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# Lentinan, A Shiitake Mushroom β-Glucan, Stimulates Tumor-Specific Adaptive Immunity through PD-L1 Down-Regulation in Gastric Cancer Cells

**Hiroko Ina[1], Masahiko Yoneda[1], Mitsuro Kanda[2], Yashiro Kodera[2], Megumi Kabeya[3], Shu Yuasa[3], Takae Kataoka[4], Ryuichi Furuta[4] and Kenji Ina[4]***

[1]*School of Nursing and Health, Aichi Prefectural University, Public University in Nagakute, Japan*
[2]*Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine, Chikusa-ku, Nagoya, Japan*
[3]*Department of Pharmacy, Department of Hospital Pharmacy, Nagoya Memorial Hospital, Japan*
[4]*Department of Medical Oncology, Nagoya Memorial Hospital and Nagoya, Japan*

## Abstract

**Background:** Despite the significant advances in chemotherapy, the prognosis of unresectable gastric cancer is still very poor and the role of immunotherapy remains to be clarified. We examined whether lentinan, a biological response modifier, could enhance the chemotherapeutic effects.

**Materials and methods:** A retrospective cohort study was conducted to evaluate the survival benefits of lentinan among the patients with gastric cancer receiving chemotherapy. To investigate the mechanisms underlying the clinical effects of lentinan, its cytotoxic activity was accessed by cell proliferation assay. The expression of molecules relevant to immune checkpoints were analyzed by real-time PCR using human gastric cancer cell lines; MKN1, MKN45, and NUGC3.

**Results:** The addition of lentinan prolonged the survival of patients with gastric cancer receiving S-1 based chemotherapy. Lentinan reduced the constitutive expression of PD-L1 in all cell lines mainly by suppressing the MAPK pathway.

**Conclusion:** Lentinan at clinical concentrations stimulates tumor-specific adaptive immunity through PD-L1 downregulation, which may enhance chemotherapy-induced tumor clearance and patient survival.

**Keywords:** Lentinan; Gastric cancer; Chemotherapy; β-glucan; PD-L1; Immune checkpoints; Mitogen-activated protein kinase

## Introduction

Cancer cells express many inhibitory signaling proteins that cause immune cell dysfunction [1]. One of these inhibitory molecules is programmed cell death ligand 1 (PD-L1), which engages programmed cell death receptor 1 (PD-1) expressed by activated T cells and subsequently triggers inhibitory signaling downstream of the T-cell antigen receptors (TCRs), blocking effector functions [2,3]. Recent evidence suggests that PD-L1 protein is abundantly expressed on the cell surface in various human cancers [4]. This molecule can shield tumor cells and protect them from lysis by cytotoxic T lymphocytes [5]. Mitogen-activated protein kinase (MAPK) signaling pathway, which plays a critical role in cell survival and proliferation, is aberrantly activated in many types of cancer through oncogenic mutations such as Ras and B-Raf [6]. The activation of the MAPK pathway promotes PD-L1 expression in melanoma cells [7]. Because loss of phosphatase and tensin (PTEN) function can directly up-regulate PD-L1 expression on cancer cells, stimulation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway caused by inactivating PTEN might be also associated with the intrinsic induction of PD-L1 [8,9]. In addition, the transcription factors, NF-β and STAT3, bind to PD-L1 promoter to regulate its expression [10,11]. Therefore, inhibition of these molecules should reduce PD-L1 expression, which may contribute to the enhancement of antitumor immune response. With the clinical success of monoclonal antibodies to either PD-L1 or PD-1 in the treatment of melanoma and non-small-cell lung cancer [12,13], blockade of immune checkpoints is actively developed for other solid tumors, including gastric cancer [14].

Gastric cancer remains the fifth most common malignancy and the third leading cause of cancer mortality worldwide [15], despite the significant advances in gastric cancer diagnosis and therapy [16,17]. For patients with metastatic gastric cancer, platinum-based and fluoropyrimidine combination regimens are considered the mainstay of the first line of treatment [18]. However, the prognosis of unresectable gastric cancer is still very poor with a median overall survival (OS) of one year [19,20]. Because immune-based therapies have the potential to elicit immune system cells, the combination of immunotherapy with cytotoxic chemotherapy might improve patient survival [21]. Lentinan, a purified β-glucan derived from Shiitake mushroom [22,23], has direct antitumor and immunomodulatory properties [24,25]. An individual patient data meta-analysis implicated that the addition of lentinan to conventional chemotherapy should prolong the survival of patients with gastric cancer over that of patients receiving chemotherapy alone [26]. We recently experienced a patient who showed complete disappearance of primary gastric tumor and multiple liver metastases [27] in response to the triple PSC chemotherapy combined with this β-glucan [28,29].

