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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Exosomal PD-L1 Retains Immunosuppressive Activity and is Associated with Gastric Cancer Prognosis

Yibo Fan, MD, PhD[1,2], Xiaofang Che, MD, PhD[1,2], Jinglei Qu, MD[1,2], Kezuo Hou, MD[1,2], Ti Wen, MD, PhD[1,2], Zhi Li, MD, PhD[1,2], Ce Li, PhD[1,2], Shuo Wang, MD[1,2], Ling Xu, MD, PhD[1,2], Yunpeng Liu, MD, PhD[1,2], and Xiujuan Qu, MD, PhD[1,2]

[1]Department of Medical Oncology, The First Hospital of China Medical University, Shenyang, China; [2]Key Laboratory of Anticancer Drugs and Biotherapy of Liaoning Province, The First Hospital of China Medical University, Shenyang, China

ABSTRACT

Background. A recent study showed that circulating exosomal PD-L1 is an effective predictor for anti-PD-1 therapy in melanomas. Exosomal PD-L1 induced immunosuppression microenvironments in cancer patients. However, its prognostic value and immunosuppressive effect in gastric cancer (GC) were poorly understood.

Methods. We retrospectively evaluated the prognostic value of exosomal PD-L1 and soluble PD-L1 in preoperative plasma of 69 GC patients. The correlation between exosomal PD-L1 and the T cell counts or cytokine in the plasma was evaluated in 31 metastatic GC patients before chemotherapy.

Results. Overall survival (OS) was significantly lower in the high exosomal PD-L1 group compared with the low exosomal PD-L1 group ($P = 0.004$). Exosomal PD-L1 was an independent prognostic factor in GC ($n = 69$, 95% confidence interval = 1.142–7.669, $P = 0.026$). However, soluble PD-L1 showed no correlation with OS ($P = 0.139$). Additionally, exosomal PD-L1 in the plasma samples of 31 *metastatic* GC patients was negatively associated with CD4+ T cell count ($P = 0.001$, $R = -0.549$), CD8+ T-cell count ($P = 0.054$, $R = -0.349$), and granzyme B ($P = 0.002$, $R = -0.537$), indicating that exosomal PD-L1 was associated with immunosuppressive status of GC patients. GC cells also secreted exosomal PD-L1 and were positively associated with the amount of PD-L1 in corresponding GC cell lines. Besides, exosomal PD-L1 significantly decreased T-cell surface CD69 and PD-1 expressions compared with soluble PD-L1 due to its stable and MHC-I expression.

Conclusions. Overall, exosomal PD-L1 predicts the worse survival and reflects the immune status in GC patients, resulting from a stronger T-cell dysfunction due to its stable and MHC-I expression.

Recent studies have shown that the immune checkpoint PD-L1 interacts with PD-1, which is an inhibitory receptor expressed on activated T cells. This in turn suppresses T-cell activation and is associated with poor prognosis in cancer patients.[1–3] Thus, currently an anti-PD-1/PD-L1 agent has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of melanoma, non-small cell lung cancer, and renal cancer.[4] Several clinical trials on gastric cancer (GC) have demonstrated that the overall response rate (ORR) of anti-PD-1/PD-L1 agent was 22%, indicating that the PD-1/PD-L1 pathway plays a pivotal role in GC.[5,6] Pembrolizumab is a monoclonal antibody directed against PD-1 and is a U.S. FDA-approved drug for the treatment of patients with advanced PD-L1 positive gastric and gastroesophageal junction adenocarcinoma and had at least two prior lines of chemotherapy. [6] Therefore, PD-L1 might be considered as a useful biomarker in predicting the therapeutic efficiency of GC patients, whereas few other studies did not consider PD-L1 as a definite therapeutic predictive factor. Also, the role of PD-L1 expression in the prognosis of GC remains controversial.[1,6–9] Besides the use of different antibodies and tested specimens for the highly heterogeneous GC, the

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Y. Liu, MD, PhD
e-mail: cmuliuyunpeng@hotmail.com

X. Qu, MD, PhD
e-mail: xiujuanqu@yahoo.com

discrepancies in the findings might be mainly explained by the complexity of the immune system. Thus, tumor PD-L1 may not accurately predict the prognosis of GC patients.

Apart from tumor membrane PD-L1, tumor cells also secrete PD-L1 into the extracellular environment and circulation. Extracellular PD-L1 includes the soluble and exosomal forms of PD-L1.[10–12] Previous studies have almost focused on the role of soluble PD-L1 in cancer patients. Indeed, the total amount of circulatory PD-L1 has been detected without distinguishing between the soluble and exosomal forms in the circulation, and emerging evidence has suggested that the exosomal form plays a more important role in the immunosuppression.[13] Exosomes are small membrane vesicles that contain functional biomolecules, including proteins, RNA and DNA, and can be horizontally delivered to the recipient cells. Currently, tumor-secreted exosomes are emerging as critical messengers in tumor progression, angiogenesis, and metastasis, modulating the host immune system.[14] Because exosomes are more stable and not easily degraded by proteolytic enzymes, the associated immunosuppressive molecules might exert stronger immunomodulatory effects in the circulation. [15] According to a recent study, exosomal PD-L1 was considered as an effective predictor for anti-PD-1 therapy.[16] Additionally, other studies showed that exosomal PD-L1 inhibited T-cell killing of breast cancer cells and promoted tumor growth.[17] Only one study discussed the prognostic effects of exosomal PD-L1 in patients with head and neck cancer.[18] However, whether the circulating exosomal PD-L1 could be used as a prognostic factor and exert important immunosuppressive effects in GC are still unknown.

Hence, in the present study, we investigated whether the circulating exosomal PD-L1 in GC patients was associated with poorer prognosis. Besides, exosomal PD-L1 was negatively correlated with CD4+ T-cell count, CD8+ T-cell count, and granzyme B and induced immunosuppression of GC. Mechanically, GC cell line-secreted exosomal PD-L1 induced stronger T-cell dysfunction compared with soluble counterpart due to its stable and MHC-I expression.

