Research Article

Role of PD-L1 expression during the progression of submucosal gastric cancer

Yasunari Ubukata[1], Kyoichi Ogata[1], Makoto Sohda[1]*, Takehiko Yokobori[1], Yuki Shimoda[2], Tadashi Handa[2], Nobuhiro Nakazawa[1], Akiharu Kimura[1], Norimichi Kogure[1], Akihiko Sano[1], Makoto Sakai[1], Hiroomi Ogawa[1], Hiroyuki Kuwano[1], Ken Shirabe[1], Tetsunari Oyama[2], Hiroshi Saeki[1]

[1] Department of General Surgery, Graduate School of Medical Sciences, Gunma University, Gunma, Japan.

[2] Department of Pathology, Graduate School of Medical Sciences, Gunma University, Gunma, Japan.

Short Title: PD-L1 expression in submucosal gastric cancer

*Corresponding Author

Makoto Sohda, MD, PhD.

Department of General Surgery, Graduate School of Medical Sciences

Gunma University

3-39-15, showamachi, maebashi

Gunma, 371-8511, Japan

Tel: +81-27-220-8230

Fax: +81-27-220-8224

E-mail: msohda@gunma-u.ac.jp

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Abstract

Introduction: PD-L1 expression is a prognostic marker for gastric cancer that correlates with tumor diameter and depth of penetration. But the role of PD-L1 and mechanism(s) employed in the initial phase of invasion in early gastric cancer is yet to be understood.

Objective: This study aims to elucidate the role of PD-L1 during the progression of gastric cancer, specifically invading the submucosa beyond the lamina muscularis mucosa.

Methods: Using 107 patients with pathological submucosal gastric cancer, we determined the expression of PD-L1 based on the staining of the cell membrane or cytoplasm of tumor cells in the central and invasive front of the tumor. Samples were categorized into three groups based on the intensity of PD-L1 expression. $CD8^+$ lymphocytes expressing PD-1 and $CD163^+$ macrophages were used to determine the number of cell nuclei at the invasive front, similar to PD-L1. CMTM6 levels were determined and used to stratify samples into three groups.

Results: PD-L1 was expressed higher in the invasive front (26.2%) than in the central portion of the tumors (7.4%; $p<0.001$). Moreover, lymphatic and vascular invasion were more frequently observed in samples with high levels of PD-L1 (lymphatic invasion: 60.7% vs. 35.4%, $P=0.0026$ and vascular invasion: 39.3% vs. 16.5%, $P=0.0018$). There was no correlation between PD-L1 expression and the levels of PD-1, CD8, CD163, and CMTM6.

Conclusions: PD-L1-expressing cancer cells at the invasive front of gastric cancer influence the initial stages of tumor invasion and lymphovascular permeation in early-stage gastric cancers. Immune checkpoint signaling may be the driving force in the invasive front during the invasion of the submucosa beyond the lamina muscularis mucosa.

Introduction

Although there has been a gradual decrease in the rate of mortality associated with gastric cancer, it remains the third most common cause of cancer-related deaths in Japan, claiming 45,000 lives annually [1]. Although gastric cancer does not usually involve venous and lymphatic permeation among the mucosal cancers, upon progression into the submucosa, patients develop lymph node and other distant metastases [2]. However, the progression of mucosal cancer into the submucosal region remains to be elucidated. Therefore, it is important to understand the mechanism(s) involved during the initial stages of invasion in gastric cancer.

Venous and lymphatic permeation is attributed to the penetration of cancer cells deeper into the mucosa and submucosa during the progression of gastric cancer [3]. Several reports have shown that clinicopathological findings, such as tumor diameter, vascular invasion, degree of differentiation, and depth of penetration, help track the progress of lymph node metastasis [4-8]. Depth of penetration includes the extent of cancer invasion; this can be determined by analyzing the gene expression of claudin 3 and claudin 18 owing to their involvement in the initial phase of gastric cancer invasion [9,10]. However, there is little mechanistic insight into the role with deep invasion during the early stages of gastric cancer.

