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Original Article

Programmed death-1 and PD-1 ligand-1 expression in early onset gastric carcinoma and correlation with clinicopathological characteristics

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Abstract: *Purpose:* Early onset gastric carcinoma (EOGC) is thought to be developed by a distinct molecular genetic profile of gastric carcinoma occurring at an older age. The aim of this study was to compare clinicopathological features and expression patterns of programmed death-1 (PD-1) and its ligand PD-L1 in young and older GC patients. *Methods:* Consecutive cases of GC presented to our hospital between 2007-2016 were collected. Clinicopathological features and overall survival data of EOGC patients (initially diagnosed at 40 years old or younger) were retrospectively reviewed from hospital records and compared with data of GC in the elderly (GC-E) (age ≥60 years). We investigated expression of PD-1 and PD-L1 by immunohistochemistry in both GC groups. Staining results were correlated with clinicopathological characteristics and overall survival. We then compared expression of PD-1 and PD-L1 with EOGC and GC-E. *Results:* Two thousands one hundred and forty five GC cases were collected. All 109 EOGC cases and 116 randomly selected GC-E cases were enrolled in the current study. Compared to GC-E patients, EOGC had a significantly higher proportion of female and gastric body location, a larger diameter, a higher proportion of low adhesive adenocarcinoma or diffuse gastric carcinoma, and a higher proportion of poorly differentiated tumors. PD-L1 was positively expressed in tumor cells in 43.6% (98/225) of GC cases and was weak to medium positive in most positive cases. The intensity of PD-L1 expression in tumor cells was significantly higher in EOGC (P=0.02). The positive rate of PD-L1 expression in tumor cells was significantly higher in EOGC than in GC-E (P=0.02). *Conclusion:* Clinicopathological characteristics of EOGC were distinctive from GC-E patients. EOGC had poorer prognosis compared to the older age group. Expression of PD-L1 in EOGC was significantly higher than in GC-E.

Keywords: Gastric carcinoma, early onset, PD-1, PD-L1, prognosis

Introduction

Gastric carcinoma (GC) is the second most common cause of cancer-related deaths worldwide [1]. Progressive GC has a very poor prognosis. Its 5-year survival rate is only 27.9%. GC mostly occurs in older adults, with an incidence peak among those older than 60 years [2, 3]. Over the past decade, GC incidence has declined every year, however, incidence of early-onset gastric carcinoma (EOGC), initially diagnosed at 40 years old or younger, is increasing gradually [4] and accounts for 2.7%-15% of GC [5, 6].

Studies have shown that EOGC is different from GC in the elderly (GC-E-, initially diagnosed at >60 years old) in many clinicopathological features and biological behaviors [7-11]. In EOGC, percentages of male and female patients are roughly equal (or women are more common) and a high percentage are diagnosed as poorly differentiated, diffuse, or infiltrative stages [6-8]. Some studies have shown that prognosis of EOGC is worse than that of GC-E [9-11]. Although some researchers have suggested that delay in diagnosis and more aggressive biological behavior has led to poor prognosis of EOGC [12, 13], more studies have indicated that different genetic changes are the main cause for poor prognosis of gastric cancer in young patients [14-16]. Further understanding of molecular differences between EOGC and GC-E will help elucidate new biomarkers to guide early diagnosis and prognosis assessment for EOGC.

