Original Article

# Serum Level of Programmed Death-Ligand 1 in Patients with Gastric Cancer in Mazandaran Province as a High-Risk Region in Iran


## Abstract

**Background:** Gastric cancer is one of the most common cancers in the world. Programmed death-ligand 1 (PD-L1) is the main ligand of the programmed death-1 receptor that plays a key role in apoptosis in patients with gastric cancer. **Materials and Methods:** In this study, forty gastric cancer patients and forty healthy controls were enrolled. Serum PD-L1 was measured by enzyme-linked immunosorbent assay. **Results:** The mean (interquartile range) value of expression level of PD-L1 was 70.6 (36.6–127.3) in patients and 47.7 (29.9–92.1) in controls. There were no significant differences in PD-L1 expression between the two groups and tumor characteristics, but there was a statistically significant difference between the high expression level of PD-L1 and gastric cancer development ($P$ = 0.044, odds ratio = 2.77, 95% confidence interval = 1.12–6.86). **Conclusions:** PD-L1 level increases in gastric cancer patients, so it could be used as a predictor factor.


**Keywords:** *Enzyme-linked immunosorbent assay, gastric cancer, programmed death-ligand 1*


**Ramin Shekarriz, Akbar Hedayatizadeh-Omran, Omolbanin Amjadi, Reza Alizadeh-Navaei, Gholamali Godazandeh**

*Gastrointestinal Cancer Research Center, Non-Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran*


## Introduction

Gastric cancer is one of the major causes of mortality worldwide and today, it is the fourth most common cancer in males (fifth in females).[1-3] It is considered the second most common cause of cancer death.[4] While the rate of gastric cancer is declining in most industrialized countries,[4] it remains vital to public health in developing nations, especially in Asia.[5] It was reported that the Middle East nations such as Iran presented a high incidence of gastric cancer with an age-standardized incidence rate (ASR) of 26.1.[6] The results from the regional studies proved that northern provinces such as Mazandaran are considered the high-risk area for gastric cancer.[6-10]

The diagnosis of gastric cancer is discovered in advanced stages due to a delay in symptom manifestations.[11] Furthermore, the heterogeneous nature of gastric cancer (involving environmental factors and genetic alterations in cancer development)[12] makes the conception of the carcinogenesis process more difficult. Satisfying results that have been released from targeted therapy focused notions to molecular biomarkers in gastric cancer development including HER2, p53, p73, mdm2, Bcl-2, pRb, MSI, GST-P, MDR1, MRP2, MUC, $p27^{Kip1}$, CCND1, and programmed death-ligand 1 (PD-L1)/programmed death-1 (PD-1).[4] PD-1 is a T cell receptor that is involved in the regulation of cell proliferation.[13] PD-L1 is the main ligand for the PD-1 receptor that induces apoptosis of activated T cells.[14] Several studies investigated the association between PD-L1 expression and various types of cancer including thymoma and thymic carcinoma, adrenocortical carcinoma, head-and-neck squamous cancer, malignant brain tumors, glioma, lung cancer, esophageal cancer, pancreatic cancer, and gastric cancer.[15]

Some studies show that the expression of PD-L1 is increased in patients with gastric carcinoma.[16-21] The anti-tumor activity of PD-1/PD-L1 inhibitors seems to be effective in gastric cancer patients' treatment.[22,23] However, the predicted effects of PD-L1 in response to PD-1/PD-L1 antibodies in gastric cancer are not conclusive;[3] determining the expression profile of PD-L1 in gastric cancer patients may be helpful for future personalized therapeutic management. Due



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***Address for correspondence:***
*Dr. Akbar Hedayatizadeh-Omran, Gastrointestinal Cancer Research Center, Non-Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran. E-mail: akbar_hedayati@yahoo.com*

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to genetic changes in different societies,[24] the prognostic value of PD-L1 expression in gastric cancer, and the paucity of information in this area, the current study was performed to determine the level of PD-L1 expression in patients with gastric cancer and its relationship with clinicopathologic features in Mazandaran province (north of Iran).

## Materials and Methods

### Patients

Blood specimens were collected from forty patients who had been diagnosed with gastric cancer in 2018 in Sari Imam Khomeini Hospital, Iran, and forty age- and gender-matched healthy controls. The blood samples were obtained before chemotherapy, radiotherapy, and surgery. The serum samples were isolated by centrifugation at 2500×*g* for 10 min, aliquoted at the desired volume, and stored at −80°C until required. All patients and controls provided written informed consent for sampling and enzyme-linked immunosorbent assay (ELISA) analysis. The diagnosis of all gastric cancer patients was pathologically confirmed. The study protocol was approved by the Ethics Committee of the Mazandaran University of Medical Sciences.

