

PD-L1 expression and soluble PD-L1 production in gastric cancer

(胃癌における PD-L1 発現と可溶性 PD-L1 分泌についての検討)

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Abstract

Programmed death-ligand 1 (PD-L1) expression on tumor cells plays an important role in escape from anti-tumor immunity in a variety of cancers including gastric cancer (GC). In this study, we investigated not only intracellular PD-L1 expression but also membrane-bound PD-L1 expression in GC cells: MKN1, MKN74, KATO III, and OCUM-1 cells. Additionally, soluble PD-L1 (sPD-L1) levels in both supernatants of GC cells and sera of GC patients were also determined by enzyme-linked immunosorbent assay (ELISA). Interferon (IFN)- γ treatment resulted in increased cytoplasmic expression of PD-L1 in GC cells except for MKN74 cells in a dose-dependent manner, but there was no relationship between tumor necrosis factor- α treatment and enhanced PD-L1 expression. Concordant with these findings, flow cytometric analyses demonstrated that membrane-bound PD-L1 expression was also increased by IFN- γ treatment in a dose-dependent manner in these cells. Among them, marked sPD-L1 production was observed only in the culture supernatant of OCUM-1 cells. Serum levels of sPD-L1 were significantly increased in GC patients, especially stage IV patients compared with controls. In conclusion, IFN- γ treatment simultaneously enhanced intracellular and membranous PD-L1 expression in GC cells. sPD-L1 was detected not only in the supernatant of GC cells but also in sera of GC patients. Further analyses of the mechanisms underlying both the regulation of PD-L1 expression and production of its soluble form would be necessary.

Introduction

Gastric cancer (GC) is the fourth most common malignancy worldwide and is the second leading cause of cancer-related death (1). The risk of GC are chronic inflammation due to *Helicobacter pylori* infection, heavy alcohol drinking, heavy smoking, and excessive salt intake. In particular, the rate of helicobacter pylori infection in Japanese has been reported to be high among developed countries (2). Although patients with early GC are curable by endoscopic surgery, patients in advanced stages are usually treated with systemic chemotherapy. In spite of the advances in anticancer agents, the overall 5-year survival rate remains at 20% (3).

Programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1) are important immune checkpoints in the tumor. It is well-known that the PD-1/PD-L1 pathway functions as adaptive immune escape machinery (4, 5). Therefore, blockade of the PD-1/PD-L1 pathway by immune checkpoint inhibitors (ICIs), such as pembrolizumab and nivolumab, has already been clinically applied for a variety of cancers including GC (6). PD-L1 is expressed on the surface of tumor cells, tumor-associated macrophages (TAMs), and T lymphocytes and its expression can be induced by cytokines such as interferons (IFNs) and tumor necrosis factors (TNFs) (7, 8). Of interest, recent reports documented that the soluble forms of PD-L1 (sPD-L1) were detected in the blood of patients with tumors (9, 10). However, the mechanism has not been fully elucidated.

In this study, we examined the expression of PD-L1 and sPD-L1 secretion in GC cells followed by treatment with cytokines. Subsequently, we conducted a sandwich enzyme-linked immunosorbent assay (ELISA) to examine the serum levels of sPD-L1 in GC patients.

Materials and Methods

Cell culture and reagents

Human GC cell lines: MKN1, MKN 74, KATO III, OCUM-1 cells, were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) or RPMI-1640 medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen). Both recombinant human interferon (IFN)- γ and human tumor necrosis factor (TNF)- α were obtained from PeproTech Inc. (Rocky Hill, NJ, USA).

Quantitative real-time PCR

Total RNA extraction was performed using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. Quantitative real-time PCR was performed with an MX3000P qPCR

system (Stratagene, San Diego, CA, USA) using the Universal Probe Library System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's directions. PCR for human *PD-L1* and *GAPDH* was conducted using the following primers: *PD-L1* (forward 5'-AAATGGAACCTGGCGAAAG-3', reverse 5'-GCTCCCTGTTTGACTCCATC-3'), *GAPDH* (forward 5'-CTGACTTCAACAGCGACACC-3', reverse 5'-TAGCCAAATTCGTTGTCATACC-3').