RESULTS

Patient Characteristics

The cohort that analyzed the effect of prognosis included 43 men and 26 women, with a median age of 64 (range 42–81) years. Thirty-one (44.92%) patients were at clinical stage I or II, and 38 (55.08%) were at stage III (Table 1). The cohort that analyzed for the correlation of exosomal PD-L1 and CD4+ T lymphocyte count or CD8+ T lymphocyte count included 25 males and 6 females, with a median age at diagnosis of 58 (range 40–79) years. The clinical characteristics of GC patients with stage IV are summarized in Table 2. The Human Ethics Review Committee of China Medical University approved this study. In accordance with the Declaration of Helsinki and subsequent revisions, the signed informed consent forms were obtained from all patients.

Prognostic Role of Circulatory Exosomal PD-L1 in GC

Extracellular vesicles from GC patient blood samples were isolated by Exosome Precipitation Solution *before surgery*. NanoSight nanoparticle tracking analysis showed that the extracellular vesicles were approximately 94 nm in diameter (Fig. 1a). Exosome biomarkers were evaluated by Western blotting (Fig. 1b). Proteins from the exosomes of GC patients included the exosomal markers TSG101, CD9, and CD63. ELISA was performed to investigate the prognostic value of serum soluble PD-L1 and exosomal PD-L1 in GC patients. The cutoff values of 82.585 ng/ml and 49.495 ng/ml by ROC curve were used to analyze the correlations and survival analysis again in the soluble PD-L1 and exosomal PD-L1. The area under the curve (AUC) values were 0.638 and 0.709, respectively (Figs. 1c, d). Soluble PD-L1 also showed no correlation with OS ($P = 0.139$; Fig. 1e). In subgroup analysis, OS was significantly poorer in high exosomal PD-L1 group than in low group in AJCC stage I and II ($P = 0.010$; Fig. 1g). There is no significant difference between the two groups in AJCC stage III ($P = 0.157$; Fig. 1h). Additionally, OS had no significant difference between the high exosomal PD-L1 group and the low group for well and moderate differentiation ($P = 0.157$; Fig. 1i). This was significantly poorer in the high exosomal PD-L1 group than in the low group ($P = 0.004$; Fig. 1f). Significant differences were observed in patients with poor differentiation ($P = 0.020$, Fig. 1j). Exosomal PD-L1 also was associated with T stage ($P = 0.028$; Table 1). Soluble PD-L1 was associated with the age and location of GC ($P = 0.022$ and $P = 0.036$; Table 1). Multivariate Cox proportional hazard model (forward) was used to fit all nine clinical pathological variables. Exosomal PD-L1 was included in the multivariate Cox proportional hazards model (forward) analysis of 69 patients along with prognostic clinic-pathologic factors. High exosomal PD-L1 expression (hazard ratio [HR] 2.960; 95% confidence interval [CI] 1.142–7.669; $P = 0.026$) and lymphatic invasion (HR 3.534; 95% CI 1.292–9.671; $P = 0.014$) were significant independent prognostic factors associated with OS (Table 3). These findings suggested that the exosomal PD-L1 existed in circulation and was associated with poorer prognosis of GC patients.

TABLE 1 Patient characteristics according to the expression of soluble PD-L1 and exosomal PD-L1

Variables	Total patients	Soluble PD-L1		*P* value	Exosomal PD-L1		*P* value
		High expression	Low expression		High expression	Low expression	
Age				**0.022**			0.804
< 60	20 (28.99%)	6 (16.67%)	14 (42.42%)		10 (32.26%)	10 (26.32%)	
≥ 60	49 (71.01%)	30 (83.33%)	19 (57.58%)		21 (67.74%)	28 (73.68%)	
Gender				0.759			0.341
Male	43 (62.32%)	24 (66.67%)	19 (57.58%)		17 (54.84%)	26 (68.42%)	
Female	26 (37.68%)	12 (33.33%)	14 (42.42%)		14 (45.16%)	12 (31.58%)	
Location				**0.036**			0.581
Lower 1/3	47 (68.12%)	3 (8.33%)	3 (9.09%)		2 (6.45%)	4 (10.53%)	
Middle 1/3	16 (23.19%)	13 (36.11%)	3 (9.09%)		8 (25.81%)	8 (21.05%)	
Upper 1/3	6 (8.69%)	20 (55.56%)	27 (81.82%)		21 (67.74%)	26 (68.42%)	
AJCC stage				0.098			0.231
I	9 (13.04%)	2 (5.56%)	7 (21.21%)		1 (3.23%)	8 (21.05%)	
II	22 (31.88%)	9 (25.00%)	13 (39.39%)		11 (35.48%)	11 (28.95%)	
III	38 (55.08%)	25 (69.44%)	13 (39.39%)		19 (61.29%)	19 (50.00%)	
Tumor classification				0.068			**0.028**
T1 + T2	12 (17.39%)	2 (5.56%)	10 (30.30%)		2 (6.45%)	10 (26.32%)	
T3 + T4	57 (82.61%)	34 (94.44%)	23 (69.70%)		29 (93.55%)	28 (73.68%)	
Lymph node metastasis				0.251			0.663
Negative	28 (40.58%)	11 (30.56%)	17 (51.52%)		11 (35.48%)	17 (44.74%)	
Positive	41 (59.42%)	25 (69.44%)	16 (48.48%)		20 (64.52%)	21 (55.26%)	
Differentiation				0.155			0.326
Well	4 (5.80%)	3 (8.33%)	1 (3.03%)		1 (3.23%)	3 (7.89%)	
Moderate	14 (20.29%)	9 (25.00%)	5 (15.15%)		5 (16.13%)	9 (23.68%)	
Mixed type	51 (73.91%)	24 (66.67%)	27 (81.82%)		25 (80.65%)	26 (68.42%)	

Bold values indicate statistical significance ($P < 0.05$)

