Prognostic value of PD-L1 expression and CD68 macrophages in tumor nest of patients with primary gastric cancer

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Abstract. The programmed death receptor 1/programmed death receptor ligand 1 axis (PD-1/PD-L1) is involved in tumor immune escape and is a potential prognostic biomarker and anti-tumor immunotherapy target in patients with gastric cancer (GC). However, the results of studies obtained in recent years have been inconsistent. The present study aimed to determine the possible predictive significance of PD-L1 in conjunction with three proteins linked with PD-L1 regulation in patients with primary GC. In the present study, the PD-L1, human epidermal growth factor receptor 2 (HER2), cluster of differentiation (CD)133 and microphage-associated CD68 expression levels were identified by multiplexed immunohistochemistry and assessed by automated pathological analysis system in 93 GC tumors and neighboring normal tissues arrayed on the same tissue microarray. All four proteins were statistically analyzed in relation to the clinicopathological characteristics. The expression levels of HER2, CD133 and CD68 were considerably higher in cancer tissues compared with neighboring normal tissues (P<0.05), however, the reverse trend was detected for PD-L1 expression (P=0.0577), particularly in tumor nest (TN; P<0.05). There was no significant correlation between the HER2 and CD133 expression levels and clinicopathological factors. However, significant relationships were found between PD-L1 expression and the TNM stage, pathological differentiation and survival status of patients (P<0.05). Moreover, survival time was prolonged in individuals with elevated PD-L1 expression in TN and GC tissues, but no significant correlation was identified (P=0.0881). The CD68 expression level in tumor stroma, but not in TN, was significantly correlated with poor pathological differentiation in patients with GC (P<0.05). However, PD-L1+CD68+ macrophages were strongly related to lower tumor size (diameter <5 cm), early TNM stage (stage I+II), good pathological differentiation and overall survival in TN (P<0.05). In conclusion, PD-L1+CD68+ macrophage infiltration in TN might be a potential indicator of prognosis in patients with primary GC and merits further investigation.

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Introduction

Gastric cancer (GC) is the most prevalent malignant tumor of the digestive tract, with over 1 million new cases identified annually and China accounts for ~50% of the global GC incidence (1). Despite a decline in incidence and mortality worldwide during the last five years, GC remains a serious public health concern in China and across the globe (2). Therefore, prevention and new therapeutic strategies are urgently needed.

At present, it is widely believed that the occurrence of GC results from a combination of cytogenetic variation accumulation and tumor immune evasion (3). The programmed death receptor 1/programmed death receptor ligand 1 (PD-1/PD-L1) axis is crucial for tumor immune escape and PD-L1 overexpression is predictive of poor prognosis (4-6). Immune checkpoint inhibitors (ICIs) targeting PD-1/PD-L1 are often used to treat patients with advanced GC and PD-L1 overexpression. However, increasing evidence has suggested contradictory findings (7-12). Therefore, the usefulness of PD-L1 expression in GC patient prognosis requires additional investigation.

PD-L1 is extensively expressed in several cell types, including tumor cells and activated immune cells associated with tumors, such as dendritic cells (DCs), macrophages and monocytes. Studies have reported that the prognosis in patients with tumors is not only related to PD-L1 expression but also to the tumor microenvironment (TME) immune cells, especially in tumor-infiltrating T cells (TILs) (13-15). Increasing evidence demonstrates that tumor-associated macrophages (TAMs) are one of the most important immune cells in TME

and serve crucial roles in tumor occurrence and progression. Macrophages in TME can secret cytokines to regulate immune response against cancer through the PD1/PD-L1 axis of tumor cells or immune cells. Therefore, macrophages in combination with PD-L1 may be a potential prognosis indicator of patients with GC. However, studies of GC in this area are still limited and the results obtained are inconsistent. This may be related to macrophage plasticity, spatial distribution and the methods used to evaluate PD-L1 expression. Based on this consideration, in the present study, mIHC combined with an automated pathological analysis system was used to quantify the expression and spatial distribution of PD-L1 and cluster of differentiation (CD)68, a biomarker of pan-microphages, in primary GC and paired adjacent normal tissues. In addition, to optimize PD-L1-based prognostic biomarkers in patients with GC, two other proteins, human epidermal growth factor receptor 2 (HER2) and CD133, closely related to the regulation of PD-L1 expression, were also evaluated in this study (16).

Materials and methods

Patient characteristics. The HStnA180Su17 tissue microarray (TMA; Xinchao Company) consisted of paired gastric adenocarcinoma tissues and neighboring normal tissues (≥5 cm from tumor tissues) derived from 94 patients with GC, of which a cohort of 83 cases with integral information and 10 cases with censored data (gastric adenocarcinoma tissues only, no adjacent normal tissues) was taken into final analyses. One case lost with multi-cutting was excluded. Patients had had surgery between March 2007 and February 2008 and follow-up information was provided between March 2007 and July 2011. Prior to the operation, patients did not receive routine radiotherapy or chemotherapy treatments. The Institutional Ethics Committee of the First People's Hospital of Yunnan Province approved the research (approval no. KHLL2019-KY037). Every process was carried out under regulations (17). Table I summarizes the clinicopathological features of patients.

Samples and TMA preparation. Based on the pathological diagnosis of each tissue, TMAs were produced. At least two independent pathologists examined formalin-fixed, dehydrated, paraffin-embedded hematoxylin and eosin (H&E) stained tumor and nearby normal tissue samples. For fixation, cut tissue blocks were fixed in 4% paraformaldehyde (PFA) at a volume ratio of 1:7 tissue block to 4% PFA for 24 h. The fixed tissues were dehydrated with graded alcohol (30 min each in 85, 90 and 100% ethanol) and cleared with xylene for 20 min. Tissue was placed in a mold with paraffin wax. Subsequently, the mold is moved to a bench at -10˚C. The paraffin wax solidified rapidly and the tissue was fixed in it. The representative tumor regions of each donor block were then identified. Next, 1 mm diameter core cylinders were punched from each of these regions and placed in a recipient paraffin block to create TMAs. Finally, 4 mm-thick TMA sections were sliced and mounted on slides coated with poly-L-Lysine for multiplexed immunohistochemistry (mIHC) analysis.

Fluorescent mIHC of TMA. For mIHC labeling, the antibody concentrations for HER2 (1:200; cat. no. BX50015; PerkinElmer, Inc.), PD-L1 (1:200; cat. no. BX00005; PerkinElmer, Inc.), CD133 (1:100; cat. no. 86781; Cell Signaling Technology, Inc.) CD68 (1:1,500; cat. no. BX50031; PerkinElmer, Inc.) were optimized and a spectrum library was constructed using single-stained slides. Then, using the PANO 7-plex IHC kit (cat. no. 0004100100, Panovue), multiplex multispectral imaging of the identified proteins and immunofluorescence staining were performed on a single TMA slide. The slides were heated for ≤1 h at 60˚C in a dry oven and deparaffinized three times for 10 min using xylene. The slide was rehydrated in a sequence of 100, 95 and 85% alcohols to distilled water. After washing for 5 min in distilled water, antigen retrieval was conducted in citrate buffer (pH 6.0) for 15 min using microwave treatment and cooled to room temperature. After washing and blocking with a 3% H_2O_2 blocking solution for 10 min, the slide was stained with the primary antibody. The Opal Polymer HRP Ms+Rb Kit (PerkinElmer, Inc.) was used for detection after overnight incubation at 4˚C with each primary antibody. The slide was then treated with a 1:100 dilution of tyramide (TSA)-conjugated fluorophore (TSA Fluorescence Kit; Panovue). The TSA was then vacuumed off and the slide was washed twice with 1X TBST for 3 min before the subsequent staining step. For each additional marker, the procedure was repeated by microwave heat-treating the slide for retrieving antigen, followed directly by one primary antibody staining in each cycle, ordered as HER2, CD133, PD-L1 and CD68, respectively and then by the aforementioned downstream procedures. After labeling all human antigens, nuclei were counterstained with 4'-6'-diamidino-2-phenylindole (DAPI; cat. no. D9542; MilliporeSigma).