Immunocytochemistry

GC cells were fixed with 2% paraformaldehyde and blocked with normal goat serum. Cells were subsequently stained with anti-PD-L1 (Cell Signaling Technology, Danvers, MA, USA) antibody, followed by incubation with Alexa 555-conjugated immunoglobulin G (IgG) (Molecular Probes, Eugene, OR, USA) antibody. The cells were coverslipped using a mounting medium that contained 40,6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA).

Flow cytometric analysis

Single-cell suspensions were stained with allophycocyanin (APC)-conjugated anti-PD-L1 antibody (Biolegend, San Diego, CA, USA). After the incubation, 1 µg/mL of propidium iodide was added to eliminate dead cells. Flow cytometric cell analyses were performed using FACSCanto II (BD Biosciences, San Jose, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

sPD-L1 levels in the culture supernatant of GC cells and sPD-L1 and IFN-γ in sera of GC patients were determined using a sandwich ELISA according to the manufacturer's instructions (R&D Systems, Inc., MN, USA). sPD-L1 production ability of the GC cell line was determined using culture supernatants collected 24 and 48 hours after plating of 100,000 cells in 6-well plates. Serum sPD-L1 and IFN-γ concentrations of the GC patients were also measured before the treatment initiation.

Patients and blood samples

Serum samples were collected before treatment initiation from 40 GC patients. Similarly, the sera of 10 non-GC patients (controls) were also collected. After obtaining informed consent, we analyzed the preserved blood samples and the data acquired from their medical records. This study was approved by the Research Ethics Committees of the Graduate School of Medicine, Chiba University (approval number: 3552).

Statistical analysis

Data are presented as the mean ± SEM. The significance of differences between 2 groups were

analyzed using Student's t-test. p -values <0.05 were considered significant.

Results

PD-L1 expression in GC cells treated with TNF- α and IFN- γ

To examine cytokine-induced PD-L1 expression, we first conducted quantitative RT-PCR analyses in GC cells: MKN1, MKN74, KATO III, and OCUM-1 cells. These cells were treated with 1, 10, or 100 ng/mL of TNF- α and 1, 10, or 100 ng/mL of IFN- γ for 24 hours. There was no relationship between TNF- α treatment at the various concentrations examined and upregulation of the mRNA expression of PD-L1 (Fig. 1A). In contrast, IFN- γ treatment increased PD-L1 mRNA expression in a dose-dependent manner in GC cells except for MKN74 cells (Fig. 1B). Concordant with these findings, immunocytochemical analyses demonstrated the enhancement of PD-L1 expression in MKN1, KATO III, and OCUM-1 cells treated with IFN- γ (Fig. 1C).

Quantification of membrane-bound PD-L1 by flow cytometry

GC cells treated with 100 ng/mL of IFN- γ for 24 hours were subjected to flow cytometric analyses. MKN1, KATO III, and OCUM-1 cells, but not MKN74 cells, showed an increase in membrane-bound PD-L1 expression on IFN- γ treatment in a dose-dependent manner (Fig. 2).

sPD-L1 detection in the culture supernatant of GC cells

To investigate the relationship between membrane-bound PD-L1 expression and sPD-L1 production, we conducted ELISA for the measurement of sPD-L1 in the culture supernatant of GC cells (Fig. 3). sPD-L1 could not be detected in supernatant cultured in GC cells without IFN- γ treatment. Concordant with flow cytometric analyses, MKN1, KATO III, and OCUM-1 cells, but not MKN74 cells, produced sPD-L1 in response to IFN- γ treatment in a time-dependent manner. Of note, the sPD-L1 levels of the supernatant cultured in OCUM-1 cells were markedly higher than in other GC cells.

Measurement of serum PD-L1 concentrations in GC patients

Next, ELISA was conducted to measure the serum PD-L1 concentration in GC patients (Table 1). The serum PD-L1 levels in GC patients were significantly higher than those in controls ($p < 0.05$, Fig. 4A). The median PD-L1 levels in controls and GC patients were 27.4 and 33.8 pg/mL, respectively. Subsequently, we compared the serum PD-L1 levels in view of stage progression. The serum PD-L1 levels in GC patients with stage IV were significantly higher than those with stages I, II, or III ($p < 0.05$, Fig. 4B).

