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Original Article

Peptidoglycan induces CXCL10 production and inhibits esophageal squamous cell carcinoma proliferation

Yoshihiro Sasaki[1,2], Yusuke Sato[1,2], Kyoko Nomura[3], Akiyuki Wakita[1,2], Yushi Nagaki[1,2], Ryohei Sasamori[1,2], Takatoshi Yoneya[1,2], Tsukasa Takahashi[1,2], Munehiro Yamada[1,2], Mayu Takahashi[1,2], Kaori Terata[2], Kazuhiro Imai[2]

[1]Division of Esophageal Surgery, Akita University Hospital, Akita 010-8543, Japan; [2]Department of Thoracic Surgery, Akita University Graduate School of Medicine, Akita 010-8543, Japan; [3]Department of Environmental Health Science and Public Health, Akita University Graduate School of Medicine, Akita 010-8543, Japan

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Abstract: Poor oral health is an independent risk factor for upper-aerodigestive tract cancers, including esophageal squamous cell carcinoma (ESCC); thus, good oral health may reduce the risk of ESCC. We previously reported that high expression of Toll-like receptor (TLR) 6, which recognizes peptidoglycan (PGN) from Gram-positive bacteria correlates with a good prognosis after esophagectomy for ESCC. Most beneficial bacteria in the mouth are Gram-positive. We therefore hypothesized that PGN affects cancer cell proliferation and disease progression in ESCC. To test that idea, we assessed the expression of cytokine and chemokine mRNA and protein in eight ESCC cell lines. We also employed a mouse xenograft model to investigate the effect of PGN on ESCC tumor progression *in vivo*. We then investigated the relationship between the combined expression profiles of TLR6 and C-X-C motif chemokine ligand 10 (CXCL10) in clinical samples and 5-year overall survival (OS) and disease-specific survival (DSS) in ESCC patients after curative esophagectomy. We found that PGN significantly inhibited cell proliferation in six of eight ESCC lines and upregulated CXCL10 production via NF-κB2. *In vivo*, subcutaneous PGN administration tended to decrease ESCC tumor volume in mice. Combined high expression of TLR6 and CXCL10 correlated with a better prognosis in ESCC patients. This suggests that PGN reduces cell proliferation and tumor progression through a PGN-TLR-CXCL10 cascade, thereby influencing prognosis after esophagectomy for ESCC, and that improving the oral environment could potentially improve the prognosis of ESCC patients after esophagectomy.

Keywords: Peptidoglycan (PGN), Toll-like receptor 6 (TLR6), C-X-C motif chemokine ligand 10 (CXCL10), esophageal cancer, esophageal squamous cell carcinoma (ESCC), prognosis

Introduction

It has been estimated that there were 604,000 new cases of esophageal cancer worldwide in 2021 and that there are 544,000 deaths related to esophageal cancer annually [1]. Esophageal cancer is divided into two histological subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC is common in Central South America, Africa and Asia [2, 3]. Cigarette smoking, heavy alcohol consumption, poor dietary habits, inactivation of alcohol dehydrogenase-1B (ADH1B) and aldehyde dehydrogenase-2 (ALDH2) are well known risk factors for ESCC [4, 5]. More recent reports suggest that poor oral health is an independent risk factor for upper-aerodigestive tract cancers, including ESCC [6-9]. In addition, our earlier findings suggest severe periodontitis is an independent risk factor contributing to a poorer long-term prognosis after esophagectomy [10].

Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) released from a wide range of viruses, bacteria, fungi and parasites [11-13]. These receptors play key roles in mediating innate and antigen-specific adaptive immunity. Humans are known to express 10 TLRs, which recognize a variety of different PAMPs [12]. Human mouths reported-

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ly contain about 700 species of bacteria, which have been classified as "bad", "beneficial" or "opportunistic" [14, 15]. Representative of bad bacteria are periodontal pathogens, most which are Gram-negative with cell walls composed of lipopolysaccharide (LPS). LPS is recognized by TLR4, and we previously reported that ESCC patients exhibiting high TLR4 expression show significantly poorer survival than patients expressing lower levels of TLR4 [16].

On the other hand, beneficial bacteria in the mouth include Streptococcus mitis, Streptococcus oralis and Streptococcus salivalis, all of which are Gram-positive. Moreover, beneficial bacteria in the gut microbiome, including Lactobacillus species, butyrate-producing bacteria and Bacillus subtilis, are all Gram-positive. Their cell walls are composed of peptidoglycan (PGN), which is recognized by TLR2/TLR6 heterodimer [17]. We previously reported that ESCC patients exhibiting high TLR6 expression show significantly better survival than patients expressing lower levels of TLR6 [18].

These findings suggest that there may be a relationship between PGN/TLR signaling and ESCC growth. However, little is known about the relationship between PGN and ESCC growth. In the present study, therefore, we assessed the effects of PGN on ESCC cell proliferation and migration *in vitro* and on progression of ESCC in an *in vivo* mouse xenograft model. We also investigated whether the combined expression statuses of TLR6 and CXCL10 in ESCC clinical samples reflect prognosis after curative surgery for ESCC.

Materials and methods

Cell lines

KYSE series (KYSE-150, KYSE-170, KYSE-220, KYSE-270, KYSE-410, KYSE-450) and TE series (TE-8, TE-10) ESCC lines were purchased from the Health Science Research Resources Bank (Osaka, Japan) and RIKEN Cell Bank (Tsukuba, Japan). All cell lines were cultured in RPMI1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (GIBCO, Grand Island, NY) and antibiotics (penicillin G/streptomycin/amphotericin B, GIBCO) under 5% CO_2 in a humidified incubator at 37°C.