PD-L1/2 is found on the surface of cancer cells that bind to the immune checkpoint receptor PD-1 on the surface of cytotoxic T cells, thereby inactivating active T cells and evading immune recognition [11-13]. Immune checkpoint inhibitors targeting PD-1/PD-L1 suppress T cell inactivation and activate tumor immunity [14,15]. PD-L1 is also involved in tumor invasion; Tsutsumi et al. reported the correlation between PD-L1 expression and poor prognosis of patients with esophageal cancer along with epithelial-mesenchymal transition [16]. Numerous other studies have shown that PD-L1 expression correlates with poor prognoses for various malignant tumors such as esophageal cancers, hepatocellular cancers, renal cell cancers, and malignant melanomas [17-20]. The expression of PD-L1, high content of infiltrated lymphocytes in tumors, and distant metastasis in breast cancer can be attributed to the levels of PD-1 [21].

PD-L1 expression is a prognostic marker for gastric cancer that correlates with tumor diameter and depth of penetration [22]. Jing Li et al. demonstrated a decrease in tumor metastasis and proliferation by the knocking down of PD-L1 in gastric cancer cell lines [23]. PD-L1 expression in gastric cancer is controlled by IFN-γ via JAK-STAT signaling and epithelial-mesenchymal transition; PD-L1 affects tumor immunity by modulating cytotoxic T lymphocyte activity [24]. However, the role of PD-L1 and mechanism(s) employed in the initial phase of invasion in early gastric cancer is yet to

be understood. We hypothesize that PD-L1 may contribute to the acquisition of invasive phenotypes of cancer cells during progression from the mucosal layer to the stromal cells-abundant submucosal layer, as it theoretically acts in immune evasion. This study aims to elucidate the role of PD-L1 during gastric cancer progression, specifically in the invasion of the submucosa beyond the lamina muscularis mucosa. Thus, we determined the expression of PD-L1 in different regions of submucosal gastric cancer. Subsequently, we investigated the correlation between PD-L1 expression, CMTM6 (in the regulation of PD-L1), and infiltration of immune cells (e.g., tumor-associated $CD163^+$ macrophages and cytotoxic $CD8^+$ T lymphocytes expressing PD-1) in gastric cancer tissues.

Materials and Methods

Patients

We performed radical surgeries on 635 gastric cancer patients at the Gunma University Hospital between April 2010 to March 2017. Among them, 107 patients who underwent surgery for primary gastric cancer with pT1b (pathological results showing tumor cell infiltration in the submucosal layer through the mucosal muscle plate) were included. Among pT1b carcinomas, pSM1 (pT1b1) is defined as when the distance from the lower end of the mucosal muscle plate to the deepest point is <500 µm, and pSM2 (pT1b2) is defined as if the distance is >500 µm. This study was approved by the Ethics Committee of Gunma University (Approval Number: HS2019-183).

Immunohistochemistry

Biomarker expression was analyzed by immunohistochemistry. Briefly, 4 µm sections were deparaffinized in xylene and dehydrated using a gradient of ethanol. The specimens were pretreated in an autoclave to retrieve the following antigens: PD-L1 and PD-1 (121°C, 10 min, Target Retrieval Solution; pH 6.1), CD8 (121°C, 30 min, boiled citric acid; pH 6.0), CD163, CMTM6, and D2-40 (98°C, 30 min, boiled citric acid; pH 6.0). Subsequently, the sections were incubated in 0.3% hydrogen peroxide dissolved in methanol to saturate and inhibit the endogenous peroxidases. Non-specific binding by antibodies were prevented by blocking the sections followed by overnight incubation at room temperature with primary antibodies against PD-L1 (1:400; 28-8, Abcam), PD-1 (1:400; NAT105, Abcam), CD8 (1:100; C8/144B, DAKO), CD163 (1:500; D6U1J, Cell Signaling Technology), CMTM6 (1:50; ab97652, Abcam), and D2-40 (1:200; M3619, DAKO).