Multispectral imaging and scoring multispectral images. At x20 magnification, ≤5 non-overlapping image fields were collected for each slide using the Vectra system (PerkinElmer, Inc.) and processed using inform softwarev2.3.0 (PerkinElmer, Inc.). Briefly, tissue autofluorescence and each fluorescein spectra were extracted from unstained and single-stained sections images, respectively. The extracted images were then used to create a spectrum library for images of sections with autofluorescence removed. For scoring, the expression of CD68, CD133, HER2 and PD-L1 levels were assessed using H-score as previously described (18).

Human protein atlas database (HPA) and gene expression profiling interactive analysis database analysis (GEPIA). HPA and GEPIA are two online databases. The PD-L1 expression levels in cellular components of GC tissues and link with 5-year survival were analyzed using the Human Protein Atlas (HPA) website (http://www.proteinatlas.org). GEPIA (http://gepia.cancer-pku.cn) is an online database making gene expression profiling and interactive analyses with cancer and normal samples. In the present study, GEPIA was used to verify the correlation between PD-L1 and CD68 protein levels and the link with disease-free survival of primary GC patients. The Spearman correlation statistical method was used to calculate the correlation coefficient.

Statistical analysis. The Mann-Whitney U test determined the expression differences of the four detected proteins in studied specimens. Clinical correlation was calculated by Spearman analysis. The Kaplan-Meier analysis assessed overall survival

Table I. Clinicopathological characteristics of a cohort of 93 patients with primary GC.

Clinical characteristics		Sex		Age		TNM stage		Lymph node		Differentiation		Disease status	
Marker	N (%)	χ^2	P-value	χ^2	P-value	χ^2	P-value	χ^2	P-value	χ^2	P-value	χ^2	P-value
Sex		1.000	-	0.043	0.532	2.245	0.204	0.025	0.873	0.059	0.808	0.818	0.366
Male	71 (76.3)												
Female	22 (23.7)												
Age (years)		0.043	0.532	1.000	-	0.788	0.375	0.012	0.914	0.527	0.468	0.075	0.784
≤60	27 (29)												
>60	66 (71)												
TNM stage		0.788	0.375	0.788	0.375	1.000	-	44.214	<0.001	9.139	0.003	14.190	<0.001
Early (I+II)	34 (36.6)												
Late (III+IV)	59 (63.4)												
Lymph node		0.025	0.873	0.012	0.914	44.214	<0.001	1.000	-	7.423	0.006	13.705	<0.001
Negative	20 (21.5)												
Positive	73 (78.5)												
Differentiation		0.059	0.808	0.527	0.468	9.139	0.003	7.423	0.006	1.000	-	1.951	0.163
Well	36 (38.7)												
Poor	57 (61.3)												
Disease status		0.818	0.366	0.075	0.784	14.190	<0.001	13.705	<0.001	1.951	0.163	1.000	-
Survival	19 (20.4)												
Succumbed	74 (79.6)												
Tumor size (cm)		0.536	0.595	0.316	0.627	10.078	0.002	7.503	0.012	0.746	0.439	3.381	0.092
L<5 cm	28 (30.1)												
L≥5 cm	65 (69.9)												

P<0.05 was considered to indicate a statistically significant difference. TN, tumor nest; TS, tumor stroma.

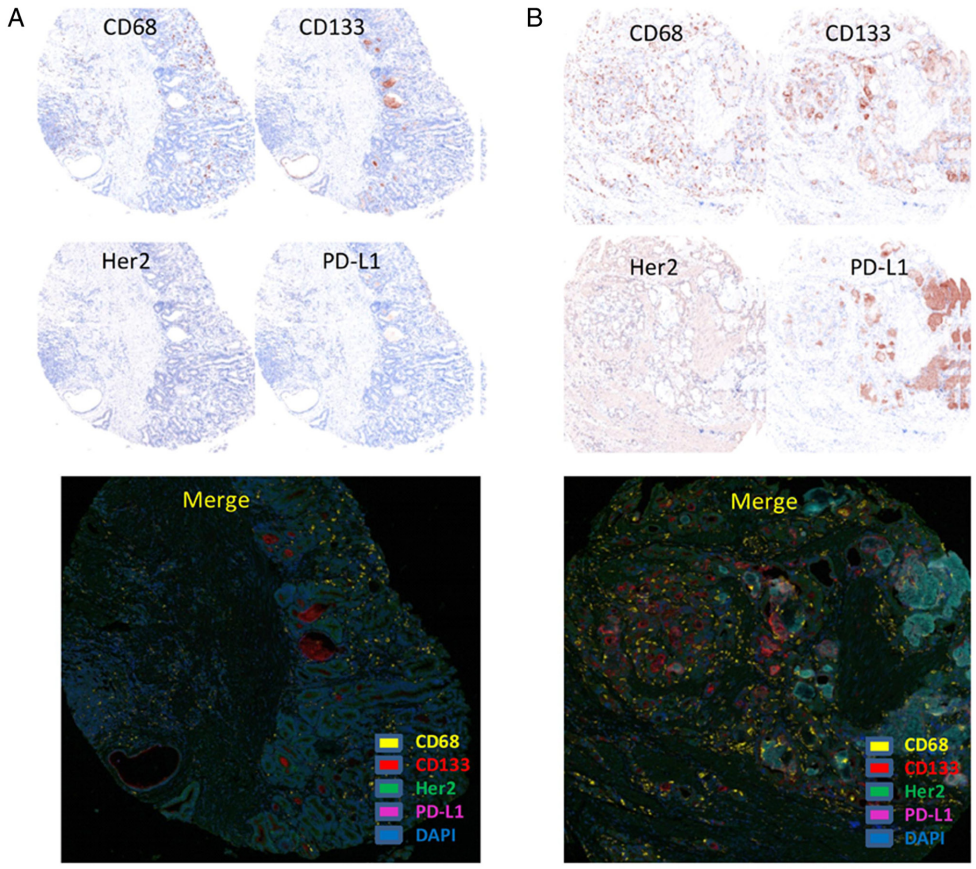

Figure 1. Representative mono- and multi-color overlapping fluorescence images of the detected proteins in tumor and adjacent normal tissues. (A) Representative images of single and multiple staining obtained from normal paracancerous tissues of primary patients with GC. (B) Representative images of single and multiple staining obtained from normal paracancerous tissues of tumor tissues of primary patients with GC. Images at 20x magnification. GC, gastric cancer; CD, cluster of differentiation; HER2, human epidermal growth factor receptor 2; PD-L1, programmed death receptor ligand 1; DAPI, 4'-6'-diamidino-2-phenylindole.

(OS) rates and the log-rank test was performed to plot survival curves. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software; Dotmatics) and SPSS 22.0 (IBM Corp.).

Results

Demographics characteristics. There were 71 male patients with an average age of 66±10 years and 22 female patients with an average age of 64±11 years. Other basic information about patients, such as sex, pathological differentiation, lymph nodes, TNM stages and survival status, is listed in Table I. Statistical analysis of the clinical variables showed no significant correlation between either age or sex and lymph node metastasis (LNMs), survival rate, pathological differentiation, or TNM stage of patients with GC (P>0.05). Pathological differentiation and tumor size were significantly correlated with the LNMs and TNM stages of patients. However, there was no statistically significant difference in the OS of the patients (P>0.01).

