-5, 2018. PMID: 29282375. DOI: 10.1038/nrd.2017.264

34 Tessier-Cloutier B, Asleh-Aburaya K, Shah V, McCluggage WG, Tinker A and Gilks CB: Molecular subtyping of mammary-like adenocarcinoma of the vulva shows molecular similarity to breast carcinomas. Histopathology *71(3)*: 446-452, 2017. PMID: 28418164. DOI: 10.1111/his.13239

35 Ma YF, Chen C, Li D, Liu M, Lv ZW, Ji Y and Xu J: Targeting of interleukin (il)-17a inhibits pdl1 expression in tumor cells and induces anticancer immunity in an estrogen receptor-negative murine model of breast cancer. Oncotarget *8(5)*: 7614-7624, 2017. PMID: 27935862. DOI: 10.18632/oncotarget.13819

36 Juric V, O'Sullivan C, Stefanutti E, Kovalenko M, Greenstein A, Barry-Hamilton V, Mikaelian I, Degenhardt J, Yue P, Smith V and Mikels-Vigdal A: MMP-9 inhibition promotes anti-tumor immunity through disruption of biochemical and physical barriers to T-cell trafficking to tumors. PLoS One *13(11)*: e0207255, 2018. PMID: 30500835. DOI: 10.1371/journal.pone.0207255

37 Mo Z, Liu J, Zhang Q, Chen Z, Mei J, Liu L, Yang S, Li H, Zhou L and You Z: Expression of PD1, PD-L1 and PD-L2 is associated with differentiation status and histological type of endometrial cancer. Oncol Lett *12(2)*: 944-950, 2016. PMID: 27446374. DOI: 10.3892/ol.2016.4744

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(This text is a combination of advice and suggestions contributed by Editors, Authors, Readers and the Managing Editor of AR).