CLINICAL RESEARCH

Association Between Intra-Tumoral Immune Response and Programmed Death Ligand 1 (PD-L1) in Gastric Cancer

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

A 1 **Rong Yan**
B 2 **Xiaobing Yang**
E 2 **Xiaolei Wang**
B 2 **Bin Wang**
D 2 **Youcai Zhao**
G 2 **Wenbin Huang**
G 2 **Wei Zhao**
ABCDEF 3 **Lizhou Jia**

1 Department of Pathology, The First People's Hospital of Lianyungang, Lianyungang, Jiangsu, P.R. China
2 Department of Pathology, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, P.R. China
3 Central Laboratory, Bayannur Hospital, Linhe, Inner Mongolia, P.R. China

Corresponding Authors: Lizhou Jia, e-mail: nmgjlz@163.com, Wei Zhao, e-mail: zhaowei_njmu@163.com
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Background: Targeting of PD-1/PD-L1 immune checkpoints exhibits excellent clinical outcomes in numerous types of solid tumors, including gastric cancer. However, the tumor microenvironment of gastric cancer is very complex and the association of PD-L1 with the tumor microenvironment in gastric cancer is still not clear.

Material/Methods: This study analyzed the characteristics of PD-L1 expression and used immunohistochemistry to assess CD8 and CD4 tumor-infiltrating leucocytes (TILs) in 478 cases of gastric cancer compared with the expression patterns in 70 matched adjacent tissues, and 32 cases of benign gastric tissues. Standardized methods for TILs assessment in gastric cancer were used.

Results: The results indicated that PD-L1 expression was increased in gastric cancer tissues (193 out of 478, 40.37%) compared with matched adjacent tissues (14 out of 70, 20.00%) and benign gastric tissues (10 out of 32, 31.25%). It was observed that in gastric cancer patients, positive PD-L1 status in tumor cells (tPD-L1) was associated with distant metastasis (χ^2=3.344, P=0.044). The positive expression pattern of tPD-L1 was associated with higher density of TILs, and this pattern was most significant in the non-metastasis group, compared to the metastasis group. We also found that tPD-L1 was not prognostic for overall survival in gastric cancer patients, but tPD-L1 and tCD8 combined positive status in gastric cancer patients was strongly associated with better overall survival rates both in the univariate analysis [hazard ratio (HR)=2.341, 95% confidence interval (CI)=1.147–3.556, P<0.001 and in the multivariate analysis (HR=1.844, 95% CI=1.136–2.592, P=0.031).

Conclusions: These data suggested an interaction between tPD-L1 expression and TILs in gastric cancer, and tPD-L1 expression positively correlated with high densities of tCD8 and indicated a better overall survival and decreased metastasis in gastric cancer patients.

MeSH Keywords: Antigens, CD274 • Antigens, CD4 • Antigens, CD8 • Stomach Neoplasms

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/916432

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Background

Gastric cancer is one of the most common cancers in Asia, particularly in China [1,2]. Despite improvements in diagnostic and treatment strategies, the overall survival of gastric cancer patients still remains poor [2]. One of the biological markers of tumor development is the ability to evade immune surveillance. The immune surveillance capacity can be recognized and destroyed by tumor cells. As a result, immunotherapy has been implemented in many types of tumors [3,4]. A previous study reported that PD-1 was a checkpoint molecule in the immune response and was mainly over-expressed on activated T/B cells monocytes and dendritic cells [5,6]. PD-L1 is the ligand for PD-1 and is mainly expressed on many types of tumor cell. PD-L1 combines with and activates PD-1 to produce inhibitory signals that decrease T cell activation [7].

However, increasing research has reported a strong association between tumor-infiltrating lymphocytes (TILs) and survival of tumor patients [8,9]. Moreover, TILs have been revealed to be predictive markers of response to novel immune checkpoint blockade therapies [10]. Therefore, it would be meaningful to explore the link between PD-L1 and TILs in gastric cancer.