Discussion

PD-1 (Programmed death-1, CD279), a single-pass type I membrane protein, belongs to the CD28/CTLA-4 family. It is predominantly expressed on the surface of immunocompetent cells such as T lymphocytes, B lymphocytes, and natural killer cells (11). Of note, high expression levels of PD-1 are closely associated with T-cell exhaustion. PD-L1 has been determined as B7 homolog 1 (B7-H1) and functions as a ligand for PD-1 (12). In normal tissues, PD-1/PD-L1 binding prevents an excessive immune response and protects tissues from damage through the induction of immune tolerance (13). On the other hand, PD-L1/PD-1-mediated tumor immune escape attenuates the immune response in cancer tissues and makes the elimination of cancer cells difficult (14). Additionally, PD-L1 is a target for hypoxia-inducible factor-1 (HIF-1) and its expression was further upregulated under hypoxia (15). Overall, PD-L1 expression is closely associated with cancer development and progression.

Both aberrant expression of PD-L1 and uncontrolled PD-L1/PD-1 signaling are observed with variable frequency in a variety of cancers (16). Previous immunohistochemical analyses demonstrated that PD-L1 expression was detected in approximately 40% of GC tissues analyzed and correlated with both an aggressive phenotype and unfavorable prognosis (17). PD-L1 expression is regulated by inflammatory signaling, oncogenic signaling, and genetic and epigenetic alterations (18, 19). Additionally, the co-existence of PD-L1-positive cancer cells and TILs was reported to be associated with a poor prognosis in patients with GC (20).

Inflammatory cytokines, such as IFNs, TNFs, and ILs, were mainly released from TILs and upregulated PD-L1 expression of cancer cells in a variety of cell types (21). Consistent with these findings, IFN- γ treatment resulted in a significant increase in PD-L1 expression in GC cell lines except MKN74 cells. TNF- α treatment modestly increased PD-L1 expression in GC cell lines except for MKN74 cells. Subsequently, we examined whether an increase in membranous PD-L1 expression was accompanied by the upregulation of PD-L1 induced by IFN- γ treatment. Flow cytometric analyses revealed that IFN- γ treatment induced membrane-bound PD-L1 expression in a dose-dependent manner. It is well-known that membranous PD-L1 expression is closely associated with PD-1/PD-L1-mediated tumor immune escape. Of interest, intracellular PD-L1, but not membranous PD-L1, plays an important role in the proliferation and migration of melanoma and ovarian cancer cells (22, 23).

A recent study successfully demonstrated that soluble forms of PD-L1 (sPD-L1) were detected not only in human serum samples but also in culture supernatants of PD-L1-expressing cell lines (24). Therefore, we aimed to detect sPD-L1 in supernatants of GC cell lines using ELISA. Although both intracellular and membrane-bound PD-L1 was upregulated in three cell lines treated with IFN- γ , sPD-L1 production was only observed in OCUM-1 cells. It is possible that MMPs were in part associated with sPD-L1 release (24). Alternatively, sPD-L1 was shown to originate from its splicing variants

lacking the transmembrane domain (25). Further analyses are needed to elucidate mechanisms underlying sPD-L1 production in GC cells.

sPD-L1 is also utilized as a prognostic biomarker in a variety of cancers (26). It has been reported that sPD-L1 functions as a decoy and attenuates the effect of ICI in lung cancer (27). Additionally, the exposure of CD4⁺ and CD8⁺ lymphocytes to sPD-L1 induces their apoptotic death (28). Even considering this background, many studies have shown that a high sPD-L1 level is closely associated with an unfavorable prognosis in many types of cancer including GC (20, 29-31). However, Zheng et al. demonstrated opposite results whereby GC patients with higher sPD-L1 levels had a better prognosis than those with lower sPD-L1 levels (32). Concordant with these findings, our results of ELISA demonstrated that sPD-L1 levels in sera of patients with Stage IV were significantly higher than in those with stage I-III. Additional analyses in a large number of patients would be of importance to determine the role of sPD-L1.

In conclusion, our study showed that IFN- γ treatment simultaneously enhanced intracellular and membranous PD-L1 expression in a part of GC cells. sPD-L1 was also detected in sera of GC patients in a variety of ranges. Further studies of the mechanisms underlying both the regulation of PD-L1 expression and production of its soluble form are needed to develop novel therapeutic approaches against GC.

