PD-L1 expression in malignant melanomas of the skin and gastrointestinal tract

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Received May 7, 2019; Accepted November 25, 2019

DOI: 10.3892/ol.2020.11325

Abstract. Gastrointestinal melanoma (GM) is a rare but aggressive type of malignant melanoma arising in the gastrointestinal tract. An anti-programmed cell death protein 1 (PD-1) antibody markedly improves prognosis in patients with melanoma. However, little is known regarding the expression of immune-oncology biomarkers in GM compared with skin melanoma (SM), especially in the Asian population. the present study examined clinicopathological characteristics, PD-L1 and HLA expression, and immune-oncology marker expression in 10 cases of GM and 31 cases of SM. Patients with GM exhibited significantly higher incidences of lymph node and distant metastases than patients with SM (P=0.0448 and P=0.0247, respectively). The infiltration of $CD8^+$ lymphocytes was significantly higher in GM than in SM (P=0.0231). The infiltration of $PD\text{-}1^+$ lymphocytes was higher in GM than in SM, but the difference was not significant (P=0.0975). PD-L1-positive melanoma exhibited a higher proportion of $BRAF^{V600E}$-positive melanoma than PD-L1-negative melanoma (P=0.0317; 39.4 and 0%, respectively). PD-L1-positive melanoma exhibited significantly higher rates of $CD8^+$ and $FOXp3^+$ lymphocyte infiltration than PD-L1-negative melanoma (P=0.0221 and P=0.0463, respectively). By contrast, $PD\text{-}1^+$ lymphocytes did not differ between PD-L1-positive and -negative cases. Furthermore, HLA-positive melanoma exhibited higher proportions of PD-1 (P=0.0101; 53.7 and 15.4%) and CD8 than HLA-negative melanoma (P=0.0818; 66.7 and 38.2%). These results provided useful information regarding tumor immunity in GM and SM and may contribute to the development of treatment strategies for GM.

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Key words: gastrointestinal melanoma, malignant melanoma, programmed death-ligand 1, programmed cell death protein 1, CD8, FOXp3, lymphocyte

Introduction

Melanoma is one of the most aggressive tumors (1,2); it is most commonly localized in the skin but can occur at any site where melanocytes exist (3). Gastrointestinal melanoma (GM) is a rare type of malignant melanoma arising in the gastrointestinal tract (4-6). We have previously reported that GM shows more aggressive features than those of skin melanoma (SM), such as a high mitotic rate and frequent metastases to lymph nodes and distant organs (7). A recently developed immune checkpoint inhibitor (ICI), the anti-programmed death 1 (PD-1) antibody nivolumab, has markedly improved patient prognosis in SM as compared to that observed with the conventional cytotoxic chemotherapeutic agent dacarbazine (8). However, predictive biomarkers for ICIs are needed owing to the potential for resistance and the high cost.

PD-1 is expressed on the surface of cytotoxic T cells, and its ligands programmed death ligand (PD-L) 1 and 2 are expressed on both tumor and immune cells (9). The inhibition of interactions between PD-1 and PD-L1/PD-L2 by an anti-PD-1 antibody causes the reactivation of cytotoxic T cells, leading to the recognition and destruction of melanoma cells (10). Diagnostic immunohistochemical assays of PD-L1 have been approved by the FDA (11), but research is ongoing to better understand the role of PD-L1 as an immune-oncology marker, both alone and in combination with other markers.

Major factors involved in tumor immunity include tumor antigens, inflammation, immune suppression, and host environment. Tumor antigens, which are fragments of DNA, RNA, and protein, are recognized as non-self by the host immune system (12). Inflamed tumors show immune cell activation, especially of $CD8^+$ cytotoxic T cells (13). Immune suppression is mainly regulated by Forkhead box protein 3 (FOXp3)-positive regulatory T cells (14). The host environment, including the microbiome, germline mutations, and human leukocyte antigen (HLA) phenotypes, modulates the immune response (15). Therefore, these factors have been reported as predictive biomarkers for ICI. However, little is

known about the expression of immune-oncology biomarkers in GM and SM, especially in the Asian population. In the present study, we investigated the clinicopathological characteristics associated with PD-L1 and HLA expression in tumor cells as well as the degree of tumor-infiltrating lymphocytes in GM and SM.