Cell proliferation assays

The effect of PGN on ESCC cell proliferation was evaluated using cells incubated with or without 10 μg/ml PGN (Invivogen, CA). ESCC cells were seeded into 96-well plates at a density of 1×10^3 cells/well and incubated first for 24 h in 100 μL of RPMI1640 with 10% FBS and then for an additional 48 h in 100 μL of RPMI1640 with or without 10 μg/ml of PGN. A CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, WI) was then used to assess cell numbers. Cells incubated without PGN served as the control, and the average number of control wells was defined as 100% and compared with the PGN group. Each sample was analyzed in eight wells, and this evaluation was performed twice.

RNA isolation, reverse transcription, and quantitative real time PCR

ESCC lines were seeded into 6-well plates with or without 10 μg/ml PGN. After reaching subconfluence, total RNA was extracted using Trizole® (Invitrogen, CA), and the quantity and purity of the RNA was evaluated using a NanoDrop 2000® spectrophotometer (Thermo Fisher Scientific, MA). cDNA was synthesized using a Transcriptor First Strand cDNA synthesis kit® (Roche Applied Science, Germany).

Quantitative real-time PCR was performed using a LightCycler 480® Real-Time PCR System (Roche Applied Science). The amplification was carried out in a total volume of 10 μL containing 1 μL of cDNA sample derived from 50 ng of total RNA, 0.4 μM each primer, 0.2 μM Universal ProbeLibrary probe, 5 μL of 2× Probes Master (Roche Applied Science) and 3.4 μL of H_2O. The cycling protocol entailed an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s and annealing and extension at 60°C for 30 s. PCR amplification efficiency and CT values were calculated using LightCycler 480 Software Version 1.5, after which the ddCT method was used to compare mRNA expression levels between samples. The sequences of the primers and probes used for real-time PCR are listed in Supplementary Table 1.

Enzyme-linked immunosorbent assay

After growing ESCC cells to subconfluence in 6-well plates, the cells were incubated for 24 h with or without PGN. The culture supernatants were then collected, and levels of CXCL10 expression were compared using an ELISA kit (Biotechne, US).

Caspase 3/7 assay

ESCC cells (KYSE-150, KYSE-270) were seeded into 96-well plates to a density of 1×10^3 cells/well and incubated first for 24 h in 100 µL of RPMI1640 with 10% FBS and then for an additional 48 h in 100 µL of RPMI1640 with or without 10 µg/ml PGN. Thereafter, 100 µl of Caspase-Glo 3/7 Reagent (Promega, WI) were added to the cultures, which were then incubated for 1 h at room temperature. Finally, the caspase 3/7 activity was determined by measuring the luminescence using a microplate reader.

Mouse xenograft model

All experiments with mice were conducted with the approval (#a-1-0568) of the Akita University Ethics Committee. KYSE-150 and KYSE-270 cells (5×10^6 cells/mouse in 100 µL of PBS) were subcutaneously injected into the backs of 5-week-old female BALB/c nude mice obtained from CLEA Japan. For mice implanted with KYSE-150 cells (five per group), treatments were administered every other day for a total of 14 days (7 treatments) starting 14 days after tumor implantation. The PGN group received an intratumoral injection of 250 µg/kg PGN in 100 µL of PBS, while the control group received a similar injection of 100 µL of PBS. Tumor volume was measured every 2 days using the formula 1/2 × length × width. The mice were sacrificed, and tumors were harvested 8 days after the last treatment. Because KYSE-270 (eight per group) tumors grow faster, treatments were administered every other day for a total of 14 days (7 treatments) starting 7 days after cell implantation. The mice were then sacrificed, and tumors were harvested 2 days after the last treatment.

Patients and procedure

This study was approved by the Ethics Committee of Akita University School of Medicine (#2324, approved date, November 20, 2019), and all experiments were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. In total, 177 patients with pT2-pT4 thoracic ESCC were enrolled in this study. All participants underwent curative esophagectomy without preoperative treatment at Akita University Hospital between January 2001 and December 2011 [19]. Our standard operative procedure was right thoracoscopic or robot-assisted thoracoscopic esophagectomy with extended three-field (mediastinal, abdominal and cervical fields) lymph node dissection. The clinical stage and treatment strategy for these patients was discussed and determined by a committee consisting of gastroenterologists, surgeons, radiologists and oncologists. The pathological stage was diagnosed according to the UICC International Union Against Cancer Tumor-Node Metastasis (TNM) Classification of Malignant Tumors (8th edition).

ESCC tissue microarray and immunohistochemical (IHC) staining

An ESCC tissue microarray was constructed at the Institute of Pathology in Toyama, Japan, using 177 paraffin-embedded blocks of primary tumor taken from the enrolled patients, as previously described [18, 20, 21]. Details about IHC staining for CXCL10 and TLR6 and the evaluation methods are described elsewhere [18, 21].

Statistical analysis

The Wilcoxon test (for continuous variables) or χ^2 and Fisher's exact tests (for categorical variables) were used to evaluate the differences in luminescence or the relative mRNA expression levels between wells treated with or without PGN. Overall survival (OS) was calculated as the time from the date of surgery to death from of any cause, while disease-specific survival (DSS) was calculated as the time from the surgery to death from ESCC. The Kaplan-Meier method was applied to construct OS curves, taking into consideration the combined CXCL10 and TLR6 expression statuses (high + high, high + low, low + high or low + low). Comparisons between curves were made using the log-rank test. Cox's proportional hazards regression model was used for univariate and multivariate analyses. To identify independent prognostic factors affecting OS, we applied a Cox proportional hazard model to calculate the hazard ratios (HRs) and 95% confidence intervals (CIs).