In recent years, much attention has been paid to the role of programmed death-1 (PD-1) and its ligand-1 (PD-L1) in tumor immune escape, as well as to immunosuppressive drugs against these molecules. PD-1 is a member of the CD28 superfamily, expressed on surfaces of immune cells such as activated T-cells and B-cells [17]. PD-L1, one of the two specific ligands of PD-1, is widely expressed on the surface of antigen-presenting cells, activated T-cells, and B-cells. The binding of PD-1 and PD-L1 can arrest $CD8^+$ cytotoxic T-cells in the G_0/G_1 phase and even induce apoptosis and functional depletion, thereby protecting cells from attacks by cytotoxic T-cells and achieving protective immunity in the body. This mechanism, however, may also be used by tumor cells. In many solid tumors, the tumor cells show normal expression of PD-L1 [18, 19], thereby triggering the PD-1/PD-L1 pathway to inhibit ability of T-cells to kill tumor cells, thus, reducing the body's ability in tumor surveillance and facilitating immune escape by the tumor [20]. High expression of PD-L1 has been shown in 23.5%-63% of gastric cancer cells. Moreover, data from most researchers have associated high PD-L1 expression in GC cells with clinicopathological features indicative of poor prognosis [21-23]. Younger people tend be more immunocompetent and immune escape of tumor cells likely helps promote rapid tumor progression. Does activating the PD-l/PD-L1 pathway lead to poor prognosis of EOGC? So far, no studies have investigated PD-l/PD-L1 expression in EOGC.

In our present study, we collected EOGC cases, summarizing their clinicopathological features and overall survival (OS). Moreover, we observed PD-L1 and PD-1 expression in GC tumor tissue, surrounding interstitium, and tumor-infiltrating immune cells and comparatively analyzed EOGC and GC-E in terms of clinicopathological features, survival, and PD-1 and PD-L1 expression.

Materials and methods

Patient data

We collected data and specimens of progressive gastric adenocarcinoma among patients that underwent radical gastrectomies from July 2007-June 2016 at Beijing Chaoyang Hospital, affiliated to the Capital Medical University of China. All of the patients were Asian. Cases were divided into two groups by age: EOGC (initially diagnosed at ≤40-year-old) and GC-E (initially diagnosed at >60-year-old). We excluded cases that were outside of the age range, had incomplete data, insufficient tissue specimens for testing, or had distant metastases at the time of surgery. One hundred and nine cases of EOGC were collected and we also randomly selected 116 cases of GC-E as the control group.

We collected basic clinical information from patient hospital medical records and pathological information including age, sex, and distant metastases. All patients were followed up by telephone. OS was defined as time between initial diagnosis and death due to any cause or the last date of follow up. The cut-off follow up time was December 31, 2016.

We also collected tumor clinicopathological features such as tumor site, size, Borrmann type [24], WHO histological classification [24], Lauren's histological type [25], invasion depth, lymph node metastasis, vein invasion, lymphatic vessel invasion, perineural invasion, surrounding organ involvement, TNM stage, and number of infiltrated immune cells in tumors. We examined sites with the highest immune-cell densities between or around the tumors. Immune cells were counted in 3 high-power fields (HPF, ×400) selected at random and then averaged. Lymphoid follicles were excluded from the count. Numbers of immune cells per HPF were defined as low (<50 cells/HPF); and medium (50-200 cells/HPF) or high: (>200 cells/HPF).

Immunohistochemical staining

Immunohistochemical staining was performed in all cases, using a conventional manual method, on two paraffin-embedded tissue blocks selected from the tumor area and from normal gastric mucosa at least 2 cm from the tumor site. Mouse anti-human monoclonal antibodies included PD-1 (clone number: 4F10, 1:150 dilution, OriGene. Tech, MD, USA), PD-L1 (clone number: 9E12, 1:500 dilution, OriGene.Tech.), and HER2 (clone number: 4B5, 1:150 dilution, Roche, Shanghai, China). Two senior physicians interpreted immunohistochemically stained sections by the double-blind method.

