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ORIGINAL RESEARCH

Comprehensive clinical and molecular characterization of claudin 18.2 expression in advanced gastric or gastroesophageal junction cancer

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Background: We conducted comprehensive clinical and molecular characterization of claudin 18.2 expression (CLDN18.2) in advanced gastric or gastroesophageal junction cancer (GC/GEJC).

Patients and methods: Patients with advanced GC/GEJC who received systemic chemotherapy from October 2015 to December 2019 with available tumor specimens were analyzed. We evaluated clinicopathological features of CLDN18.2 expression with four molecular subtypes: mismatch repair deficient, Epstein—Barr virus-positive, human epidermal growth factor receptor 2-positive, and others. In addition, programmed death-ligand 1 (PD-L1) combined positive score (CPS), genomic alterations, and the expression of immune cell markers were assessed. Clinical outcomes of standard first- or second-line chemotherapy and subsequent anti-programmed cell death protein 1 (anti-PD-1) therapy were also investigated according to CLDN18.2 expression.

Results: Among 408 patients, CLDN18.2-positive (moderate-to-strong expression in $\geq$75%) was identified in 98 patients (24.0%) with almost equal distribution in the four molecular subtypes or CPS subgroups. CLDN18.2-positive was associated with Borrmann type 4, *KRAS* amplification, low CD16, and high CD68 expression. Overall survival with first-line chemotherapy was not significantly different between CLDN18.2-positive and -negative groups [median 18.4 versus 20.1 months; hazard ratio 1.26 (95% confidence interval 0.89-1.78); $P = 0.191$] regardless of stratification by PD-L1 CPS $\geq$5. Progression-free survival and objective response rates of first- and second-line chemotherapy, and anti-PD-1 therapy also showed no significant differences according to CLDN18.2 status.

Conclusions: CLDN18.2 expression in advanced GC/GEJC was associated with some clinical and molecular features but had no impact on treatment outcomes with chemotherapy or checkpoint inhibition. CLDN18.2-positive also had no impact on overall survival. This information could be useful to interpret the results from currently ongoing clinical trials of CLDN18.2-targeted therapies for advanced GC/GEJC and to consider a treatment strategy for CLDN18.2-positive GC/GEJC.

Key words: gastric cancer, claudin 18.2, zolbetuximab, mismatch repair deficiency, Epstein—Barr virus

INTRODUCTION

Gastric cancer (GC), including gastroesophageal junction cancer (GEJC), is the fifth most common type of cancer and the fourth leading cause of cancer-related deaths globally.[1] Combination chemotherapy with fluoropyrimidines and platinum agents [with or without anti-programmed cell death protein 1 (anti-PD-1) inhibitor for human epidermal growth factor receptor 2 (HER2)-negative cases, or with trastuzumab for HER2-positive cases] is the standard first-line treatment of patients with advanced GC/GEJC.[2-7] Taxane agents with or without ramucirumab are most frequently used in second-line treatment.[8,9] Third- or later-line treatment options include anti-PD-1 inhibitors, trifluridine/tipiracil, irinotecan and trastuzumab deruxtecan (for HER2-positive GC/GEJC).[10-14] Despite the recent developments in treatment options, the prognosis remains poor [median overall survival (OS) <15 months].

Claudin 18 isoform 2 (CLDN18.2), a member of the claudin family, is an important component of tight junction proteins that regulates tissue permeability, paracellular transport, and signal transduction. CLDN18.2 is predominantly present in stomach mucosa and is retained during

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malignant transformation. Moreover, it may become more exposed and accessible in malignant tissues with disruption of tight junctions, which may make CLDN18.2 an attractive target for cancer treatment.[15,16]

Recently, zolbetuximab is being developed for GC/GEJC. It is a novel chimeric immunoglobulin G1 antibody highly specific for CLDN18.2.

Zolbetuximab binds to CLDN18.2 on the tumor cell surface to stimulate cellular and soluble immune effectors that activate antibody-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity.[17] The single-arm MONO study demonstrated preliminary efficacy with a manageable safety profile of zolbetuximab monotherapy.[18] The FAST trial, a randomized phase II study, showed a significant improvement of OS with zolbetuximab in combination with first-line chemotherapy especially in GC/GEJC patients with CLDN18.2 high expression.[19] These results led to two ongoing phase III studies (SPOTLIGHT: NCT03504397[20] and GLOW: NCT03653507[21]) for CLDN18.2-positive (moderate-to-strong expression in $\geq$75% of tumor cells) GC/GEJC. The SPOTLIGHT trial was a global double-blinded phase III trial which aimed to verify the efficacy of modified FOLFOX6 (mFOLFOX6) plus zolbetuximab compared with mFOLFOX6 plus placebo for CLDN18.2-positive (hereinafter, called CLDN-positive) advanced GC/GEJC. Most recently, the result was announced to be positive: both the primary endpoint of progression-free survival (PFS) and the secondary endpoint of OS were significantly improved with mFOLFOX6 plus zolbetuximab.[22]

The prevalence of CLDN positivity is reported to be 30%-33% in patients with GC/GEJC and associated with diffuse type.[23,24] The clinicopathological features of CLDN-positive GC/GEJC and its impact on treatment outcomes with current standard chemotherapy or anti-PD-1 therapy, however, remain unclear. Therefore, we conducted comprehensive clinical and molecular characterization of CLDN18.2 expression in advanced GC/GEJC.