All statistical analyses were two-sided and performed using JMP Pro 17 (SAS Institute, NC). Values of $P \leq 0.05$ were considered significant.

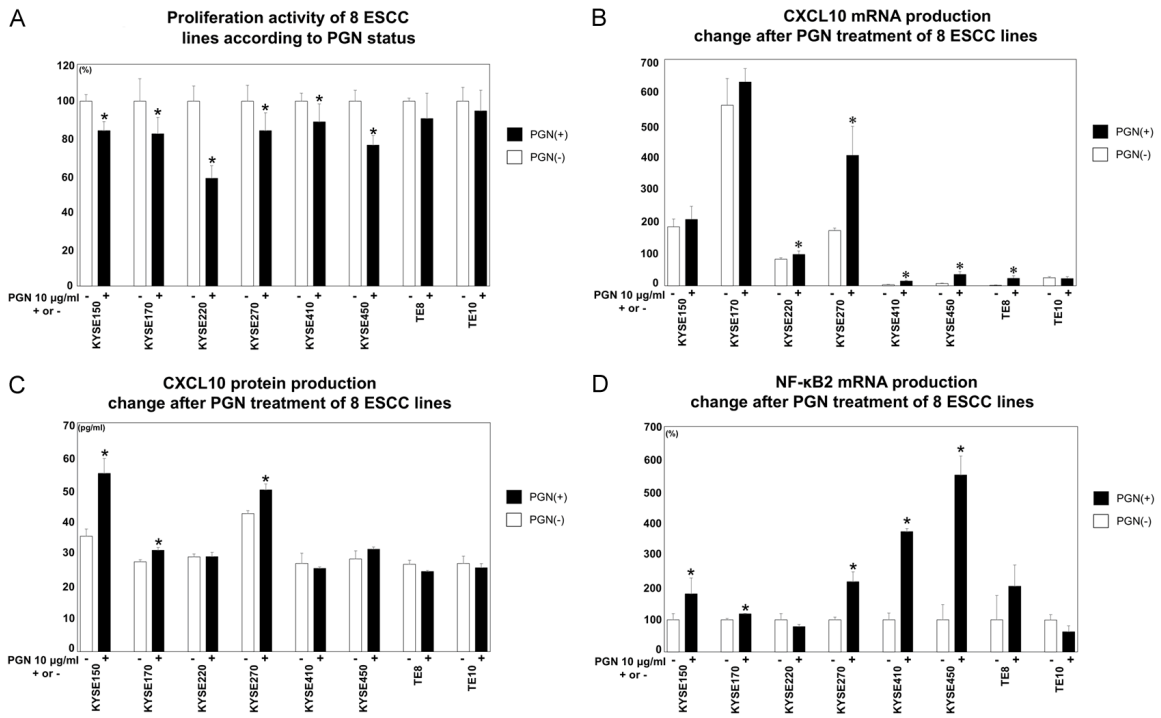

Figure 1. PGN induces ESCC cell proliferation by activating CXCL10 through NF-κB2. (A) Effect of PGN (10 μg/ml) on cell proliferation in the indicated ESCC lines. Control cells grown in the absence of PGN were assigned a value of 100. (B, C) Comparison of expression of CXCL10 mRNA (B) and protein (C) between ESCC cell lines treated with 10 μg/ml PGN and untreated controls. (D) Levels of NF-κB2 mRNA expression in ESCC lines. *P<0.05.

Results

Effect of PGN on cell proliferation in ESCC lines

The effect of PGN (10 μg/mL) on ESCC cell proliferation was investigated in 8 cell lines. Cell proliferation assays revealed that PGN stimulation for 48 h significantly decreased cell proliferation to 90-60% of control in all six cell lines in the KYSE series but not in the two TE series cell lines (**Figure 1A**).

PGN inhibits ESCC cell proliferation by activating CXCL10 through NF-κB2

Quantitative RT-PCR was performed to investigate expression of mediators potentially involved in the PGN-induced inhibition of cell proliferation (**Figure 1B**, **1D** and Supplementary Figure 1A-H). We found that expression of CXCL10 mRNA was slightly increased as compared to control in PGN-treated KYSE150 and KYSE-170 cells and was significantly increased in PGN-treated KYSE-220, KYSE-270, KYSE-410, KYSE-450 and TE-8 cells (**Figure 1B**). Moreover, ELISAs revealed that expression CXCL10 protein was also significantly increased in PGN-treated KYSE-150, KYSE-170 and KYSE-270 cells (**Figure 1C**). Expression of NF-κB2 mRNA was increased by PGN treatment in KYSE-150, KYSE-170, KYSE-270, KYSE-410, KYSE-450 and TE-8 cells (**Figure 1D**). This suggests that, in ESCC cells, PGN induces CXCL10 production through TLR2/TLR6 signaling with NF-κB2 as a mediator.

Caspase 3/7 assays

Caspase-Glo 3/7 Reagent was used to evaluate cell apoptosis. These assays revealed that exposing cells to PGN for 48 h significantly increased caspase 3/7 activity to 120-130% of control in KYSE-150 and KYSE-270 cells (**Figure 2**).