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Table 1 Baseline characteristics of the patients

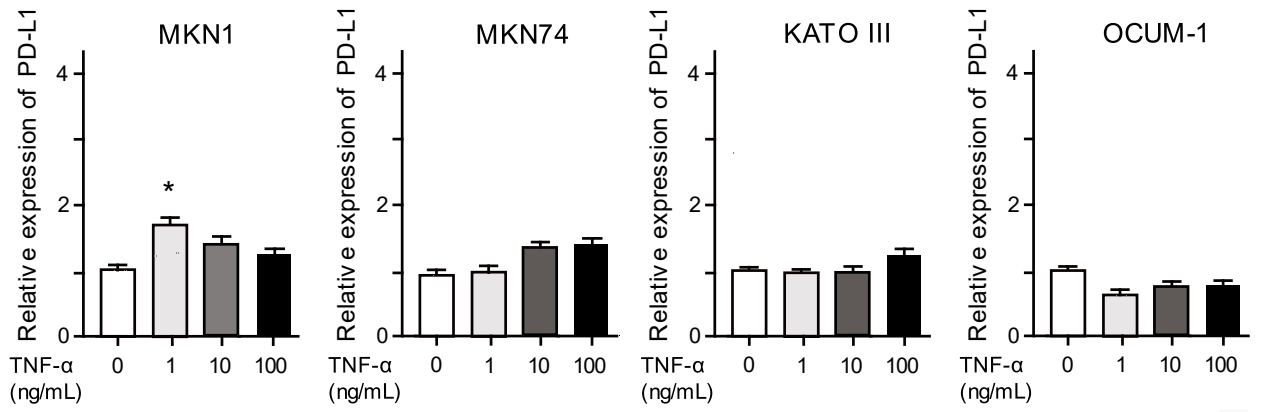
Characteristics	Value (n=40)
Age (years) ^a	71.5 (47-94)
Sex (male/female)	26/14
BMI (kg/m ²) ^a	21.2(15.6-32.0)
Smoking (Yes/No)	25/15
Alcohol intake (Yes/No)	24/16
<i>Helicobacter pylori</i> (Positive/Negative)	20/20
Tumor markers	
CEA (ng/mL) ^a	2.85 (0.5-86.9)
CA19-9 (ng/mL) ^a	19.1 (0-719.0)
Stage ^b	
I/II/III/IV	10 (25%)/10 (25%)/10 (25%)/10 (25%)
Tumor ^b	
T1/T2/T3/T4	8 (20%)/4 (10%)/14 (35%)/14 (35%)
Lymph node metastasis ^b	
N1/N2/N3/N4	14 (35%)/5 (12.5%)/8 (20%)/13 (32.5%)
Distant metastasis ^b	
M0/M1	30 (75%)/10 (25%)
Histological finding	
Diffuse/Intestinal/Mix	19 (47.5%)/17 (42.5%)/4 (10.0%)

^aMedian (range).

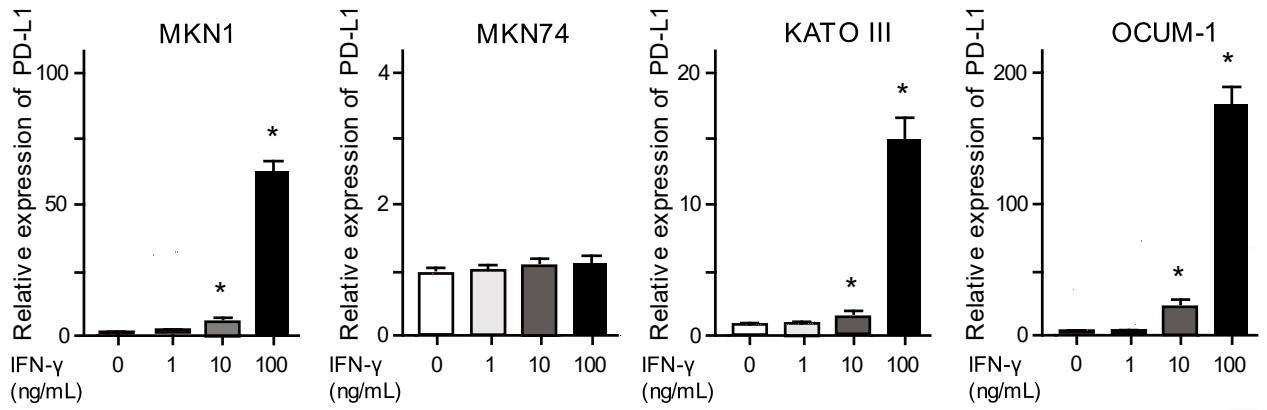
^bTumor, lymph node metastasis, distant metastasis, and stage are described according to the TNM classification for gastric cancer (UICC, 8th edition).