Materials and methods

Patients and tissues. Tissue samples [GM (n=10) and SM (n=31)] were obtained from patients who underwent surgical treatment at our hospital between 1997 and 2015 (7). This study was conducted in accordance with the principles in the Declaration of Helsinki (2008). Approval for the study was obtained from the human research ethics committees at the Tokyo Metropolitan Geriatric Hospital (No. R17-33) and the Nippon Medical School Hospital (no. 29-07-805).

Tissue processing and histological assessment. Tissues were fixed in formalin and subjected to standard processing and paraffin embedding. They were sliced into 3-μm-thick sections for hematoxylin and eosin (H&E) staining and immunohistochemical analyses. Diagnoses of pathological specimens were made by more than two pathologists based on the American Joint Committee on Cancer (AJCC, 2009) guidelines for SMs and the Union for International Cancer Control (UICC, the 7th edition) guidelines for GMs.

Immunohistochemistry and mitosis findings. Paraffin-embedded tissue sections were immunostained using Histofine Simple Stain MAX PO (Nichirei) kits. After deparaffinization, endogenous peroxidase activity was blocked by incubating sections with 0.3% hydrogen peroxide in methanol for 30 min. Sections were incubated for 1 h at room temperature with an anti-CD8 antibody (713201; Nichirei), anti-PD-1 antibody (diluted 1:100, clone NAT105; ab52587; Abcam), anti-FOXp3 antibody (diluted 1:200, ab22510; Abcam), anti-BRAF V600E antibody (diluted 1:50, E19290; Spring Bioscience), HLA-DR-DP-DQ-DX, major histocompatibility complex class-II in melanomas (16) (diluted 1:1000, sc-53302; Santa Cruz Biotechnology, Inc.), and anti-PD-L1 antibody (diluted 1:100, clone 28-8; ab205921; Abcam). Bound antibodies were detected using diaminobenzidine tetrahydrochloride as a chromogen.

An immunohistochemical review was performed separately by two of the authors (MA and YM), who were blinded to clinical and outcome data. To evaluate the immunostaining results, any tumor cell showing the expression of PD-L1, $BRAF^{V600E}$, or HLA was interpreted as positive. If none of the tumor cells expressed PD-L1, $BRAF^{V600E}$, or HLA, the sample was negative. For the evaluation of CD8, PD-1, and FOXp3, the number of positive lymphocytes in the tumor area was scored as follows: 0, negative; <25%; 1+, low; 25-50%; 2+, intermediate; and >50% 3+, high. Scores of 0 and 1 were low, and scores of 2 and 3 were high.

Statistical analysis. Clinicopathological features were analyzed using χ^2 tests and Student's t-tests. The level of significance was set to $P<0.05$ for all analyses. Statistical analyses were performed using StatViewJ version 5.0 (SAS Institute, Inc.).

Results

Comparison of SM and GM. The clinicopathological characteristics of patients with SM and GM are summarized in Table I. Consistent with our previous findings (7), patients with GM showed significantly higher proportions of lymph node and distant metastases than those of patients with SM (P=0.0448 and 0.0247, respectively).

SM and GM showed PD-L1 and HLA expression in tumor cells (Fig. 1). As compared to SM, GM showed a higher proportion of PD-L1-positive cases (77.4 and 90.0%, respectively, Table I) and a higher proportion of HLA-positive cases (32.3 and 50.0%, respectively, Table I).

GM showed a significantly greater degree of infiltration of $CD8^+$ lymphocytes than SM (P=0.0231, Table I and Fig. 2). As compared to SM, GM showed higher infiltration of PD-1^+ lymphocytes, but this difference was not significant (P=0.0975). FOXp3^+ lymphocytes did not differ between SM and GM.

Comparison of PD-L1-positive and -negative melanomas. We did not detect statistically significant differences between PD-L1-positive and -negative cases in SM or GM owing to the small sample sizes; therefore, we compared PD-L1-positive and -negative cases in both GM and SM. Patients with PD-L1-positive melanoma were younger than those with PD-L1-negative melanoma and were predominantly female (Table II). PD-L1-positive melanoma showed a higher proportion of $BRAF^{V600E}$ than that of PD-L1-negative melanoma (P=0.0317, 39.4 and 0%). PD-L1-positive melanomas showed significantly higher $CD8^+$ or FOXp3^+ lymphocyte infiltration than that of PD-L1-negative melanomas (P=0.0221 and P=0.0463, respectively). In contrast, PD-1^+ lymphocytes did not differ between PD-L1-positive and -negative cases.