METHODS

Patients

We carried out a single-institute study to evaluate the clinicopathological features of CLDN18.2 expression with four molecular subtypes: mismatch repair deficient (MMR-D), Epstein—Barr virus (EBV)-positive, HER2-positive, and others (all negative), along with programmed death-ligand 1 (PD-L1) combined positive score (CPS), and other molecular alterations in Japanese patients with advanced GC/GEJC. Clinical outcomes of standard first- (fluoropyrimidine + platinum) or second-line (taxanes ± ramucirumab) chemotherapy and subsequent anti-PD-1 therapy were also investigated according to CLDN18.2 expression. The eligibility criteria were as follows: (i) an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 to 2; (ii) unresectable, locally advanced or metastatic GC/GEJC; (iii) histologically proven adenocarcinoma; (iv) adequate bone marrow, hepatic, and renal function; (v) received systemic chemotherapy from October 2015 to December 2019; (vi) with available molecular features of HER2, MMR, and EBV; and (vii) archival tissue sample from primary tumor. All patients provided written informed consent for biomarker analysis. The study protocol was approved by the Institutional Review Board of the National Cancer Center Japan. The datasets which were analyzed in the current study are not publicly available because of privacy concerns.

Molecular characteristics

Molecular characteristics such as CLDN18.2, HER2, PD-L1, MMR, EBV, mesenchymal-epithelial transition factor, and epidermal growth factor receptor (EGFR) were analyzed using formalin-fixed paraffin-embedded (FFPE) tissue specimens from primary tumor. CLDN18.2 expression was assessed by immunohistochemistry (IHC) using CLDN18 (Clone 43-14A, Roche Ventana, Oro Valley, AZ). CLDN positivity was defined as moderate-to-strong expression in $\geq$75% of tumor cells. IHCs of HER2, MMR, EBV, EGFR, and mesenchymal-epithelial transition factor were evaluated as previously reported.[25] PD-L1 expression was assessed by IHC using an anti-PD-L1 rabbit monoclonal antibody (clone SP142 or SP263; Ventana, Tucson, AZ) and measured using CPS, defined as the ratio of the number of PD-L1-positive cells (tumor cells, lymphocytes, and macrophages) to the total number of tumor cells multiplied by 100. In patients who received subsequent anti-PD-1 antibody, IHCs of immune cell markers (CD3, CD8, CD16, CD56, CD68, granzyme B) were also assessed, using archival FFPE tissue. IHC staining images of these immune cell markers were captured using a pathology imaging device (Aperio AT2, Leica, Nussloch, Germany) and assessed using automatic analysis (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100762). All specimens in this study were reviewed by TK. The details of the IHCs are shown in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100762.

The *CLDN18-ARHGAP26/6* fusion was assessed by RNA sequencing and quantitative PCR in patients with adequate archival tissue samples. Genomic alterations were analyzed using the Oncomine Comprehensive Assay version 3 or Oncomine Cancer Research Panel (Thermo Fisher Scientific, Waltham, MA).

Outcomes and statistical analysis

We evaluated outcomes including tumor response, PFS, and OS. Tumor response was assessed in patients with measurable lesions using Response Evaluation Criteria in Solid Tumors version 1.1. The objective response rate (ORR) was defined as the proportion of patients with the best overall response of complete response (CR) or partial response (PR). Disease control rate (DCR) was defined as the proportion of patients with the best overall response to CR, PR, or stable disease. PFS was defined as the time from treatment initiation to disease progression or death from any cause. The OS was defined as the time from initiation of first-line chemotherapy to death due to any cause. Fisher's exact test or *t*-test was used to compare the baseline

characteristics and ORR. PFS and OS were estimated using the Kaplan—Meier method and compared using the Cox proportional hazards model, presented as hazard ratios (HRs) with 95% confidence intervals (CIs). The prediction models for OS and PFS with anti-PD-1 antibody were analyzed using univariate and multivariate Cox regression analyses. The covariates in the multivariate analysis included age ($\geq$65 versus $<$65 years), sex (male versus female), ECOG PS (1, 2 versus 0), prior gastrectomy (yes versus no), primary lesion (gastric versus GEJ), Borrmann classification (type 4 versus non-type 4), metastatic site (liver, peritoneum, lung, and lymph node), MMR (deficient versus proficient), EBV status (positive versus negative), CLDN18.2 status (positive versus negative), CPS ($\geq$5 versus $<$5). Differences in the levels of immune cell markers according to molecular characteristics were analyzed using the Mann—Whitney *U* test. All tests were two-sided and $P < 0.05$ was considered to indicate statistical significance. Statistical analyses were carried out using the statistical program R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient characteristics

A total of 408 patients who received systemic chemotherapy from October 2015 to December 2019 with archival tissue samples, were enrolled in this study (Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2022.100762). All specimens were biopsy specimens collected from the primary tumors. Comprehensive molecular features including molecular subtypes and PD-L1 CPS according to CLDN18.2 expression are shown in Figures 1 and 2, respectively.