Exosomal PD-L1 Reflected Immunosuppression in Metastatic GC Patients

To investigate whether exosomal PD-L1 reflects the immune status in GC, exosomal PD-L1 was extracted from the serum of 31 *metastatic* GC patients before *chemotherapy*. The cutoff value by ROC curve was 65.62 ng/ml (Fig. 2a), and the AUC values were 0.457. The correlation between exosomal PD-L1 and T-lymphocyte count were analyzed. The high exosomal PD-L1 group demonstrated lower CD4+ T-lymphocyte count and CD8+ T-lymphocyte count. The exosomal PD-L1 was significantly negatively correlated with CD4+ T-lymphocyte count ($P = 0.001$, $R = -0.549$; Fig. 2b). Although statistical significance was not achieved, exosomal PD-L1 was negatively correlated with CD8+ T-lymphocyte count ($P = 0.054$, $R = -0.349$; Fig. 2b). A previous study showed that the engagement of PD-1 on T-cell surface with PD-L1 inhibited the effector functions and promoted the differentiation of CD4+ T cells into Treg.[2] Because cytokines IL-10 and TGF-β are secreted by Treg, the correlation between exosomal PD-L1 and L-10 and TGF-β were analyzed. Although there is no significant difference, the high exosomal PD-L1 group had higher cytokine levels IL-10 and TGF-β ($P = 0.117$, $R = 0.287$ and $P = 0.303$, $R = 0.191$ respectively; Fig. 2c). Additionally, exosomal PD-L1 was negatively correlated with serum granzyme B ($P = 0.002$, $R = -0.537$), indicating that exosomal PD-L1 reflects the immunosuppression status in GC patients.

Correlation Between Exosomal PD-L1 Production and PD-L1 Expression in GC Cells

Flow cytometry and Western blotting were used to assess PD-L1 levels in human GC cell lines. Interestingly, MGC803 showed low PD-L1 amounts, while MKN74 showed highly expressed PD-L1 (Fig. 3a, b). Next, the GC cell-derived exosomes were isolated by centrifugation. Electron microscopy and NanoSight nanoparticle tracking analysis confirmed the shape and diameter of exosomes (Fig. 3c, d). To evaluate the relationship between exosomal PD-L1 production and PD-L1 expression in GC cell lines,

TABLE 2 Clinical characteristics of 31 *metastatic* GC patients

Variables	No. of patients
Age (years)	
< 60	18 (58.06%)
≥ 60	13 (41.94%)
Gender	
Male	25 (80.65%)
Female	6 (19.35%)
Histology grade	
G2	12 (38.71%)
G3	19 (61.29%)
No. of distant metastatic site	
1	21 (67.74%)
2	4 (12.90%)
3	3 (9.68%)
4	2 (6.45%)
5	1 (3.23%)
Metastasis	
Liver	10 (32.26%)
Ascitic	8 (25.81%)
Lung	2 (6.45%)
Other	11 (35.48%)

the exosomal PD-L1 in MGC803 and MKN74 was shown by Western blotting and flow cytometry (Fig. 3e, f), indicating that the exosomal PD-L1 was found in cell lines that highly express PD-L1, including MKN74, but hardly detected in MGC803.

Immunosuppressive Effects of Exosomal and Soluble Forms of PD-L1

To determine whether exosomal PD-L1 has suppressive functions on CD8+ effector T cells, we activated normal human PMBCs using anti-CD3/anti-CD-28 antibodies for 6 h, which was confirmed by increased PD-1 expression (Fig. 4a). To further assess the effects of exosomal PD-L1 on T-cell function, MKN74 cells were transiently transfected with siRNA by targeting PD-L1. Decreased PD-L1 expression was confirmed by Western blotting (Fig. 4b). Exosomes from the nonsilencing control and PD-L1 knockdown groups were treated with activated T cells for 16 h, respectively. As expected, exosomes derived from the cells with PD-L1 knockdown significantly reversed the decrease of surface CD69 and PD-1 expression (Fig. 4c). To compare the immunosuppressive effects between exosomal and soluble forms of PD-L1, flow cytometry was performed to quantify the surface expressions of CD69 and PD-1 in T cells induced by exosomal or soluble PD-L1. ELISA confirmed that the exosomal and soluble forms of PD-L1 had equal amounts. Interestingly, a similar amount of PD-L1 on exosomes induced higher CD69 and decreased expression of PD-1 compared with the soluble forms (Fig. 4d). This indicated that the exosomal PD-L1

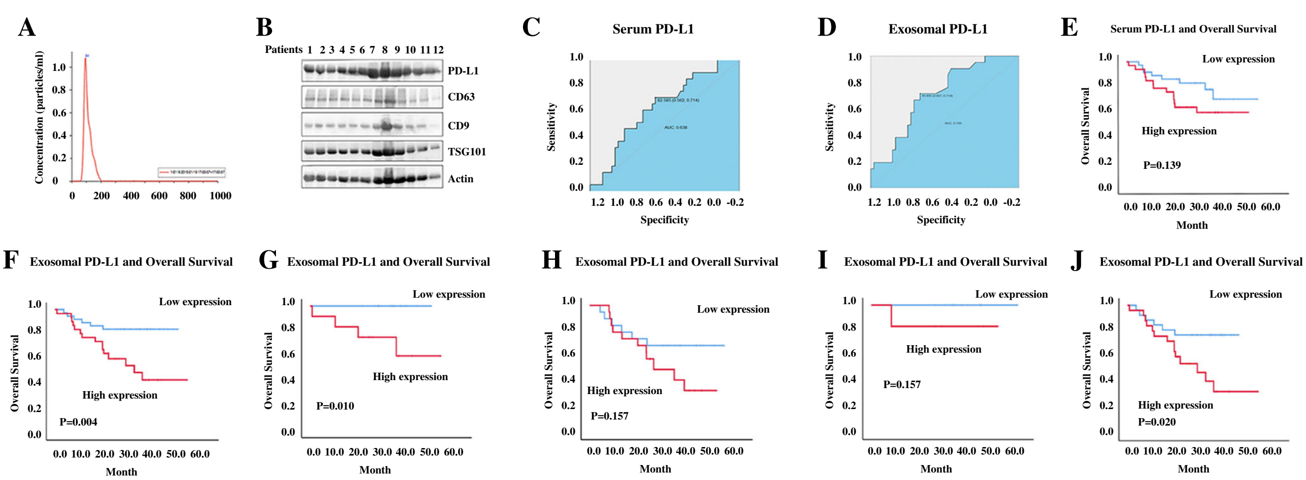

FIG. 1 Exosomal PD-L1 was associated with prognosis of GC patients. **a** Exosome concentration and size distribution by NanoSight analysis in circulation of GC patients. **b** Western blot analyzed the biomarker of exosomes in circulation of GC patients. **c**, **d** ROC curve for soluble PD-L1 and exosomal PD-L1 in GC. **e**, **f** KM survival curve and log-rank test for patients classified as showing either high or low expression of soluble PD-L1 and exosomal PD-L1 in GC according to ROC curve cutoff value. **g**, **h** KM survival curve and log-rank test for patients classified as showing either high or low expression of exosomal PD-L1 in GC according to ROC curve cutoff value in AJCC stage I + II and AJCC stage III, respectively. **i**, **j** KM survival curve and log-rank test for patients classified as showing either high or low expression of exosomal PD-L1 in GC according to ROC curve cutoff value in well and moderate differentiation and poor differentiation, respectively