Comparison of HLA-positive and -negative melanomas. We compared HLA-positive and -negative cases in both GM and SM. Patients with HLA-positive melanoma were older than patients with HLA-negative melanoma and were predominantly male (Table III). HLA-positive melanoma showed higher proportions of PD-1 (P=0.0101, 53.7 and 15.4%) and CD8 than those of HLA-negative melanoma (P=0.0818, 66.7 and 38.2%). In contrast, HLA$^+$ lymphocytes did not differ between FOXp3, $BRAF^{V600E}$, and PD-L1-positive and -negative cases.

Discussion

We characterized the expression of immune-oncology markers in SM and GM. Compared with SM, GM exhibited greater degrees of infiltration of $CD8^+$ and PD1-positive lymphocytes and higher levels of PD-L1 and HLA in melanoma cells. Furthermore, patients with PD-L1-positive melanoma were younger, female-predominant, and had a higher proportion of $BRAF^{V600E}$ positivity and a higher infiltration rate of $CD8^+$ or FOXp3^+ lymphocytes as compared to those of patients with PD-L1-negative melanomas. Patients with HLA-positive melanoma were older, male-predominant, and had higher infiltration of PD-1-positive lymphocytes as compared to

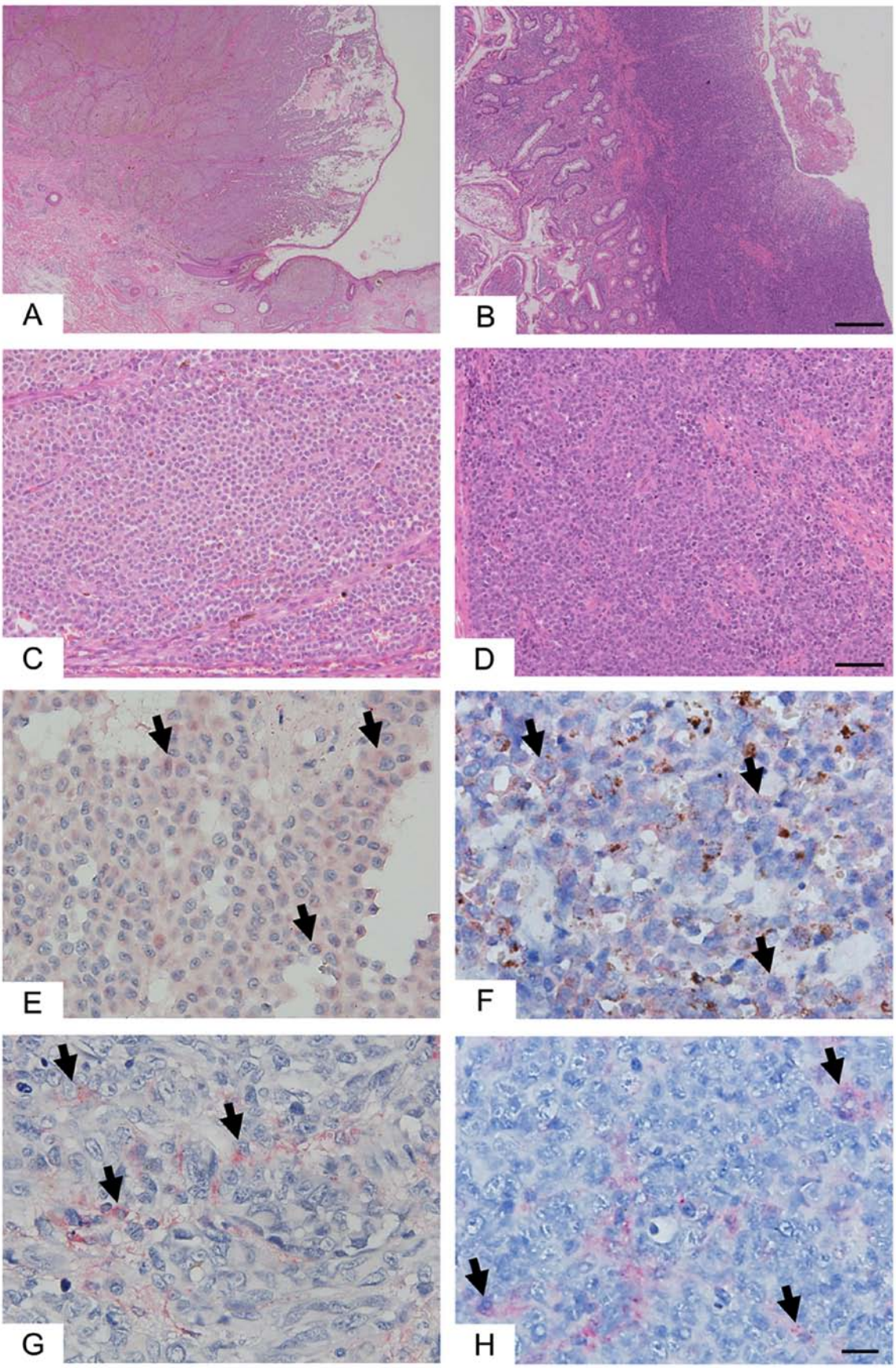

Figure 1. Histological findings and immunohistochemical staining for PD-L1 and HLA. (A) T4 SM tissue sample taken from the mandibular region. (B) Malignant melanoma of the rectum. Scale bar, 1 mm. (C) Magnified image of (A). Melanoma cells exhibiting hyperchromatic nuclei with melanin deposition in the cytoplasm. (D) Magnified image of (B). Hematoxylin and eosin (H&E) staining. Scale bar, 100 μm. (E) SM exhibited PD-L1 expression. (F) GM exhibited PD-L1 expression. Arrows indicate membranous and cytoplasmic PD-L1 expression in the tumor cells. (G) SM and (H) GM exhibited HLA expression. Arrows indicate membranous HLA expression in the tumor cells. Counterstaining, hematoxylin. (E-H) Scale bar, 25 μm. HLA, human leukocyte antigen; PD-L1, programmed death ligand 1; SM, skin melanoma; GM, gastrointestinal melanoma.