The sections were washed with phosphate-buffered saline, and staining by the primary antibodies was visualized using the Histofine Simple Stain MAX-PO (Multi) Kit (Nichirei, Tokyo, Japan) according to the instructional manual. The chromogen (0.02%), 3,3-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan), was dissolved in 0.005% H_2O_2 and 50 mM Tris-HCl buffer (pH 7.6). Subsequently, the sections were lightly counterstained with Mayer's hematoxylin and mounted.

PD-L1, PD-1, CD8, CD163, and CMTM6 levels were independently determined by two investigators, including one general pathologist. PD-L1 expression was analyzed based on the staining of the cell membrane or cytoplasm of tumor cells in the central and invasive tumor samples. The immunostaining score was calculated based on the number of positive cells and staining intensity, as described previously [25]. Samples with PD-L1 expression were categorized into three groups (no, low, and high expression) based on the Allred score [26]. PD-1, CD8, and CD163 expressions were analyzed using the number of positive cell nuclei at the invasive front of the tumor (similar to PD-L1), as described previously [27, 28]. CMTM6 expression was analyzed as per a previous study [29].

Clinicopathological features and PD-L1 expression in patients

The patients were stratified in two different ways. First, we assigned patients into no/low expression and high expression groups based on PD-L1 expression at the invasive portion of the tumor. Second, we assigned patients into the center-dominant (stronger or equal expression of PD-L1 in the central rather than invasive front) and invasive front-dominant (stronger PD-L1 expression in the invasive front rather than the central portion) groups. Clinicopathological features (such as age, gender, differentiation of gastric cancer, tumor size, pathological lymph node metastasis, lymphatic permeation, venous permeation, postoperative recurrence, and cancer death) were comparatively analyzed in each subgroup.

Statistical analysis

The differences between the two groups were compared using the Student's *t*-test and Chi-square test. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using EZR (2.4-0). Standard deviations, instead of standard errors of the mean, were preferred. All significant results necessarily included the test value, degree(s) of freedom, and probability level.

Results

PD-L1 expression in the central and invasive portion of submucosal gastric cancer

Table 1 shows the immunohistochemical profile of the central and invasive portions of the tumor. PD-L1 was expressed in the cell membrane and cytoplasm of tumor cells in the submucosal gastric cancer specimens (Fig. 1A, B). Among the 107 patient samples, PD-L1 was observed to be higher in the invasive front (26.2%) than in the central portion (7.4%; $p<0.001$). Similarly, in the pathological SM1 and pathological SM2 subgroups, we observed a higher expression of PD-L1 in the invasive front than in the central portion (pSM1: 16.7% vs. 8.3%, $P=0.002$; pSM2: 28.9% vs. 7.2%, $P<0.001$).

Clinicopathological features of the patient cohort based on PD-L1 expression

Table 2 summarizes the clinicopathologic features based on the expression of PD-L1 in the invasive front of the gastric tumors. Samples with high PD-L1 expression correlated with a higher proportion of lymphatic and vascular invasion (lymphatic invasion: 60.7% vs. 35.4%, $P=0.0026$ and vascular invasion: 39.3% vs. 16.5%, $P=0.0018$). There were no significant differences in patient age and gender, tumor size and differentiation, lymph node metastases, postoperative recurrence, and cancer death between the two groups.

Clinicopathological features of the patient cohort based on the dominant pattern of PD-L1 expression

Table 3 shows the clinicopathologic features based on the dominant pattern of PD-L1 expression. Pathological lymph node metastasis and lymphatic and vascular invasion were more frequently observed in the invasive front-dominant group (pathological lymph node metastasis: 25.4% vs. 6.8%, $P=0.019$; lymphatic invasion: 52.4% vs. 27.3%, $P=0.011$; and vascular invasion: 30.2% vs. 11.4%, $P=0.033$). There were no significant differences in patient age, gender, tumor size, differentiation, postoperative recurrence, and cancer death between the two groups.

