

Original Article



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Discordance in Claudin 18.2 Expression Between Primary and Metastatic Lesions in Patients With Gastric Cancer

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ABSTRACT

Purpose: Claudin 18.2 (CLDN18.2) has emerged as a promising therapeutic target for CLDN18.2-expressing gastric cancer (GC). We sought to examine the heterogeneity of CLDN18.2 expression between primary GC (PGC) and metastatic GC (MGC) using various scoring methods.

Materials and Methods: We retrospectively analyzed data from 102 patients with pathologically confirmed paired primary and metastatic gastric or gastroesophageal junction adenocarcinomas. CLDN18.2 expression was evaluated through immunohistochemistry on formalin-fixed paraffin-embedded tissue samples. We assessed CLDN18.2 positivity using multiple scoring approaches, including the immunoreactivity score, H-score, and the percentage of tumor cells showing moderate-to-strong staining intensity. We analyzed the concordance rates between PGC and MGC and the association of CLDN18.2 positivity with clinicopathological features.

Results: CLDN18.2 positivity varied from 25% to 65% depending on the scoring method, with PGC consistently showing higher expression levels than MGC. Intratumoral heterogeneity was noted in 25.5% of PGCs and 19.6% of MGCs. Intertumoral heterogeneity, manifesting as discordance in CLDN18.2 positivity between PGC and MGC, was observed in about 20% of cases, with moderate agreement across scoring methods ($\kappa=0.47$ to 0.60). In PGC, higher CLDN18.2 positivity correlated with synchronous metastasis, presence of peritoneal metastasis, poorly differentiated grade, and biopsy specimens. In MGC, positivity was associated with synchronous metastasis, presence of peritoneal metastasis, and metastatic peritoneal tissues.

Conclusions: CLDN18.2 expression demonstrates significant heterogeneity between PGC and MGC, with a 20% discordance rate. Comprehensive tissue sampling and reassessment of CLDN18.2 status are crucial, especially before initiating CLDN18.2-targeted therapies.

Keywords: Claudin; Gastric cancer; Metastasis

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

The cornerstone of palliative first-line systemic therapy for patients with locally advanced unresectable or metastatic gastric or gastroesophageal junction (G/GEJ) adenocarcinomas is a combination of a fluoropyrimidine and a platinum-based agent. Despite this standard treatment, prognosis remains poor [1]. The advent of targeted therapies has markedly improved outcomes and has become integral to standard palliative care for these patients [1-4]. For instance, human epidermal growth factor receptor 2 (HER2)-targeted therapies like trastuzumab, used alongside fluoropyrimidine plus platinum doublet chemotherapy, are now standard for HER2-positive G/GEJ adenocarcinoma, significantly enhancing overall survival (OS) [1-6]. Additionally, immune checkpoint inhibitors targeting programmed death-1 (PD-1), such as nivolumab and pembrolizumab, are increasingly being incorporated into standard fluoropyrimidine plus platinum doublet chemotherapy, particularly for patients with programmed death-ligand 1 (PD-L1)-positive tumors, or those with mismatch repair deficient (dMMR) or microsatellite instability (MSI)-high tumors [1-4,7-9].

Recently, Claudin 18 isoform 2 (CLDN18.2) has emerged as a promising therapeutic target in systemic therapy for locally advanced unresectable or metastatic G/GEJ adenocarcinomas. CLDN18.2, a specific isoform within the claudin family, is essential for the formation and maintenance of tight junctions in epithelial and endothelial tissues [10,11]. Although typically localized at tight junctions in normal gastric epithelium, CLDN18.2 is predominantly expressed on the surface of gastric adenocarcinoma cells during malignant transformation, rendering it an accessible target for therapy [10,11]. Zolbetuximab, a chimeric immunoglobulin G1 monoclonal antibody, binds to CLDN18.2, boosting the immune system's ability to attack cancer cells via antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity [10,11]. The recent phase 3 SPOTLIGHT and GLOW studies, which focused on patients with untreated, locally advanced unresectable or metastatic G/GEJ adenocarcinoma that was HER2-negative and expressed high levels of CLDN18.2, [12,13], demonstrated statistically significant improvements in progression-free survival and OS when zolbetuximab was combined with chemotherapy. Thus, in determining the most effective palliative systemic therapy regimens for GC, CLDN18.2 expression, alongside HER2, PD-L1, and MMR/MSI status, should be considered a crucial predictive biomarker.