Figure 1.

A



B



C

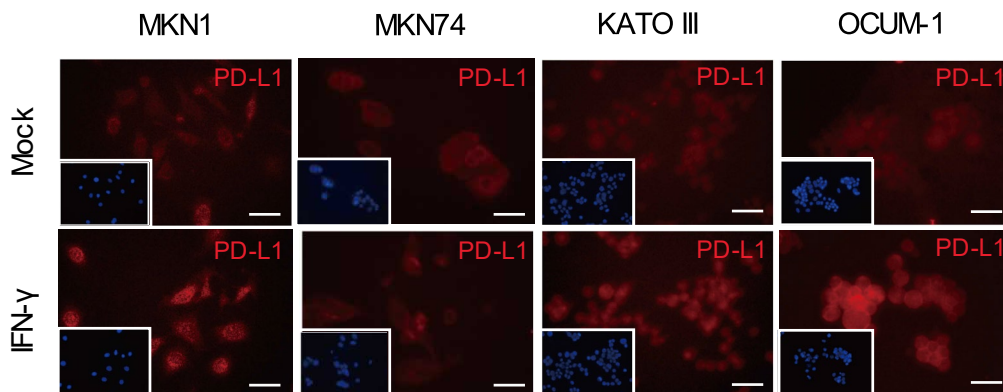
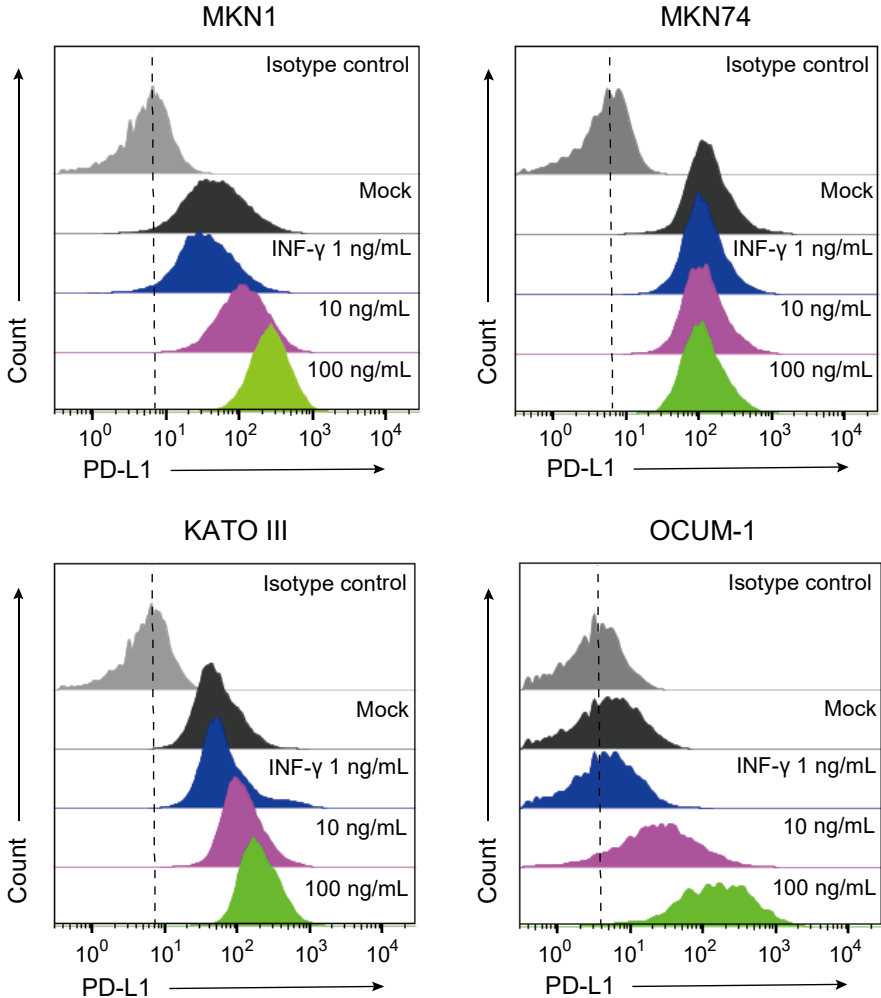


Figure 2.