Table I. Clinicopathological characteristics of patients with melanoma of the skin and gastrointestinal tract.

Variable	Skin, n (%)	Gastrointestinal tract, n (%)	P-value
Age, years (mean ± SD)	66.7±16.8	75.7±14.9	0.1384
Sex			
Male	17 (54.8)	5 (50.0)	0.7896
Female	14 (45.2)	5 (50.0)	
Location			
Acral/CSD/mucosal/non-CSD	13/4/2/12 (41.9/12.9/6.5/38.7)		
Esophagus/rectum/anal canal/small intestine		1/4/4/1 (10.0/40.0/40.0/10.0)	
T-classification			
1	8 (25.8)	3 (30.0)	0.0747
2	11 (35.5)	0 0.0	
3	11 (35.5)	5 (50.0)	
4	1 (3.2)	2 (20.0)	
N-lymph node			
Negative	21 (67.7)	4 (40.0)	0.0448[a]
Positive	10 (32.3)	6 (60.0)	
M-metastasis			
Negative	30 (96.8)	8 (80.0)	0.0247[a]
Positive	1 (3.2)	2 (20.0)	
UICC stage			
I	8 (25.8)	3 (30.0)	0.0747
II	11 (35.5)	0 (0.0)	
III	11 (35.5)	5 (50.0)	
IV	1 (3.2)	2 (20.0)	
$BRAF^{V600E}$			
Positive	12 (38.7)	1 (10.0)	0.0898
Negative	19 (61.3)	9 (90.0)	
PD-L1			
Positive	24 (77.4)	9 (90.0)	0.3827
Negative	7 (22.6)	1 (10.0)	
HLA			
Positive	10 (32.3)	5 (50.0)	0.3111
Negative	21 (67.7)	5 (50.0)	
CD8(+) lymphocyte			
High	12 (25.8)	8 (60.0)	0.0231[a]
Low	19 (74.2)	2 (40.0)	
PD-1(+) lymphocyte			
High	7 (6.5)	5 (30.0)	0.0975
Low	24 (93.5)	5 (70.0)	
FOXp3(+) lymphocyte			
High	26 (32.3)	8 (22.2)	0.7105
Low	5 (67.7)	1 (77.8)	

[a]P<0.05, skin vs. gastrointestinal tract in χ^2 test. The number of FOXp3-GM cases was 9. The data are presented as the number of patients with percentages in the parentheses. The data for age are presented as the mean ± SD. CSD, chronic sun damaged; PD-1, programmed cell death protein 1; HLA, human leukocyte antigen; UICC, Union for International Cancer Control.

those of patients with HLA-negative melanoma. These results indicate that GM shows greater activation of tumor immunity than SM, and thus GM might exhibit a greater response to ICIs.