Tumor heterogeneity in G/GEJ adenocarcinoma presents a significant challenge due to substantial variations in molecular and cellular characteristics both within a single tumor (intratumoral heterogeneity) and across different tumor sites (intertumoral heterogeneity) [14]. Such heterogeneity is well-documented for established predictive biomarkers like HER2 and PD-L1, underscoring the complexity of accurately targeting these markers in clinical settings [15-21]. With the emerging role of CLDN18.2 as a novel predictive biomarker, it is critical to determine whether similar variability exists for CLDN18.2 expression in G/GEJ adenocarcinoma. Given that palliative systemic therapy addresses both primary and metastatic tumors, understanding the differences in CLDN18.2 expression between primary and metastatic G/GEJ tumor sites is essential for optimizing treatment strategies. Consequently, this study aims to analyze and compare CLDN18.2 expression levels in primary and metastatic G/GEJ adenocarcinomas to guide clinical decisions regarding the use of CLDN18.2-targeted therapies.

MATERIALS AND METHODS

Patients and tumor samples

We reviewed cases using a database of patients with paired primary and metastatic G/GEJ adenocarcinoma, pathologically confirmed at Chungbuk National University Hospital between March 2006 and December 2023. Formalin-fixed paraffin-embedded (FFPE) tissue samples were collected to accurately assess CLDN18.2 expression. Two experienced pathologists examined hematoxylin and eosin-stained slides from each FFPE block, selecting the slide with the largest viable tumor cell area for evaluation. We gathered clinicopathological data, including age, sex, timing of metastatic disease onset, number of metastatic sites, presence of peritoneal metastasis, tumor location, World Health Organization histological classification, Lauren classification, and tissue specimen of primary gastric cancer (PGC); tumor location and tissue specimen of metastatic gastric cancer (MGC). Additionally, HER2, PD-L1, and MMR statuses for both PGC and MGC, the duration between the acquisition of PGC and MGC tissues, and any anticancer systemic therapy administered before the acquisition of MGC tissue were also obtained from medical records and pathology reports.

The study protocol adhered to the ethical guidelines of the 1975 Declaration of Helsinki. This study was reviewed and approved by the Institutional Review Board (IRB) of Chungbuk National University Hospital (CBNUH 2024-04-002), which waived the requirement for written informed consent from the patients.

Immunohistochemistry (IHC)

IHC was performed on FFPE tissue sections to assess the expression of HER2, PD-L1, MMR, and CLDN18.2. Tumor specimens were sectioned at 4- μ m thickness and subsequently stained. Fully automated immunostaining for HER2, MMR, and CLDN18.2 was conducted using the BenchMark XT autostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) with the following antibodies: anti-HER2/neu rabbit monoclonal primary antibody (clone 4B5; Ventana Medical Systems, Inc.), mouse anti-MLH1 (Ventana/Roche M1, pre-dilute), mouse anti-MSH2 (Roche G219-1129, pre-dilute), rabbit anti-MSH6 (Roche SP93, pre-dilute), mouse anti-PMS2 (Roche A16-4, pre-dilute), and anti-CLDN18.2 antibody (clone 43-14A, Ventana). Silver *in situ* hybridization (SISH) for *HER2* used Inform HER2 DNA and chromosome 17 probes (Ventana Medical Systems, Inc.), followed by 2-color chromogenic ISH on FFPE specimens. PD-L1 expression in tumor cells was analyzed using the 28-8 pharmDx assay (Agilent Technologies, Santa Clara, CA, USA) on the DAKO Autostainer Link 48.