A



B

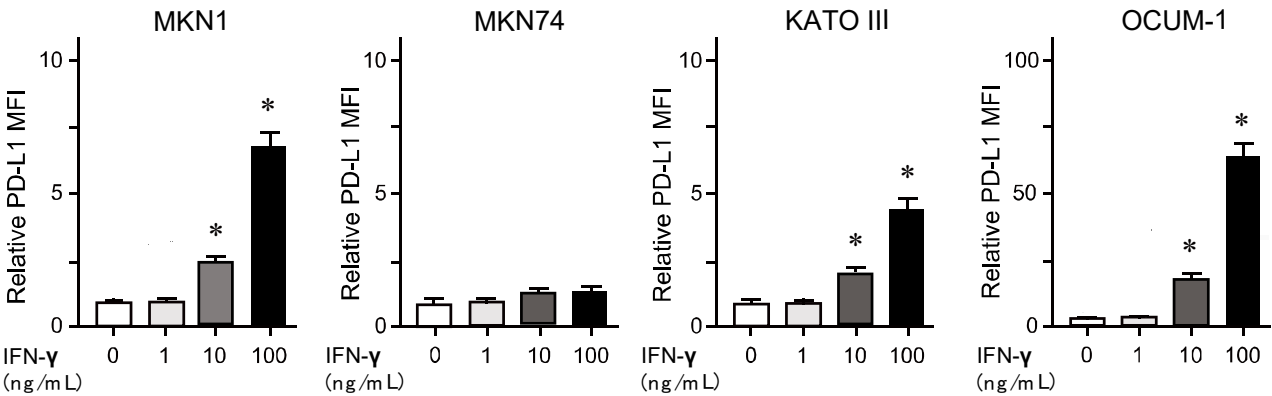


Figure 3.