CLDN positivity was identified in 98 of 408 patients (24.0%). The prevalence of CLDN positivity was almost equal among molecular subtypes; 5 of 24 (20.8%) in MMR-D, 4 of 15 (26.7%) in EBV-positive, 4 of 15 (26.7%) in HER2-positive, and 74 of 311 (23.8%) in all-negative. The frequency of macroscopic type 4 tumor by Borrmann classification was significantly higher in the CLDN-positive group compared with the CLDN-negative group (28.6% versus 17.1%, $P = 0.019$). The frequency of CPS $\geq$5 was $\sim$10% lower in the CLDN-positive group compared with the CLDN-negative group (41.9% versus 51.5%, $P = 0.122$), although the difference was not statistically significant (Table 1).

In an exploratory analysis, the accordance rate of CLDN positivity between before and after first-line chemotherapy was 75.1% in 17 patients whose specimens were obtained both before and after first-line chemotherapy (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2022.100762), although we could not rule out the possibility that this result was affected by intratumoral heterogeneity.

Among 408 patients, genomic analysis was conducted in 218 patients. The frequency of *KRAS* amplification tended to be higher in CLDN-positive patients than in CLDN-negative patients, although there were no differences in the frequencies of other gene alterations between the two groups (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2022.100762).

After excluding patients with MMR-D, EBV-positive, and HER2-positive subtypes, gene alterations between the CLDN-positive and CLDN-negative groups were still not different. The *CLDN18-ARHGAP26/6* fusion was detected in five cases (1.3%) in 388 patients with a sufficient amount of tumor samples for RNA analysis. Among the five cases

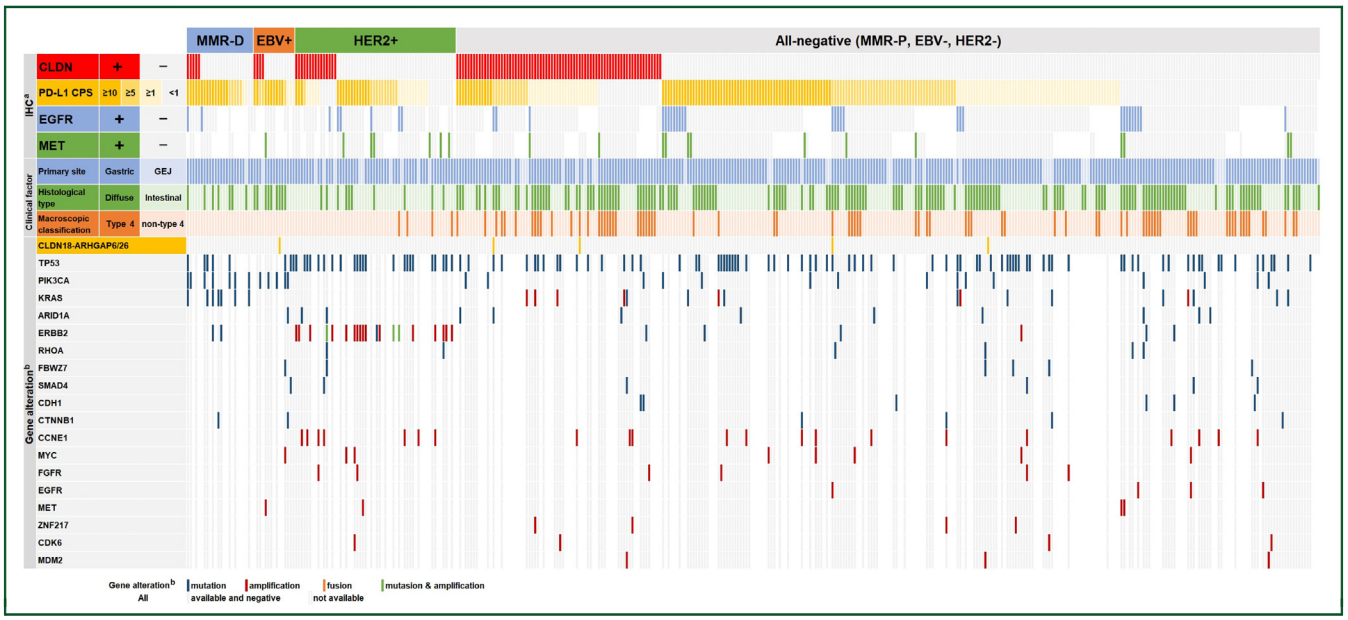

Figure 1. Association between CLDN expression and molecular characteristics. CLDN, claudin; CPS, combined positive score; EBV, Epstein—Barr virus; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; MMR-D, mismatch repair deficient; MMR-P, mismatch repair proficient; PD-L1, programmed death-ligand 1.
[a]CLDN+, 2+/3+ $\geq$75%; EGFR+, 2+/3+ $\geq$50%; MET+, 2+/3+ $\geq$50%. [b]The top 10 most frequent gene mutations, top 10 most frequent amplifications, and *CLDN18-ARHGAP6/26* fusion.