PGN inhibits ESCC cell proliferation in vivo

To assess the effects of PGN on ESCC *in vivo*, KYSE-150 and KYSE-270 cells were subcutaneously transplanted into BALB/c nude mice. These cells exhibited large increases in cell pro-

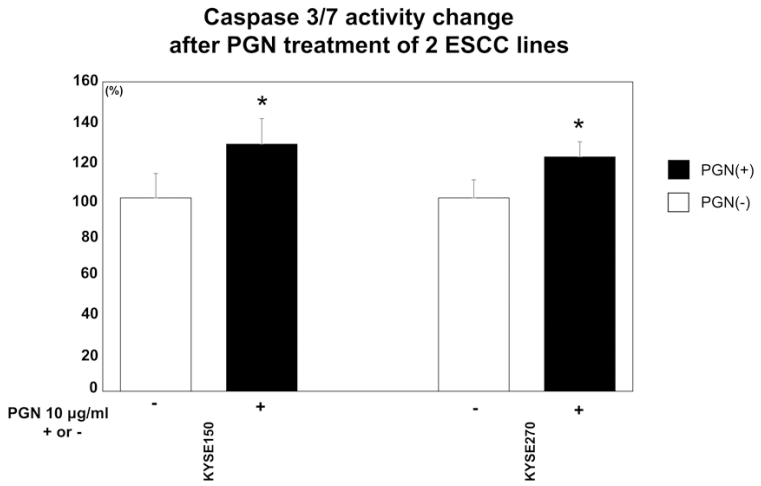

Figure 2. PGN induces apoptosis in KYSE-150 and KYSE-270 cells. Apoptosis was assessed based on levels of caspase 3/7 activity in cells treated with PGN (10 μg/ml) for 48 h (*P<0.05).

liferation and in expression of both CXCL10 mRNA and protein *in vitro*. About 5 × 10^6 tumor cells were subcutaneously injected into the backs of the mice, after which the engrafted tumors were allowed to grow for 2 weeks. We then intratumorally injected PBS or PGN every other day for 2 weeks and evaluated the increase in tumor size (**Figure 3A** and **3B**). We found that PGN tended to reduce ESCC xenograft tumor growth *in vivo*, though the effect did not reach the level of statistical significance.

TLR6 and CXCL10 expression correlates with prognosis of ESCC patients

Based on the results of our *in vitro* experiments, we investigated the relationship between the combined TLR6 and CXCL10 expression statuses and prognosis in ESCC patients. We previously performed IHC staining for TLR6 and CXCL10 separately in a tissue microarray containing samples from 177 primary tumors collected from ESCC patients who underwent curative esophagectomy without preoperative treatment. Representative images assigned IHC scores of 3+, 2+, and 1+ are shown elsewhere [18, 21]. The clinicopathological characteristics of the TLR6 high + CXCL10 high, TLR6 high + CXCL10 low, TLR6 low + CXCL10 high, and TLR6 low + CXCL10 low groups are summarized in **Table 1**. Kaplan-Meier curves showed that both 5-year OS (P=0.0012) and DSS (P=0.0158) significantly increased in ESCC patients with increasing TLR6 and CXCL10 scores (**Figure 4A** and **4B**). When univariate analysis was performed in the three aforementioned groups, TLR6 high + CXCL10 high, age, lymph node metastasis, pathological stage and tumor differentiation were found to be significantly associated with 5-year OS in ESCC patients (**Table 2A**) and TLR6 high + CXCL10 high, lymph node metastasis, pathological stage and tumor differentiation were found to be significantly associated with 5-year DSS in ESCC patients (**Table 2B**). Importantly, multivariate analysis showed that TLR6 high + CXCL10 high expression status was an independent prognostic factor in every combination with age, sex, depth of invasion, lymph node metastasis status, pathological stage and tumor differentiation. Moreover, the combined TLR6 high + CXCL10 high expression status correlated with a better prognosis in ESCC patients. This suggests that CXCL10 signals mediated via PGN/TLR6 are an important factor associated with prognosis in ESCC patients.

Discussion

In this study, we demonstrated that PGN treatment inhibits proliferation of ESCC cells *in vitro* by increasing CXCL10 production and apoptosis. We also showed that PGN treatment tends to reduce the progression of ESCC tumors *in vivo* in a mouse model. Moreover, the combined expression statuses of TLR6 and CXCL10 was an independent prognostic factor affecting 5-year OS and DSS.

The PGN used in this experiment was derived from Bacillus subtilis, which is recognized to be a beneficial bacterium in the human gut microbiota, and it exhibits both anticancer and antibacterial effects [22-24]. Several reports have shown a relationship between PGN and cancer. Patidar et al. reported that PGN-activated dendritic cells induce robust host-protective antitumor T cells that completely suppress tumor growth and recurrence in prostate tumor cells [25]. Li et al. reported that the effects of PGN