Table 1. Comparison of clinicopathological features of gastric carcinoma between early-onset (EOGC) and elderly (GC-E) groups

	EOGC n=109 (%)	GC-E n=116 (%)	*P* value
Gender			0.010
Female	56 (51.4)	79 (68.1)	
Male	53 (48.6)	37 (31.9)	
Localization			0.001
Cardia/pylorus	11 (10.1)	32 (27.6)	
Gastric body	33 (30.3)	18 (15.5)	
Pyloric canal	64 (58.7)	63 (54.3)	
Diffuse	1 (0.9)	3 (2.6)	
Gross type			0.297
Polypoid	3 (2.8)	1(0.9)	
Fungating	15 (13.8)	11 (9.5)	
Ulcerated	74 (67.9)	91 (78.4)	
Infiltrative	17 (15.6)	13 (11.2)	
Tumour size			0.001
≤5 cm	60 (55.0)	89 (76.7)	
>5 cm	49 (45.0)	27 (23.3)	
WHO histologic type			0.011
Tubular	23 (21.1)	41 (35.3)	
Papillary	0 (0.0)	4 (3.4)	
Poorly cohesive	70 (64.2)	51 (44.0)	
Mixed	16 (14.7)	19 (16.4)	
Other rare types	0 (0.0)	1 (0.9)	
Lauren type			0.001
Intestinal	20 (18.3)	46 (39.7)	
Diffuse	72 (66.1)	46 (39.7)	
Mixed	17 (15.6)	23 (19.8)	
Grading			0.000
Well-differentiated	4 (3.7)	6 (5.2)	
Moderately differentiated	13 (11.9)	39 (33.6)	
Poorly differentiated	92 (84.4)	71 (61.2)	

Interpretational methods

Staining intensities and percentages were interpreted in tumor cells, stromal, and immune cells. For PD-L1 staining of tumor cells, membrane staining alone was taken as positive and further subjected to the following immunoreactivity scoring system (IRS) [27]. A: percentage of stained cells-0 (negative), 1 (≤1% positive), 2 (2%-10% positive), 3 (11%-50% positive), and 4 (>50% positive cells); and B: staining intensity-0 (no immunostaining), 1 (weak staining/light yellow), 2 (moderate staining/brown), and 3 (strong staining/dark brown). The addition of category A and B resulted in an IRS ranging from 0 and 7. A total score of ≤2 was considered negative and >2 was considered positive.

PD-L1 expression in interstitial cells was evaluated as present (positive) or absent (negative). Only percentages of positive cells were calculated for PD-L1 expression in tumor-infiltrating immunocytes (TII): 0 (negative), 1 (1%-5% positive), 2 (6%-20% positive), and 3 (>20% positive).

PD-1 staining in immune cells was evaluated in TII and lymph follicles and percentages of positive cells were calculated. Membrane staining was interpreted as positive. Scoring criteria were: 0 (negative), 1 (1%-5% positive), 2 (6%-20% positive), or 3 (>20% positive) points. Whole sections were observed at low magnification to find sites with highest densities of immune cells; 5 HPFs (×400) were then selected at random. Percentages of PD-l-positive cells in 100 lymphocytes were counted in each HPF and the average was taken as the positive percentage for each case.

Statistical analysis

Statistical analysis was mainly performed using IBM SPSS Statistics for Windows, version 20.0 (IBM SPSS, Somers, NY, USA). PD-l and PD-L1 data were analyzed using Chi-square test to compare groups and evaluate their associations with various histological and clinicopathological factors. $P<0.05$ (two-tailed) was considered statistically significant. 5-year survival rate was calculated using the Kaplan-Meier method. Survival curve