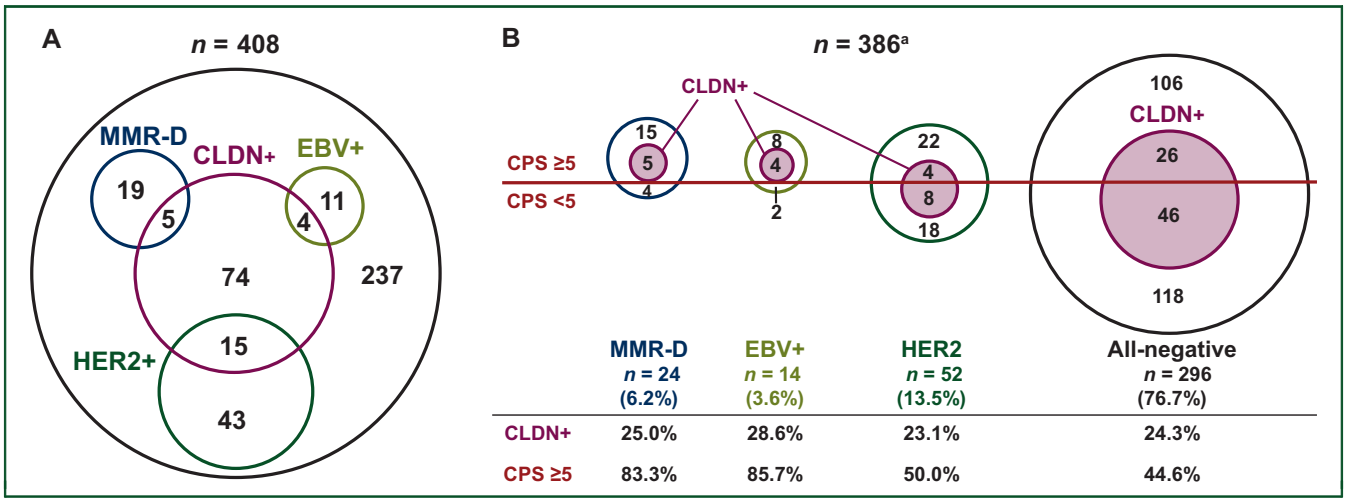

Figure 2. Relationship between CLDN and other biomarkers (A) and PD-L1 CPS (B). All-negative: negative for neither MMR-D, EBV nor HER2.
CLDN, claudin; CPS, combined positive score; EBV, Epstein—Barr virus; HER2, human epidermal growth factor receptor 2; MMR-D, mismatch repair deficient; MMR-P, mismatch repair proficient.
[a]Patients with available CPS results.

Table 1. Patient characteristics according to CLDN expression (n = 408)[a]

		CLDN+ n = 98 (24.0%)	CLDN- n = 310 (76.0%)	P value[b]
Age, years	Median (range)	65 (32-85)	67 (23-89)	0.23[c]
	<65	45 (45.9%)	124 (40.0%)	0.347
	≥65	53 (54.1%)	186 (60.0%)	
Sex, *n* (%)	Male	66 (67.3)	215 (69.4)	0.709
	Female	32 (32.7)	95 (30.6)	
PS, *n* (%)	0	86 (87.8)	235 (75.8)	0.011
	1-2	12 (12.2)	75 (24.2)	
Primary site, *n* (%)	Gastric	85 (86.7)	278 (89.7)	0.459
	GEJ	13 (13.3)	32 (10.3)	
Histological type, *n* (%)	Intestinal	51 (52.0)	173 (55.8)	0.561
	Diffuse	47 (48.0)	137 (44.2)	
Macroscopic classification[d], *n* (%)	Type 4	28 (28.6)	53 (17.1)	0.019[e]
	Non-type 4	70 (71.4)	257 (82.9)	
	Type 3	29 (29.6)	126 (40.6)	
	Type 2	33 (33.7)	115 (37.1)	
	Type 1	4 (4.1)	11 (3.5)	
	Type 0	4 (4.1)	4 (1.3)	
	Unclassifiable	0 (0)	1 (0.3)	
Site of metastasis, *n* (%)	Liver	21 (21.4)	81 (26.1)	0.422
	Lung	7 (7.1)	23 (7.4)	1.000
	Peritoneum	48 (49.0)	132 (42.6)	0.294
	Lymph node	64 (65.3)	227 (73.2)	0.158
Organs with metastases, *n* (%)	1	48 (49.0)	150 (48.4)	1.000
	≥2	50 (51.0)	160 (51.6)	
MMR, *n* (%)	Deficient	5 (5.1)	19 (6.1)	0.810
	Proficient	93 (94.9)	291 (93.9)	
EBV, *n* (%)	Positive	4 (4.1)	11 (3.5)	0.763
	Negative	94 (95.9)	299 (96.5)	
HER2, *n* (%)	Positive	15 (15.3)	43 (13.9)	0.741
	Negative	83 (84.7)	267 (86.1)	
PD-L1,[f] *n* (%)	CPS <1	24 (25.8)	68 (23.2)	0.675
	CPS ≥1	69 (74.2)	225 (76.8)	
	CPS <5	54 (58.1)	142 (48.5)	0.122
	CPS ≥5	39 (41.9)	293 (51.5)	