In this study, we first reviewed the clinical data of patients with gastric cancer receiving chemotherapy in order to re-evaluate the clinical efficacy of lentinan. As the survival benefits of lentinan were strongly supported by our retrospective analysis, we then investigated whether this agent can modulate PD-L1/PD-1 axis using *in vitro* experiments. Our results showed that lentinan treatment significantly reduced PD-L1 expression at the transcriptional levels in gastric cancer

***Corresponding author:** Kenji Ina, Department of Medical Oncology, Nagoya Memorial Hospital, 4-305 Hirabari, Tenpaku-ku, Nagoya, 468-8520, Japan, Tel: +0528041111; Fax: +0528038830; E-mail: kina@hospy.or.jp

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cells without inhibiting cell proliferation, which may explain the novel mechanism of lentinan for the treatment of gastric cancer.

## Materials and Methods

### Patients

A retrospective chart review of patient with gastric cancer receiving chemotherapy was performed at Nagoya Memorial Hospital during 5 years from 2011 to 2015. Patients were eligible if they provided informed consent for chemotherapy and met the following criteria: (1) pathologically proven inoperable gastric cancer; (2) age 20 to 85 years; (3) white blood cell count between 3,000 and 12,000 /mm$^3$, platelet count >75,000/mm$^3$, hemoglobin >8 g/dL; serum bilirubin <3.0 mg/dL, aspartate aminotransferase and alanine aminotransferase <3 times the upper limit of normal; and serum creatinine <1.3 mg/dL. OS was calculated from the beginning of chemotherapy until death or the most recent follow-up day. The Kaplan-Meier method was used to plot OS curves. Doses of chemotherapeutic agents were adjusted at the initiation of subsequent cycles, if severe toxicity (grade 3-4) was present; 2 mg of lentinan was intravenously administered every 2 or 3 weeks in combination with S-1 based chemotherapy. In some patients the serum concentrations of β-glucan were chronologically determined. The chart review was approved by ethics committee of Nagoya Memorial Hospital.

### Reagents and cell culture

Lentinan was purchased from Ajinomoto Co., Ltd. (Tokyo, Japan). Stock solutions of this agent were prepared in sterile distilled water and dissolved in culture medium immediately before their use. The human gastric cancer cell lines (MKN1, MKN45, and NUGC3) were kindly provided by the Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine (Professor Yasuhiro Kodera) and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies Corp., Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies Corp.). The cells were maintained at 37°C in a humidified incubator under an atmosphere containing 5% $CO_2$. Cells were exposed to various concentrations of lentinan.

### Evaluation of cytotoxicity

Gastric cancer cells in the logarithmic phase of growth were seeded in 96-well plates at a density of $1.5 \times 10^4$ cells per well in 200 μL of medium and grown for 48 h. The cells were then treated with increasing concentrations of lentinan, from 0 to 100 μg/mL, for 40 h. Cytotoxic activity was measured by a colorimetric assay that is based on the cleavage of tetrazolium salts (Premix WST-1 cell proliferation assay system; Takara Bio Inc., Ohtsu, Japan) [23,30]. Cells were reacted with water-soluble tetrazolium salts (WST) -1 at 37°C for 2 h. Absorbance of each well was measured at 450 nm using a microplate reader. Assays were performed in triplicate and were repeated three times. The ratio of cell proliferation inhibition was determined according to the following formula:

Ratio of cell proliferation inhibition in cancer cells (%)=1-[(OD of treatment group–OD of blank group)/ (OD of control group–OD of blank group)] × 100 [31]