Differential expression of the potential proteins between the tumor tissues and adjacent normal tissues. HER2, CD133 and PD-L1 antigens were mainly expressed on the cell membrane, particularly CD133 expression on glandular-luminal tumor cell membrane surface (luminal expression, L-type), but CD68 is found in both the cell membrane and cytoplasm (Fig. 1). Statistical analysis revealed that the HER2, CD133 and CD68 expression levels were considerably greater in tumor tissues than in surrounding normal tissues (P<0.05), while no substantial link was observed for PD-L1 expression (P=0.0560; Fig. 2A). The spatial distribution revealed that the CD133, HER2 and CD68 expression levels were greater in TN and tumor stroma (TS) than in the neighboring normal tissues (P<0.05). In contrast, PD-L1 expression was considerably lower in TN of tumor tissues than in adjacent areas of normal tissue, although no significant difference was observed in TS. (Fig. 2B and C). In addition, although the proportion of double-positive and triple-positive antigens is low, there is also a significant difference in the expression level between cancer and adjacent normal tissues (Table II).

Correlation of the detected proteins expressed in GC tissues. The correlation analysis showed that the PD-L1 and CD68 and HER2 and CD68 expression were positively correlated in both the whole tumor tissue and the TN (P<0.01). In the TS, only the HER2expression was positively correlated with CD68 (P<0.01), while no significant correlation was found between the expression of PD-L1 and CD68 (P>0.05; Table II).

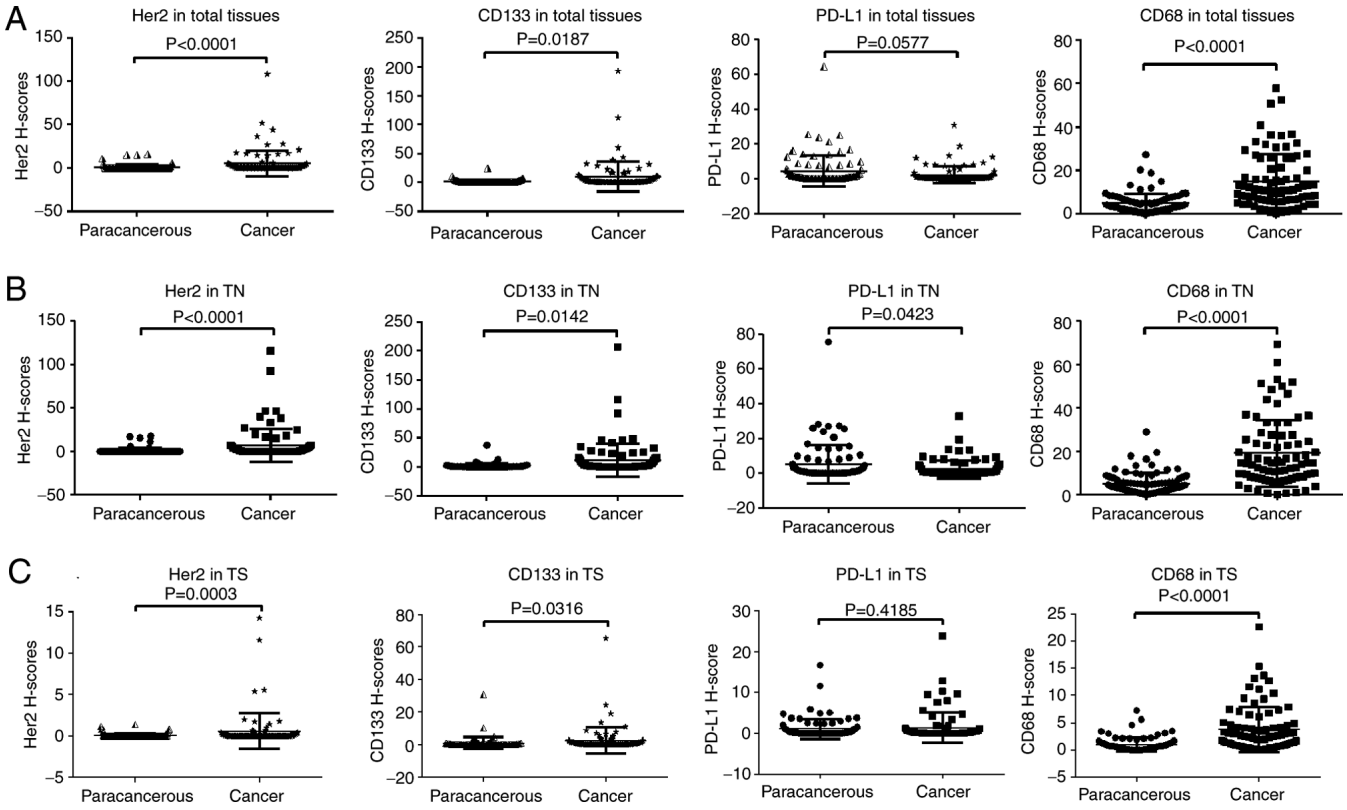

Figure 2. Different spatial distribution of HER2, CD133, CD68 and PD-L1 in gastric cancer tissues and paired adjacent normal tissues of a cohort of 84 patients with primary GC. (A) Comparing expression of single stained proteins in whole tissues. (B) Comparing expression of single stained proteins in tumor nests. (C) Comparing expression of single stained proteins in stromal areas. All the data were analyzed with the Mann-Whitney U test. HER2, human epidermal growth factor receptor 2; CD, cluster of differentiation; PD-L1, programmed death receptor ligand 1; GC, gastric cancer.

The relationship between the detected proteins and clinical features. Although the CD133 and HER2 proteins expression levels were significantly elevated in GC tissues than in neighboring normal tissues, there was no significant correlation between the expression levels of these two proteins in tumor tissues and clinical variables such as sex, age, survival status, pathological differentiation, TNM stage and LNMs. The PD-L1 expression level had no significant correlation with sex and age but had a significant correlation with TNM stage and pathological differentiation no matter in whole tumor tissues or TN ($P<0.05$), with a marginally significant association with survival status in TN ($P=0.056$) and significantly associated with pathological differentiation in TS (Fig. 3A-C). Survival curve analysis revealed that patients with GC with high PD-L1 expression survived longer than those with low expression; however, the difference between the two groups was not statistically significant ($P=0.088$; Fig. 4A and B). The degree of CD68 expression in TS was substantially associated with pathological differentiation of patients with GC ($P=0.0376$), while there was no significant association in TN (Fig. 3C). However, PD-L1^{+}CD68^{+} macrophages in TN were significantly related to tumor size, TNM stages, pathological differentiation and survival status ($P<0.05$; Fig. 3D). To obtain support for the results of the present study from clinical samples, the correlation of PD-L1 and CD68 expression in tumor tissues from patients with GC was analyzed with clinical outcomes using two public online databases (HPA and GEPIA). PD-L1 was expressed on fibroblasts, endothelial cells, neutrophils and macrophages of GC tissues (Fig. 5A). Moreover, PD-L1 expression was significantly correlated with CD68 (Fig. 5B). Although there was no statistical difference between PD-L1 expression and disease-free survival (DFS) or 5-year survival, patients with high PD-L1 expression had a prolonged survival compared with patients with low PD-L1 expression (Fig. 5C and D). These results are consistent with the present study.