CLDN, claudin; CPS, combined positive score; EBV, Epstein—Barr virus; GEJ, gastroesophageal junction; HER2, human epidermal growth factor receptor 2; MMR, mismatch repair; PD-L1, programmed death-ligand 1; PS, performance status.
[a]Characteristics at first diagnosis of recurrence or metastatic disease.
[b]Using Fisher's exact test except for age (range).
[c]Using *t*-test.
[d]Borrmann classification.
[e]*P* value between type 4 and non-type 4.
[f]PD-L1 was evaluated in 93 of CLDN-positive (94.9%) and 293 of CLDN-negative patients (94.5%).

harboring *CLDN18-ARHGAP26/6* fusion, all cases demonstrated diffuse type, four cases had peritoneal metastases, and one had EBV-positive status (Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2022.100762).

Clinical outcomes of standard first- or second-line chemotherapy and subsequent anti-PD-1 therapy according to CLDN18.2 expression

First-line chemotherapy. Among 408 patients, 226 patients with HER2-negative GC/GEJC received standard first-line chemotherapy (fluoropyrimidine + platinum). The ORRs were 42.9% and 48.7% ($P = 0.742$) (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2022.100762), and the median PFS times were 8.6 and 7.1 months {HR 1.02 [95% confidence interval (CI) 0.73-1.43]; $P = 0.895$} in CLDN-positive and CLDN-negative groups, respectively (Figure 3A). Among 215 HER2-negative patients with available CPS status, there were also no significant differences in the efficacy (ORR and PFS) of standard first-line chemotherapy according to CPS status alone (CPS $\geq$5 versus CPS <5) or CLDN status combined with CPS (CPS $\geq$5/CLDN-positive versus CPS $\geq$5/CLDN-negative or CPS <5/CLDN-positive versus CPS <5/CLDN-negative) (Supplementary Figure S3A and B, Supplementary Table S6, available at https://doi.org/10.1016/j.esmoop.2022.100762).

The OS in patients who received standard first-line treatment was also not significantly different between CLDN-positive and -negative groups [median 18.4 versus 20.1 months; HR 1.26 (95% CI 0.89-1.78); $P = 0.191$] (Figure 4). The OS was not significantly different between CPS $\geq$5 and CPS <5 (median 20.1 versus 18.8 months; HR 0.91; 95% CI 0.67-1.24; $P = 0.555$) (Supplementary Figure S3B, available at https://doi.org/10.1016/j.esmoop.2022.100762). When we stratified patients by CPS status and CLDN status, there were also no significant differences in OS between CPS $\geq$5/CLDN-positive and CPS $\geq$5/CLDN-negative groups or between CPS <5/CLDN-positive and CPS <5/CLDN-negative groups (Supplementary Figure S3D, available at https://doi.org/10.1016/j.esmoop.2022.100762). In the multivariate analysis, CLDN status was not associated with OS. Meanwhile, ECOG PS 1 or 2, ALP $\geq$ upper limit of normal, and peritoneal dissemination were associated with poor OS (Supplementary Table S7, available at https://doi.org/10.1016/j.esmoop.2022.100762).

Second-line chemotherapy. In 275 patients who received standard second-line chemotherapy (taxanes ± ramucirumab), the efficacy was not significantly different between CLDN-positive and CLDN-negative groups [ORR 28.8% versus 30.1%; $P = 1.000$; median PFS 4.2 months versus 4.0 months; HR 1.08 (95% CI 0.80-1.44); $P = 0.625$] (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2022.100762, Figure 3B).

Anti-PD-1 therapy. Among 408 patients, 164 received subsequent anti-PD-1 antibody as a second- or later-line treatment. The ORRs were 14.3% and 19.0% ($P = 0.810$), and the DCRs were 31.7% and 48.0% ($P = 0.301$) in the CLDN-positive and CLDN-negative groups, respectively (Supplementary Table S5, available at https://doi.org/10.1016/j.esmoop.2022.100762). The median PFS with anti-PD-1 antibody were 1.8 and 1.9 months in the CLDN-positive and CLDN-negative groups, respectively [HR 1.07 (95% CI 0.75-1.52); $P = 0.725$] (Figure 3C). In the CLDN-positive group, the ORR and DCR were numerically lower than those in the CLDN-negative group with no statistically significant differences. These trends were maintained when the MMR-D, EBV-positive, and HER2-positive subtypes were excluded from the analyses (Supplementary Figure S4 and Supplementary Table S8, available at https://doi.org/10.1016/j.esmoop.2022.100762). Among 158 patients with available CPS status, CPS $\geq$5 was significantly associated with longer PFS with anti-PD-1 antibody [HR 0.67 (95% CI 0.48-0.94); $P = 0.019$] (Supplementary Figure S5A, available at https://doi.org/10.1016/j.esmoop.2022.100762). In both the CPS $\geq$5 and CPS <5 groups, the DCRs in CLDN-positive patients were ∼10%-15% lower than those in CLDN-negative patients (Supplementary Table S9, available at https://doi.org/10.1016/j.esmoop.2022.100762). There were, however, still no significant differences in PFS times between CLDN-positive and CLDN-negative patients in both CPS $\geq$5 and CPS <5 groups (Supplementary Figure S5B, available at https://doi.org/10.1016/j.esmoop.2022.100762). In multivariate analysis of PFS with anti-PD-1 antibody, CLDN positivity was not associated with PFS. Male and MMR-D were associated with longer PFS, whereas Borrmann type 4, liver metastasis, and peritoneal metastasis were associated with shorter PFS with anti-PD-1 antibody (Supplementary Table S10, available at https://doi.org/10.1016/j.esmoop.2022.100762).