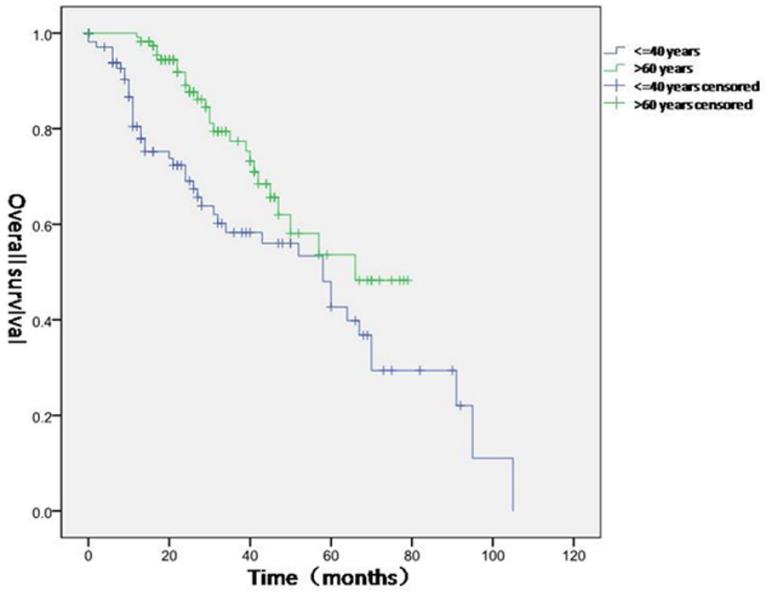

Figure 1. Comparison of overall survival of EOGC group and GC-E group. EOGC patients (blue) had poorer overall survival than GC-E patients (green) (P<0.05).

test was conducted using the two-stage test method.

Results

Patients

Out of 2,145 GC patients that underwent radical gastrectomy during the study time frame, 109 (5.0%) were EOGC patients (male: female was 1.06:1; median age: 36 years; range: 19-40 years). One hundred and sixteen cases of GC-E were selected at random for comparison (male: female, 2.1:1; mean age: 69 years; median age: 68 years: range: 60-89 years).

Comparison of clinicopathological features between EOGC and GC-E

The EOGC group had a significantly higher percentage of women than the GC-E group (31.9% vs 48.6%, *P*=0.010). Compared with GC-E, EOGC had a significantly higher percentage that occurred in the gastric body (P=0.003) and diameter of tumor >5 cm (P=0.001), whereas GC-E had lower percentage that occurred in the gastric cardiac (P=0.003). There were significant differences between the two groups in terms of histological type (P=0.011) and grade (P=0.001). No significant differences were found between the groups in ratio of gross tumor type, invasion depth, lymphatic metastasis, veins, lymphatic vessel, neural invasion, tumour stage, or HER2 expression (*P*>0.05 for all). Detailed data is shown in **Table 1**.

All patients were followed up for 6-108 months (median: 32 months). 5-year OS rate was 42.7% in the EOGC group and 53.6% in GC-E group. The difference in OS was statistically significant between the two groups (*P*=0.005, **Figure 1**).

Expression of PD-L1 and PD1

Although PD-L1 was expressed in tumor cells, interstitial cells, and tumor-infiltrating immune cells of GC, it was hardly expressed in non-neoplastic gastric epithelial cells. Of the 225 patients in both groups, PD-L1 membrane staining was observed to varying degrees in tumor cells of 156 (69.3%) patients (**Figures 2** and **3**). Among them, 96% of cases had ≤10% of PD-L1-positive tumor cells. According to the IRS system, PD-L1 positive cases accounted for 43.6% (98/225) of all cases. PD-L1 positive expression in immune cells was seen in 49.8% cases (112/225) and positive expression in surrounding stroma was seen in more than half of the cases (120/225, 53%).

Unlike PD-L1, PD-1 was not expressed in tumor cells but was diffused or scattered expressed in TII and lymphoid follicles in all cases with different degrees (median positive expression: 15%; range: 1%-80%) (**Figure 4**).

Comparison of PD-L1 and PD-1 expression in EOGC and GC-E

Expression intensity of PD-L1 in the EOGC group was significantly higher than in GC-E group (*P*=0.016). IRS score, based on cell numbers and staining intensity, also significantly differed between the two groups (*P*=0.044). Although EOGC group had a higher percentage of specimens with PD-L1-positive tumor cells (*P*=0.022), no significant difference was found between the two groups with regard to PD-L1 expression in the stroma, immune cells, or normal gastric tissue (**Table 2**).