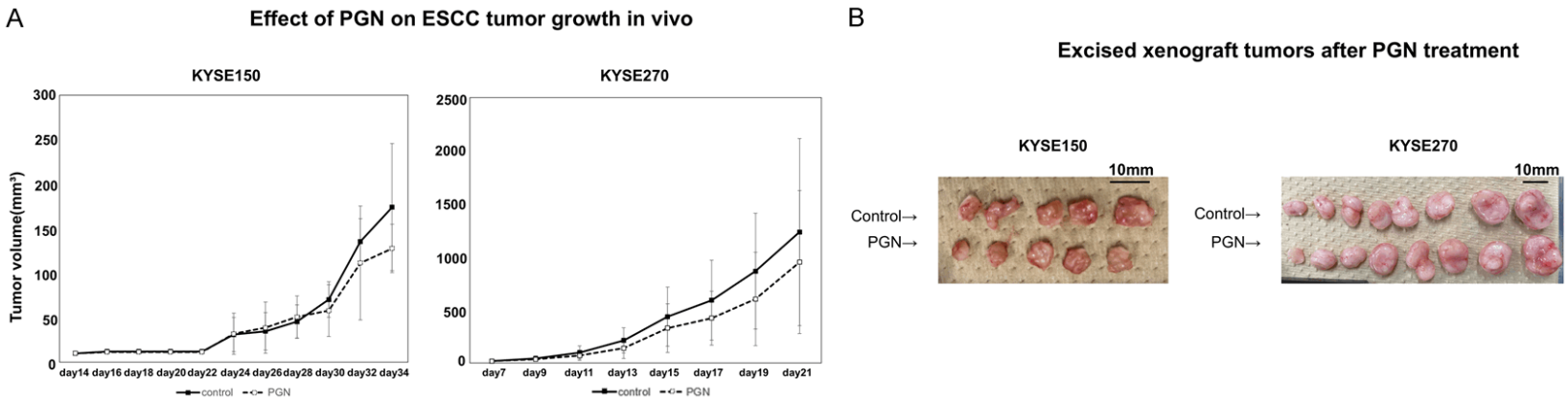

Figure 3. Effect of PGN on ESCC tumor growth *in vivo*. A. Graph showing the time-dependent increases in tumor volume in each group. The treatment start date was set as the measurement start date. B. Photo of excised xenograft tumors after treatment.

Table 1. The clinicopathological characteristics of 177 ESCC patients according to combination of TLR6 and CXCL10

Characteristics	TLR6 & CXCL10 low n=55 (31.1%)	TLR6 low CXCL10 high TLR6 high CXCL10 low n=83 (46.9%)	TLR6 & CXCL10 high n=39 (22.0%)	*P* value
Sex				0.082
Female	7 (12.7%)	14 (16.9%)	3 (7.7%)	
Male	48 (87.3%)	69 (83.1%)	36 (92.3%)	
Age at surgery	66 (38-82)	65 (38-78)	64 (50-76)	0.489
Habitual smoking				0.867
Current	31 (56.4%)	49 (59.0%)	20 (51.3%)	
Past	12 (21.8%)	18 (21.7%)	8 (20.5%)	
Never	12 (21.8%)	16 (19.3%)	11 (28.2%)	
Habitual alcohol consumption				0.634
Current	44 (80.0%)	59 (71.1%)	29 (74.4%)	
Past	4 (7.3%)	13 (15.7%)	6 (15.4%)	
Never	7 (12.7%)	11 (13.3%)	4 (10.3%)	
Tumor location				0.095
Upper (esophagus)	1 (1.8%)	5 (6.0%)	0 (0.0%)	
Middle	32 (58.2%)	53 (63.9%)	31 (79.5%)	
Lower	22 (40.0%)	25 (30.1%)	8 (20.5%)	
Depth of invasion (pT)				0.140
pT2	11 (20.0%)	13 (15.7%)	7 (18.0%)	
pT3	43 (78.2%)	67 (80.7%)	27 (69.2%)	
pT4a	1 (1.8%)	3 (3.6%)	5 (12.8%)	
Lymph node metastasis (pN UICC)				0.063
pN0	10 (18.2%)	24 (28.9%)	15 (38.5%)	
pN1	5 (9.1%)	7 (8.4%)	0 (0.0%)	
pN2	14 (25.5%)	17 (20.5%)	15 (38.5%)	
pN3	13 (23.6%)	14 (16.9%)	3 (7.7%)	
M1 Lymph (supraclavicular)	13 (23.6%)	21 (25.3%)	6 (15.4%)	
Number of dissected LN	55 (19-131)	83 (9-126)	39 (25-118)	0.099
Pathological stage				0.472
pStage IIA	3 (5.5%)	12 (14.5%)	5 (12.8%)	
pStage IIB	7 (12.7%)	11 (13.3%)	9 (23.1%)	
pStage IIIA	3 (5.5%)	3 (3.6%)	1 (2.6%)	
pStage IIIB	26 (47.3%)	39 (47.0%)	17 (43.6%)	
pStage IVA	7 (12.7%)	10 (12.1%)	6 (15.4%)	
pStage IVB (M1 Lymph)	9 (16.4%)	8 (9.6%)	1 (2.6%)	
Tumor differentiation				0.423
Well	7 (12.7%)	13 (15.7%)	9 (23.1%)	
Moderate	26 (47.3%)	44 (53.0%)	21 (53.9%)	
Poor	22 (40.0%)	26 (31.3%)	9 (23.1%)	
Adjuvant chemotherapy				0.946
Positive	32 (58.2%)	49 (59.0%)	24 (61.5%)	
Negative	23 (41.8%)	34 (41.0%)	15 (38.5%)	
Recurrence of ESCC				0.632
Positive	26 (47.3%)	39 (47.0%)	15 (38.5%)	
Negative	29 (52.7%)	44 (53.0%)	24 (61.5%)	
Prognosis				0.036*
Alive	16 (29.1%)	35 (42.1%)	25 (64.1%)	
Deceased from ESCC	24 (43.6%)	34 (41.0%)	9 (23.1%)	
Deceased from other cancer	2 (3.6)	3 (3.6%)	0 (0.0%)	
Deceased from other diseases	13 (23.6%)	11 (13.2%)	5 (12.8%)	

*p<0.05.

