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Article

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Abstract: Lipopolysaccharides are a type of polysaccharide mainly present in the bacterial outer membrane of Gram-negative bacteria. Recent studies have revealed that lipopolysaccharides contribute to the immune response of the host by functioning as a cancer antigen. We retrospectively recruited 198 patients with gastric cancer who underwent surgery. The presence of lipopolysaccharides was determined using immunohistochemical staining, with the intensity score indicating positivity. The relationship between lipopolysaccharides and CD8, PD-L1, TGFBI (a representative downstream gene of TGF-β signaling), wnt3a, and E-cadherin (epithelial–mesenchymal transition marker) was also investigated. Thereafter, we identified 20 patients with advanced gastric cancer receiving nivolumab and investigated the relationship between lipopolysaccharides and nivolumab sensitivity. After staining for lipopolysaccharides in the nucleus of cancer cells, 150 negative (75.8%) and 48 positive cases (24.2%) were found. The lipopolysaccharide-positive group showed increased cancer stromal TGFBI expression (p < 0.0001) and PD-L1 expression in cancer cells (p = 0.0029). Lipopolysaccharide positivity was significantly correlated with increased wnt3a signaling (p = 0.0028) and decreased E-cadherin expression (p = 0.0055); however, no significant correlation was found between lipopolysaccharide expression and overall survival rate (p = 0.71). In contrast, high TGFBI expression in the presence of LPS was associated with a worse prognosis than that in the absence of LPS (p = 0.049). Among cases receiving nivolumab, the lipopolysaccharide-negative and -positive groups had a disease control rate of 66.7% and 11.8%, respectively (p = 0.049). Lipopolysaccharide positivity was associated with wnt3a, TGF-β signaling and epithelial–mesenchymal transition and was considered to promote therapeutic resistance to nivolumab.

Keywords: epithelial–mesenchymal transition **(**EMT); Gastric cancer; lipopolysaccharides (LPS); Nivolumab; TGF-β

1. Introduction

Gastric cancer (GC), one of the most common digestive cancers is the leading cause of cancer-related death worldwide [1]. The clinical outcomes of patients with GC, particularly overall survival (OS), has gradually improved following the introduction of chemotherapy and immune checkpoint

inhibitors (ICIs). In fact, ATTRACTION-2, the first phase 3 study on patients with GC receiving two or more chemotherapy regimens, showed that the anti-PD-1 antibody nivolumab improved overall survival (OS) [2]. Furthermore, the CheckMate 649 study recommended nivolumab plus chemotherapy as the primary treatment for GC [3]. However, patients with GC have shown unsatisfactory response rates to ICIs. Concerns regarding the impact of expensive ICIs on the health care economy have also emerged. As such, a robust biomarker is needed to more effectively assess the positive clinical therapeutic effects of nivolumab in patients with GC.

Nejman et al. showed that intratumor bacteria were present in cancer cells and were mostly intracellular. They also showed that bacterial lipopolysaccharides (LPS) were present in tumor cells and that intratumor bacteria could affect responses to immunotherapy [4]. The cell wall of Gram-negative bacteria is composed of LPS. Reports have shown that LPS were associated with the development of GC by regulating cell proliferation, autophagy, and epithelial–mesenchymal transition (EMT) [5-7]. In particular, Helicobacter pylori LPS have been found to activated both NFkB and STAT3 signaling pathways [8-10]. NFkB induces PD-L1 expression and EMT by activating transforming growth factor β (TGF-β) signaling [11].

TGF-β plays a critical role in EMT, which causes therapeutic resistance to ICIs [12,13]. In particular, TGF-β activates a pathway mediated by small mother against decapentaplegic (SMAD) proteins that induces the downstream activation of EMT, including N-cadherin and vimentin, via SNAIL, TWIST, and ZEB1 [14,15]. However, the appropriate method for evaluating TGF-β signaling in patients with cancer remains elusive given the genetic variations in TGF-β pathway genes across several cancers [16,17]. Our previous report showed that high stromal transforming growth factor-beta-induced protein (TGFBI) in lung cancer was associated with poor prognosis and therapeutic resistance to ICIs [18]. TGFBI had initially been described as a protein strongly induced by TGF-β and one of the representative downstream genes in TGF-β signaling [19]. However, few studies have addressed the relationship between LPS and TGFBI, as well as the therapeutic effects of the anti-PD-1 antibody nivolumab in GC.