TABLE 3 Multivariate Cox regression analysis, including exosomal PD-L1 expression levels and overall survival in 69 GC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (years) (≤ 60 vs. > 60)	2.93	0.862–9.962	0.085			
Gender (male vs. female)	1.176	0.495–2.794	0.713			
Location	0.836	0.454–1.539	0.565			
AJCC	3.819	1.432–10.182	**0.007**			
Tumor classification	5.337	0.715–39.822	0.102			
Lymphatic invasion	5.022	1.476–17.087	**0.010**	3.534	1.292–9.671	**0.014**
Differentiation	6.536	0.964–44.335	0.055			
Total PD-L1	2.727	1.057–7.037	**0.038**			
Exosome PD-L1	3.624	1.402–9.364	**0.008**	2.960	1.142–7.669	**0.026**

Bold values indicate statistical significance ($P < 0.05$)

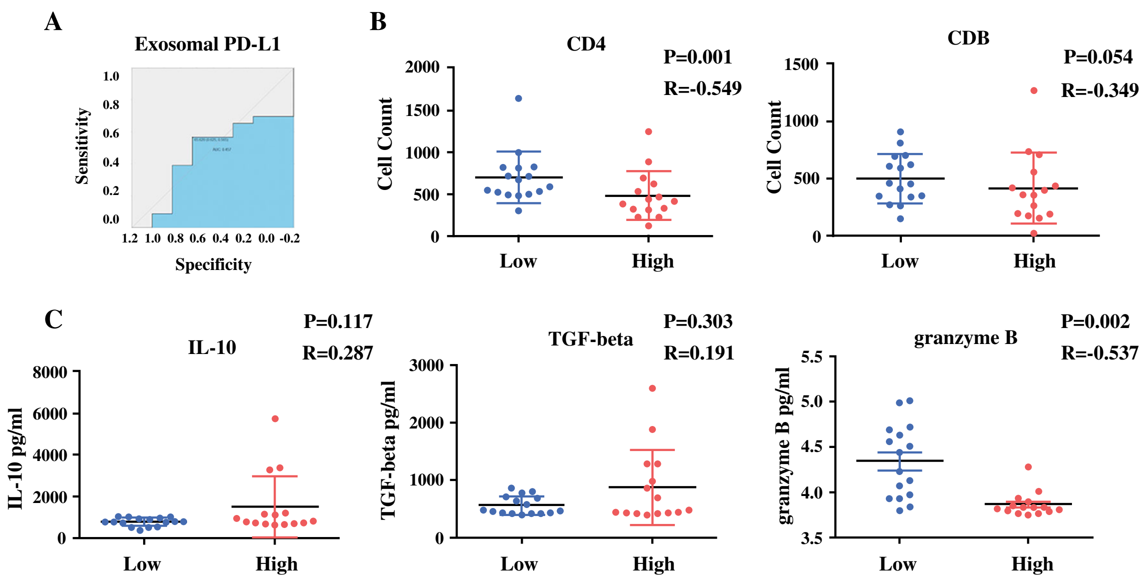

FIG. 2 Exosomal PD-L1 reflected the immunosuppression in metastatic gastric cancer patients. **a** ROC curve for exosomal PD-L1 in 31 metastatic GC. **b** Spearman test was used to analysis the correlation of exosomal PD-L1 and CD4+ T lymphocyte count, CD8+ T lymphocyte count. **c** Spearman test was used to analysis the correlation of exosomal PD-L1 and serum IL-10, TGF-beta, and granzyme B

exerted stronger immunosuppressive effect in the microenvironment than soluble PD-L1.

Stability of Exosomal and Soluble PD-L1 Forms

To verify the stability of PD-L1 on exosomes, the exosomes were treated with trypsin as described in the previous study.[15] The same amount of PD-L1 on exosomes and soluble counterparts were exposed to trypsin for 30 min. Western blot analysis confirmed the expression of PD-L1. The expression of PD-L1 on soluble form was significantly decreased compared with exosomes treated with trypsin for 30 min (Fig. 5a). Moreover, this stability was associated with intact membranes of exosomes, as disruption of exosomal membrane by 1% triton resulted in decreased amounts of PD-L1 and TSG101 (Fig. 5b). These results indicated that exosomal PD-L1 was more stable and not easily degraded by proteolytic enzymes in the microenvironment.

Exosomal MHC-I Promoted Exosomal PD-L1 Inducing T-Cell Dysfunction

MHC molecules are essential ligands for TCR. MHC-I/II interacts with TCR and induces the first signal activation of T cells. The second signal includes the co-stimulatory factors and co-inhibitors. PD-L1 is a co-inhibitor. It was supposed that PD-L1 exerts inhibitor signals after MHC interaction with TCR. Exosomes also carried MHC molecules, but soluble PD-L1 did not. Thus, it was hypothesized that exosomal MHC played a role in exosomal PD-L1 induction of immunosuppression. To investigate the effect