### RNA isolation and reverse transcription-polymerase chain reaction

Cells were seeded in 6-well plates at a density of $5 \times 10^5$ cells per well in 1 mL of culture medium. Two days later, cells were treated with lentinan for 40 h. Total RNA was extracted from gastric cancer cells using SV total RNA isolation system (Promega Inc, Tokyo, Japan) and RNA concentrations were quantified using a spectrometer (GeneQuant pro; GE Healthcare UK Ltd., Buckinghamshire, England). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with PrimeScript™ RT Master Mix (Takara Bio Inc.). Real-time polymerase chain reaction (PCR) analysis was performed using double-strand DNA-specific dye with Thermal Cycler Dice; Version 4.02, Code TP900/ TP960 (Takara Bio Inc.). The reaction mixtures (20 μL) included: cDNA 1 μL, primer 1 μL each, dd$H_2O$ 9.5 μL, SYBR premix EX TaqII (Takara Bio Inc.) 12.5 μL. The amplification conditions were 95°C for 5 minutes, 45 PCR cycles at 95°C for 15 s, 60°C 1 s, and then 95°C for 15 s, 60°C for 30 s, 95°C for 15 s. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. To quantify gene expression, the ΔΔCt method was developed for the comparison of expression of a gene of interest among different samples [32]. Based on the concept of this method, each datum of real-time PCR was expressed as a relative quantity, compared to the fluorescence intensity of β-actin, a house keeping gene, in the same samples. The following primer pairs were used for the cDNA amplification. Three independent experiments were performed to determine the mean and standard error (SE) of gene expression.

*PD-L1*: Forward primer; 5′-GGACAAGCAGTGACCATCAAG-3′, Reverse primer; 5′-CCCAGAATTACCAAGTGAGTCCT-3′

*MAPK*: Forward primer; 5′-CGTTGGTACAGGGCTCCAGAA-3′, Reverse primer; 5′-CTGCCAGAATGCAGCCTACAGA-3′

*AKT*: Forward primer; 5′-AGCGACGTGGCTTTGTGAA-3′, Reverse primer; 5′-CACGTTGGTCCACATCCTG-3′

*NF-κβ*: Forward primer;

5′-ACGAATGACAGAGGCGTGTATAAGG-3′, Reverse primer; 5′-CAGAGCTGCTTGGCGGATTAG-3′

*STAT3*: Forward primer;

5′-TGCCTTATCAGGGCTGGGATAC-3′, Reverse primer; 5′-GGGACCTTTAGACACGCAAGGA-3′

β-actin: Forward primer; 5′-CATGTACGTTGCTATCCAGGC-3′, Reverse primer; 5′-CTCCTTAATGTCACGCACGAT-3′

### Statistical analysis

The rates of survival in patients with or without lentinan were compared with the use of either unadjusted log-rank test or a matched pair analysis using McNemar's test. Experimental data sets were tested by one-way ANOVA followed by Dunnett's multiple comparison test and Wilcoxon-signed rank test for the comparison between two groups. Differences were considered statistically significant when *P* values were less than 0.05. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [33]. More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

## Results

### Clinical findings

Among 81 eligible patients with gastric cancer receiving S-1 based chemotherapy, 52 patients were classified into stage 4. Thirty-four

patients were intravenously administered lentinan at their request, while the other 18 patients did not receive this agent during the 5 years. A retrospective study of stage-4 gastric cancer patients showed that median OS was significantly longer in the group that received lentinan in combination with chemotherapy than that in the chemotherapy alone group (401 days [95% confidence interval (CI), 257-747 days]) versus 148 days [95% CI, 79–225 days]), *P*=0.000243) (Figure 1). However, the chemotherapeutic regimens used were different between the above two groups (S-1 alone, 12 versus 15; S-1/ cisplatin, 13 versus 3; PSC triple therapy, 9 versus 0). To neglect the bias of the difference of chemotherapeutic regimens as well as age and gender, a matched pair analysis was conducted between patients who were or were not administered lentinan, showing that lentinan treatment significantly increased the rate of one-year survival (matched odd's ratio 8.0; *P*=0.0455) (Table 1). The serum β-glucan concentrations of patients with gastric cancer ranged from 0.24 to 16.7 ng/mL during 3 weeks after intravenous infusion of lentinan (Table 2).