The current study therefore aimed to clarify the significance of LPS in patients with GC. Moreover, we investigated whether LPS could be a biomarker for predicting treatment resistance in patients receiving nivolumab for GC.

2. Results

2.1. Presence of LPS in GC surgical tissues

LPS were stained in the nucleus of cancer cells (Figure 1), with our results showing 150 (75.8%) and 48 (24.2%) negative and positive cases, respectively.
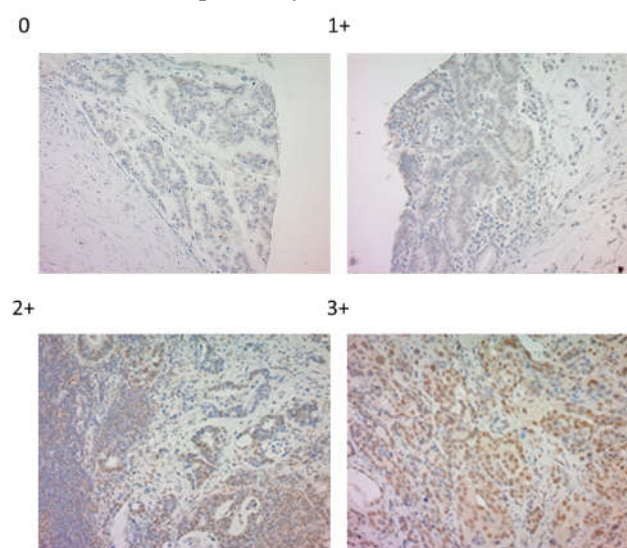
Figure 1. Representative images of the immunohistochemical staining of lipopolysaccharides.

2.2. Relationship between LPS and clinicopathological factors of patients with GC

The results for the relationship between LPS and clinicopathological factors are summarized in Table 1. Accordingly, no significant difference in age, sex, tumor size, depth, differentiation, presence of lymph node metastasis, and stage were observed between positive and negative cases. The LPS-positive group showed significantly greater cancer stromal TGFBI expression (p < 0.0001), more HER2-negative cases (p = 0.012), greater PD-L1 expression (p = 0.0029), increased wnt3a signaling (p = 0.0028) and lower E-cadherin expression (p = 0.0055) (EMT marker) than did the LPS-positive group.

Table 1. Relationship between lipopolysaccharides and clinicopathological factors.

Variable	Presence of LPS		p-Value
	Negative n = 150 No. (%)	Positive n = 48 No. (%)	
Age (years)			
	65	63	0.20
Sex			
Male	108 (72.0%)	30 (62.5%)	0.22
Female	42 (28.0%)	18 (37.5%)	
Tumor size (mm)			
	65.0±2.88	63.4±5.01	0.78
Depth			
m, sm, mp	41 (27.3%)	9 (18.8%)	0.22
ss, se, si	109 (72.7%)	39 (81.2%)	
Differentiation			
tub1,tub2,pap	58 (38.7%)	15 (31.3%)	0.35
por,sig	92 (61.3%)	33 (68.7%)	
Lymph node metastasis			
Absent	48 (32.0%)	15 (31.3%)	0.92
Present	102 (68.0)	33 (68.7%)	
Stage			
I II	77 (51.3%)	19 (39.6%)	0.13
III IV	71 (48.7%)	29 (60.4%)	
TGFBI in stromal			
Low expression	105 (70.0%)	16 (33.3%)	<.0001*
High expression	44 (30.0%)	32 (66.7%)	
CD8			
Low expression	50 (33.3%)	12 (25.0%)	0.27
High expression	100 (66.7%)	36 (75.0%)	
HER2 score			
0	103 (68.7%)	38 (79.2%)	0.012*
1,2,3	32 (31.3%)	3 (20.8%)	
PD-L1			
Low expression	127 (84.7%)	31 (64.6%)	0.0029*
High expression	22 (15.3%)	17 (35.4%)	
Wnt3a			
Low expression	70 (46.7%)	11 (22.9%)	0.0028*
High expression	80 (53.3%)	37 (77.1%)	
E-cadherin			
Low expression	69 (46.0%)	33 (68.8%)	0.0055*
High expression	81 (54.0%)	15 (31.2%)	

* *p*<0.05.