Table II. Clinicopathological characteristics of patients with PD-L1-positive melanoma.

Variables	PD-L1 positive, n (%)	PD-L1 negative, n (%)	P-value
Age, years (mean ± SD)	67.4±17.6	75.0±10.1	0.2520
Sex			
Male	16 (48.4)	6 (75.0)	0.1677
Female	17 (51.5)	2 (25.0)	
Location of lesion			
SM	24 (77.4)	7 (22.6)	0.3827
GM	9 (90.0)	1 (10.0)	
UICC stage			
I	8 (24.2)	3 (37.5)	0.7054
II	9 (27.3)	1 (12.5)	
III	14 (42.4)	3 (37.5)	
IV	2 (6.1)	1 (12.5)	
$BRAF^{V600E}$			
Positive	13 (39.4)	0 (0.0)	0.0317[a]
Negative	20 (60.6)	8 (100.0)	
HLA			
Positive	13 (39.4)	2 (25.0)	0.4483
Negative	20 (60.6)	6 (75.0)	
CD8(+) lymphocyte			
High	19 (57.6)	1 (12.5)	0.0221[a]
Low	14 (42.4)	7 (87.5)	
PD-1(+) lymphocyte			
High	10 (30.3)	2 (25.0)	0.7674
Low	23 (69.7)	6 (75.0)	
FOXp3(+) lymphocyte			
High	29 (90.6)	5 (62.5)	0.0463[a]
Low	3 (9.4)	3 (37.5)	

[a]P<0.05, PD-L1 positive vs. PD-L1 negative in χ^2 test. SM, skin melanoma; GM, gastrointestinal melanoma; PD-L1, programmed death ligand 1; PD-1, programmed cell death protein 1; HLA, human leukocyte antigen; UICC, Union for International Cancer Control.

Our results have several potential explanations. (1) GM cases represented a more advanced stage than that of SM cases owing to the difficulty of early diagnosis; therefore, advanced melanoma might induce the activation of tumor immunity depending on the disease duration. (2) GM tended to have higher incidences of $PD\text{-}L1^+$ and HLA^+ than those of SM; therefore, the characteristics of tumor cells might differ between GM and SM depending on tumor origin. (3) The tumor microenvironment must differ between GM and SM. Immune cells are more abundant in the gastrointestinal tract than in the skin.

Previously, we have reported that GMs were significantly more likely than SMs to be amelanotic and display round cells and aggressive features (lymph node and distant metastasis) (7). Further research should analyze the important differences in gene expression or response to therapy based on race and histological subtype, with a larger cohort of melanoma patients. However, we have not performed these analyses in the current study due to the small number of cases.

Patients with PD-L1-positive sarcoma are younger than those with PD-L1-negative sarcoma (17), as observed for melanoma in the present study. In contrast, patients with HLA-positive melanomas were older than those with HLA-negative melanomas in the present study. These results suggested that aging influences tumor immunity by decreasing tumor-specific memory T cells and increasing immune-suppressive cells (18). Different treatment strategies might be needed for elderly patients. Thus, older patients with melanoma reportedly responded better to ICI treatment than younger ones (19). Furthermore, it is important to consider the physical condition of elderly individuals when deciding to perform a surgical intervention. Therefore, ICI treatment may be recommended for the elderly.