This study used standardized methods for TILs assessment in gastric cancer and explored the association between PD-L1 expression and TILs (CD8 and CD4) to investigate potential mechanism of immune response in gastric cancer.

Material and Methods

Gastric cancer tissue samples and clinical pathologic information

All of the 478 gastric cancer cases included in this study were diagnosed from 2009 to 2012. The range of tissue samples available included cancer tissues (n=478) and matched adjacent tissues (n=70), as well as benign gastric tissues (n=32). All tissue samples were collected from the Department of Pathology, Nanjing Hospital affiliated to Nanjing Medical University. Age at diagnosis, sex, tumor node metastasis (TNM) stage, histological type, differentiation grade, and overall survival were recorded. We re-staged the patients according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (Eighth Edition). Patients did not receive any treatment prior to surgical resection. This research protocol was approved by the Human Research Ethics Committee of Nanjing Medical University (No. 2016-113).

Tissue microarray (TMA) construction and immunohistochemistry (IHC)

The tissue microarrays (TMAs) were produced in the Department of Pathology, Nanjing Hospital affiliated to Nanjing Medical University using a tissue system Quick-Ray (Unitma Co., Ltd, Seoul, Korea), according to the manufacturer's protocol. All cases were initially selected from the pathology database, and then re-reviewed to select tumor areas. Core tissue biopsies (2 mm in diameter) were taken from 70 individuals. A total of 8 gastric TMAs was made. PD-L1 (28-8) (Roche Diagnostics, Basel, Switzerland), CD8 (OTI7C10; OriGene Technologies, Inc., Beijing, China) and CD4 (OTI1D6; OriGene Technologies, Inc.) monoclonal antibodies were used to stain the TMAs. Envision™ peroxidase kits (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) were used to detect the reaction.

Immunohistochemistry (IHC) scores and assessments of TILs were evaluated by 2 experienced pathologists under blinded experimental conditions. All antigen expression was scored using the semi-quantitative H-score method; TILs markers assessed were CD4 and CD8 through direct counting of positive cells, and both stromal and intra-tumoral TILs were evaluated in the research setting. Necrotic areas were excluded. For statistical analysis, CD8 TIL levels in tumors were categorized into a low or high TIL status based on the median of the CD8/CD4 TIL density scores (divided into tumor cells and stromal TIL density scores).

Statistical analysis

All statistical analysis was evaluated by SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL, USA). The statistical significance of differences between the groups were used by Student's t-test and χ^2 test. The overall survival of patients was evaluated by Kaplan-Meier method and a log-rank test. Univariate and multivariate hazard ratios (HR) for the variables were calculated by a Cox proportional hazards model. All tests used were 2-sided, $P<0.05$ was defined to a statistically significant difference.

Results

PD-L1 protein was overexpressed in gastric cancer tissue samples

IHC analysis was conducted to detect PD-L1 expression in all gastric cancer tissue samples and the results demonstrated that the PD-L1 positive status was detected more frequently in gastric cancer tissues (193 out of 478 samples, 40.37%) compared to matched adjacent tissue samples (14 out of 70 samples, 20.00%) and benign gastric tissue samples (10 out of 32, 31.25%), as presented in Table 1 (χ^2=43.70, P=0.001).

Table 1. PD-L1 expression in gastric tissues.

Characteristic	n	PD-L1 expression (%)		Pearson χ²	P
		Low or no	High		
	580			43.70	0.001
GC tissues	478	285 (60.25)	193 (40.37)		
Matched adjacent tissues	70	56 (80.00)	14 (20.00)		
benign gastric tissues	32	22 (68.75)	10 (31.25)		

* $P<0.05$.