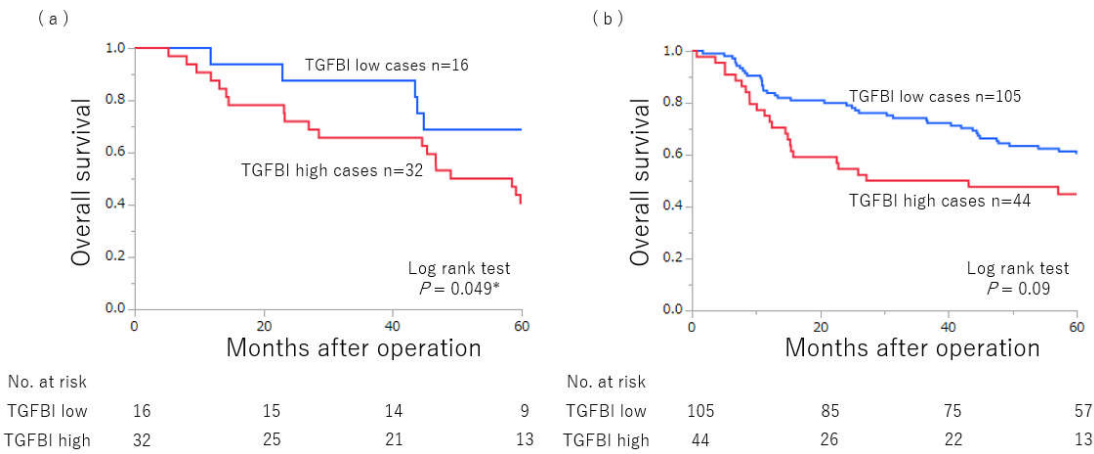*2.3. Kaplan–Meier curve for overall survival according to the presence of LPS in surgical cases with GC*

Figure 2. presents the Kaplan–Meier curve for overall survival according to the presence of LPS in surgical cases with GC. Accordingly, the presence of LPS was not significantly associated with overall survival (p = 0.71).
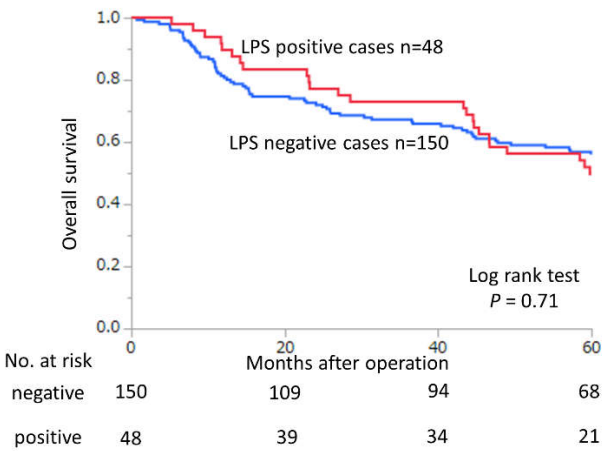
Figure 2. Kaplan–Meier analysis of 5-year overall survival in relation to lipopolysaccharides.

2.4. Kaplan–Meier curve for overall survival with TGFBI expression and prognosis according to the presence or absence of LPS in surgical cases with GC

Figure 3 also presents the Kaplan–Meier curve for overall survival with TGFBI expression and prognosis according to the presence or absence of LPS in surgical cases with GC. High TGFBI expression in the presence of LPS was associated with a worse prognosis than that in the absence of LPS (p = 0.049).

Figure 3. Kaplan–Meier analysis of 5-year overall survival in relation to TGFBI expression and prognosis according to the presence or absence of lipopolysaccharides. (a) presence of lipopolysaccharides, and (b) absence of lipopolysaccharides.

2.5. Relationship between LPS and nivolumab sensitivity

Table 2 outlines the results for the relationship between LPS and nivolumab sensitivity. Notably, the LPS-negative and -positive groups showed a disease control rate of 66.7% and 11.8%, respectively (p = 0.049).

Table 2. Relationship between lipopolysaccharides and nivolumab sensitivity.

Variable	Presence of LPS		*p*-Value
	Negative n = 3 No. (%)	Positive n = 17 No. (%)	
PR+SD	2 (66.7%)	2 (11.8%)	0.049*
PD	1 (33.3%)	15 (88.2%)	