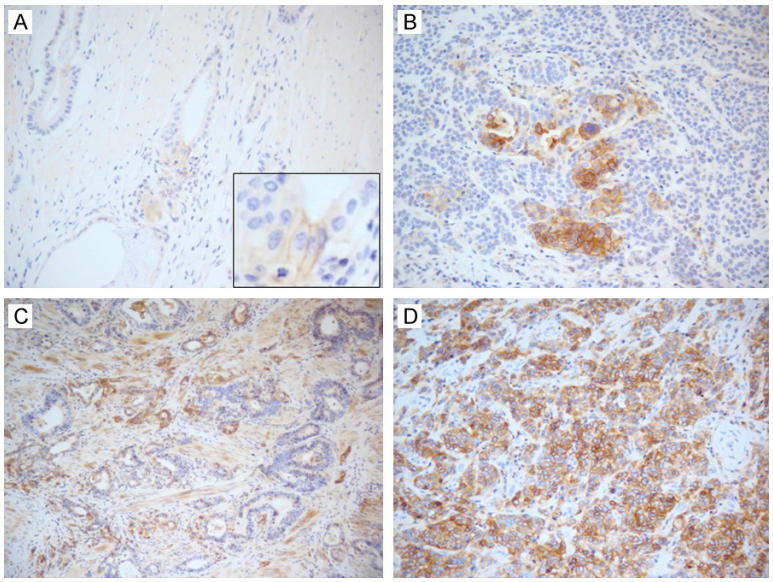

Figure 2. PD-L1 expression in gastric carcinoma tumor cells shown by brownish-yellow cell membrane. A. 1 point (≤1% positive tumor cells); lower right corner: zoomed area of positive cells; B. 2 points (2%-10% positive tumor cells); C. 3 points (11%-50% positive tumor cells); and D. 4 points (>50% positive tumor cells) (Immunohistochemical staining; A and C. 100×; B and D. 200×).

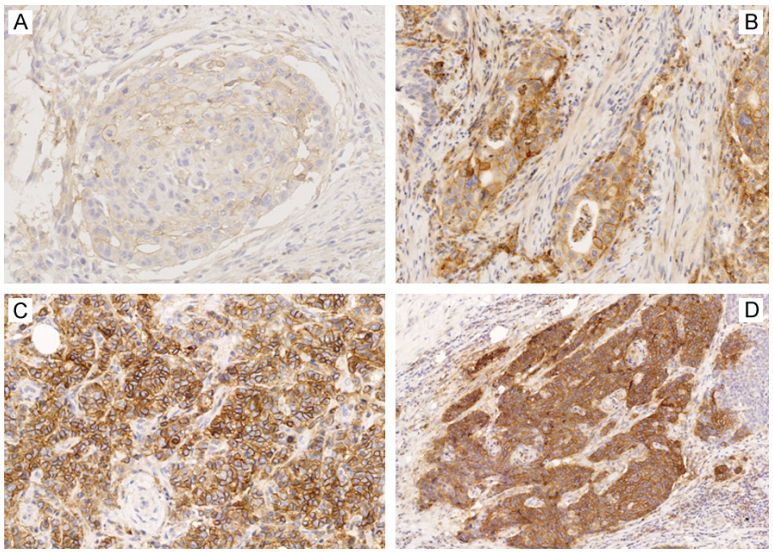

Figure 3. Intensity score of PD-L1 expression in tumor of gastric carcinoma. A. 1 point, weak staining; B. 2 points, moderate staining; C and D. 3 points, strong staining (Immunohistochemical staining; 400×).

Discussion

EOGC accounted for only 5.0% of all gastric cancers in our cohort study. This is close to the results of previous studies of EOGC [9, 26-28] and, thus, did not suggest an increasing trend in EOGC. Recently, an epidemiological survey and final outcome of U.S. National Cancer Institute showed a markedly increasing trend in incidence of EOGC with age of onset between 25 and 39 years, from 0.27 to 0.45 per 100,000 individuals per year. However, in our study, all included patients had noncardia and oesophagogastric junction carcinoma and were selected from a Western population whose incidence is significantly lower than that of Asians [29]. In contrast, epidemiological data from South Korea has shown a significant decline in incidence of EOGC. At present, no epidemiological surveys of EOGC among Chinese population have been performed. Chinese researchers Zhou et al. [28] collected 4,671 cases of gastric cancer diagnosed by surgery or endoscopic biopsy in the decade from 2004 to 2014. EOGC accounted for 3.2% (152 cases), similar to data presented in our present study. All of our patients had progressive GC and underwent radical gastrectomy. Since we did not include early and stage IV cases, the data may not provide the actual percentage of EOGC in GC.