Discussion

At present, PD-L1 expression is an important molecular marker for the prognosis of GC and the selection of targeted PD-1/PD-L1 ICIs (4-6). However, the results reported in studies are often inconsistent (7-12). The inconsistent results are related to the choice of assays and assessment methods in different studies on the one hand and suggest that the molecular markers for prognosis of patients with GC based on PD-L1 still need to be optimized on the other hand.

In the present study, mIHC and an automated pathological analysis system were applied to detect the expression level and spatial distribution of PD-L1, CD68, CD133 and HER2 in tumor tissues of primary patients with GC arranged on TMA and its potential prognostic value was explored.

In recent years, mIHC has emerged as an important technique in the field of pathology research, allowing for the detection of multi-targets *in situ* on cell or tissue samples, as well as the quantitative pathological analysis of spatial

Table II. Spearman analysis for the correlation of each protein distributed in tumor tissues.

		CD133		HER2		PD-L1		CD68	
Measured region	Marker	r	P-value	r	P-value	r	P-value	r	P-value
Total	CD133	1.000	-	0.055	0.598	-0.161	0.122	0.065	0.534
	HER2	0.055	0.598	1.000	-	0.062	0.555	0.446	<0.001
	PD-L1	-0.161	0.122	0.062	0.555	1.000	-	0.345	0.001
	CD68	0.065	0.534	0.446	<0.001	0.345	0.001	1.000	-
TN	CD133	1.000	-	0.048	0.649	-0.162	0.120	-0.015	0.884
	HER2	0.048	0.649	1.000	-	-0.065	0.537	0.377	<0.001
	PD-L1	-0.162	0.120	0.065	0.537	1.000	-	0.368	<0.001
	CD68	-0.015	0.884	0.377	<0.001	0.368	<0.001	1.000	-
TS	CD133	1.000	-	-0.009	0.932	-0.136	0.193	0.177	0.090
	HER2	-0.009	0.932	1.000	-	0.009	0.928	0.480	<0.001
	PD-L1	-0.136	0.193	0.009	0.928	1.000	-	-0.024	0.821
	CD68	0.177	0.090	0.480	<0.001	-0.024	0.821	1.000	-

P<0.05 was considered to indicate a statistically significant difference. TN, tumor nest; TS, tumor stroma; CD, cluster of differentiation; HER2, human epidermal growth factor receptor 2; PD-L1, programmed death receptor ligand 1.

localization and quantification of each target and its interactions *in situ* on tissue cells (19). In addition, all tissue samples in the present study were prepared into TMA, which can further reduce the influence of human factors on the results during the experimental process.

In the present study, the PD-L1 expression level in tumor tissues was not statistically different from that in adjacent normal tissues; nevertheless, PD-L1 expression was considerably downregulated in TN relative to normal tissues. PD-L1 expression was also associated with a favorable prognosis in GC, including early TNM stage, excellent tumor differentiation and prolonged overall survival. This conclusion contradicts earlier evidence that the PD-L1 expression in patients with GC is associated with a worse prognosis (20-23). Indeed, the PD-L1 expression-based prognosis in GC remains controversial (8,9,22,24). Böger *et al* (9) revealed that a high PD-1/PD-L1 expression was strongly related to an improved prognosis for patients with GC and PD-L1 became an independent survivor prognosticator in Western patients. Although Asian and non-Asian GCs have unique tumor immunity patterns associated with T-cell activity, this may affect the association between PD-1/PD-L1 expression and patient survival. Rha *et al* (8) found that PD-L1 expression was not linked with OS in patients with GC, regardless of whether they originated from Asia or the West.

It was hypothesized that those controversial results might relate to the means of PD-L1 detection and assessment methods. Not only do tumor cells express PD-L1, but so do immune cells in the TME. Moreover, over the last several years, an increasing number of studies have found that PD-L1 expressed on immune cells in the TME is closely related to patient prognosis (13,25). The present study showed that high PD-L1 expression in TN of tumor tissues and a high number of PD-L1+CD68+ macrophages were significantly associated with good prognosis of primary patients with GC. The results appear that PD-L1 expression in cancer cells may not be a critical factor in GC and that immune cell deprivation in the TME may be a more critical factor in GC occurrence. This is also consistent with the study that the combined positive score of PD-L1 scoring systems in GC is more prognostically valuable than tumor proportion score (26). However, as CD68 is an pan-macrophage marker, which separates at least MI and M2 subtypes, which serve different roles in immunity. Therefore, more research will be conducted to further explore the prognostic potential of macrophage subtypes co-expressed with PD-L1 in primary patients with GC.

HER2 is an epithelial cell-expressed ligand-independent receptor tyrosine kinase involved in cell differentiation. HER2 has been documented to be amplified and overexpressed in a variety of human cancers, which is correlated with a worse prognosis, higher recurrence rates and shorter OS (27). A number of studies established that HER2 controls the abnormal expression of PD-L1 in the stomach and the combination of anti-HER2 and anti-PD-1 has proven synergistic anticancer effects in animal models (16,28,29). Further studies disclosed that dimerization of the HER2 receptor activates two major intracellular signaling pathways: the mitogen-activated protein kinase (MAPK) (Ras/Raf/MEK/ERK) and phosphatidylinositol 3'-kinase (PI3K)/Akt, inducing cell-cycle progression, proliferation and survival (30-32). PD-L1 is a dynamic marker that can be expressed constitutively (driven by endogenous oncogenic pathways) or in an inducible fashion (motivated by exogenous signals) (33). Constitutive PD-L1 is mainly regulated via MAPK (Ras/Raf/MEK/ERK) and PI3K/Akt pathways. Therefore, both molecular routes are involved in the HER2 intracellular signaling pathway. Inhibiting these routes can regulate PD-L1 expression, which can modify the efficacy of HER2 target treatments (34). Inducible PD-L1 is regulated by extracellular signals such as cytokines, epidermal growth factors and hypoxic conditions. Interferon-γ (IFN-γ) is a cytokine which is known to regulate PD-L1 expression (35). Yamashita *et al* (26) showed that trastuzumab can