* *p*<0.05.
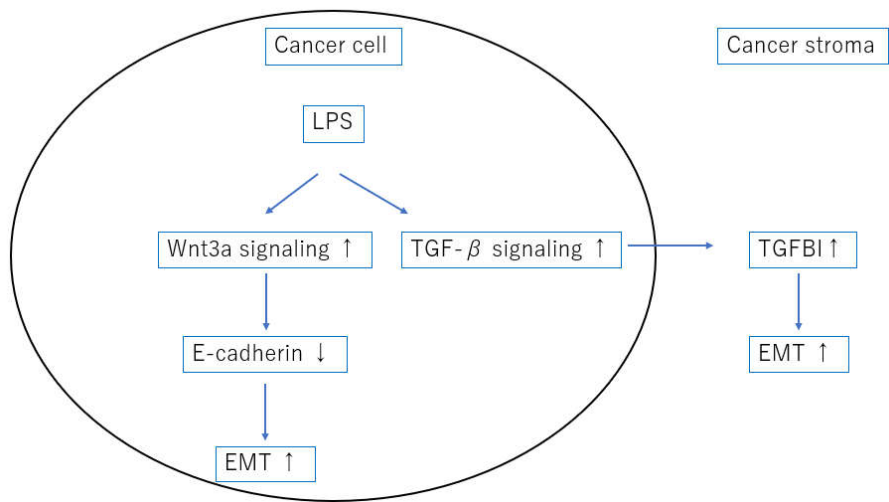
Figure 4. We hypothesized that LPS triggered EMT by activating wnt3a and TGF-β signaling.

3. Discussion

The current study showed that LPS in GC cells were associated with enhanced TGF-β signaling, wnt3a signaling, PD-L1 expression, and EMT. Although the presence of LPS did not contribute to the prognosis of GC, it did contribute to therapeutic resistance in patients receiving nivolumab for unresectable advanced recurrent GC (p = 0.049). Furthermore, high TGFBI expression in the presence of LPS was associated with a worse prognosis than that in the absence of LPS.

A previous report revealed that intratumor bacteria were present in both cancer and immune cells and were mostly intracellular. Evidence has also shown that intratumor bacteria or their predicted functions were correlated with tumor types and subtypes, patients' smoking status, and responses to immunotherapy [4]. Moreover, recent studies have revealed that LPS contributes to the immune response of the host by functioning as a cancer antigen [20]. In line with this, our study showed that LPS contributed to therapeutic resistance in patients receiving nivolumab for GC.

Epithelial cells can acquire a mesenchymal phenotype through a process called EMT. More specifically, EMT can be described as a process of cell plasticity in which epithelial cells acquire mesenchymal characteristics, such as fibroblast-like morphology, increased motility, and increased expression of mesenchymal markers such as vimentin, while simultaneously decreasing cell-to-cell contact, E-cadherin and claudin expression, and other epithelial characteristics [21]. Cytotoxin-associated gene A (CagA) is the major virulence factor and oncoprotein of H. pylori [22]. Previous reports have shown that CagA promoted EMT by reducing GSK-3 activity [23], and downregulating programmed cell death protein 4 [24]. The current study showed that LPS, a constituent molecule of Gram-negative bacteria, were present in cancer cells, suggesting a new possibility for inducing EMT. We also showed that the activation of wnt3a signaling may downregulate E-cadherin expression and induce EMT during these processes [Figure 4].

TGF-β signaling is considerably complex in addition to being time- and context-dependent, with different signaling processes occurring in cancerous and noncancerous areas [25]. Hence, evaluating

TGF-β signaling is quite challenging. TGFBI is a representative downstream gene of TGF-β signaling and a protein whose expression is induced downstream of SMAD [19]. In other words, TGFBI expression can be a good indicator of TGF-β signaling activation. Furthermore, one study showed that TGFBI expression is highly induced in the process of EMT [26]. LPS induced IL-6 and TGF-β1 secretion in esophageal cancer cell lines, and E-cadherin was significantly decreased after LPS treatment, promoting EMT [27]. Interestingly, the current study showed that cancer stromal TGFBI expression was strongly correlated with the presence of LPS in cancer cells. This indicates that bacterial-derived LPS activate TGF-β signaling, suggesting that LPS regulation may be useful for regulating TGF-β signaling and, consequently, EMT [Figure 4].

Our previous study reported that cancer stromal TGFBI expression was significantly associated with therapeutic resistance in patients receiving nivolumab for lung cancer [18]. Similarly, the present study found that the presence of LPS was significantly correlated with cancer stromal TGFBI expression, which may have contributed to therapeutic resistance in patients with GC using nivolumab. Furthermore, reports of increased EMT and resistance to ICI treatment have been observed in other cancers [28,29]. Although the presence of LPS was positively correlated with PD-L1 expression in cancer cells in present study, but the aforementioned EMT findings suggest that the presence of LPS may have caused treatment resistance in patients receiving nivolumab for GC.