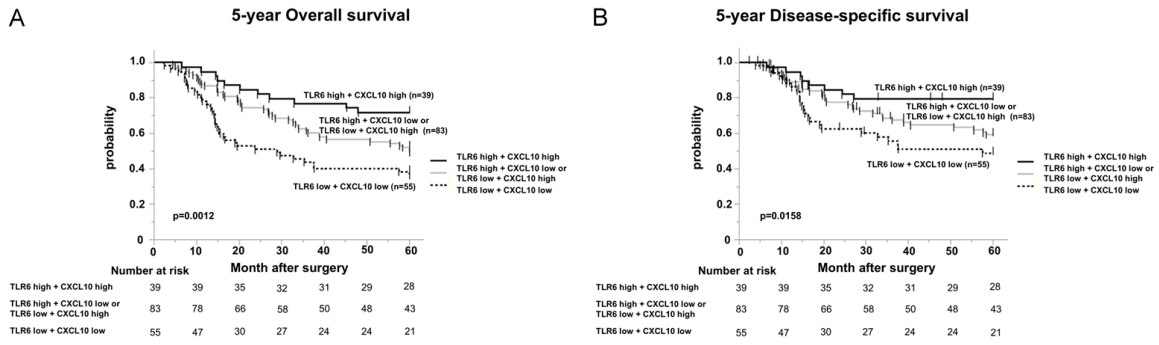

Figure 4. (A, B) Kaplan-Meier survival curves illustrating the association between combined TLR6 and CXCL10 expression statuses (TLR6 high + CXCL10 high, TLR6 high + CXCL10 low with TLR6 low + CXCL10 high, TLR6 low + CXCL10 low) and 5-year OS (A) and DSS (B) in ESCC patients after curative esophagectomy. The log-rank test was used to compare differences among the three groups (5-year OS: P=0.0012, DSS: P=0.0158).

Table 2A. Univariate and Multivariate analysis of the hazard ratios for 5-year OS in the tissue microarray cohort

5-year overall survival	Univariate Cox PH Model		Multivariable Cox PH Model	
Variable	HR	95% CI	HR	95% CI
TLR6 & CXCL10				
Low & low vs. high & high	3.275	1.661-6.457	2.559	1.285-5.096
High & low vs. high & high	2.033	1.047-3.950	1.869	0.959-3.642
Low & low vs. high & low	1.611	1.028-2.525	1.369	0.867-2.162
Sex: male vs. female	1.825	0.882-3.776		
Age at Surgery: 70 and older vs. younger	1.575	1.035-2.396	1.410	0.915-2.172
Depth of invasion: T3-4 vs. T2	1.257	0.710-2.227		
Lymph node metastasis: N+ vs. N0	5.770	2.785-11.953	2.332	0.680-8.000
Pathological stage: III-IV vs. I-II	5.162	2.667-9.991	2.346	0.764-7.203
Tumor differentiation: poorly vs. not poorly	1.900	1.240-2.910	1.265	0.809-1.977

Table 2B. Univariate and Multivariate analysis of the hazard ratios for 5-year DSS in the tissue microarray cohort

5-year disease-specific survival	Univariate Cox PH Model		Multivariable Cox PH Model	
Variable	HR	95% CI	HR	95% CI
TLR6 & CXCL10				
Low & low vs. high & high	3.085	1.384-6.876	2.553	1.140-5.717
High & low vs. high & high	2.112	0.973-4.584	2.004	0.921-4.357
Low & low vs. high & low	1.461	0.860-2.482	1.274	0.744-2.181
Sex: male vs. female	1.491	0.680-3.269		
Age at Surgery: 70 and older vs. younger	1.378	0.442-1.193		
Depth of invasion: T3-4 vs. T2	1.655	0.788-3.472		
Lymph node metastasis: N+ vs. N0	8.570	3.111-23.610	1.513	0.268-8.545
Pathological stage: III-IV vs. I-II	9.821	3.564-27.062	6.191	1.101-34.802
Tumor differentiation: poorly vs. not poorly	2.004	1.218-3.297	1.371	0.825-2.279

on colorectal tumors and intestinal tissue during pelvic radiotherapy suggest PGN administration may be a useful adjuvant therapy with radiation [26]. In addition, Griffin et al. observed that enterococcal PGN remodeling promotes checkpoint inhibitor immunotherapy [27]. The-

se studies support the idea that PGN exerts antitumor effects.

PGN is known to induce CXCL10 via the TLR2/6 pathway [28]. CXCL10 is a chemokine involved in chemotaxis, induction of apoptosis, regulation of cell growth and mediation of angiostatic effects [29]. We previously investigated CXCL10 in patients who had undergone curative esophagectomy and found that those exhibiting high CXCL10 expression had significantly better 5-year OS and DSS than those with low CXCL10 expression [21]. Consistent with a relationship between CXCL10 and esophageal cancer, basal circulating CXCL10 levels appeared to be a robust predictor of responses in a phase II trial of pembrolizumab in refractory esophageal cancer [30]. Additionally, use of probiotics during ICI treatment appears to improve patient prognosis [31, 32], further demonstrating the potential importance of beneficial bacteria in these cases. The present results provide additional evidence of the beneficial effects of PGN derived from beneficial bacteria in ESCC.

On the other hand, PGN may also promote tumors. Xie et al. reported that PGN promoted breast cancer cell invasiveness and adhesiveness by targeting TLR2 [33]. This tumor-promoting effect is probably due in part to the fact that the PGN used in this study was derived from Staphylococcus aureus, a pathogenic bacterium. In addition, Suarez et al. reported that peptidoglycan enhances NOD1 activation and promotes gastric cancer [34]. This tumor-promoting effect is likely due in part to the fact that the PGN was derived from a Gram-negative pathogenic bacterium, Helicobacter pylori. These results are consistent with the results of the present study in that it appears PGN must be derived from a beneficial bacterium to have beneficial effects. We recently demonstrated that LPS, derived from Gram-negative bacteria, induces CCL2 through TLR4 signaling and promotes ESCC cell proliferation [35]. Therefore, maintaining a favorable oral environment that suppresses growth of Gram-negative bacterial and, in turn, decreases release of LPS while fostering growth of beneficial Gram-positive bacteria and production of PGN would be expected to improve ESCC patient prognosis.