Immune cell markers

Among 164 patients who received subsequent anti-PD-1 antibody, 149 patients with sufficient specimens were additionally analyzed for IHCs of immune cell markers (CD3, CD8, CD16, CD56, CD68, and granzyme B) in the tumors. In the CLDN-positive group, the number of CD16-positive cells was significantly lower ($P = 0.028$), whereas the number of CD68-positive cells was significantly higher, compared with those in the CLDN-negative group (Supplementary Figure S6A, available at https://doi.org/10.1016/j.esmoop.2022.100762). When we analyzed them separately from the MMR-D, EBV, and HER2-positive groups, the trends did not change, although statistically significant differences disappeared (Supplementary Figure S6B, available at https://doi.org/10.1016/j.esmoop.2022.100762).

DISCUSSION

We evaluated the clinicopathological features and clinical outcomes with standard chemotherapy and checkpoint inhibition according to CLDN18.2 expression in patients with unresectable advanced GC/GEJC. Additionally, we investigated genomic alterations and immune cell markers

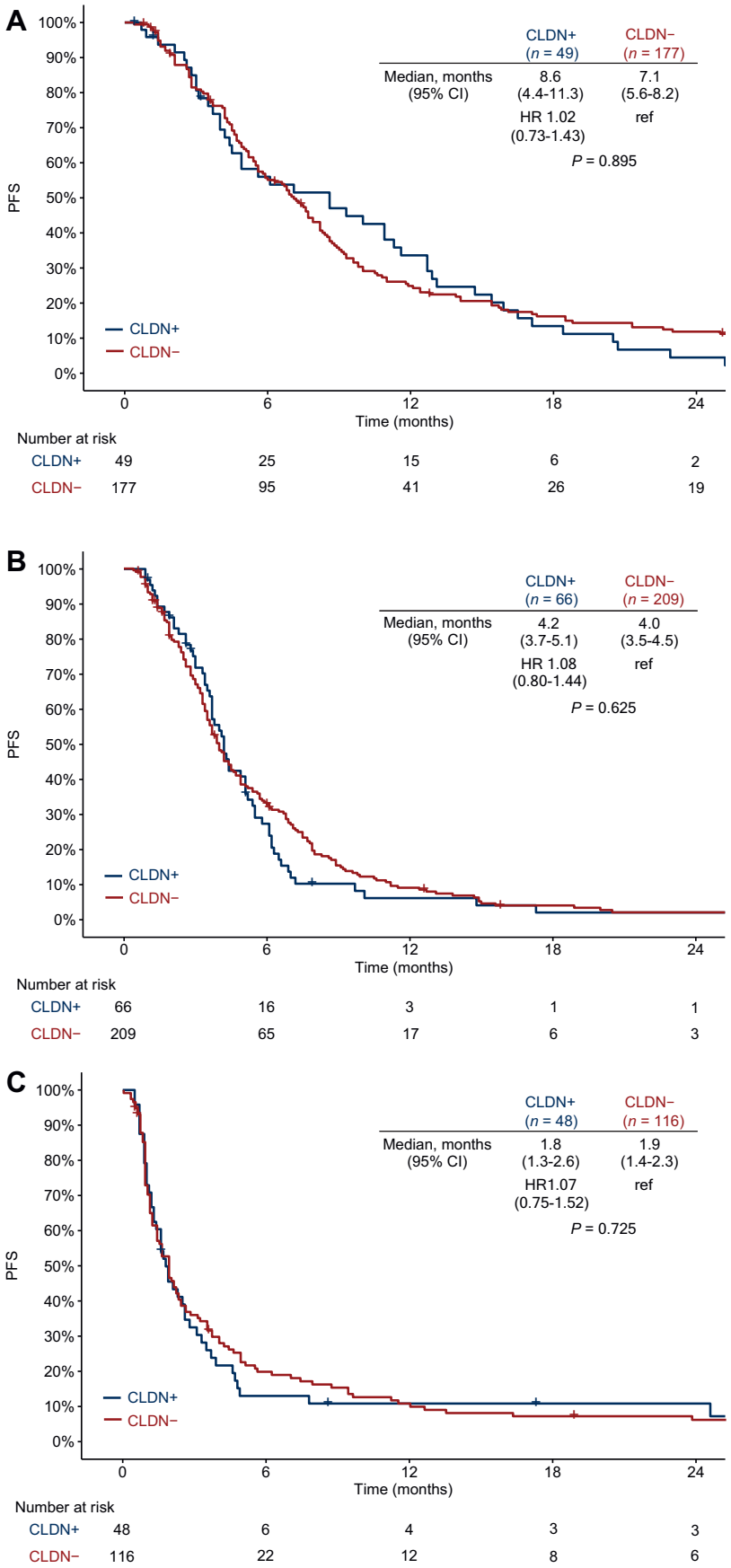

Figure 3. Kaplan–Meier plots of progression-free survival (PFS) with each line treatment according to CLDN expression. (A) first-line chemotherapy (platinum + fluoropyrimidine, $n = 226$), (B) second-line chemotherapy (taxanes ± RAM, $n = 275$), (C) anti-PD-1 antibody ($n = 164$).