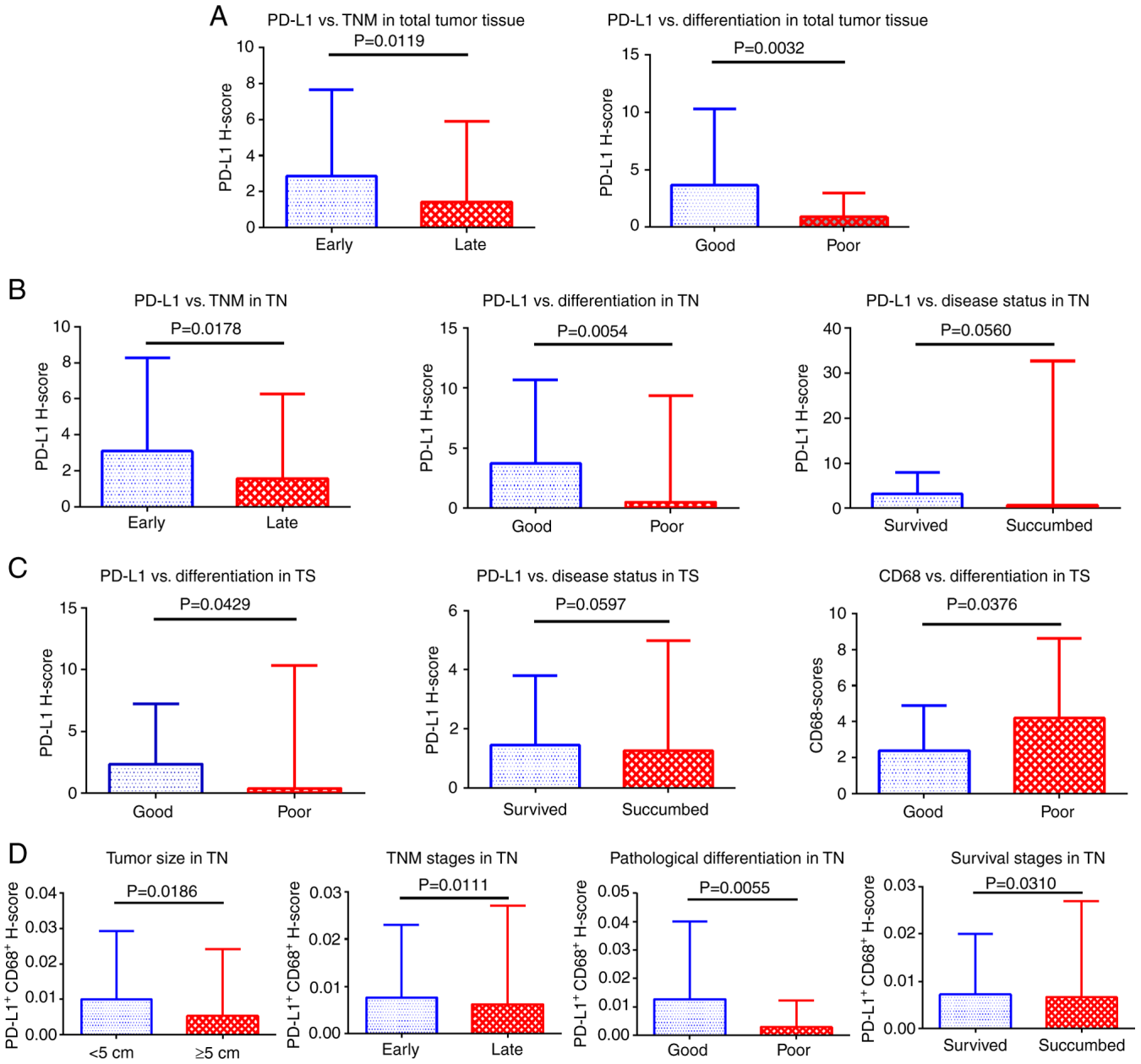

Figure 3. Correlation analysis of each protein expression with single clinical variables. (A) In the whole tumor tissues, PD-L1 expression was significantly associated with the TNM stages and pathological tissue differentiation. (B) In the TNs, PD-L1 expression was not only significantly correlated with aforementioned two clinical parameters, but also with survival status of patients. However, no significant correlation was observed between the other proteins and clinical variables of patients. (C) In the TS, both PD-L1 and CD68 expression was only significantly correlated with pathological differentiation. (D) The levels of PD-L1$^+$CD68$^+$ in the TNs (but not in the TS) were significantly correlated with the patients' tumor size, TNM stage, pathological differentiation and survival status. No correlation was found between other protein combinations and clinical variables. Spearman analysis was used to assess the correlation between protein expression and clinical variables. PD-L1, programmed death receptor ligand 1; TN, tumor nest; TS, tumorstroma; CD, cluster of differentiation.

upregulate PD-L1 in HER2-amplified GC cells by interacting with NK cells through the secretion of IFN-γ. Altogether, there is a complex regulatory network between HER2 and PD-L1 expression, which is not only affected by the tumor cells themselves, but also closely related to the TME (36). The relationship between molecular subtypes of GC and PD-L1, HER2 and combined HER2 and PD-L1 expression, requires further investigation.

According to the present study, HER2 expression was substantially greater in GC tissues than in nearby normal tissues. However, HER2 expression did not correlate significantly with PD-L1 nor with clinical prognostic parameters in the TN. Notably, HER2 expression was significantly linked with CD68 expression in the TS and poor tumor differentiation. This result is in accordance with Chen *et al* (37) on breast ductal carcinoma *in situ*. The spatial distribution of CD68 in GC tissues appeared quite different and associated with clinicopathological parameters. For instance, CD68 expression in TS was markedly linked with poor tumor differentiation, but co-expression of CD68 with PD-L1 in TN was significantly associated with excellent tumor differentiation. Similar results have been observed for a number of tumor types, including non-small cell lung cancer and breast cancer (38,39). Several growth factors and proteases important in angiogenesis,

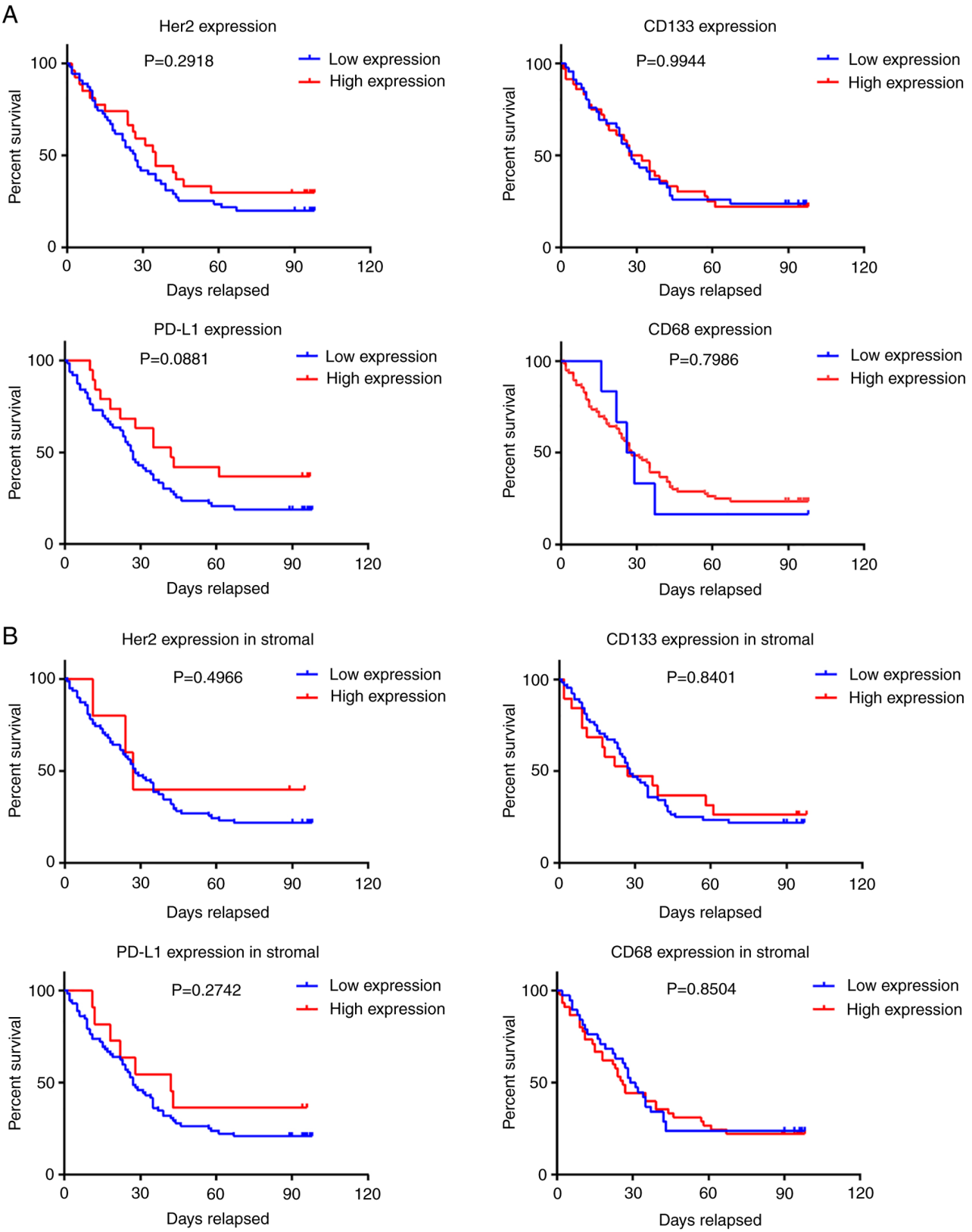

Figure 4. Kaplan-Meier analysis of overall survival with four proteins expression in tumor nest of primary patients with GC. (A) The four markers expressed were associated with eight-year survival status in total cells expressed in TN of 93 primary patients with GC. (B) The four markers expressed were associated with eight-year survival status in total cells expressed in TS of 93 primary patients with GC. GC, gastric cancer; TN, tumor nest; HER2, human epidermal growth factor receptor 2; CD, cluster of differentiation; PD-L1, programmed death receptor ligand 1.