### *In vitro* experiments

As lentinan presented survival benefits in the patients with stage-4 gastric cancer, the mechanism underlying these effects were investigated *in vitro*. WST assay demonstrated that lentinan suppressed the proliferation of gastric cancer cells as its concentration increased. However, this β-glucan had minimum anti-proliferative activities at concentrations less than 10 ng/mL (Figure 2). We then conducted real-time PCR to examine whether lentinan treatment affects the PD-L1/ PD-1 pathway. Constitutive levels of *PD-L1* expression were different among the cell lines and the highest level of expression was detected in NUGC3 cells (Figure 3). Lentinan treatment at either 1 or 10 ng/ mL, concentrations that are compatible with the serum concentrations observed in patients, reduced *PD-L1* mRNA expression in each cell line. In contrast to the low concentrations, treatment with 100 ng/mL lentinan significantly increased *PD-L1* expression in NUGC3 cells. To analyze how lentinan modulates *PD-L1* expression, upstream signaling molecules were examined using NUGC3 cells. The levels of *MAPK* mRNA decreased after treatment with 1 ng/mL lentinan and then increased after treatment with 100 ng/mL lentinan, while *AKT* mRNA expression was not altered in parallel with that of PD-L1 (Table 3). The mRNA expression of the transcription factors, *NF-κβ* and *STAT3*, was also decreased after treatment with either 1 or 10 ng/mL lentinan, although this reduction was not significant.

## Discussion

Our retrospective analysis of 52 patients with stage-4 gastric cancer showed that median OS was significantly longer in the group that received lentinan in combination with S-1 based chemotherapy, which was further supported by a pair matching comparison to adjust the confounding factors such as age, gender, and chemotherapeutic regimens. These findings were consistent with the previous meta-analysis that the addition of lentinan to oral fluoropyrimidine-based chemotherapy prolonged the survival of patients with gastric cancer, when compared to chemotherapy alone [26].

*In vitro* experiments showed that gastric cancer cell lines differ in the mRNA level of tumor-intrinsic expression of *PD-L1*. Epstein-Barr virus infection is one of the major causes of stomach cancer [34], in which viral integration is implicated in the aberrant PD-L1 transcription. Genomic aberrations in tumor cells are also associated with constitutive expression of PD-L1 [35], leading to the cancer immune escape from the host immune system. PD-L1, which binds to PD-1 on T cells and dendritic cells, is expressed in a broad range of malignant tumors [4]. The present study implicated that treatment

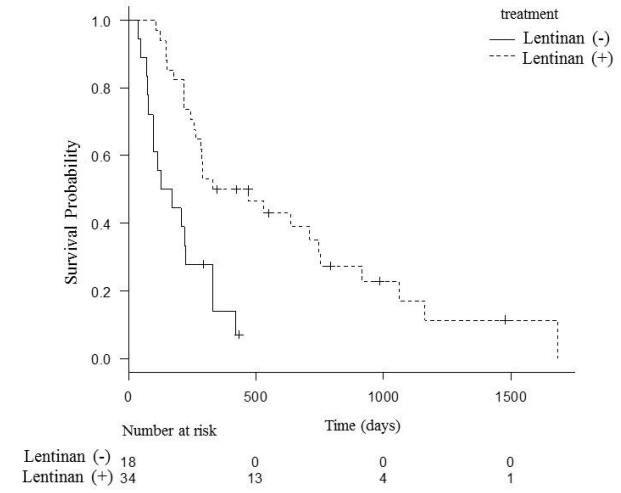


**Figure 1:** Survival benefits of lentinan. Retrospective analysis of stage-4 gastric cancer patients showed that median overall survival (OS) was significantly longer in the group that received lentinan in combination with S-1 based chemotherapy (N=34) than that in the chemotherapy alone group (N=18) (401 days [95% confidence interval (CI), 257-747 days] versus 148 days [95% CI, 79-225 days]) (*P* value=0.000243 determined by log-rank test).

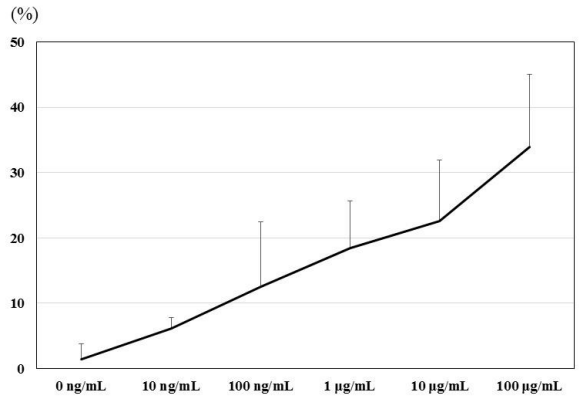


**Figure 2:** Proliferation assay using WST-1 assay. Lentinan increased the inhibition ratio of MKN1 cell proliferation in a dose-dependent manner. However, the difference was little between cells cultured without or with 10 ng/mL lentinan.