This study has several limitations. First, BALB/c nude mice were used to promote tumor engraftment. Because T- or B-cell immunity was not considered, the findings do not fully reflect actual human effects. Second, there is a lack of data on the patients' oral cavity environment and oral flora prior to surgery. We only began preoperative assessment of the oral cavity environment by dentists in patients waiting for esophagectomy in 2009. Consequently, those data were not available for most of the patients in the cohort used for this study. Whether the oral cavity environment and its flora are associated with expression of TLR6 and/or CXCL10 remains to be determined.

We demonstrated that PGN inhibits ESCC cell proliferation *in vitro* by enhancing CXCL10 production via TLR signaling and tends to inhibit ESCC tumor progression *in vivo*. Moreover, we showed that high expression of both CXCL10 and TLR6 is an independent prognostic factor favorably affecting 5-year OS and DSS. These results suggest that improving the oral environment has the potential to improve the prognosis of ESCC patients.

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Disclosure of conflict of interest

None.

Abbreviations

PGN, Peptidoglycan; CXCL10, C-X-C motif chemokine ligand 10; TLRs, Toll-like receptors; ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma.

Address correspondence to: Dr. Yusuke Sato, Department of Thoracic Surgery, Akita University Graduate School of Medicine, 1-1-1, Hondo, Akita 010-8543, Japan. Tel: +81-18-884-6132; Fax: +81-18-836-2615; E-mail: yusuke@doc.med.akita-u.ac.jp

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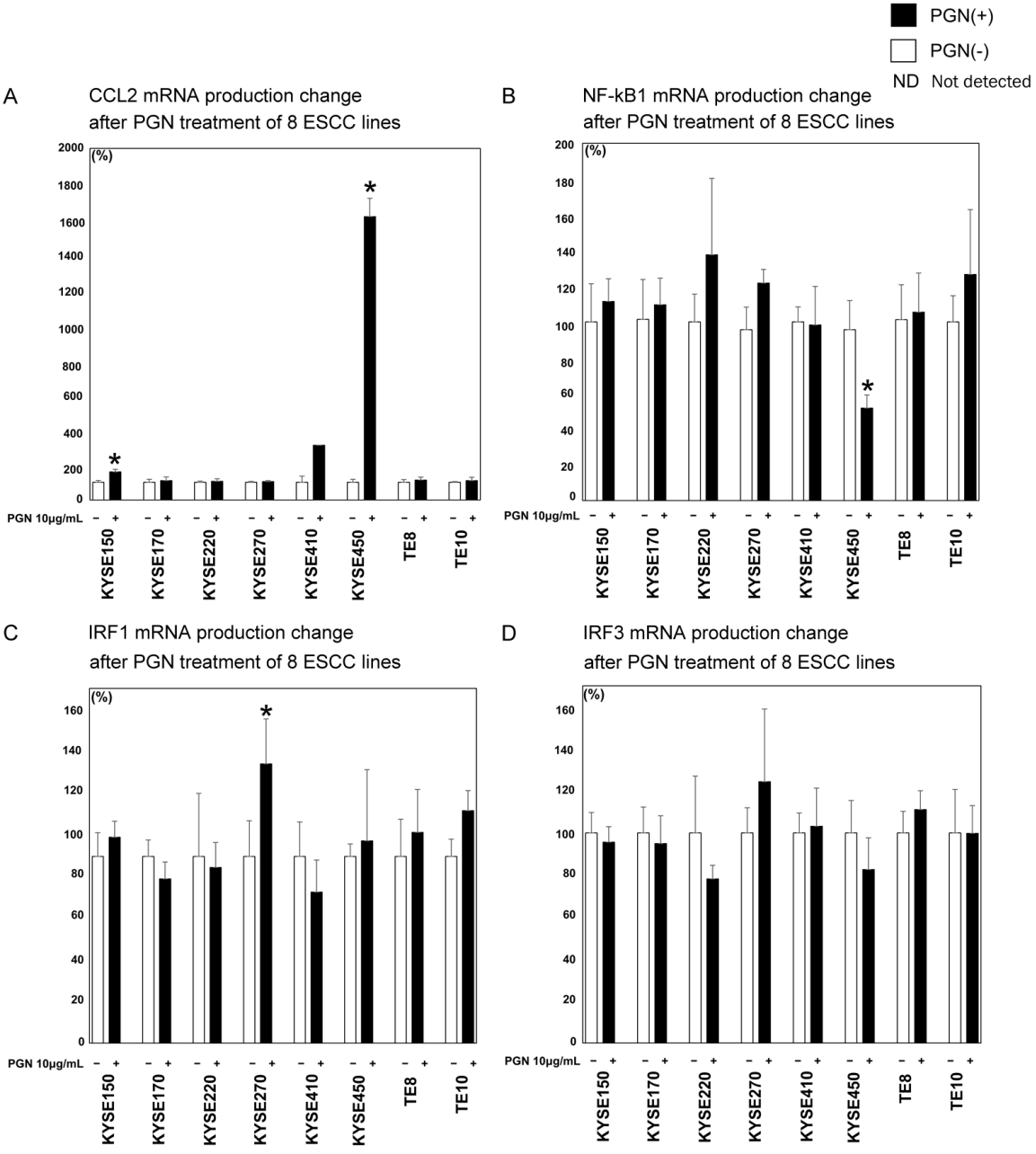
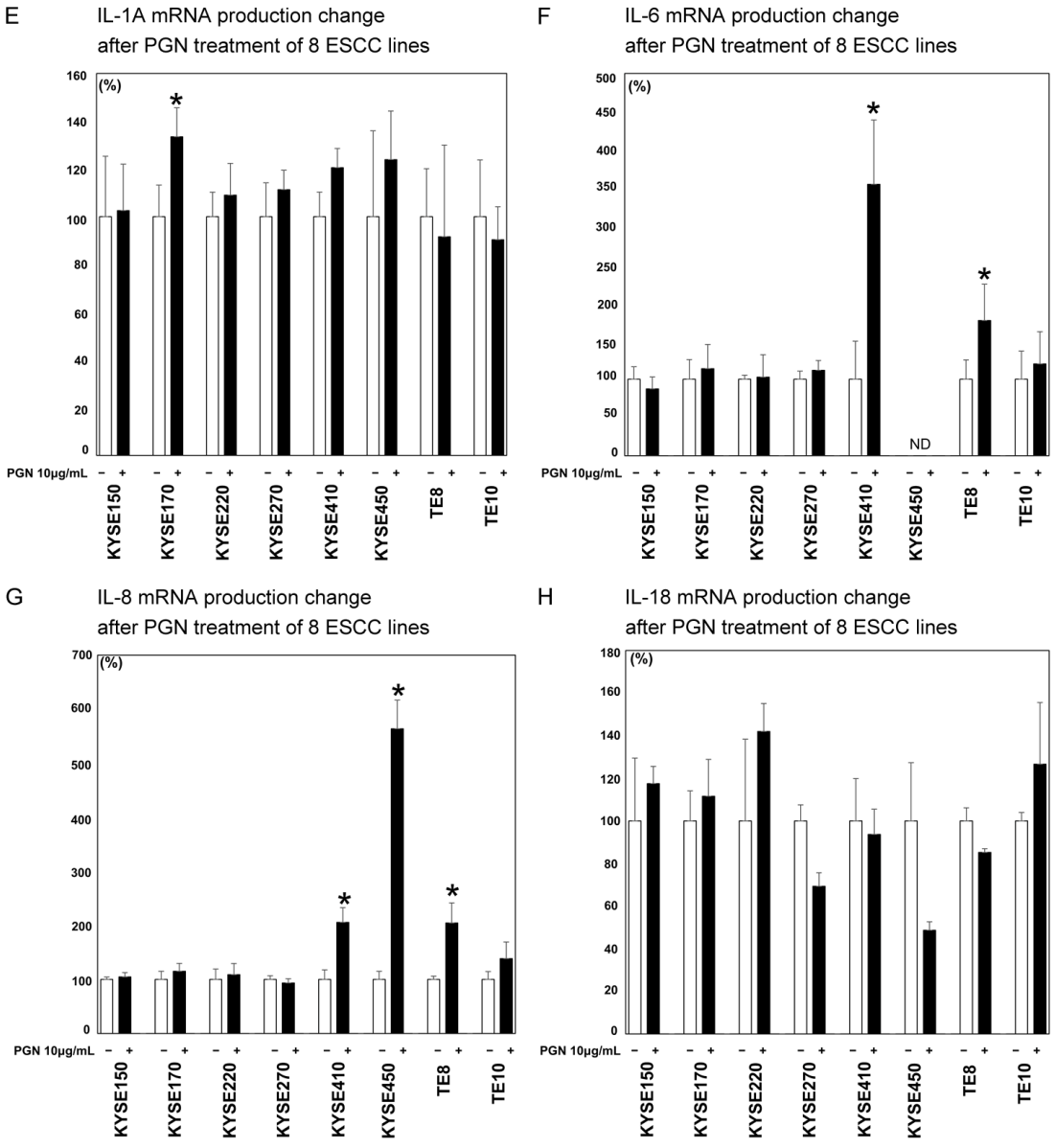
Supplementary Table 1. Sequences of primer and Universal Probe Library