Our present study and other research data has shown a higher percentage of women with EOGC than with GC-E [10]. A study found that the risk of developing gastric cancer was higher in patients after ovarian resection and suggested that development of GC was associated with the role of estrogen [30]. Subsequently, this was confirmed by researchers from two countries [31, 32]. Animal experiments have shown that estrogen can block the cell cycle, leading to emergence of numerouspolyploidcellsandmalignanttransformation [33]. However, the molecular mechanism

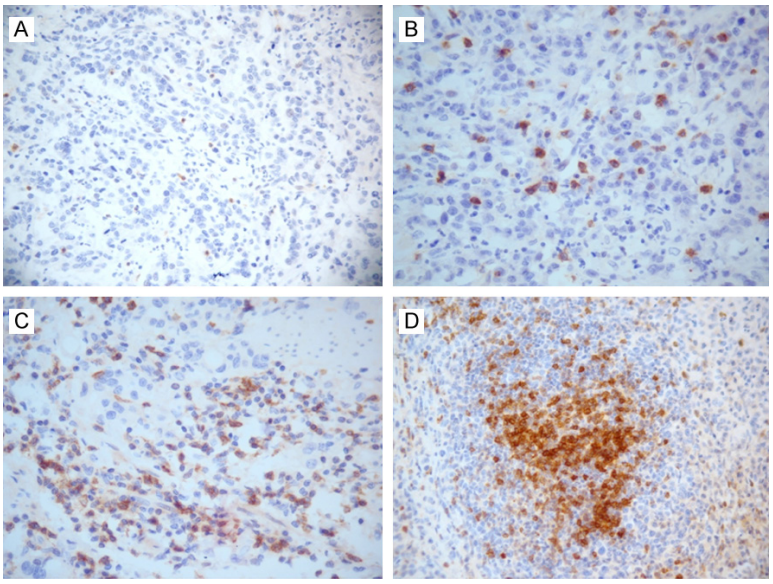

Figure 4. PD1 expression in immune cells and lymphoid follicles of gastric carcinoma. (A) 1%-5% positive cells; (B) 6%-20% positive cells; (C) >20% positive cells; and (D) PD-I expression of some cells within lymphoid follicle (Immunohistochemical staining; A. 200×; B-D. 400×).

underlying such an association and the putative role of estrogen in women with EOGC awaits further investigation. Moreover, our results demonstrate that the percentage of early gastric cancer located in the gastric body is significantly higher in EOGC than that of gastric cancer in the elderly, similar to findings reported in the literature [8]. This further suggests that EOGC is more associated with genetic changes and family genetic factors than GC and less associated with environmental stimuli. Due to limitations of medical history collection, we did not analyze family medical histories of patients in our cohort. Reportedly, ~25% of early gastric cancer is derived from familial factors and this percentage is significantly higher than for family-related gastric cancer in the elderly [26]. E-cadherin gene (*CDH1*) is associated with hereditary diffuse gastric cancer [34]. However, only 30% of families with inheritance-associated diffuse gastric cancer carry *CDH1* germline mutations. The mechanism of development remains unclear in a large portion of early-onset gastric cancer. Numerous studies have also shown that diffuse histological type is more common in EOGC [6]. Similarly, our study also showed that the percentage of diffuse or low-adherence gastric cancer was significantly higher in the EOGC group than in the GC-E group, whereas the latter had a higher percentage of intestinal gastric cancer. One reason for the association between age and histological type may be that gastric cancer develops through different pathways in these two age groups. Gastric cancer development is commonly considered to be promoted by long-term stimulation of chronic gastritis. Gastric mucosa is repeatedly repaired, followed by gastric mucosal atrophy (atrophic gastritis) and intestinal metaplasia, and subsequent occurrence of gastric epithelial dysplasia and carcinogenesis [43]. However, prevalence of diffuse gastric cancer in young patients is more likely associated with genetic changes such as E-cadherin gene mutations [6].