PD-L1 expression in gastric cancer and associated clinicopathological characteristics

Next, the present study examined the association between PD-L1 expression and clinicopathological characteristics. PD-L1 protein expression was localized to the membrane and endomembrane of the tumor cells. PD-L1 staining was also observed in stromal cells. The results indicated that PD-L1 expression in tumors was observed in 193 of the 478 cases of gastric cancer in gastric cancer tissue samples (Figure 1). PD-L1 expression was associated with distant metastasis and PD-L1 exhibited increased expression in the M0 group (170 out of 413 cases, 41.16%) compared with the M1 group (19 out of 65 cases, 29.23%) (χ^2=3.344, P=0.044). However, there was no significant correlation between PD-L1 and other clinicopathological variables, including age, gender, differentiation, tumor size, lymph node metastasis, TNM stage and histological type.

Association between immune response and PD-L1 expression

To explore the importance of the immune response in tumors, the present study analyzed the association between PD-L1 expression location, expression levels, and localization of CD8 and CD4 TILs. To assess CD8+ or CD4+ TILS number, 5 separate fields of view were randomly selected for viewing under a 200× high-power magnification (Olympus BX43) and the number of positive status cells were counted. Results indicated that the PD-L1 positive pattern was associated with increased density of TILs, and pattern was most significant in the non-metastasis group, compared with the metastasis group (Figure 2).

### Association of tPD-L1 expression with CD8 TILs and clinical outcomes

The study also used univariate and multivariate analyses to detect the relationship between tPD-L1 expression and prognostic factors in gastric cancer patients. It was observed that tPD-L1 was not prognostic for overall survival in gastric cancer patients (HR=0.732, 95% CI=0.506–1.061, P=0.230), but tPD-L1 and tCD8 positive status were associated with better

overall survival in gastric cancer patients both in the univariate analysis (HR=2.341, 95% CI=1.147–3.556, P<0.001) and multivariate analysis (HR=1.844, 95% CI=1.136–2.592, P=0.031).

Additionally, Kaplan-Meier survival curves revealed that tPD-L1 was not prognostic for overall survival in gastric cancer patients (log rank chi square=2.246, P=0.134), although positive status in gastric cancer patients revealed a slightly better survival compared with tPD-L1 negative status of gastric cancer patients. In addition, tPD-L1 and tCD8 positive expression were significantly associated with better overall survival (log rank chi square=65.732, P<0.001) (Figure 3).