CI, confidence interval; CLDN, claudin; FP, fluoropyrimidine; HR, hazard ratio; RAM, ramucirumab; ref, reference.

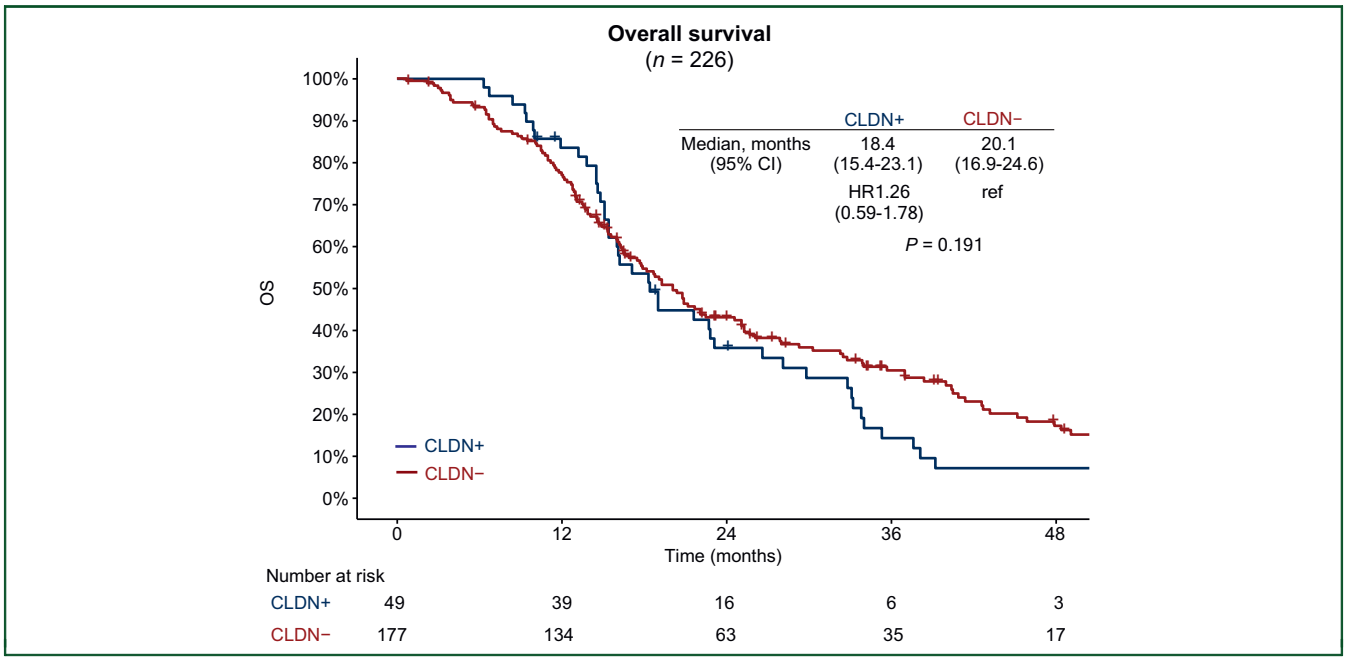

Figure 4. Kaplan–Meier plots of overall survival (OS) in patients who received standard first-line chemotherapy (platinum + fluoropyrimidine, *n* = 226).
HR, hazard ratio; ref, reference.

according to CLDN18.2 expression. To our knowledge, this is the first report to assess comprehensive clinical and molecular characterization of CLDN18.2 expression using the same definition as ongoing phase III trials and evaluate its impact on treatment outcomes in patients with unresectable advanced GC/GEJC.

In our patient cohort, CLDN positivity was associated with Borrmann Type 4. Previous single cohort studies demonstrated CLDN18.2 expression in GC/GEJC was associated with diffuse type[26-29] and EBV-positive,[24,29,30] whereas a recent meta-analysis showed CLDN18.2 expression had no significant correlation with Lauren classification.[30] In contrast to previous reports, the prevalence of CLDN positivity in EBV subtype was not higher than that of other subtypes in our cohort, which might be due to the small number and warrants further investigations. Importantly, CLDN-positive tumors distributed among various molecular subtypes such as MMR-D, EBV-positive, HER2-positive, and all negative. This suggests that CLDN18.2 could be targetable regardless of the molecular subtype.