HER2 positivity was defined as either IHC 3+ or IHC 2+, with *HER2* amplification confirmed through SISH. PD-L1 expression was evaluated using the combined positive score (CPS), which is calculated by dividing the number of PD-L1-positive cells (including tumor cells, lymphocytes, and macrophages) by the total number of viable tumor cells, then multiplying the result by 100. Tumors lacking MLH1, MSH2, PMS2, or MSH6 expression were classified as dMMR, whereas those expressing all 4 proteins were considered to have proficient MMR. CLDN18.2, a transmembrane protein crucial for forming tight junctions between epithelial cells, was deemed positive only in cases showing membranous, linear staining. Granular expression in the cytoplasm or nucleus was deemed non-specific and was excluded from the assessment of CLDN18.2 positivity.

Scoring methods for CLDN18.2 expression in IHC

CLDN18.2 expression was scored semi-quantitatively based on staining intensity and the percentage of positive staining. Intratumoral heterogeneity was defined as the presence of at least 2 distinct staining intensities (ranging from 0 to 3+) within a single tumor specimen, with more than 10% of the tumor exhibiting a different staining intensity. Two of the scoring methods, the immunoreactivity score (IRS) and H-score, considered all tumor cells with a staining of $\geq 1+$ intensity in any fraction of tumor cells ("any positivity"). In contrast, the third method assessed the percentage of tumor cells with a staining intensity of $\geq 2+$ (indicating moderate-to-strong membrane staining intensity).

IRS

The staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong) was multiplied by the percentage of stained tumor cells (0, negative; 1, 1%–25%; 2, 26%–50%; 3, 51%–75%; 4, 76%–100%) to produce the IRS of each sample (score 0–12). Samples with an IRS > 8 were assessed as CLDN18.2-positive, while those with an IRS ≤ 8 were CLDN18.2-negative [22,23].

H-score

The H-score was calculated using the following formula: $H\text{-score} = \sum (\text{Percentage of Staining} \times \text{Staining Intensity})$. In brief, the H-score was defined as: $(\text{Percentage of CLDN18.2 } 1+ \text{ Tumor Cells} \times 1) + (\text{Percentage of CLDN18.2 } 2+ \text{ Tumor Cells} \times 2) + (\text{Percentage of CLDN18.2 } 3+ \text{ Tumor Cells} \times 3)$. Thus, this composite score can range from 0 (indicating a completely negative tumor) to a maximum of 300 (indicating a tumor in which all the cells exhibit 3+ staining). Scores were categorized as negative (0: 0–50) or positive (1: 51–300) [22,24,25].

Scoring method implying moderate-to-strong staining intensity

In the 2 pivotal phase III trials, CLDN18.2 positivity was defined as an IHC score of $\geq 2+$ staining intensity across $\geq 75\%$ of the tumor cell surface area [12,13]. More recently, newly developed CLDN18.2-targeting agents, characterized by improved affinity and enhanced antitumor activity, have been applied to a broader range of tumor cells ($\geq 1\%$ or $\geq 40\%$) showing $\geq 2+$ staining intensity.

Statistical analysis

Data are presented as numbers and percentages for categorical variables, and means with standard deviations (SDs) for numerical variables. The χ^2 test was utilized to compare percentages, while the paired t-test was employed to compare mean values between PGC and paired MGC. Normality assumptions for the IRS and H-score were verified before applying parametric methods, including the paired t-test. The concordance rate in CLDN18.2 positivity between PGC and paired MGC was determined by calculating the ratio of concordant cases to total cases. Additionally, the level of concordance in CLDN18.2 positivity was assessed using Cohen's kappa coefficient (κ -value) statistics [26], with concordance classified as follows: poor ($\kappa < 0.00$), slight ($\kappa = 0.00$ – 0.20), fair ($\kappa = 0.21$ – 0.40), moderate ($\kappa = 0.41$ – 0.60), substantial ($\kappa = 0.61$ – 0.80), or almost perfect ($\kappa = 0.81$ – 1.00).