with lentinan should reduce the expression of tumor-intrinsic *PD-L1*. This inhibition was suspected to be mediated through MAPK signaling, because the mRNA expression of *PD-L1* and *MAPK* was similarly modulated in the presence of lentinan. More importantly, the suppressive effects of this β-glucan on PD-L1 were observed at either 1 or 10 ng/ mL, concentrations that are in the clinical range, which should be helpful for improving the clinical outcome of gastric cancer. The PI3K/AKT pathway has been reported to be activated by β-glucan via scavenger receptor [25,36]. However, in this study, the addition of lentinan did not cause any changes in the constitutive expression of *AKT*. With regard to transcription factors, *NF-κβ* as well as *STAT3* appeared to be down-regulated by lentinan at either 1 or 10 ng/mL lentinan, almost in parallel with the reduction of *PD-L1* expression, but this change did not reach statistical significance.

Currently, the prognosis of patients with advanced gastric cancer remains poor, despite recent therapeutic improvements [16,17]. This preliminary study implies that lentinan might sufficiently reduce the mRNA expression of *PD-L1* and related signaling molecules to boost the therapeutic antitumor immunity of the host [37], although the precise mechanism of PD-L1 expression in protein levels remains to be clarified. Lentinan possesses several distinct characteristics from targeting therapy against the PD-L1/ PD-1 pathway. Of note, the use of lentinan has an advantage in terms of health care economics, partly because this β-glucan has been approved as a biological response modifier for the treatment of gastric cancer for more than 20 years. In addition, lentinan has little adverse effects, while immune checkpoint inhibitors sometimes induce immunological disorders such as type 1 diabetes mellitus, interstitial pneumonitis, and colitis [12-14]. Accordingly, the combination therapy of targeting PD-L1 with very low levels of lentinan might be possible with no serious adverse event to further control the PD-L1/PD-1 axis. It has been reported that chemotherapeutic agents such as platinum compounds up-regulated PD-L1 expression in tumor cells, resulting in decreased activation of T cells [38,39]. Our results support the hypothesis that lentinan treatment restore the chemo-sensitivity to cisplatin via suppressing the expression of PD-L1, which might be considered as a synergistic mechanism of chemo-immunotherapy. Further investigations are necessary to establish a basis for the rational design of the optimized combinational regimens for the treatment of gastric cancer.

| S No | Age | | Gender | Regimen | Overall survival (OS) days | |
|---|---|---|---|---|---|---|
| | (+) | (-) | | | (+) | (-) |
| 1 | 51 | 51 | Male | S-1 | 160 | 83 |
| 2 | 54 | 59 | Male | S-1 | 843* | 55 |
| 3 | 55 | 60 | Male | S-1 | 101 | 68 |
| 4 | 66 | 65 | Male | S-1 | 835* | 359 |
| 5 | 67 | 72 | Female | S-1 | 210 | 123 |
| 6 | 70 | 67 | Male | S-1 | 117 | 78 |
| 7 | 73 | 73 | Male | S-1 | 525* | 120 |
| 8 | 74 | 74 | Male | S-1 | 440* | 276 |
| 9 | 78 | 81 | Female | S-1 | 1591* | 275 |
| 10 | 79 | 76 | Male | S-1 | 140 | 215 |
| 11 | 80 | 81 | Male | S-1 | 615* | 193 |
| 12 | 82 | 84 | Male | S-1 | 166 | 207 |
| 13 | 76 | 75 | Male | S-1/ cisplatin | 329 | 407* |
| 14 | 76 | 76 | Male | S-1/ cisplatin | 541* | 69 |
| 15 | 78 | 76 | Male | S-1/ cisplatin | 1039* | 278 |

In order to adjust the confounding factors such as age, sex, and the difference of chemotherapeutic regimens, a matched pair analysis was conducted between patients with or without intravenous administration of lentinan, which showed that the treatment with this agent significantly increased the rate of one-year survival (P=0.0455 determined by McNemar's test).
(+): Chemo-immunotherapy in combination with lentinan; (-): Chemotherapy alone; *: OS was more than one year

**Table 1:** A matched pair analysis of stage-4 gastric cancer patients according to the presence or absence of lentinan.