Correlation between PD-L1 expression and other factors

We determined the expression of PD-1, CD8, CD163, and CMTM6 in the invasive front of the submucosal tumors and compared them with PD-L1 expression (Fig. 2). We used two different ways of subgrouping and found that there was no significant correlation between PD-L1 expression and other patient factors in either subgroup (PD-1: *P*=0.37; CD8: *P*=0.39; CD163: *P*=0.6; and CMTM6: *P*=0.23; Table 4).

PD-L1 and D2-40 profiles in samples with lymphatic permeation

We performed PD-L1 and D2-40 staining using serial sections of the representative patient sample with lymphatic permeation (Fig. 3). PD-L1 was expressed in the cancer cells that permeated into lymphatic vessels.

Discussion/Conclusion

Cancer cells that penetrate the lamina muscularis mucosa and invade into the submucosa (with an abundance of stromal cells) are exposed to host immune response. Therefore, we hypothesized that PD-L1-expressing cancer cells at the invasive front escape immune recognition and infiltrate deep into the host tissues to enhance tumor progression. In this study, we observed high levels of PD-L1 within the pSM1 subgroup, indicating the importance of PD-L1 in the initial stages of gastric cancer invasiveness.

Venous and lymphatic permeation occurs as cancers progress, thereby enabling metastasis to the lymph nodes and distant organs. Akt, mTOR, VEGF-C, and VEGF-D are also involved during this process [30]. In this study, we found that PD-L1 expression correlated with venous and lymphatic permeation in submucosal gastric cancer samples. PD-L1 and D2-40 staining of the tissue sections of a representative patient with lymphatic permeation revealed the presence of PD-L1 expression in cancer cells that permeated into the lymphatic vessels (Fig. 3). These findings suggest that cancer cells expressing a high content of PD-L1 in submucosal invasive cancers have the ability of deep invasion and vascular permeation.

$CD8^+$ T cells influence tumor immunity by differentiating into cytotoxic T lymphocytes. CD8 cell infiltration has been shown to correlate with PD-L1 expression in tumor cells and stroma [30, 31]. $CD8^+$ T cell infiltration correlates with PD-L1 expression and poor prognosis in gastric cancer [32]. CD163 is expressed on the surface of M2 macrophages; M2 macrophages stimulate the formation of

the tumor microenvironment and early vascular invasion in gastric cancer [33]. CD163 expression on M2 macrophages has been reported to correlate with PD-L1 expression in gastric cancer. PD-1 is expressed on $CD4^+$ and $CD8^+$ T cells and interacts with PD-L1/2 in avoiding immune responses. Marian et al. reported that CMTM6 stabilizes PD-L1 from degradation by ubiquitination [34]. In this study, we did not observe a correlation between the expression of PD-L1 in cancer cells and that in other proteins. Shen et al. revealed there was no correlation with CD8 and PD-L1, suggesting that an adaptive immune resistance mechanism may be inactive in early-stage cancer [35]. Okabe et al. reported that $CD8^+$ T cell density was not associated with PD-L1 expression in early breast cancer [36]. Concerning the relationship between PD-L1 and CD163, to our knowledge, no previous study has focused on early-stage cancer. The studies that examined the relationship between PD-L1 and CD163 in gastric [28] and colorectal cancers [37] showed a positive correlation between PD-L1 and CD163; probably, this could be attributed to the use of samples with mixed stages of cancer (mainly T2 or deeper cancers). We speculated that tumor microenvironment might be immature in pT1b gastric cancer regardless of high PD-L1 expression on cancer cells, compared to advanced cancer. Therefore, it is important to understand the interaction between the tumor and stroma in the future, especially in early-stage cancer.