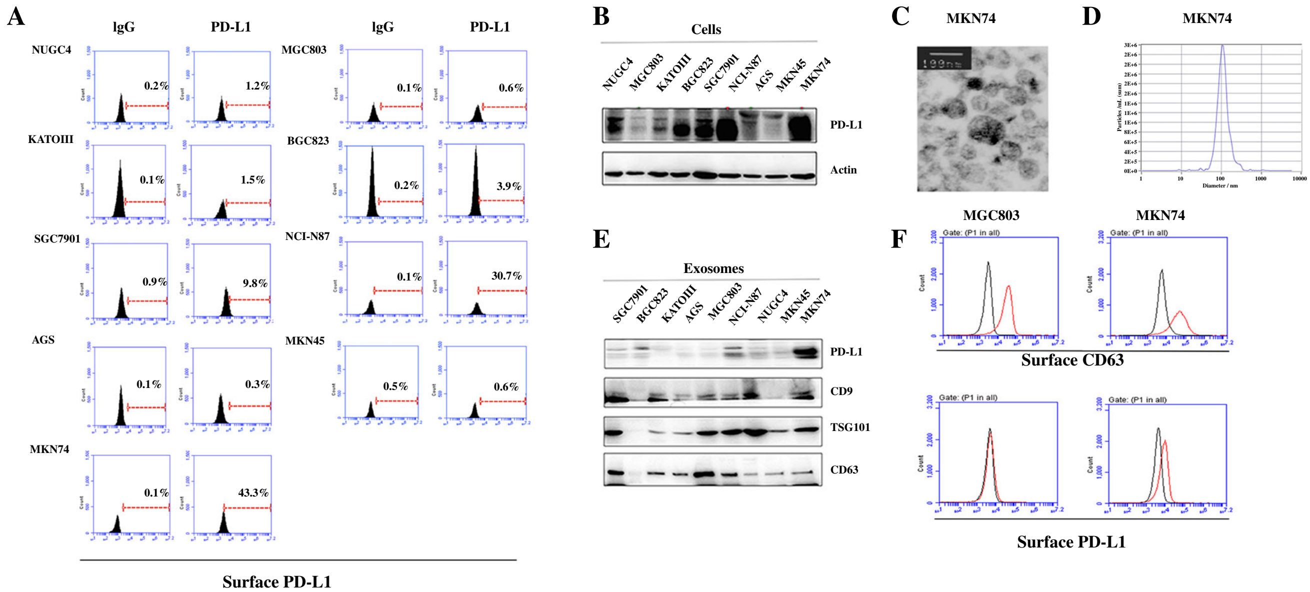

FIG. 3 Exosomal PD-L1 was positively associated with the expression of PD-L1 in GC cell lines. **a** Flow cytometry analysis the expression of PD-L1 in nine GC cell lines. **b** Western blot analysis the expression of PD-L1 in nine GC cell lines. **c** and **d** TEM micrograph and nanosight validated MKN74-derived exosomes. The results are representative of three independent experiments. **e** Western blot analysis the expression of exosomal PD-L1 in nine GC cell lines. **f** Flow cytometry analysis the expression of exosomal PD-L1 in MKN74 and MGC803

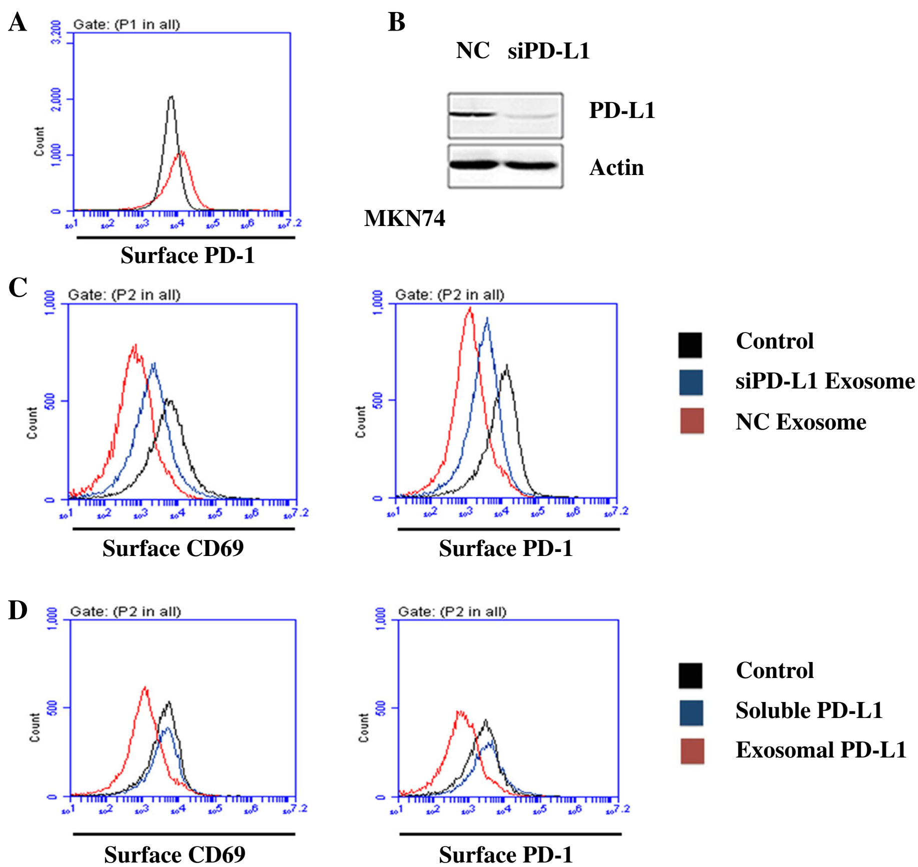

FIG. 4 Exosomal PD-L1 exerted stronger immunosuppressive effect than soluble PD-L1. **a** Flow cytometry analysis the expression of PD-1 in T cells. **b** Western blot analysis the expression of PD-L1 in NC and siPD-L1 MKN74 cell lines. **c** Exosomes from the non-silencing control and PD-L1 knockdown groups were treated with activated T cell for 16 h, respectively. The activation of T cells was analyzed by Flow cytometry. **d** Exosomal and soluble forms of PD-L1 were treated with activated T cell for 16 h, respectively. The activation of T cells was analyzed by flow cytometry. The results are representative of three independent experiments