| | Median (range) (ng/mL) | N |
|---|---|---|
| Immediately after | 13.4 (5.43-16.7) | 5 |
| 12 h | 9.18 (5.65-15.7) | 4 |
| Day 3 | 9.48 (2.92-9.97) | 4 |
| Day 7 | 6.85 (3.33-9.72) | 9 |
| Day 14 | 3.14 (1.00-6.99) | 16 |
| Day 21 | 2.20 (0.24-10.0) | 11 |

Intravenous administration of 2 mg of lentinan was conducted every 2 or 3 weeks in combination with S-1 based chemotherapy. Serum concentrations of β-glucan were determined at day 0, 0.5, 3, 7, 14, and 21 after infusion of lentinan.

**Table 2:** Serum concentrations of β-glucan in patients receiving lentinan.

| Lentinan | 0 | 0.1 ng/mL | 1 ng/mL | 10 ng/mL | 100 ng/mL |
|---|---|---|---|---|---|
| *MAPK* ($\times 10^{-5}$) | 169 ± 22 | 159 ± 31 | 104 ± 10 (*$P$=0.036) | 130 ± 21 | 234 ± 78 |
| *AKT* ($\times 10^{-5}$) | 150 ± 27 | 151 ± 12 | 109 ± 21 ($P$=0.313) | 146 ± 47 | 160 ± 16 |
| *NF-kβ* ($\times 10^{-5}$) | 110 ± 8.7 | 118 ± 8.9 | 67 ± 15 ($P$=0.078) | 54 ± 7.6 | 118 ± 23 |
| *STAT3* ($\times 10^{-7}$) | 174 ± 60 | 161 ± 37 | 104 ± 38 ($P$=0.156) | 106 ± 23 | 132 ± 38 |

Real-time PCR revealed that mRNA expression of *MAPK*, but not *AKT*, was significantly decreased in NUGC3 cells after treatment with 1 ng/mL lentinan. The mRNA expression of transcriptional factors, *NF-κβ* and *STAT3*, appeared to be decreased after treatment with 1 and 10 ng/mL lentinan, but this change did not reach statistical significance. Real-time PCR data were shown as the relative quantity in comparison with the fluorescent signal of β-actin, a house keeping gene, based on the concept of the ΔΔCt method [31]. Three independent experiments were performed to determine the mean and standard error (SE) of gene expression (*$P$<0.05 compared to control cultures; Wilcoxon-signed rank test).

**Table 3:** The expression of PD-L1 related signaling molecules after lentinan treatment determined by real-time PCR analysis.

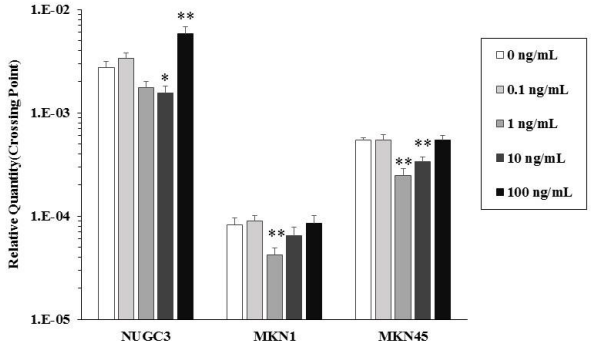


**Figure 3:** Real-time PCR analysis of *PD-L1* in three gastric cancer cell lines. Real-time PCR revealed that constitutive *PD-L1* expression was the highest in NUGC3 cells among the three cell lines examined. Relative quantity of *PD-L1* in each sample was determined by comparison to the fluorescent signal of β-actin, a house keeping gene, based on the concept of the ΔΔCt method (31). The expression of *PD-L1* was reduced after 1 or 10 ng/ mL lentinan treatment in each cell line. On the other hand, after treatment with 100 ng/mL lentinan, *PD-L1* expression was increased in NUGC3 cells. Three independent experiments were performed to determine the mean and standard error (SE) of gene expression (*$P$<0.05, **$P$<0.01 compared to control cultures; Dunnett's multiple comparison test).

## Conclusion

Lentinan can stimulate tumor-specific adaptive immunity through PD-L1 down-regulation in the range of clinically feasible concentrations and may enhance chemotherapy-induced tumor clearance and the survival of gastric cancer patients.

### Conflict of Interest Statement

The authors have no conflict of interest to declare.

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