All statistical analyses were conducted using IBM SPSS Statistics version 21 (IBM Corporation, Armonk, NY, USA). Tests were 2-sided, with a significance level set at $P < 0.05$.

RESULTS

Clinicopathological characteristics

We identified 102 patients with pathologically confirmed paired primary and metastatic G/GEJ adenocarcinoma in our archive. The clinicopathological characteristics of these patients are summarized in **Table 1**.

Synchronous metastatic disease was present in 71.6% of the patients, with 41.2% exhibiting peritoneal metastasis. Among the PGCs, 58.8% were of poorly differentiated histologic grade, classified as either poorly differentiated tubular adenocarcinoma or poorly cohesive carcinoma. Additionally, 32.4% were categorized as diffuse type, and 56.9% were resected specimens. The most common source of MGC tissue was the omentum or peritoneum, accounting for 42.2% of specimens, and 70.6% were large-sized tumor specimens (>25 mm²). All tissues from PGCs were obtained at the initial diagnosis of G/GEJ adenocarcinoma. Metastatic tissues from MGCs were collected either synchronously at the time of initial diagnosis (63.7%) or more than 3 months after the acquisition of PGC tissue (36.3%). Additionally, 24.5% of MGC tissue samples were obtained following the administration of anticancer drugs.

HER2, PD-L1, and MMR status in PGC and MGC

The comparison of HER2, PD-L1, and MMR status in PGC and MGC is detailed in **Supplementary Table 1**. HER2 positivity was observed in 13.7% of PGCs and 10.8% of MGCs, with substantial concordance between the 2 (κ value of 0.682). For PD-L1 CPS using a cutoff of 1, the positivity rates were 79.4% for PGC and 76.5% for MGC, while both showed a positivity rate of 47.1% when a cutoff of 5 was used. However, concordance levels were evaluated as slight, with κ values of 0.060 and 0.095, respectively. dMMR was observed in the same 3 patients in both PGC and MGC.

CLDN18.2 expression in PGC and MGC

Intratumoral heterogeneity was observed in 25.5% of PGC and 19.6% of MGC samples ($P < 0.001$) (**Fig. 1**). The recent phase 3 SPOTLIGHT and GLOW studies on zolbetuximab adopted a cutoff level of 75% of tumor cells with a staining intensity of $\geq 2+$ for CLDN18.2 positivity [12,13]. Various scoring methods have been introduced to more comprehensively evaluate the heterogeneous expression of CLDN18.2 [22-25]. The proportions of CLDN18.2 positivity in PGC and MGC according to each scoring method are presented and compared in **Table 2** and **Fig. 2**. The IRS (mean $\pm$ SD) showed a tendency to be higher in PGC (5.06 ± 4.67) compared to MGC (4.39 ± 4.68) (**Fig. 2A**, $P = 0.080$; **Supplementary Fig. 1A**). The H-score (mean $\pm$ SD) was higher in PGC (112.25 ± 115.28) compared to MGC (93.43 ± 110.31) (**Fig. 2B**, $P = 0.045$; **Supplementary Fig. 1B**). The percentage of tumor cells with a staining intensity of $\geq 2+$ (mean $\pm$ SD) was 41.02 ± 41.70 for PGC and 34.36 ± 41.10 for MGC (**Fig. 2C**, $P = 0.048$; **Supplementary Fig. 1C**). When applying cutoff levels of IRS > 8 , H-score > 50 , and $\geq 75\%$, $\geq 40\%$, and $\geq 1\%$ for the percentage of tumor cells with a staining intensity of $\geq 2+$, CLDN18.2 positivity was statistically significantly higher in PGC compared to MGC (all $P < 0.001$, **Fig. 2D**; **Supplementary Fig. 1**).