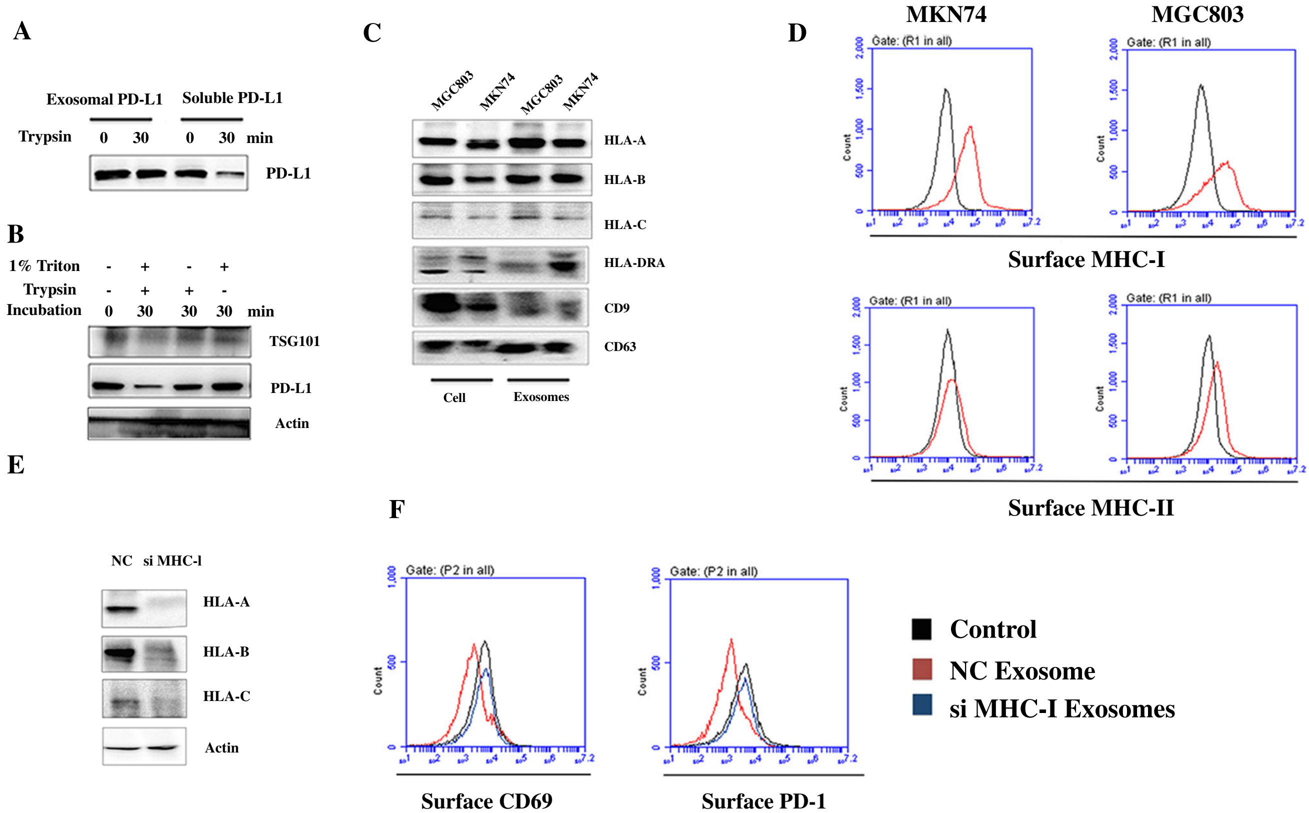

FIG. 5 Exosomal PD-L1 was more stable and Exosomal MHC I expression induced Exosomal PD-L1 has stronger immunosuppression effect. **a** The same amount of PD-L1 on exosomes (40 μg) and PD-L1 (6 ng) were exposed by trypsin for 30 min. Western blot analysis the expression of PD-L1. **b** Exosomes were disrupted by 1% Triton X-100 for 30 min at 4 °C. Equal amount of disrupted and intact exosomes were then subjected to trypsin digestion at 37 °C for 30 min. Western blot analysis the expression of TSG101 and PD-L1. **c** Western blot analysis the expression HLA-A, HLA-B, HLA-C, and HLA-DRA in MGC803 and MKN74 and its derived exosomes. **d** Flow cytometry analysis the expression HLA-A, HLA-B, HLA-C, and HLA-DRA in MGC803 and MKN74. **e** Western blot analysis detected the effect of knockdown HLA-A, HLA-B, HLA-C in MKN74. **f** Exosomes from the non-silencing control and HLA-A, HLA-B, HLA-C knockdown groups were treated with activated T cell for 16 h, respectively. The activation of T cells was analyzed by flow cytometry. The results are representative of three independent experiments

of exosomal MHC molecules in promoting exosomal PD-L1 induced T-cell dysfunction, Western blot and flow cytometry were performed. Results showed that HLA-A, HLA-B, and HLA-C were highly expressed in MGC803 and MKN74 cells and derived exosomes compared with HLA-DRA (Fig. 5c, d). MKN74 cells were transiently transfected with siRNA targeting HLA-A, HLA-B, and HLA-C. Decreased HLA-A, HLA-B, and HLA-C expressions were confirmed by western blotting (Fig. 5e). Knockdown of HLA-A, HLA-B, and HLA-C derived exosomes reversed the decreased expression of surface CD69 and PD-1 molecules compared with nonsilencing controls (Fig. 5f), indicating that the exosomal MHC I promoted exosomal PD-L1 induced T-cell dysfunction. These might be the reasons for exertion of stronger of immunosuppressive effects by exosomal PD-L1 than the soluble form.

MATERIALS AND METHODS

Patients

This study was conducted on two independent cohorts of GC patients who were admitted to The First Hospital of China Medical University, Shenyang City, Liaoning Province, China. This study involved human subjects and conducted according to the Declaration of Helsinki. The patients analyzed for the correlation of prognosis and exosomal PD-L1 were enrolled if they met the following criteria: GC with stage 1–3; D2 lymphadenectomy; and had no neoadjuvant therapy before surgery. Sixty-nine patients from May 2013 to December 2015 formed cohort 1 according to the random number generated by R/sample function. The median follow-up period was 26.9 (range 0.8–51.2) months. The clinical information of all the patients was retrieved from HIS (Tables 1 and 2). Patients analyzed for the correlation of exosomal PD-L1 and CD4+

T-cell count or CD8+ T-cell count were enrolled if they met the following criteria: availability of clinicopathological data and CD4+ T lymphocyte count and CD8+ T lymphocyte count during the initiation of chemotherapy. Thirty-one patients from March 2012 to December 2012 formed cohort 2 according to the random number generated by R/sample function. Clinical information of all the patients was retrieved from HIS (Table 3).