Discussion

A literature review highlighted the importance of considering several factors in analyzing the expression of PD-L1 and its association with the immune response, including the differences in antibody clones, scoring cutoff definitions, and assessment approach. Wang et al. [11] used SP142 rabbit monoclonal antibody, which recognizes C-terminus of human PD-L1 protein. The selected cutoff point corresponds to the upper quartile of expression levels in the cohort to determine PD-L1 tumor cell expression status and its relation to outcome in breast cancer. Huang et al. [12] selected PD-L1 (E1L3N) XP rabbit mAb, which recognizes endogenous levels of total PD-L1 protein. The scored PD-L1 expression corresponds to several types of methods. Cells demonstrating membranous staining for PD-L1 were scored as ≥5%, 0–5%, or 0% based on the proportion of PD-L1 positive cells, but any expression (>0%) of PD-L1 on tumor infiltrating and stromal immune cells was considered present. Otherwise, cases were considered absent of PD-L1 expression in immune stroma. Thompson et al. [13] selected PD-L1 (5H1 clone) mAb to detect PD-L1 expression in gastric adenocarcinomas. PD-L1 expression was scored as ≥5% of PD-L1 expression in the surface (membranous) of malignant cells. In the present study, according to the clinical treatment and the quality of the samples in the hospital, we selected anti-PD-L1 antibody (28-8), which contains the specific extracellular domain of hPD-L1. Any expression (>5%) of PD-L1 on tumor cells
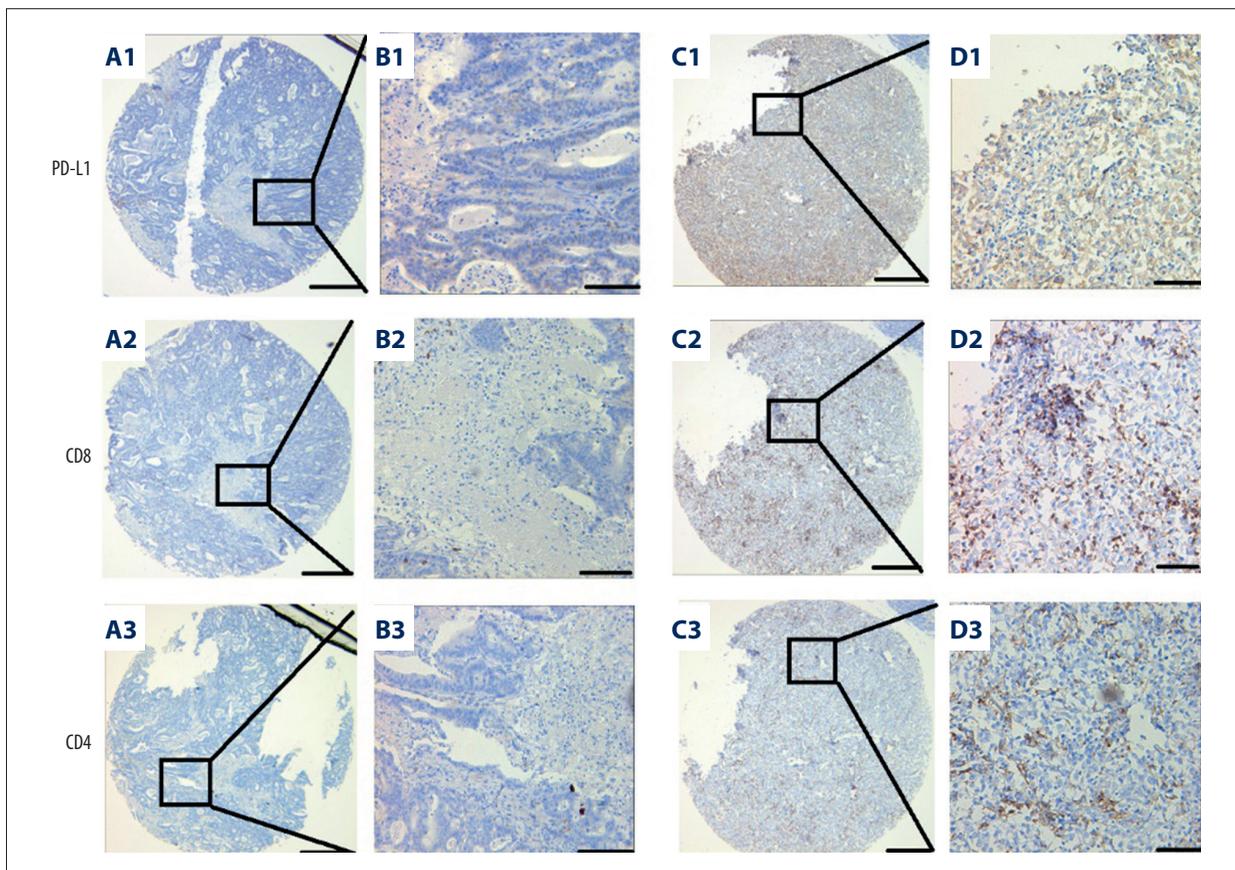