Concordance of CLDN18.2 expression between PGC and MGC

The comparison of CLDN18.2 positivity between PGC and MGC according to each scoring method is shown in **Table 3** and **Fig. 3**. The concordance levels between PGC and MGC indicated moderate agreement (κ value = 0.47–0.60) (**Table 3**). According to the 5 scoring methods—IRS, H-score with staining intensity of $\geq 1+$, and high, moderate, and

Table 1. Clinicopathological characteristics

Characteristics	Total (n=102)
Age at metastatic disease diagnosis (yr)	
Median (range)	60 (28–87)
Sex	
Male	81 (79.4)
Female	21 (20.6)
Timing of metastatic disease	
Synchronous	73 (71.6)
Metachronous	29 (28.4)
Extent of metastatic disease	
No. of metastatic sites	
1	54 (52.9)
≥2	48 (47.1)
Presence of peritoneal metastasis	
No	60 (58.8)
Yes	42 (41.2)
PGC	
Primary tumor location	
GEJ	6 (5.9)
Gastric	96 (94.1)
Histological grade	
Well/moderately differentiated	42 (41.2)
Poorly differentiated	60 (58.8)
Lauren	
Intestinal	60 (58.8)
Diffuse	33 (32.4)
Mixed	9 (8.8)
Tissue specimen	
Biopsy	44 (43.1)
Resection	58 (56.9)
MGC	
Metastatic tumor location	
Omentum or peritoneum	43 (42.2)
Another GI organ	15 (14.7)
Liver	13 (12.7)
Distant lymph node	12 (11.8)
Lung	5 (4.9)
Ovary	4 (3.9)
Other*	10 (9.8)
Tissue specimen†	
Small	30 (29.4)
Large	72 (70.6)
Duration between the acquisition dates of PGC and MGC tissues‡	
Simultaneous‡	65 (63.7)
Staged	37 (36.3)
Anticancer drug administration before the acquisition of MGC tissue	
No	77 (75.5)
Yes	25 (24.5)

Values are presented as number (%).

PGC = primary gastric cancer; GEJ = gastroesophageal junction; MGC = metastatic gastric cancer; GI = gastrointestinal.

*The other metastatic tumor locations were included the abdominal wall (5 cases), spine (2 cases), adrenal gland (1 case), prostate (1 case), and brain (1 case).

†Small and large specimens were classified based on a criterion of 25 mm².

‡Simultaneous acquisition was defined as obtaining both PGC and MGC tissues within a period of 3 months.

low expression with staining intensity of ≥2+—the rates of CLDN18.2-positive PGC and CLDN18.2-negative MGC were 11.8%, 13.7%, 10.8%, 13.7%, and 13.7%, respectively (**Table 3, Figs. 3, 4A and B**). Conversely, the rates of CLDN18.2-negative PGC and CLDN18.2-positive MGC were 9.8%, 6.9%, 5.9%, 6.9%, and 6.9%, respectively (**Table 3, Figs. 3, 4C and D**). The overall discordance rates were 20.6%, 20.6%, 16.7%, 20.6%, and 20.6% for IRS, H-score