This study demonstrated that PD-L1 was highly expressed at the invasive front of submucosal invasive gastric cancer, indicating heterogeneous expression of PD-L1 in gastric cancer. However, biopsies from patients in a clinical trial that studied the effect of anti-PD-1 as a therapy against advanced gastric cancer revealed that PD-L1 expression in the mucosal surface could not serve as a biomarker for determining therapeutic efficacy [38]. Based on the heterogeneous results of this study, it was speculated that biopsy specimens might be inappropriate to qualify the status of PD-L1 in patients with gastric cancer. Thus, it is important to understand the basis for heterogeneous PD-L1 expression in gastric cancer in the future.

There are some limitations associated with this study. First, this was a single facility-based study with a small cohort. Second, PD-L1 expression was determined using a single antibody and did not consider the differences in staining potentials by multiple antibodies. Further, the study only focused on submucosal cancer owing to surgical resections. To preliminarily investigate PD-L1 expression in pT1a and pT2 cancers, we randomly stained 20 samples for each group, using our archived samples. We found that there was no difference in expression between the central portion and invasive front in pT1a cases, while the expression of PD-L1 in the invasive front was stronger than in the central portion in pT2 cases (data not shown). This may motivate further detailed studies on PD-L1 expression in the initial progressive stages. Thus, it is imperative to analyze samples from mucosal

cancer obtained by endoscopy in the future to elucidate the mechanism(s) employed by early gastric cancer in evading immune responses.

In conclusion, PD-L1-expressing cancer cells at the invasive front of tumors are important during the initial stages of invasion and lymphovascular permeation in early gastric cancer. Studying the immune checkpoint mechanisms employed during the initial phases of invasion may be a promising area of cancer research in the future.

Statements

Acknowledgment

Statement of Ethics

This research complies with the guidelines for human studies and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Subjects (or their parents or guardians) have given their WRITTEN informed consent.

The study was approved by the Ethics Committee of Gunma University (Approval Number: HS2019-183).

Disclosure Statement

No potential conflicts of interest were disclosed.

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Author Contributions

Conception and design of the study: Hiroyuki Kuwano, Ken Shirabe, Tetsunari Oyama, Hiroshi Saeki

Acquisition of data: Yasunari Ubukata, Yuki Shimoda, Tadashi Handa

Analysis and/or interpretation of data: Yasunari Ubukata, Kyoichi Ogata, Takehiko Yokobori

Drafting the manuscript: Yasunari Ubukata, Hiroshi Saeki

Revising the manuscript critically for important intellectual content: Kyoichi Ogata, Makoto Sohda, Takehiko Yokobori, Nobuhiro Nakazawa, Akiharu Kimura, Norimichi Kogure, Akihiko Sano, Makoto Sakai, Hiroomi Ogawa

Approval of the version of the manuscript to be published: Yasunari Ubukata, Kyoichi Ogata, Makoto Sohda, Takehiko Yokobori, Yuki Shimoda, Tadashi Handa, Nobuhiro Nakazawa, Akiharu Kimura, Norimichi Kogure, Akihiko Sano, Makoto Sakai, Hiroomi Ogawa, Hiroyuki Kuwano, Ken Shirabe, Tetsunari Oyama, Hiroshi Saeki

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Figure Legends

Fig. 1. Expression of PD-L1 in the central, invasive, and submucosal regions of gastric carcinoma.

Representative immunohistochemistry images of samples stained with anti-PD-L1 antibodies (100×, A: central portion; B: invasive front; and 50×, C: submucosal region).

Fig. 2. Expression of PD-1, CD8, CD163, and CMTM6 at the invasive front of the tumor.

Representative immunohistochemistry images of samples stained with anti-PD-1 (200×, A), anti-CD8 (200×, B), anti-CD163 (200×, C), and anti-CMTM6 (200×, D) antibodies.

Fig. 3. Expression of PD-L1 in the tumor cells present in the lymphatic vessel at the invasive front of the submucosal gastric cancer.

Representative immunohistochemistry images of samples stained with D2-40 (400×, A) and anti-PD-L1 (400×, B) antibodies.