### Enzyme-linked immunosorbent assay

An anti-human PD-L1 precoated 96-well ELISA plate (Human PD-L1 Platinum ELISA, Invitrogen by Thermo Fisher Scientific, Austria) was used for quantitative detection of human PD-L1. The plates were washed twice with 400-μL wash buffer using a microplate washer (BioTek ELx50, Winooski, VT, USA). Patients' serum was diluted with sample diluent at 1:1 vol ratio. After plate washing, the diluted samples and standards were added to the wells and incubated for 2 h at room temperature on a microplate shaker (GFL, Burgwedel, Germany) set at 400 rpm. The specific binding protein was detected with Biotin-conjugate anti-human PD-L1 monoclonal antibody (Invitrogen, Thermo Fisher Scientific, Austria), followed by streptavidin-HRP (Invitrogen, Thermo Fisher Scientific, Austria) and incubated at room temperature for 30 min on a microplate shaker (GFL, Germany) set at 400 rpm. Color reactions were developed using tetramethyl-benzidine-ELISA substrate solution (Invitrogen, Thermo Fisher Scientific, Austria). The enzymatic reaction was stopped by adding 1 M phosphoric acid stop solution (Invitrogen, Thermo Fisher Scientific, Austria) to each well. Optical density was read at 450 nm as the primary wavelength (optically 620 nm as the reference wavelength) and quantified using Synergy™ HTX Multi-Mode Microplate Reader (BioTek; USA) equipped with a specialized software Gen5 (BioTek; USA). The human PD-L1 standard was diluted in serial two-fold steps in a sample diluent, used as the standard. The standard curve was created by plotting the mean absorbance for each standard concentration on the ordinate against the human PD-L1 concentration on the abscissa. A 5-parameter curve was drawn as the kit instruction recommended. The minimum detectable concentration of PD-L1 was 4.7 pg/mL, and the quantitative range was 4.7–300 pg/mL.

### Statistical analysis

All the statistical analyses were performed in SPSS 16 (SPSS Inc., Chicago, IL, USA). The Chi-square, Fisher's exact, *t*-test, Mann–Whitney, and partial correlation were applied to evaluate the association between PDL1 expression and clinicopathological characteristics. Histogram was plotted to estimate data distribution. $P < 0.05$ was considered statistically significant.

## Results

Overall data from forty gastric patients and forty healthy controls in Sari Imam Khomeini Hospital, Iran, were analyzed. The serum level of PDL1 was analyzed by ELISA. The mean age of the patients with gastric adenocarcinoma was 66.3 ± 14.6 and that of controls was 49.1 ± 12.1 ($P$ = 0.000). Tumor characteristics of the patients are shown in Table 1. The majority of tumors were intestinal type (75.5%) with poor growth of cancer cells (63.3%) located in the cardia (35%).

The study population was divided into two groups according to the expression level of PD-L1 (pg/mL); the mean (interquartile range) value of expression level of PD-L1 was 70.6 (36.6–127.3) in patients and 47.7 (29.9–92.1) in controls. Mann–Whitney test showed no statistically significant differences in PD-L1 expression between the two groups ($P$ = 0.106) [Figure 1]. There were considerable differences between the age of cases and controls, so the partial correlation was used to measure the correlation between age and PD-L1 expression level ($r$ = 0.165, $P$ = 0.147). The mean value

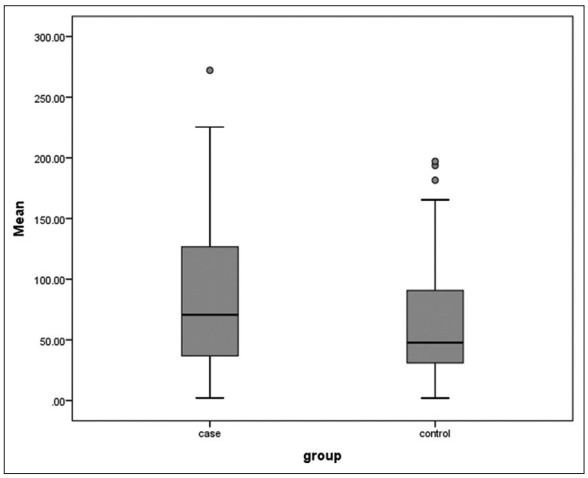


**Figure 1: Distribution of programmed death-ligand 1 expression level (pg/mL) in cases and controls**

of the PD-L1 level was used to categorize the samples into two groups (higher than 58.5 pg/mL and lower than 58.5 pg/mL) [Table 2]. The calculated severity index demonstrated a statistically significant association between the high expression level of PD-L1 and gastric cancer development ($P$ = 0.044, odds ratio [OR] = 2.77, 95% confidence interval [CI] = 1.12–6.86). The association between high and low levels of PD-L1 expression in gastric cancer patients and tumor characteristics is shown in Table 3. There were no significant differences between the high and low levels of PD-L1 expression and tumor characteristics.