Whether prognoses of EOGC and GC-E differ has been debated for years. More data shows that younger patients with gastric cancer have worse prognosis and survival [8, 35]. Our data showed that the EOGC group had a larger tumor maximal diameter and histological types were also dominated by low-adhesion adenocarcinoma (WHO classification) or diffuse (Lauren classification) gastric cancer, with a higher proportion of poorly differentiated cancers. All of these differences in clinicopathological features indicate more aggressive behavior for EOGC. Similarly, Japanese researcher Maehara [36] reported a comparative study of clinicopathological data from gastric carcinoma patients aged younger than 40 years and older than 70 years. GC-E was found to have smaller and higher differentiated tumors than gastric carcinoma in younger patients. Similar results have been obtained by many researchers [37, 38]. Some researchers believe that delayed diagnosis leads to these differences, as young people often feel symptoms less intensely and are less likely to undergo routine physical examinations. However, pathological features (except for tumor size) could not be explained by growth time in our data. We, therefore, believe that EOGC and GC-E have different tumorigenic mechanisms and genetic characteristics, one of which may be PD-L1.

Sun et al. [39], for the first time, reported PD-L1 expression in gastric cancer and observed 102

Table 2. Comparision of expression of PD-L1 and PD-1 in EOGC and GC-E

	EOGC n=109 (%)	GC-E n=116 (%)	*P* value
PD-L1 Quantity Score			0.139
0 (0%)	33 (30.3%)	36 (31.0%)	
1 (≤1%)	54 (49.5%)	63 (54.3%)	
2 (2%-10%)	20 (18.3%)	10 (8.6%)	
3 (11%-50%)	2 (1.8%)	6 (5.2%)	
4 (>50%)	0 (0%)	1 (0.9%)	
PD-L1 Intensity Score			0.016
0 (negative)	33 (30.3%)	36 (31.0%)	
1 (weak)	25 (22.9%)	39 (33.6%)	
2 (medium)	40 (36.7%)	22 (19.0%)	
3 (strong)	11 (10.1%)	19 (16.4%)	
PD-L1 IRS (Sum Score)			0.044
0	33 (30.3%)	36 (31.0%)	
2	20 (18.3%)	36 (31.0%)	
3	32 (29.4%)	18 (15.5%)	
4	20 (18.3%)	15 (12.9%)	
5	3 (2.8%)	6 (5.2%)	
6	1 (0.9%)	4 (3.4%)	
7	0 (0.0%)	1 (0.9%)	
Tumor cells PD-L1 expression			0.022
Negative (IRS ≤2)	53 (48.6)	74 (63.8)	
Positive (IRS >2)	56 (51.4)	42 (36.2)	
Stroma PD-L1 expression			0.269
Absent	55 (50.5)	50 (43.1)	
Present	54 (49.5)	66 (56.9)	
TII PD-L1 expression			0.089
Negative	19 (17.4)	9 (7.8)	
1-5%	43 (39.4)	44 (37.9)	
6-20%	28 (25.7)	32 (27.6)	
>20%	19 (17.4)	31 (26.7)	
Normal gastric epithelial PD-L1 expression			0.411
Negative (0-2)	105 (96.3)	109 (94.0)	
Positive (3-7)	4 (3.7)	7 (6.0)	
TII PD1 expression			0.050
1-5%	53 (48.6)	45 (38.8)	
6-20%	35 (38.8)	32 (27.6)	
>20%	21 (19.3)	39 (33.6)	
Lymphoid follicles PD-1expression			0.245
Negative	0 (0.0)	3 (2.6)	
1-5%	55 (50.5)	53 (45.7)	
6-20%	39 (35.8)	38 (32.8)	
>20%	15 (13.8)	22 (19.0)	