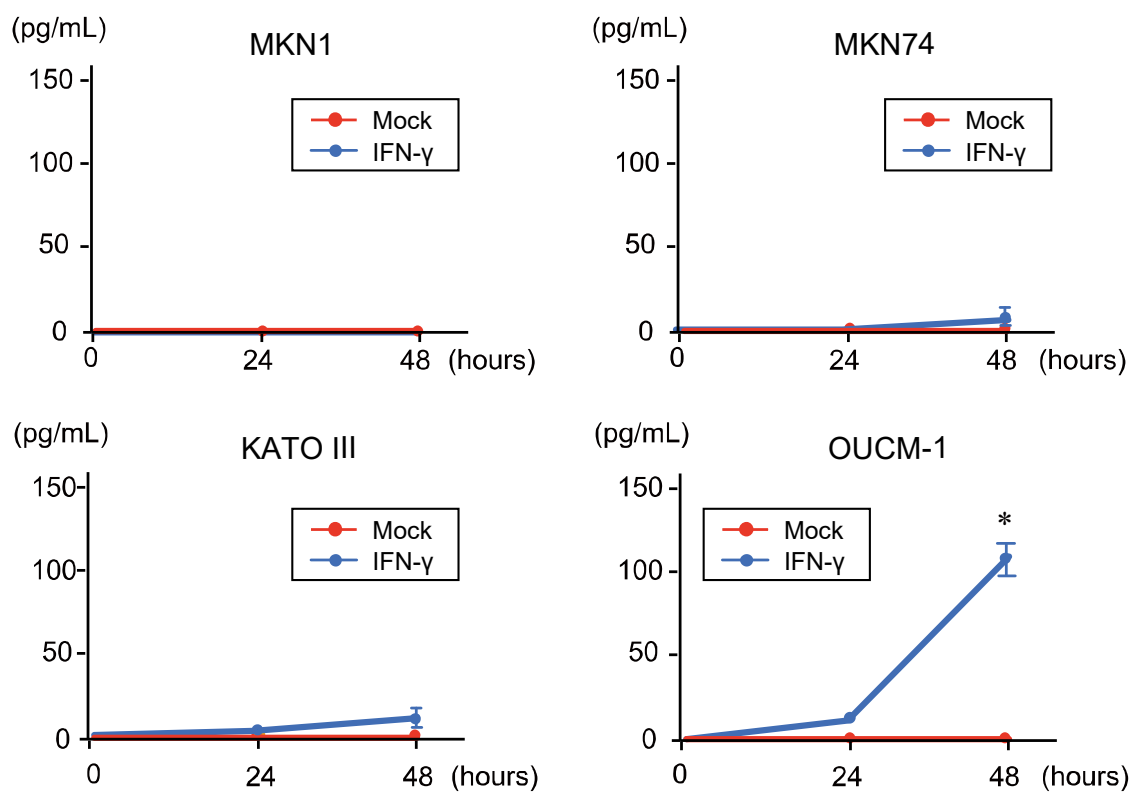
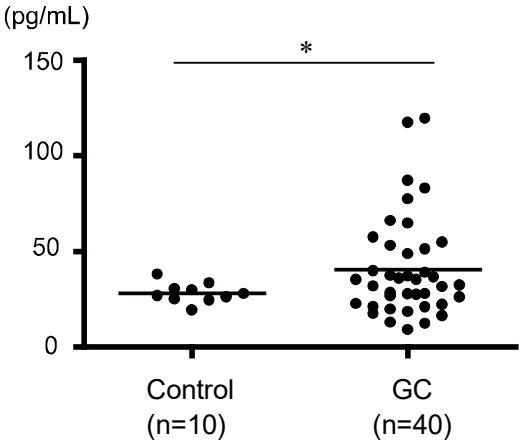


Figure 4.

A



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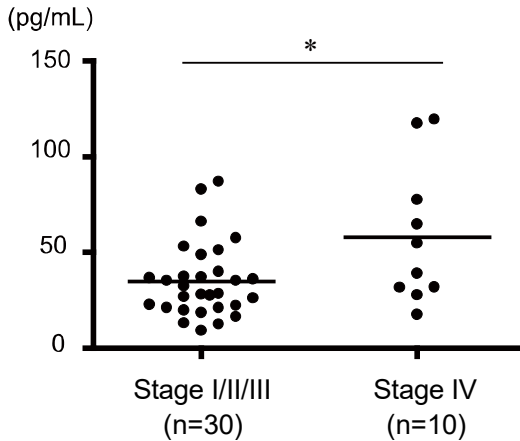


Figure legends

Figure 1 Basal and cytokine-induced PD-L1 expression in GC cells.

(A) PD-L1 mRNA expression in the presence of various concentrations of TNF- α for 24 hours were examined by quantitative RT-PCR analyses. *, significant ($p < 0.05$). (B) PD-L1 mRNA expression in the presence of various concentrations of IFN- γ for 24 hours were examined by quantitative RT-PCR analyses. *, significant ($p < 0.05$). (C) Cells treated with IFN- γ (100 ng/mL) for 24 hours were subjected to immunocytochemistry and PD-L1 (red) expression was determined. Nuclei stained with DAPI (blue) are also shown in insets. Scale bar = 100 μ m.

Figure 2 Flow cytometric profiles of membrane-bound PD-L1 and detection of soluble PD-L1 in GC cells.

(A) Histogram analyses of PD-L1 expression in GC cells treated with various concentrations of IFN- γ for 24 hours. (B) Mean fluorescent intensity (MFI) based on the data shown in (A). *, significant ($p < 0.05$).

Figure 3 Detection of sPD-L1 in GC cells.

sPD-L1 in supernatant in culture of GC cells treated with IFN- γ (100 ng/mL) for 24 or 48 hours were measured by ELISA. *, significant ($p < 0.05$).

Figure 4 Measurement of sPD-L1 in sera of GC patients.

(A) sPD-L1 in sera of controls and GC patients. *, significant ($p < 0.05$). (B) sPD-L1 levels in sera of GC patients in view of stage progression. *, significant ($p < 0.05$).