V Gopalakrishnan et al., who studied the oral and gut microbiota of patients with melanoma receiving anti-PD-1 immunotherapy, reported significant differences in the diversity and composition of patient gut bacteria between responders and non-responders. Immune profiling also suggested enhanced systemic and antitumor immunity in reactive patients with favorable gut microbiota and in sterile mice transplanted with feces from reactive patients [30]. Given that H. pylori infections have been suggested to cause GC, we would like to examine the history of H. pylori eradication and sensitivity to ICIs in the future.

Our study has several limitations worth noting. First, this is a single-center, retrospective study with small sample size. In particular, the GC samples treated by nivolumab might not be enough to indicate strong evidence of LPS as a promising biomarker for ICIs treatment. Further prospective studies are needed to clarify the importance of LPS evaluation as an ICI sensitivity marker for GC patients in the clinic. Second, no in vitro or in vivo studies were conducted herein. Third, this study did not examine the association between LPS and H. pylori. In the future, we would like to use surgical specimens to investigate the relationship between 16srDNA and LPS at cancer sites. Finally, lymphocytes were also stained for LPS in immunostaining. The significance of this staining has not been examined.

In conclusion, the current study found that LPS was associated with wnt3a, TGF-β signaling and EMT and that its expression affected therapeutic resistance to nivolumab in patients with GC.

4. Materials and Methods

4.1. Patients

This study included 198 patients who underwent potentially curative surgery for GC at the Department of General Surgical Science, Gunma University Hospital between 1996 and 2006. In this study, we performed tissue microarray analysis. Preoperatively treated cases and endoscopic mucosal resection cases were excluded, and multiple blocks containing preinvasive sites were collected and subjected to tissue microarray. Clinicopathological factors, such as age, sex, tumor size, depth, differentiation, presence of lymph node metastasis, stage, and HER2 status, were determined. Furthermore, we analyzed 20 patients receiving nivolumab for postoperative recurrent or unresectable advanced GC from 2017 to 2021. Nivolumab was used after third-line treatment in all cases. Among such cases, the response to nivolumab was evaluated using the Response Evaluation Criteria in Solid Tumors version 1.1 [31]. The resected specimens and pretreatment biopsy specimens were included in nivolumab sensitivity consideration. Our study was approved by the institutional review board of Gunma University (approval no. HS2021-085).

4.2. Immunohistochemistry

All specimens were cut into 4-μm-thick sections and mounted on glass slides. Their sections were deparaffinized with xylene, hydrated, and incubated in 0.3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was performed in ImmunoSaver (Nisshin EM, Tokyo, Japan) at 98°C for 45 min. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free (Dako, Carpinteria, CA) for 30 min at room temperature. The primary antibody for LPS (Hycult Biotech, HM6011, Mouse mAb, 1:100 dilution) was diluted with Dako REAL Antibody Diluent and incubated overnight at 4°C. The Histofine Simple Stain MAX-PO (Multi) Kit (Nichirei, Tokyo, Japan), which was used as the secondary antibody, was incubated for 30 min at room temperature. The stain 3,3-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution in 50 mM of ammonium acetate-citrate acid buffer (pH 6.0) containing 0.005% hydrogen peroxide. Sections were lightly contrast-stained with hematoxylin and then mounted. We divided LPS into four groups: negative, weak, moderate, and strong, with moderate and strong indicating positivity. For TGFBI, CD8, and PD-L1, we employed the same staining and evaluation methods as previously report [18]. And we also employed the same staining and evaluation method for wnt3a [32]. We performed immunohistochemical staining using E-cadherin antibody (HECD-1, mouse monoclonal, 1:500 dilution), with sections boiled in 10 mM citrate buffer (pH 6.0) at 98°C for 30 min for E-cadherin activation. A positive case was defined as that in which ≥50% of the cancer cells had moderate staining intensity in E-cadherin.

4.3. Statistical analysis

Statistically significant differences were analyzed using the Mann–Whitney U test for continuous variables and the Chi squared test for categorical variables. Survival rates were calculated using the Kaplan–Meier method, with statistical significance being determined by the log rank test. All analyses were performed using JMP Pro 15.0 software (SAS Institute Inc., Cary, NC, USA), with $p < 0.05$ indicating statistical significance.

5. Conclusions

The current study showed that lipopolysaccharides positivity was associated with wnt3a, TGF-β signaling and epithelial–mesenchymal transition and was considered to promote therapeutic resistance to nivolumab.

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