Ethics Statement

The study was approved by the local Ethics Committee, and all procedures were conducted in accordance with the ethical principles of China Medical University (Scientific Ethics NO. 2016-118).

Exosomes Isolation from Serum Samples of GC Patients

Serum samples of 200 µl from GC patients were ice thawed. Protein extraction from exosomes was isolated by using Exosome Precipitation Solution (ExoQuick-TC, System Biosciences) without ultracentrifugation.

Materials and Antibodies

Antibodies against Actin (Catalog #1452), TSG101 (Catalog #E0508), HLA-A (Catalog #390473), HLA-C (Catalog #166088), and HLA-DRA (Catalog #53319) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to PD-L1 (Catalog #13684S) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies specific to CD9 (Catalog #ab92726) and CD63 (Catalog #ab193349) were obtained from Abcam (Danvers, MA). Antibodies specific to HLA-B (Catalog #PA5-29929) were obtained from ThermoFisher (Waltham, MA).

Cell Culture

The human gastric adenocarcinoma cell lines MGC803, AGS, SGC7901, and BGC823 were obtained from the Chinese Academic Science (Shanghai, China). MKN45, NCI-N87, and KATO III cell lines were obtained from ATCC (Maryland, USA). NUGC4 and MKN74 were obtained from JCRB (Osaka, Japan). Cells were grown in RPMI 1640 (Rosewell Park Memorial Institute) medium containing 10% heat-inactivated FCS at 37 °C in a humidified incubator with a mixture of 95% air and 5% CO_2. All cell lines have been tested for mycoplasma contamination.

Exosomal Isolation from Human GC Cell Lines

Cells were grown in exosome-depleted FBS RPMI media until they reached a confluency of 80%. The human GC cell lines-secreted exosomes was isolated as previously described.[19] The media was collected and centrifuged at 2000*g* for 20 min, followed by a centrifugation step of 10,000*g* for 30 min to discard the cellular debris. Then, the media was filtered using a 0.2-mm pore filter. The collected media was then ultracentrifuged at 100,000*g* for 70 h at 4 °C. The exosomal pellet was then washed with 40 ml of PBS, followed by a second step of ultracentrifugation at 100,000*g* for 70 h at 4 °C. Afterwards, the supernatant was discarded, and the exosomes were collected.

ELISA

The expression of granzyme B (R&D, Catalog #DY2906-05), IL-10 (R&D, Catalog #DY417), and TGF-beta (R&D, Catalog #DY240) were detected by enzyme-linked immunosorbent assay (ELISA). Exsomal PD-L1 and soluble PD-L1 were detected by ELISA according to the previously study.[16] Human PD-L1/B7-H1 DuoSet ELISA was obtained from (R&D, Catalog #DY156). For detection of exosomal PD-L1 from GC patients' plasma, 96 well microplates (R&D Systems, Catalog #DY990) were coated with Human PD-L1 Capture Antibody overnight at 4 °C. Free binding sites were blocked with 200 µl of blocking buffer for 1 h at room temperature. A total of 100 µl of exosomes from plasma were added to each well for 2 h at room temperature. Then, human PD-L1 Detection Antibody was added to each well for another 2 h at room temperature. A total of 100 µl per well of Streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP) diluted in 1% BSA in PBS, pH 7.2–7.4, (R&D, Catalog #DY995) was then added and incubated for 20 min at room temperature. Then, 100 µl of substrate solution 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) (R&D, Catalog #DY999) was added and incubated for 20 min at room temperature and stopped with 2 N H2SO4 (R&D, Catalog #DY994). Human PD-L1standard was used to make a standard curve. The result of standard curve demonstrated that the established ELISA exhibited a reliable linear detection range from 18.75 to 1200 pg/ml. The results were quantitated by Bio-RADi Mark (Bio-RAD Laboratories Inc, Kyoto Japan). The absorbance was measured using a microplate reader at 450 nm.

Size-Distribution Analysis of Exosomes

Real-time, high-resolution particle detection, counting, and sizing were performed on the NanoSight NS300

(Malvern Instruments, Malvern, UK) according to the previous study.[19]

Electron Microscopy

Exosomes-derived from MKN74 were fixed in 4% paraformaldehyde and stored at 4 °C. The morphology of exosomes was detected by electron microscopy as previously described.[20] Exosomal size was measured by using the scale bar.

Flow Cytometry Analysis of Exosomes-Bound Beads

Exosomes were attached to 4-μm aldehyde/sulphate latex beads (Invitrogen, Catalog #A37304) by mixing 50 μg of exosomes in a 5-ml volume of beads for 20 min at room temperature with continuous rotation. This suspension was then diluted to 1 ml with PBS and left for 30 min for constant rotation at room temperature as described in the previous study.[17] Exosomes incubated with anti-PD-L1 (BD, Catalog #5061804) and CD63 (BD, Catalog #557288) were detected for its expression by flow cytometry analysis.

Western Blotting

Cells were seeded at 1×10^5 per well in 6-well plates and incubated overnight. The protein from cells and exosomes were detected by Western blotting as previously described.[20] The final result was analyzed by NIH Image J software.

Surface Expression Analysis

Surface molecular expression was determined by flow cytometry as described previously.[21] The following antibodies were used: PE Anti-Human-PD-L1 (BD, Catalog #5061804); PerCP-CyAnti-Human-PD-1 (BD, Catalog #5147730); PE Anti-Human HLA-ABC (BD, Catalog #565291); PerCP-Cy Anti-Human HLA-DR (BD, Catalog #552764). Fluorescence was assessed using Flow Cytometer (BDAccuri™, NJ, USA).

Functional Analysis

PBMCs harvested from healthy donors were centrifuged on Ficoll-Hypaque gradients and activated using anti-CD3/anti-CD-28 antibody (25 μl/ml, 10971, Stemcell Technologies) for 6 h at 37 °C. Cell-derived exosomes were added to PBMC cells and incubated for 16 h. The activated T cells were harvested and stained for CD8 (FITC Anti-Human-CD8 (BioLegend, Catalog #300906). The gates were set on CD8+ T cells. The expression levels of CD69 [FITC Anti-Human CD69 (BD, Catalog #555530)] and PD-1 [PerCP-Cy Anti-Human-PD-1 (BD, Catalog #5147730)] on CD8+ T cells were measured by flow cytometry.