Figure 1. Expression patterns of PD-L1 and CD4/CD8 TILs in gastric cancer tissues. (**A1–A3, B1–B3**) represents negative expression patterns of PD-L1 and CD4/CD8 in the same tumor tissues. (**A1–A3**) Staining with 4× (bar=500 um). (**B1–B3**) Staining with 20× (bar=100 um). (**C1–C3, D1–D3**) represents positive expression patterns of PD-L1 and CD4/CD8 in the same tumor tissues: (**C1–C3**) staining with 4× (bar=500 um); (**D1–D3**) staining with 20× (bar=100 um). TILs, tumor infiltrating leucocytes.
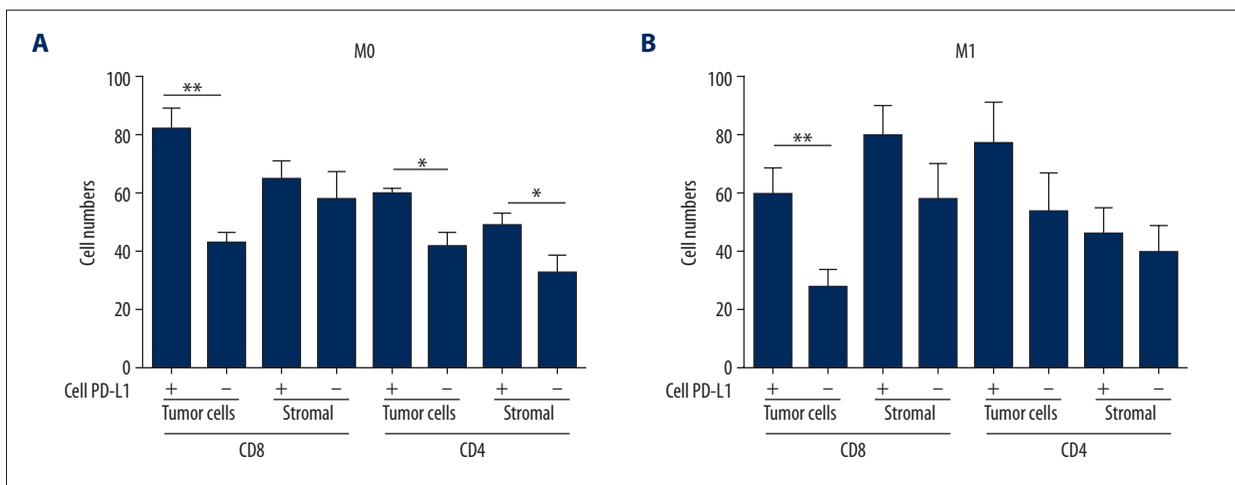
Figure 2. Association between tPD-L1 expression and CD8/CD4 TILs densities. (**A**) Gastric cancer non-metastasis patient group and (**B**) gastric cancer metastasis patient group. Bars represent means ± standard error. Four types of bars represent cell numbers of CD8, CD4 positive TILs expression in intra-tumor cell and intra-stromal respectively. The "+" represent positive expression patterns of PD-L1 in intra-tumor or intra-stromal cells; the "−" represent negative expression patterns of PD-L1 in tumor or stromal cells. M0=non-metastasis of gastric cancer; M1=metastasis of gastric cancer; * $P<0.05$, ** $P<0.001$. TILs – tumor infiltrating leucocytes.
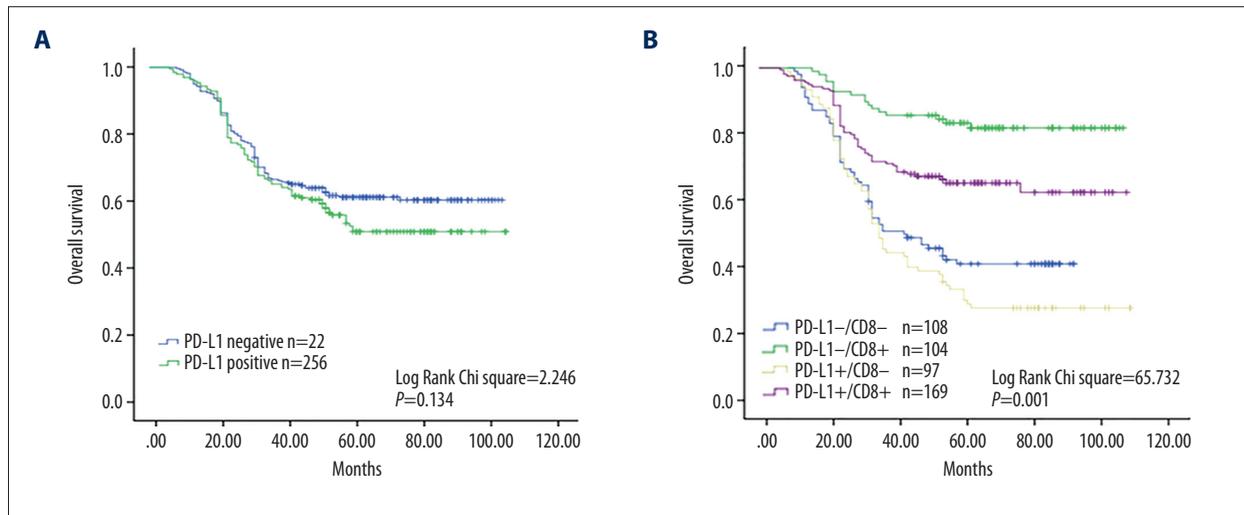
Figure 3. Survival curves for gastric cancer using the Kaplan-Meier method and log-rank test. (**A**) Overall survival curves for patients with tPD-L1 expression. The tPD-L1 negative expression is represented by the blue line, 1. The tPD-L1 positive expression is represented by the green line, 2. (**B**) Overall survival curves for gastric cancer patients with tPD-L1 expression and CD8 tumor infiltrating leucocytes. PD-L1 negative expression and CD8 negative expression are represented by the blue line, 1. The PD-L1 negative and CD8 positive expression are represented by the green line, 2. The PD-L1 positive and CD8 negative expression are represented by the gray line, 3. The PD-L1 positive and CD8 positive expression are represented by the purple line, 4.