**Table 1: Tumor characteristics in gastric cancer patients**

| Tumor characteristics | Frequency (%) |
|---|---|
| Histology subtype | |
| Intestinal | 29 (75.5) |
| Diffuse | 11 (27.5) |
| Differentiation* | |
| Well/moderately | 11 (36.7) |
| Poorly | 19 (63.3) |
| Tumor site | |
| Cardia | 14 (35) |
| Body | 6 (15) |
| Antrum | 13 (32.5) |
| Overlapping | 7 (17.5) |

*10 cases missing

**Table 2: Sample grouping according to high or low levels of programmed death-ligand 1 expression**

| Group | Low-level PDL-1 *n* (%) | High-level PDL-1 *n* (%) | *P* |
|---|---|---|---|
| Gastric cancer | 15 (37.5) | 25 (62.5) | 0.044 |
| Control group | 25 (62.5) | 15 (37.5) | |

PDL: Programmed death ligand

**Table 3: Association between high and low levels of programmed death-ligand 1 expression and tumor characteristics in gastric cancer patients**

| Tumor characteristics | Low-level PDL-1 *n* (%) | High-level PDL-1 *n* (%) | *P* |
|---|---|---|---|
| Histology subtype | | | |
| Intestinal | 13 (44.8) | 16 (55.2) | 0.158 |
| Diffuse | 2 (18.2) | 9 (81.8) | |
| Differentiation | | | |
| Well/moderately | 6 (54.5) | 5 (45.5) | 0.238 |
| Poorly | 5 (26.3) | 14 (73.7) | |
| Tumor site | | | |
| Cardia | 5 (35.7) | 9 (64.3) | 0.869 |
| Body | 2 (33.3) | 4 (66.7) | |
| Antrum | 6 (46.2) | 7 (53.8) | |
| Overlapping | 2 (28.6) | 5 (71.4) | |

PDL: Programmed death ligand

## Discussion

Programmed cell death protein 1 (PD-1) is one of the inhibitory immune checkpoint molecules that trigger T cell activation via the antigen-independent signaling pathway.[15] PD-1 (CD279) is a receptor of the PD-L1 which is expressed on $CD4^+$ and $CD8^+$ T cells, monocytes, natural killer T cells, B cells, and dendritic cells. Normally, the PD-1/PD-L1 pathway takes part in T cell immune responses to attenuate attack to the surrounding tissue and controls the development of autoimmunity responses against self-antigens.[15] The vital immune-regulatory role of PD-1/PD-L1 leads to preserving the balance in T cell activation, peripheral tolerance, and immunopathology by distributing inhibitory signals.[25] Binding of PD-L1 to PD-1 receptor results in the constitution of the inhibitory molecule, PD-1/TCR, and blocks T cell activation via stimulation of apoptosis, decreased cell proliferation, and inhibition of cytokine release.[15] The overexpression of PD-L1 was detected in gastric cancer tissue by immunohistochemical (IHC) staining of tumor tissues. It was also found that $FOXP3^+$ $T_{regs}$ infiltration is related to the upregulation of PD-L1 expression. Increased expression of $FOXP3^+$ $T_{regs}$ and PD-L1 affects gastric cancer progression and prognosis.[26] The role of PD-L1 expression in *Helicobacter pylori* infection and gastric cancer was investigated by Silva *et al*.[27] They reported the significant role of PD-L1 in persistent *H. pylori* infections and gastric cancer progression. Evidence also suggests significant associations between overexpression of PD-L1 and gastric cancer progression, prognosis, and clinicopathologic factors.[28,29] Indeed, PD1/PD-L1 pathway is introduced as an immuno-target for clinical application. Coutzac *et al*. focused on different clinical trials that had evaluated blocking the immune checkpoint of PD1/PD-L1 as immunotherapy for advanced gastric cancer.[30] They reported promising results of PD1/PD-L1 blockade in gastric adenocarcinoma. Therefore, molecular analysis can manage and improve personalized immunotherapy in gastric cancer. Gastric cancer is one of the most prevalent malignancies in both males and females in Iran.[31] The northern and northwestern provinces of Iran such as Mazandaran are considered high-risk regions.[6,32] Gastric cancer remains an important health issue in Iran, owing to the high incidence, mortality rates, and poor prognosis.[7] In the current study, we identified high levels of PD-L1 expression in the serum of gastric cancer patients compared with that of controls ($P$ = 0.044, OR = 2.77, 95% CI = 1.12–6.86). These results are consistent with those of Zheng *et al*. that found an elevated level of PD-L1 in advanced gastric cancer.[16] They also reported correlations between a high level of PD-L1 and tumor cell differentiation, lymph node metastasis, and other clinicopathological variables. We did not find any correlation between PD-L1 expression and tumor characteristics. Ma *et al*. used PD-L1 expression and its cutoff value in gastric cancer to predict its prognosis