Many studies have shown that the infiltration of $CD8^+$ cytotoxic T cells plays key roles in the cancer-initiating cell (CIC) response (20), but the roles and clinical impact of $FOXp3^+$ regulatory T cells on the CIC response are not fully understood (21). A previous report has shown that PD-L1 expression in SM (22,23), and all types of melanoma (24)

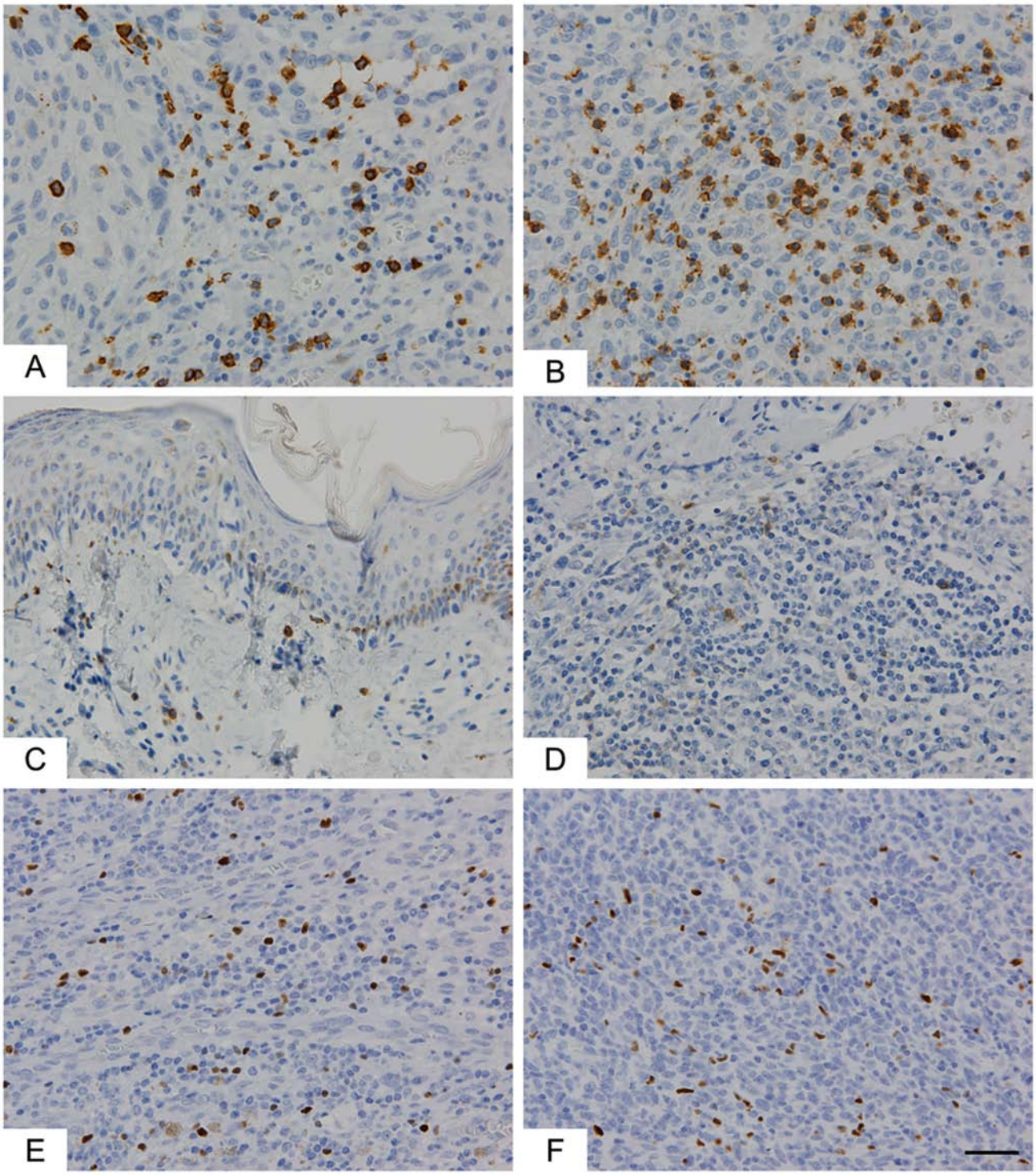

Figure 2. Immunohistochemical staining for $CD8^+$, $PD\text{-}1^+$ and $FOXp3^+$ lymphocytes in tumor areas of SM and GM. (A) Expression of CD8 in SM. (B) Strong expression of CD8 in GM. (C) Expression of PD-1 in SM. (D) Strong expression of PD-1 in GM. (E) Expression of FOXp3 in SM. (F) Expression of FOXp3 in GM. Counterstaining, hematoxylin. Scale bar, 50 μm. SM, skin melanoma; GM, gastrointestinal melanoma; PD-1, programmed cell death protein 1.

is associated with $CD8^+$ lymphocytes, consistent with our findings. Furthermore, PD-L1 expression is associated with $FOXp3^+$ lymphocytes in sarcoma (17) and breast cancer (25), consistent with our results. $CD8^+$ lymphocytes as well as PD-L1 expression in tumor cells might be candidate predictive biomarkers for the CIC response.