invasiveness and migration of cancer cells are secreted by cancer stromal macrophages, hence supporting cancer development (40). By contrast, cancer-associated macrophages release cytotoxic cytokines, such as IL-1a, IL-1b, IL-6 and TNF-a, which may inhibit tumor development (41-43). Therefore, TN macrophages and TS macrophages may have

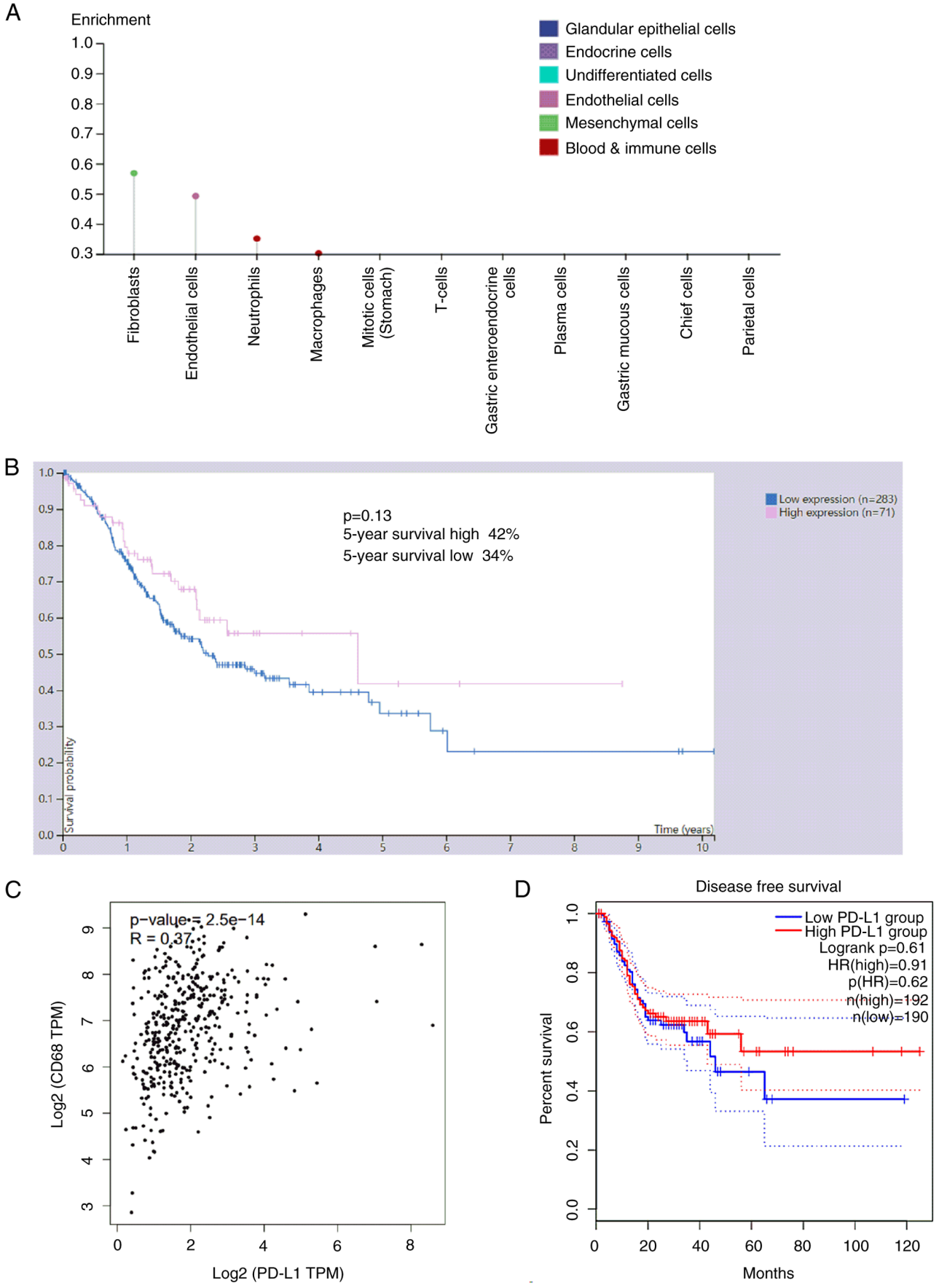

Figure 5. Online databases used analyze the correlation between the expression of PD-L1/CD68 and clinical outcomes of patients with GC. Human Protein Atlas database analyzed (A) the expression of PD-L1 in cellular components of GC tissues and (B) the correlation between PD-L1 expression and patients' 5-year survival. Gene Expression Profiling Interactive Analysis2 database analyzed (C) the correlation between the expression of PD-L1 and CD68 in gastric cancer tissue samples (D) and the relationship between PD-L1 expression and patients' disease-free survival. PD-L1, programmed death receptor ligand 1; CD, cluster of differentiation; HR, hazard ratio.

distinct biological activities in relation to tumor growth. Moreover, the predictive significance of CD68 in the clinic must take into account not only its expression level but also its spatial localization. Although the western blotting assay can reflect the relative expression of PD-L1 proteins in the whole tumor tissue, it is not able to show spatial differences in TN or TS. Therefore, the western blotting method was not used in the present study to further validate its results. To obtain support for the results of this study from clinical samples, the present study analyzed the correlation of PD-L1 and CD68 expression in tumor tissues from patients with GC with clinical outcomes using two public databases. The results were consistent with the present study.

CD133 is one of the most often used markers for cancer stem cells. Mounting evidence has shown that over-expression of CD133 is strongly related to tumor progression, treatment resistance and tumor recurrence (44-48). However, the clinical and prognostic value of CD133 in GC remains debatable (44,49,50). CD133 expression in tumor tissues was much greater than in nearby normal tissues, as shown by the present study. However, no link between CD133 expression and clinical outcome was discovered. Moreover, two types of CD133 expression were detected in tumor cells: glandular-luminal cell membrane surface expression (L-type luminal expression) and cytoplasmic expression (C-type). CD133 expression can be broadly divided into two types that have been reported in several tumors (50-52). Hashimoto *et al* (50) noted that the expression of CD133in C-type GC cells had a higher malignant potential than that in L-type GC cells. Hashimoto *et al* (50) reported that the expression of CD133 in C-type GC cells predicted a higher malignant potential than in L-type GC cells. Similar results were also observed in hepatocellular carcinoma and rectal cancer (53,54). Based on these studies, it was hypothesized that no significant association was observed between CD133 and clinical relevance because the overall CD133 expression in gastric cancer was evaluated without dividing the cases into expression types. As the automated pathological analysis system used in the present study could not distinguish the two types of CD133 expression in cancer cells. Therefore, the prognostic value of CD133 protein in GC needs to be further investigated by distinguishing these two cell types and correlating them with clinical parameters in future studies.