or >1% of PD-L1 on TILs and TAM (intra-tumoral and immune stromal) was considered positive.

An increasing number of scientists recognize the importance of assessing immune response of tumors as the prognostic value in different types of tumors [14,15]. However, many methods have been randomly selected to assess and describe the immune response, and non-standard methods may inhibit efforts at approaches such as a routine clinical biomarker. Therefore, in accordance with Hendry et al. [8,9], the present study used standard methodology to assess TILs in gastric cancer, and then analyzed the association between PD-L1 expression and clinicopathological characteristics of gastric cancer patients.

The present study examined a microarray that contained 478 cases of gastric cancer tissues, 70 cases of matched adjacent tissues, and 32 cases of benign gastric tissues. It was revealed that the expression level of PD-L1 was increased in gastric cancer tissues compared with adjacent tissues and benign gastric tissues. It has recently been reported that genomic aberrations in tumor cells lead to aberrant PD-L1 expression which has a predictive role in cancer patients [16]. The association between tPD-L1 expression and clinicopathological characteristics in gastric cancer patients was explored and the results indicated that PD-L1 expression was increased in the M0 group (170 out of 413 cases, 41.16%) compared with the M1 group (19 out of 65 cases, 29.23%). Baptista et al. [17] reported that increased PD-L1 expression in patients with breast cancer may predict a better overall survival. However, Muenst et al. [18]

reported the opposite result. Therefore, the role of PD-L1 remains controversial in predicting the prognosis of tumor patients. PD-L1 is expressed in many types of cells and in the tumor microenvironment [17–22]. PD-L1 may inhibit the anti-tumor ability of TILs via PD-1, and may generally be seen as an immunosuppressive molecular biomarker. In the present study, it was demonstrated that tPD-L1 expression positively correlated with high densities of TILs and these expression pattern indicated that gastric cancer patients may have a better overall survival. Notably, it was demonstrated that in the non-distant tissues group, tPD-L1 positive status was associated with a higher density of TILs. Faget et al. [23] reported that there is a negative correlation between the proportion of immune cells and tumor size, and further reported that immune cells play an important role in controlling tumor growth. This phenomenon may be caused by the inhibition of immune regulation in the microenvironment from the cancer metastasis group, and should be explored in future studies.

Conclusions

The present study was a retrospective observation and *in vivo* studies exploring the tumor microenvironment and immune response. In addition, a mouse model of gastric cancer should be established to study the association between PD-L1 and the intratumoral immune response.

Ethics approval and consent to participate

The study protocol was approved by the Human Research Ethics Committee of Nanjing Medical University [No. (2016)118].

Conflict of interest

None.

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