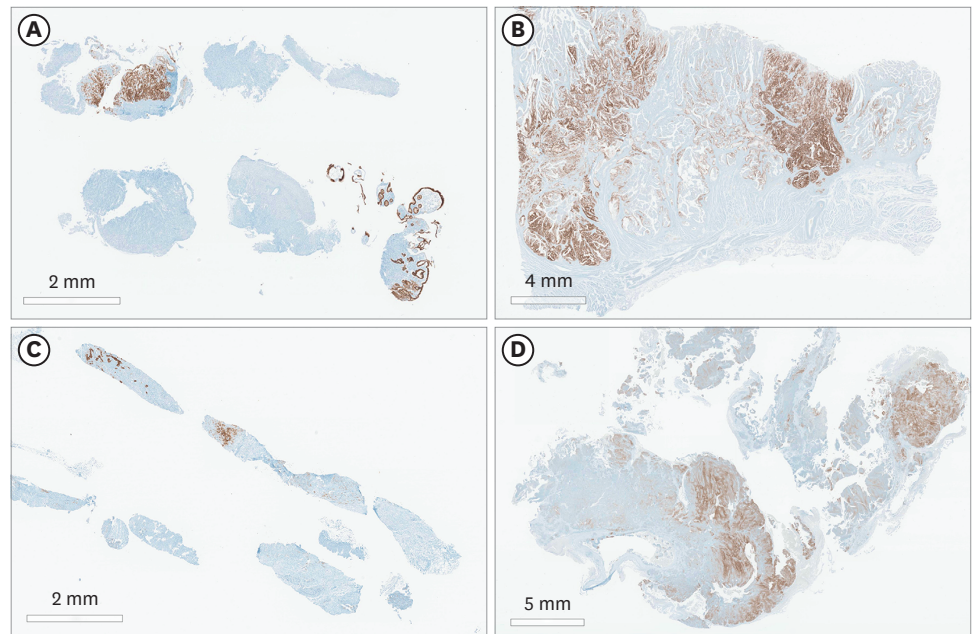


Fig. 1. Representative cases of intratumoral heterogeneous expression patterns of CLDN18.2 in a single tumor specimen. (A) A biopsy specimen of a primary gastric tumor showed strong membranous staining (3+ intensity) in some areas and no CLDN18.2 expression in others (original magnification, $\times 15$; scale bar, 200 μm). (B) A gastrectomy specimen revealed strong CLDN18.2 expression (3+ intensity) in certain areas and very weak membranous staining (1+ intensity) in others (original magnification, $\times 5$; scale bar, 400 μm). (C) A needle biopsy of a supraclavicular lymph node demonstrated areas of strong (3+ intensity), moderate (2+ intensity), and weak (1+ intensity) membranous staining (original magnification, $\times 15$; scale bar, 200 μm). (D) A metastatic brain tumor from gastric cancer exhibited strong membranous staining (3+ intensity) in some areas and weak staining (1+ intensity) in others (original magnification, $\times 5$; scale bar, 500 μm). CLDN18.2 = claudin 18.2.

Table 2. CLDN18.2 expression in the PGC and MGC according to scoring criteria

Scoring criteria	Total (n=102)		
	PGC	MGC	P-value
Score			
IRS*	5.06 $\pm$ 4.67	4.39 $\pm$ 4.68	0.080
H-score†	112.25 $\pm$ 115.28	93.43 $\pm$ 110.31	0.045
% of staining intensity of $\geq 2+$	41.02 $\pm$ 41.70	34.36 $\pm$ 41.10	0.048
CLDN18.2 positivity			
Staining intensity of $\geq 1+$			
IRS* (>8)	28 (27.5)	25 (24.5)	<0.001
H-score† (>50)	54 (52.9)	47 (46.1)	<0.001
Staining intensity of $\geq 2+$			
High ($\geq 75\%$)	33 (32.4)	28 (27.5)	<0.001
Moderate ($\geq 40\%$)	46 (45.1)	39 (38.2)	<0.001
Low ($\geq 1\%$)	66 (64.7)	59 (57.8)	<0.001

Values are presented as mean $\pm$ standard deviation or number of patients (%).

CLDN18.2 = claudin 18.2; PGC = primary gastric cancer; MGC = metastatic gastric cancer; IRS = immunoreactivity score.

*IRS was calculated by multiplying the percentage of stained tumor cells (0: 0%, 1: 1%–25%, 2: 26%–50%, 3: 51%–75%, 4: 76%–100%) by the staining intensity (score 0–3 = no staining to strong staining), resulting in the IRS for each sample (score 0–12).

†H-score was calculated according to the formula: H-score = (0 $\times$ Percentage of Stained Tumor Cells) + (1 $\times$ Percentage of Weakly Stained Tumor Cells) + (2 $\times$ Percentage of Intermediately Stained Tumor Cells) + (3 $\times$ Percentage of Strongly Stained Tumor Cells). The maximum possible H-score was 300 (if all cells of a given tumor sample showed a strong staining: (0 $\times$ 0%) + (1 $\times$ 0%) + (2 $\times$ 0%) + (3 $\times$ 100%) = 300).

with staining intensity $\geq 1+$, and high, moderate, and low expression with staining intensity of $\geq 2+$, respectively (Table 3, Fig. 3).