In conclusion, the present study investigated the prognostic value of PD-L1 in combination with CD68, CD133 andHER2 in primary GC. It was found that PD-L1+CD68+ macrophage infiltration in TN might be a potential indicator of prognosis in patients with primary GC which deserves further exploration.

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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

ZS, QG and HT designed the project; YZ analysed and interpreted the data and wrote the manuscript; JW analyzed survival data; JZ performed the experiments and collected the data; HT and QG confirmed the authenticity of all the raw data. All authors participated in critical revision of the manuscript and all authors read and approved the final manuscript.

Ethics approval and consent to participate

The patients with GC for TMA (HStnA180Su17; Xinchao Company) gave informed consent. The present study was conducted under the approval of the Institutional Ethics Committee (approval no. KHLL2019-KY037). All procedures were performed in accordance with the relevant guidelines and regulations (17).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Xie W, Yang T, Zuo J, Ma Z, Yu W, Hu Z and Song Z: Chinese and global burdens of gastrointestinal cancers from 1990 to 2019. Front Public Health 10: 941284, 2022.
3. Iwasaki A, Shinozaki-Ushiku A, Kunita A, Yamazawa S, Sato Y, Yamashita H, Fukayama M, Seto Y and Ushiku T: Human leukocyte antigen class I deficiency in gastric carcinoma: An adaptive immune evasion strategy most common in microsatellite instable tumors. Am J Surg Pathol 45: 1213-1220, 2021.
4. Qing Y, Li Q, Ren T, Xia W, Peng Y, Liu GL, Luo H, Yang YX, Dai XY, Zhou SF and Wang D: Upregulation of PD-L1 and APE1 is associated with tumorigenesis and poor prognosis of gastric cancer. Drug Des Devel Ther 9: 901-909, 2015.
5. Saito H, Kono Y, Murakami Y, Shishido Y, Kuroda H, Matsunaga T, Fukumoto Y, Osaki T, Ashida K and Fujiwara Y: Highly activated PD-1/PD-L1 pathway in gastric cancer with PD-L1 expression. Anticancer Res 38: 107-112, 2018.
6. Zhang M, Dong Y, Liu H, Wang Y, Zhao S, Xuan Q, Wang Y and Zhang Q: The clinicopathological and prognostic significance of PD-L1 expression in gastric cancer: A meta-analysis of 10 studies with 1,901 patients. Sci Rep 6: 37933, 2016.