EOGC: early onset gastric carcinoma (initial diagnosis ≤40 years); GC-E: gastric carcinoma in elderly (initial diagnosis >60 years); TII: tumor-infiltrating immunocytes.

cases of gastric adenocarcinoma. Immunohistochemical staining revealed high PD-L1 expression in 42.2% of the cases, with no positive expression in non-neoplastic gastric epithelium and weak expression in gastric adenoma. They believed that PD-L1 expression was associated with shorter survival. Subsequently, several studies have reported on PD-L1 expression in gastric cancer, with positive rates ranging from 30.1% to 63% [40, 41]. Moreover, we found PD-L1 to show generally focal and low expression. Currently, studies have shown that expression of PDL1 in tumors is related to the effect of PD-1/PD-L1 inhibitor drug therapy [42]. If PD-L1 is used as a biomarker to guide treatment, the characteristic of its focal expression needs to be paid more attention. Whether biopsy specimens can be used to test for PD-L1 expression remains to be studied. This may also be why some clinical trials failed to prove an association between tumor PD-L1 expression and therapeutic effectiveness. These clinical trials often chose patients with advanced cancers, most of whom had no opportunity for surgery and whose PD-L1 detection was often performed using endoscopic biopsy specimens. This also implies that tissue-array is unsuitable for testing of PD-L1 expression. In a study of 345 patients with gastric cancer, when analyzed by

tissue-array, PD-L1 expression was only seen in 1.7% of cases but 35.6% showed PD-L1 expression in parallel analysis of the whole section [43].

Here, we focused on differences in PD-L1 and PD-1 expression between EOGC and GCE. Despite having similar numbers of positive cells (P=0.169), the EOGC group showed higher intensity of PD-L1 expression (P=0.016) and had a higher percentage of positive PD-L1 expression (P=0.022), as shown by scores based on cell numbers and expression intensity. According to literature review, this is the first study of PD-L1 and PD-1 expression in young patients with gastric cancer. Although several studies have shown that expression of PD-L1 and PD-1 is not related to age in gastric cancer, very few cases of EOGC were included and 60-70 years of age was often the cut-off boundary for "younger" patients. Thus, those studies did not actually analyze gastric cancer in young people. Additionally, several studies have shown a higher percentage of PD-L1 expression in gastric cancer at a younger age, however, the difference was not significant, perhaps due to sample size or grouping [44]. With regard to high PD-L1 expression in EOGC, a possible reason is that high PD-L1 expression and EOGC are both associated with some clinicopathological features that are indicative of poor prognosis, such as tumor size and lymph node metastasis. This also indicates that immune escape mediated by high PD-L1 expression may be another reason for poor prognosis for EOGC and may reflect a gastric cancer cause that is more common in younger patients. Reportedly, EOGC cell molecular phenotypes such as cell adhesion molecules, E-cadherin, and mucin differ from those of GC-E [16]. Our results provide a new study point for analysis of gastric carcinogenesis in young people. Moreover, PD-1/PD-L1 pathway inhibitors are the subject of increasing research, some entering phase III of clinical trials. Several studies have shown that high PD-L1 expression is associated with therapeutic effectiveness [37]. Whether PD-l/PD-L1 inhibitors would be more effective against EOGC should be tested and verified by more clinical trials.

Conclusion

In this study, we have shown that the percentage of EOGC is relatively low among GC cases. EOGC shows different clinicopathological features and shorter OS compared with GC-E. PD-L1 is highly expressed in GC tissue. Its expression is significantly higher in EOGC tumor tissue than in GC-E.

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Disclosure of conflict of interest

None.

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