Gene	ID	Forward primer	Reverse primer	Universal Probe Library
B2M	NM_004048.2	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTC	CATCCAGC (#42)
NFkB1	NM_003998.2	ACCCTGACCTTGCCTATTTG	AGCTCTTTTTCCCGATCTCC	AGGTGGAG (#39)
NFkB2	NM_001077494.1	ACACCGTTGTACAAAGATACGC	GCCCGGCTCTGTCTAGTG	GGAAGCAG (#38)
IRF1	NM_002198.2	GGGCTGTCAGTTGATTCTGG	CTATGGCACATGCCTCAAAA	CTGGGGCC (#57)
IRF3	NM_001571.4	CTTGGAAGCACGGCCTAC	CGGGAACATATGCACCAGT	CAGCAGGA (#18)
IL-6	NM_000600.3	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT	CAGCAGGC (#40)
IL-8	NM_000584.2	AGACAGCAGAGCACACAAGC	ATGGTTCCTTCCGGTGGT	GCCAGGAA (#62)
IL-18	NM_001562.2	GCTTCCTCTCGCAACAAACT	TGATGCAATTGTCTTCTACTGGTT	ATGGCTGC (#46)
IL-1A	NM_000575.3	ACAAAAGGCGAAGAAGACTGA	GGAACTTTGGCCATCTTGAC	CTGGCTGG (#20)
CCL2	NM_002982.3	AGTCTCTGCCGCCCTTCT	GTGACTGGGGCATTGATTG	GCCTGCTG (#40)
CXCL10	NM_001565.2	GAAAGCAGTTAGCAAGGAAAGGT	GACATATACTCCATGTAGGGAAGTGA	CTGCCTCT (#34)





Supplementary Figure 1. (A-H) CCL2 (A), NF-kB1 (B), IRF1 (C), IRF3 (D), IL-1A (E), IL-6 (F), IL-8 (G) and IL-18 (H) mRNA expressions in 8 ESCC lines treated with 10 μg/ml PGN (*p<0.05).