There were no significant differences between the CLDN-positive and CLDN-negative groups in efficacy of standard first- and second-line chemotherapy, respectively. In HER2-negative patients, CLDN status combined with CPS status was also not related to the efficacy of the standard first-line chemotherapy. In addition, CLDN-positive had no impact on the efficacy of anti-PD-1 antibody. In the analysis of immune cell markers, only the level of CD8-positive cells was associated with better clinical outcomes with anti-PD-1 antibody. The level of CD8 was not significantly different according to CLDN status, which might be one of the reasons why CLDN status was not associated with the efficacy of anti-PD-1 antibody. These efficacy data might be useful when considering treatment strategy of CLDN-positive GC/GEJC.

The OS in this cohort was not significantly different between the CLDN-positive and CLDN-negative groups. A meta-analysis showed CLDN positivity was not associated with survival in surgically resected GC/GEJC,[31] although this study had limitations including a different definition of CLDN positivity such as expression in $\geq$40% of tumor cells. Recently, Pellino et al.[24] demonstrated that CLDN positivity by the recent definition (moderate-to-strong expression in $\geq$75% of tumor cells) was not related to survival in advanced GC/GEJC, but this study also included stage I-III. Our data firstly demonstrated that CLDN positivity (cut-off by 75%) was not a prognostic factor in advanced unresectable or metastatic GC/GEJC, however, the prognostic significance of CLDN positivity needs further investigations considering the results of prospective trials. These results might be useful for indirectly comparing treatment efficacies or survival benefits in previous randomized trials of chemotherapy plus anti-PD-1 therapies[6,7] and ongoing trials with zolbetuximab which only enrolled CLDN-positive patients. Furthermore, nearly 50% of CLDN-positive tumors also showed PD-L1 CPS $\geq$5, which supports an ongoing trial to investigate zolbetuximab combined with chemotherapy plus anti-PD-1 antibody in one of the cohorts (ILUSTRO: NCT03505320).

In the analysis of immune cell markers, CLDN positivity was significantly associated with higher levels of CD68. CD68 is a pan-macrophage marker, and the higher level of CD68 in the CLDN-positive group may reflect higher infiltration of tumor-related macrophages (TAMs) in CLDN-positive tumors. TAMs have two conflicting functions: they have antitumor functions including direct cytotoxicity and ADCC, whereas they are related to tumor progression by promoting angiogenesis and suppressing T cells.[32-36] The detailed phenotype of TAMs in CLDN-positive tumors and its therapeutic relevance requires further investigation. Previous study from China

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demonstrated that CLDN positivity (moderate-to-strong expression in ≥40% of tumor cells) was associated with a higher number of CD8-positive T cells in advanced GC,[37] which was inconsistent with the observations in our study. This might come from several differences including CLDN cut-off value, tumor stage, and number of patients. Tumor microenvironment including CD8-positive T cell according to CLDN expression warrants further investigations.

The *CLDN18-ARHGAP26/6* fusion was detected in 5 of 388 (1.3%) patients and was associated with diffuse type and peritoneum metastasis. The *CLDN18-ARHGAP26/6* fusion was firstly detected in 4.4% of GC by The Cancer Genome Atlas Research Network (TCGA) in 2014, and most frequent in genomically stable type (15%).[38] Some studies demonstrated that the *CLDN18-ARHGAP26/6* fusion was associated with younger age, diffuse type, lymph node metastases, distant organ metastases, and worse prognosis,[39-41] a part of which was consistent with the characteristics in this cohort. The frequency was relatively lower in our cohort compared with previous reports, which might be due to the differences in specimen type (endoscopic biopsy versus surgical specimen) or clinical stage (stage IV versus all stage). Nevertheless, the CLDN positivity by IHC in our cohort was similar to previous reports[23,24] and supported it as a relevant target for drug development.[20,21,42]

This study has some limitations. First, it was a single-institution study with a limited sample size. Second, although we evaluated treatment outcomes after first-line chemotherapy and subsequent anti-PD-1 treatment, no patients in this cohort were treated with chemotherapy plus anti-PD-1 as the current standard of care because this research was conducted before the approval of nivolumab plus chemotherapy as a first-line treatment in Japan. Third, we evaluated PD-L1 CPS using relatively old archival specimens, which might affect the positive rate.[43] Finally, PD-L1 CPS or gene alterations were not analyzed in all of the patients who received systemic chemotherapy.

In summary, CLDN18.2 expression in advanced GC/GEJC was associated with some clinical and molecular features but was not a predictive factor of chemotherapy or checkpoint inhibition. CLDN positivity also had no impact on OS. This information could be useful for interpreting the results from currently ongoing clinical trials of CLDN18.2-targeted therapies for advanced GC/GEJC and considering treatment strategy for CLDN-positive GC/GEJC.

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