7. Pereira MA, Ramos MFKP, Dias AR, Ribeiro R, Cardili L, Zilberstein B, Cecconello I, Ribeiro U Jr, de Mello ES and de Castria TB: Scoring systems for PD-L1 expression and their prognostic impact in patients with resectable gastric cancer. Virchows Arch 478: 1039-1048, 2021.
8. Rha SY, Ku GY, Kim HS, Chung HC, Amlashi FG, Maru DM, Fein CA, Tang LH, Zhou W, Wu T, *et al*: PD-L1 expression and overall survival in Asian and western patients with gastric cancer. Future Oncol 18: 2623-2634, 2022.
9. Böger C, Behrens HM, Mathiak M, Krüger S, Kalthoff H and Röcken C: PD-L1 is an independent prognostic predictor in gastric cancer of western patients. Oncotarget 7: 24269-24283, 2016.
10. Zhang F, Zhang J, Zhao L, Zhai M, Zhang T and Yu D: A PD-L1 negative advanced gastric cancer patient with a long response to PD-1 blockade after failure of systematic treatment: A case report. Front Immunol 12: 759250, 2021.
11. Ribeiro HSC, Menezes JN, da Costa WL Jr, F de Jesus VH, Diniz AL, Godoy AL, de Farias IC, Torres SM, Neotti T, Mello CAL, *et al*: PD-L1 expression in gastric and gastro-esophageal junction cancer patients treated with perioperative chemotherapy. J Surg Oncol 126: 150-160, 2022.
12. Wu Y, Cao D, Qu L, Cao X, Jia Z, Zhao T, Wang Q and Jiang J: PD-1 and PD-L1 co-expression predicts favorable prognosis in gastric cancer. Oncotarget 8: 64066-64082, 2017.
13. Ju X, Shen R, Huang P, Zhai J, Qian X, Wang Q and Chen M: Predictive relevance of PD-L1 expression with pre-existing TILs in gastric cancer. Oncotarget 8: 99372-99381, 2017.
14. Park IH, Kong SY, Ro JY, Kwon Y, Kang JH, Mo HJ, Jung SY, Lee S, Lee KS, Kang HS, *et al*: Prognostic implications of tumor-infiltrating lymphocytes in association with programmed death ligand 1 expression in early-stage breast cancer. Clin Breast Cancer 16: 51-58, 2016.
15. Wang Q, Lou W, Di W and Wu X: Prognostic value of tumor PD-L1 expression combined with $CD8^+$ tumor infiltrating lymphocytes in high grade serous ovarian cancer. Int Immunopharmacol 52: 7-14, 2017.
16. Chakrabarti J, Koh V, Steele N, Hawkins J, Ito Y, Merchant JL, Wang J, Helmrath MA, Ahmad SA, So JBY, *et al*: Disruption of Her2-induced PD-L1 inhibits tumor cell immune evasion in patient-derived gastric cancer organoids. Cancers (Basel) 13: 6158, 2021.
17. China Medical Biotechnology Association Biobank (Trial). Chin Med Biotechnol 6: 71-79, 2011 (In Chinese).
18. Zou Y, Zhang J, Zhang L and Yan X: Interferon-induced protein 16 expression in colorectal cancer and its correlation with proliferation and immune signature markers. Onco Lett 22: 687, 2021.
19. Taube JM, Akturk G, Angelo M, Engle EL, Gnjatic S, Greenbaum S, Greenwald NF, Hedvat CV, Hollmann TJ, Juco J, *et al*: The society for immunotherapy of cancer statement on best practices for multiplex immunohistochemistry (IHC) and immunofluorescence (IF) staining and validation. J Immunother Cancer 8: e000155, 2020.
20. Wu C, Zhu Y, Jiang J, Zhao J, Zhang XG and Xu N: Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. Acta Histochem 108: 19-24, 2006.
21. Hassen G, Kasar A, Jain N, Berry S, Dave J, Zouetr M, Priyanka Ganapathiraju VLN, Kurapati T, Oshai S, Saad M, *et al*: Programmed death-ligand 1 (PD-L1) positivity and factors associated with poor prognosis in patients with gastric cancer: An umbrella meta-analysis. Cureus 14: e23845, 2022.
22. Chen L, Wang L, Li X, Zhang G, Li Z and Wang Y: Clinic-pathological characteristics and prognostic value of PD-L1 and HER2 in gastric cancer. DNA Cell Biol 40: 405-413, 2021.
23. Amatatsu M, Arigami T, Uenosono Y, Yanagita S, Uchikado Y, Kijima Y, Kurahara H, Kita Y, Mori S, Sasaki K, *et al*: Programmed death-ligand 1 is a promising blood marker for predicting tumor progression and prognosis in patients with gastric cancer. Cancer Sci 109: 814-820, 2018.
24. He PX, Ma ZL, Han H, Zhang XY, Niu SH, Du LN, Zheng YC and Liu HM: Expression of programmed death ligand 1 (PD-L1) is associated with metastasis and differentiation in gastric cancer. Life Sci 242: 117247, 2020.
25. Zeng D, Li M, Zhou R, Zhang J, Sun H, Shi M, Bin J, Liao Y, Rao J and Liao W: Tumor microenvironment characterization in gastric cancer identifies prognostic and immunotherapeutically relevant gene signatures. Cancer Immunol Res 7: 737-750, 2019.
26. Yamashita K, Iwatsuki M, Harada K, Eto K, Hiyoshi Y, Ishimoto T, Nagai Y, Iwagami S, Miyamoto Y, Yoshida N, *et al*: Prognostic impacts of the combined positive score and the tumor proportion score for programmed death ligand-1 expression by double immunohistochemical staining in patients with advanced gastric cancer. Gastric Cancer 23: 95-104, 2020.
27. Fong C and Chau I: HER2 inhibition in gastric cancer-novel therapeutic approaches for an established target. Cancers (Basel) 14: 3824, 2022.
28. Oki E, Okano S, Saeki H, Umemoto Y, Teraishi K, Nakaji Y, Ando K, Zaitsu Y, Yamashita N, Sugiyama M, *et al*: Protein expression of programmed death 1 ligand 1 and HER2 in gastric carcinoma. Oncology 93: 387-394, 2017.
29. Attia S, Abd El Hafez A, Abdel-Aziz A, Elmetwaly S and Mokhtar N: Prognostic value of PD-L1 immunohistochemical marker in gastric carcinoma and its correlation with HER2 status. Asian Pac J Cancer Prev 23: 1466-1444, 2022.
30. Pous A, Notario L, Hierro C, Layos L and Bugés C: HER2-positive gastric cancer: The role of immunotherapy and novel therapeutic strategies. Int J Mol Sci 24: 11403, 2023.
31. Yoon SO, Shin S, Lee HJ, Chun HK and Chung AS: Isoginkgetin inhibits tumor cell invasion by regulating phosphatidylinositol 3-kinase/Akt-dependent matrix metalloproteinase-9 expression. Mol Cancer Ther 5: 2666-2675, 2006.
32. Fink MY and Chipuk JE: Survival of HER2-positive breast cancer cells: Receptor signaling to apoptotic control centers. Genes Cancer 4: 187-195, 2013.
33. Pardoll DM: The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12: 252-264, 2012.
34. Jiang X, Zhou J, Giobbie-Hurder A, Wargo J and Hodi FS: The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. Clin Cancer Res 19: 598-609, 2013.
35. Chen J, Jiang CC, Jin L and Zhang XD: Regulation of PD-L1: A novel role of pro-survival signalling in cancer. Ann Oncol 27: 409-416, 2016.
36. Wang D, Chen X, Du Y, Li X, Ying L, Lu Y, Shen B, Gao X, Yi X, Xia X, *et al*: Associations of HER2 mutation with immune-related features and immunotherapy outcomes in solid tumors. Front Immunol 13: 799988, 2022.
37. Chen XY, Thike AA, Md Nasir ND, Koh VCY, Bay BH and Tan PH: Higher density of stromal M2 macrophages in breast ductal carcinoma in situ predicts recurrence. Virchows Arch 476: 825-833, 2020.
38. Liu Y, Zugazagoitia J, Ahmed FS, Henick BS, Gettinger SN, Herbst RS, Schalper KA and Rimm DL: Immune Cell PD-L1 colocalizes with macrophages and is associated with outcome in PD-1 pathway blockade therapy. Clin Cancer Res 26: 970-977, 2020.
39. Wang ZQ, Milne K, Derocher H, Webb JR, Nelson BH and Watson PH: PD-L1 and intratumoral immune response in breast cancer. Oncotarget 8: 51641-51651, 2017.
40. Tan HY, Wang N, Zhang C, Chan YT, Yuen MF and Feng Y: Lysyl oxidase-like 4 fosters an immunosuppressive microenvironment during hepatocarcinogenesis. Hepatology 73: 2326-2341, 2021.
41. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishiwaki Y, Gemma A, *et al*: Predominant infiltration of macrophages and CD8(+) T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer 113: 1387-1395, 2008.
42. Ohno S, Inagawa H, Dhar DK, Fujii T, Ueda S, Tachibana M, Suzuki N, Inoue M, Soma G and Nagasue N: The degree of macrophage infiltration into the cancer cell nest is a significant predictor of survival in gastric cancer patients. Anticancer Res 23: 5015-5022, 2003.
43. Medrek C, Pontén F, Jirström K and Leandersson K: The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer 12: 306, 2012.
44. Attia S, Atwan N, Arafa M and Shahin RA: Expression of CD133 as a cancer stem cell marker in invasive gastric carcinoma. Pathologica 111: 18-23, 2019.
45. Jiang Y, He Y, Li H, Li HN, Zhang L, Hu W, Sun YM, Chen FL and Jin XM: Expressions of putative cancer stem cell markers ABCB1, ABCG2, and CD133 are correlated with the degree of differentiation of gastric cancer. Gastric Cancer 15: 440-450, 2012.
46. Zhu Y, Yu J, Wang S, Lu R, Wu J and Jiang B: Overexpression of CD133 enhances chemoresistance to 5-fluorouracil by activating the PI3K/Akt/p70S6K pathway in gastric cancer cells. Oncol Rep 32: 2437-2444, 2014.
47. Chen XL, Chen XZ, Wang YG, He D, Lu ZH, Liu K, Zhang WH, Wang W, Li CC, Xue L, *et al*: Clinical significance of putative markers of cancer stem cells in gastric cancer: A retrospective cohort study. Oncotarget 7: 62049-62069, 2016.

48. Razmi M, Ghods R, Vafaei S, Sahlolbei M, Saeednejad Zanjani L and Madjd Z: Clinical and prognostic significances of cancer stem cell markers in gastric cancer patients: A systematic review and meta-analysis. Cancer Cell Int 21: 139, 2021.
49. Yiming L, Yunshan G, Bo M, Yu Z, Tao W, Gengfang L, Dexian F, Shiqian C, Jianli J, Juan T and Zhinan C: CD133 overexpression correlates with clinicopathological features of gastric cancer patients and its impact on survival: A systematic review and meta-analysis. Oncotarget 6: 42019-42027, 2015.
50. Hashimoto K, Aoyagi K, Isobe T, Kouhuji K and Shirouzu K: Expression of CD133 in the cytoplasm is associated with cancer progression and poor prognosis in gastric cancer. Gastric Cancer 17: 97-106, 2014.
51. Ishigami S, Ueno S, Arigami T, Uchikado Y, Setoyama T, Arima H, Kita Y, Kurahara H, Okumura H, Matsumoto M, *et al*: Prognostic impact of CD133 expression in gastric carcinoma. Anticancer Res 30: 2453-2457, 2010.
52. Zhao P, Li Y and Lu Y: Aberrant expression of CD133 protein correlates with Ki-67 expression and is a prognostic marker in gastric adenocarcinoma. BMC Cancer 10: 218, 2010.
53. Sasaki A, Kamiyama T, Yokoo H, Nakanishi K, Kubota K, Haga H, Matsushita M, Ozaki M, Matsuno Y and Todo S: Cytoplasmic expression of CD133 is an important risk factor for overall survival in hepatocellular carcinoma. Oncol Rep 24: 537-546, 2010.
54. Jao SW, Chen SF, Lin YS, Chang YC, Lee TY, Wu CC, Jin JS and Nieh S: Cytoplasmic CD133 expression is a reliable prognostic indicator of tumor regression after neoadjuvant concurrent chemoradiotherapy in patients with rectal cancer. Ann Surg Oncol 19: 3432-3440, 2012.