Small Interfering RNA Transfections

PD-L1 small interfering RNA (siRNA) was obtained from Shanghai GeneChem Co. Ltd. (China). PD-L1siRNA was synthesized: PD-L1 5′-CCAGCACACUGAAUCA ATT-3′. HLA-A siRNA was synthesized: HLA-A 5′-UCU CACACCAUCCAGAUAAttUUAUCUGGAUGGUGUG AGAtt-3′. HLA-B siRNA was synthesized: HLA-B5′-GG AACACACAGAUCUACAAttUUGUAGAUCUGUGUGU UCCtt-3′. HLA-C 5′-UCUGGACAAGAGCAGAGAUttA UCUCUGCUCUUGUCCAGAtt-3′.

Lipofectamine 3000 (Invitrogen, Catalog #1857476) was diluted in RPMI 1640 and incubated at room temperature for 5 min. Then, 10 μl PD-L1, HLA-A, HLA-B, and HLA-CsiRNA was added to dilute lipofectamine 3000 and then incubated for 20 min. After 48 h of transient transfection, the cells were detected by western blotting for PD-L1 or HLA-A, HLA-B, and HLA-CRNAsiRNA effects.

Statistical Analysis

Patient characteristics were compared using Spearman test for categorical variables. OS was calculated from the time of diagnosis until death or at the last follow-up visit, i.e., July 11 2017. Survival analysis was performed using a Kaplan–Meier method, and the differences were assessed by a two-tailed log-rank test.

DISCUSSION

Exosomes are small secreted membrane vesicles that are developed into intracellular multi-vesicular endosomes. These are eventually released into the extracellular microenvironment and entered into the systemic circulation.[19] Because exosomes contain proteins and nucleic acids of the original cells, tumor-derived exosomes may reflect the molecular signature of a cancer. Therefore, exosomes could serve as markers in cancer diagnosis and help to monitor the therapeutic responses and predict the prognosis in cancer patients.[22,23] Recent findings indicated that circulating exosomes represent a less invasive and more readily available alternatives to conventional immunohistochemical assays, requiring tumor biopsies for the prediction of prognosis of cancer patients.[22] Because the prognostic value of tumor PD-L1 remains controversial, validation of exosomal PD-L1 for the prognosis of cancer patients is highly necessary. In the present study, exosomal PD-L1 was an independent prognostic factor in GC ($n = 69$, 95% confidence interval [CI] = 1.142–7.669, $P = 0.026$). Exosomal PD-L1 content was significantly associated with T stage ($P = 0.028$). Meanwhile, OS was significantly poorer in high exosomal PD-L1 group ($P = 0.004$). In subgroup analysis, OS was significantly poorer in high exosomal PD-L1 group than in low group in AJCC stage I and II ($P = 0.010$) but not in AJCC stage III ($P = 0.157$), indicating that the exosomal PD-L1 might predict the survival in the early stage of GC. The reason might due to the fact that there was more complex influence factors in the late AJCC stage of GC patients.

Previous studies assessing cell–cell communication between T cells and cancer cells focused on soluble PD-L1 in circulation.[10,24,25] According to a study, the circulatory soluble PD-L1 retains its receptor-binding domain and induces apoptosis in T cells.[10] In addition, other studies showed that the soluble PD-L1 content in circulation was associated with prognosis in GC and aggressive diffusion of large B-Cell lymphoma.[24,25] However, the total amounts of circulatory PD-L1 were detected but do not distinguish between the soluble and exosomal forms. In the present study, NanoSight nanoparticle tracking analysis, Western blot, and ELISA revealed the amounts of exosomal PD-L1 in the systemic circulation of GC patients as well as culture supernatants of GC cell lines, indicating that the exosomal PD-L1 was found in circulation and extracellular environment.

Interestingly, previous findings indicated that exosomal TGF-β induces stronger and long-term immunosuppressive effects, because it cannot be easily degraded by the extracellular proteolytic enzymes.[15] As shown above, exosomal PD-L1 induced stronger T-cell dysfunction than the soluble form and was stable but not easily degraded by trypsin. Moreover, MHC-II expression represents a tumor-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy.[26] To investigate the exosomal MHC molecules in exosomal PD-L1 induced apoptosis, MHC-I knockdown MKN74-derived exosomes induced less T-cell dysfunction than NC. These might explain the reasons for the induction of stronger T-cell dysfunction by exosomal PD-L1 than soluble form. Besides, the tumor derived exosomes affects immune homeostasis mostly by triggering the immunosuppressive changes that protect the tumor.[27–29] Tumor-derived exosomes expresses FasL and TRAIL on their membrane and directly induces the apoptosis of CD8+ T cells, while activating and expanding the Tregs and MDSCs, which in turn inhibits the CD8+ T-cell-mediated targeting of the tumor.[13,30] Thus, our findings suggested that exosomal PD-L1 might exert more pronounced immunosuppressive effects. This might be due to the synergistic effects of exosomes and PD-L1, further suppressing the immune system and causing peripheral immune tolerance in GC patients. Additionally, exosomal PD-L1 was negatively correlated with CD4+ T-cell count and CD8+ T-cell count, indicating that the exosomal PD-L1 induced immunosuppressive effects in GC. The limitation for the present study is lacking validation cohort to further validation the prognostic effect of exosomal PD-L1 in GC patients.

In summary, the current study provided evidences that exosomal PD-L1 was an independent prognostic factor and predicts the survival of GC patient in the early AJCC stage and was negatively correlated with CD4+ T-cell count and CD8+ T-cell count. Exosomal PD-L1 induces higher levels of T-cell dysfunction due to its higher stability and MHC I expression. These findings provide new insights in predicting patient prognosis and suggest that the exosomal PD-L1 can be used to assess patient eligibility for anti-PD-1 antibody therapy in GC.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE This study was approved by the Human Ethics Review Committee of the First Hospital of China Medical University. All patients agree to participate in our study.

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