by different antibody clones. They observed higher PD-L1 expression to be related to a poor 5-year survival rate. Furthermore, the higher density of PD-L1 + CD8 + T cells is associated with shorter survival time.[33] Another study evaluated PD-L1 expression levels in 132 surgically resected gastric cancer specimens by microarray and IHC methods, in which a positive correlation was seen between PD-L1 expression and poor survival in Stage II–III gastric adenocarcinoma patients. The study came to the same conclusion as ours, no association between PD-L1 expression and clinicopathologic features.[34] Yu *et al*. reported that PD-L1 overexpression in gastric cancer may be a helpful prognostic factor.[35] The PD-L1 mRNA expression was also detected in circulating tumor RNA of different types of cancer patients. The frequency of PD-L1 expression is reported to be significantly high in gastric cancer and other types of cancer.[36] The PD-L1 mRNA expression was assessed by quantitative reverse transcriptase-polymerase chain reaction (PCR) in blood samples of gastric cancer patients. The mRNA expression in advanced gastric cancer patients is significantly higher than that of those in early stages. They also demonstrated correlations between PD-L1 expression and the depth of tumor invasion, distant metastasis, stage, and disease prognosis.[37] Tamura *et al.* examined PD-L1 expression in a large number of gastric cancer patients ($n$ = 431). High expression of PD-L1 was proved in 30% of patients. Because PD-L1 was deeply related to tumor infiltration of PD1 and poor overall survival, they considered PD-L1 as an independent prognostic factor for patients with Stage II/III gastric cancer.[38] Morihiro *et al*. estimated combined markers of PD-L1 and MSI or CD8 + TILs in 283 gastric cancer patients. Their findings revealed a significant correlation between PD-L1 expression and tumor invasiveness, poor prognosis, and lymph node metastasis. Therefore, PD-L1 overexpression was suggested as an independent prognostic factor in gastric cancer.[18] The overexpression of PD-L1 was reported not only in gastric cancer but also in other malignancies such as non-small cell lung cancer,[39] oral squamous cell carcinoma,[40] colorectal cancer,[41,42] breast cancer,[43-45] ovarian cancer,[46,47] melanoma,[48,49] urothelial carcinoma,[50] bladder cancer,[51] and prostate cancer.[52-55] Overall, all gastric cancer studies reported the overexpression of PD-L1, which is consistent with the current findings. However, there is no full agreement in the correlation between PD-L1 expression and gastric cancer clinicopathological features. These differences may be due to diversity in race/ethnicity, etiology, geographical area, and tumor biology of different studies. Although most studies reported the same outcomes, there were some limitations that need to be clarified and determined: (1) sufficient large sample size can produce accurate results; (2) different methods (such as ELISA, quantitative real-time PCR, and IHC) and kits were used to identify the expression level of PD-L1, so the sensitivity and specificity of results varied in relation to one another; (3) both cancerous tissues and blood samples could be used to detect PD-L1 expression level; (4) every technique has several detection limits that cannot reflect the actual expression status of PD-L1; and (5) there is no validated and predictive cutoff value for detection of PD-L1-positive expression rate. Consequently, further carefully designed studies seem to be necessary in order to synchronize and standardize the methods of detection, detection limit, and protocols. Functional and randomized clinical trials must be verified for considering PD-L1 as a diagnostic tool and therapeutic target in immunotherapy.

## Conclusion

The identification of novel and effective biomarkers is vital to enhance the diagnosis of gastric cancer, evaluate the prognosis of GC, and found a novel and effective treatment decision for gastric cancer patients. This study described and validated the importance of the PD-1/PD-L1 pathway for the treatment of gastric cancer. PD-L1 overexpression was found to be a predictive factor in patients with gastric cancer. It is believed that the immune checkpoint blockade targeting the PD-1/PD-L1 pathway provides a potential strategy for effective immunotherapy in gastric cancer. An assessment of such studies on larger sample size and different types of cancer is also of interest.

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## Conflicts of interest

There are no conflicts of interest.

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