Previous studies have demonstrated PD-L1 expression on tumor cells using immunohistochemical staining for melanoma subtypes (24) and *PD-L1* expression and copy number in primary vaginal melanomas utilizing fluorescence in situ hybridization (FISH) (26). The existence of differences between patients from Asia and other geographical areas is controversial (22,24,26-28). Further studies, analyzing cohorts of individuals stratified by race and histological type are necessary to clarify the presence of differences in rare melanoma types.

Table III. Clinicopathological characteristics of patients with HLA-positive melanoma.

Variables	HLA positive, n (%)	HLA negative, n (%)	P-value
Age, years (mean ± SD)	72.5±15.9	66.8±17.0	0.3024
Sex			
Male	9 (60.0)	13 (50.0)	0.5362
Female	6 (40.0)	13 (50.0)	
Location of lesion			
SM	10 (32.3)	21 (67.7)	0.3111
GM	5 (50.0)	5 (50.0)	
UICC stage			
I	3 (20.0)	8 (30.8)	0.6495
II	4 (26.7)	6 (23.1)	
III	6 (40.0)	11 (42.3)	
IV	2 (13.3)	1 (3.8)	
$BRAF^{V600E}$			
Positive	5 (33.3)	8 (30.8)	0.8651
Negative	10 (66.7)	18 (69.2)	
PD-L1			
Positive	13 (86.7)	20 (76.9)	0.4483
Negative	2 (13.3)	6 (23.1)	
CD8(+) lymphocyte			
High	10 (66.7)	10 (38.2)	0.0818
Low	5 (33.3)	16 (61.5)	
PD-1(+) lymphocyte			
High	8 (53.3)	4 (15.4)	0.0101[a]
Low	7 (46.7)	22 (84.6)	
FOXp3(+) lymphocyte			
High	14 (93.3)	20 (80.0)	0.2529
Low	1 (6.7)	5 (20.0)	

[a]P<0.05, HLA positive vs. HLA negative in χ^2 test. SM, skin melanoma; GM, gastrointestinal melanoma; PD-L1, programmed death ligand 1; PD-1, programmed cell death protein 1; HLA, human leukocyte antigen; UICC, Union for International Cancer Control.

Several companion assays are on the market to assess PD-L1 expression by immunohistochemistry, each of which is linked to a different drug. Tests for the expression of PD-L1 are not required for use of ICI in melanoma but may provide physicians and patients more information. PD-L1 expression in SM as detected by the PD-L1 clone 28-8 is correlated with the magnitude of the treatment effect of nivolumab with respect to progression-free survival (8). Our results suggest that PD-L1 28-8 testing is useful in GM.

Our study had a few limitations. Primarily, the number of cases was small, and most of the patients were elderly, especially in the GM group. Second, we examined the difference between regions but not the type of disease. Third, the quantification of expression levels depended solely on the histochemistry technique. Finally, the homogeneity of cases and heterogeneity of tissues might have affected the results.

In conclusion, our results provide useful information regarding tumor immunity in GM and SM. Further studies are needed to enable accurate predictions of the effect of immunotherapy.

Acknowledgements

The authors would like to thank Dr Seiichi Shinji (Surgery for Organ Function and Biological Regulation, Nippon Medical School, Tokyo, Japan) for his support in case presentations. The authors would also like to thank Ms. Yasuko Hasegawa (Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan) for her immunohistochemical work.

Funding

The present study was supported by a Grant-in-Aid for Young Scientists (B) (grant no. 15K19705 to MA).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MA, YM and HS were involved in the conception and design of the study. MA collected the data and performed the experiments. MA, YM and TA analyzed the data. MA and YM wrote the paper. TA and HS critically revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Approval for the study was obtained from the Human Research Ethics Committees at the Tokyo Metropolitan Geriatric Hospital (approval no. R17-33) and the Nippon Medical School Hospital (approval no. 29-07-805). Written informed consent for the anonymous use of their data and tissue samples for study purposes was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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