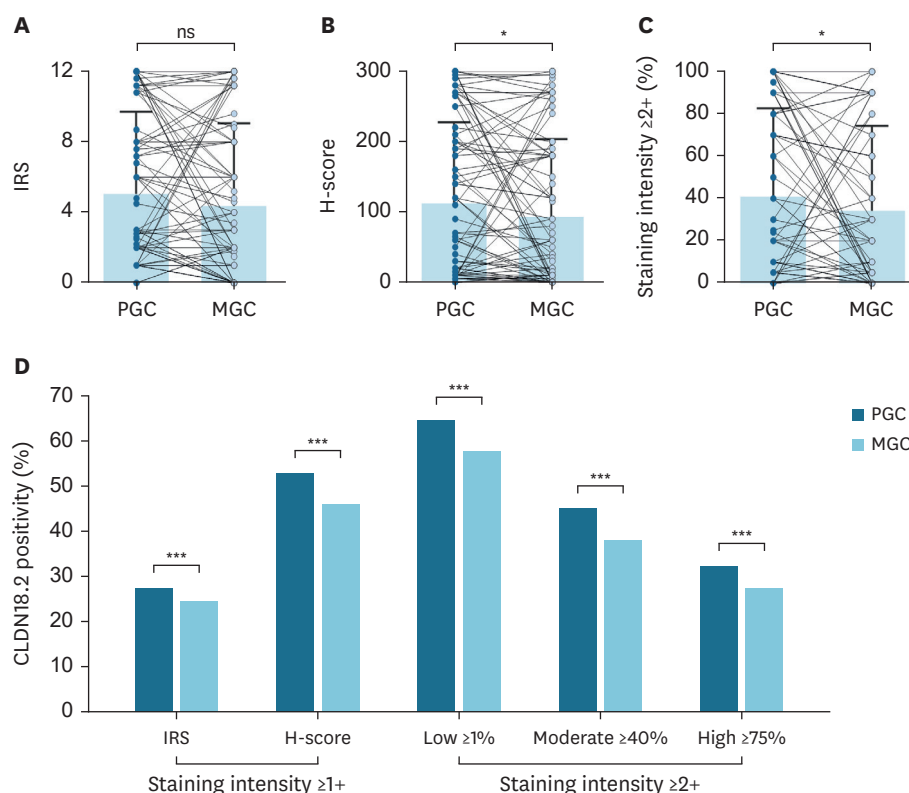


Fig. 2. CLDN18.2 expression in PGC and MGC according to scoring criteria. (A) IRS, (B) H-score, (C) percentage of tumor cells with staining intensity $\geq 2+$, and (D) percentage of CLDN18.2-positivity according to scoring criteria. CLDN18.2 = claudin 18.2; PGC = primary gastric cancer; MGC = metastatic gastric cancer; IRS = immunoreactivity score; ns = not significant. * $P < 0.05$; *** $P < 0.001$.

Table 3. Concordance of CLDN18.2-positivity between PGC and MGC according to immunohistochemistry scoring criteria

CLDN18.2 positivity	No. of patients (%)			κ-value
	PGC	MGC		
		Negative	Positive	
Staining intensity of ≥1+				
Positive IRS (>8)	Negative	65 (63.7)	9 (8.8)	0.47
	Positive	12 (11.8)	16 (15.7)	
Positive H-score (>50)	Negative	41 (40.2)	7 (6.9)	0.59
	Positive	14 (13.7)	40 (39.2)	
Staining intensity of ≥2+				
High (≥75%)	Negative	63 (61.8)	6 (5.9)	0.60
	Positive	11 (10.8)	22 (21.6)	
Moderate (≥40%)	Negative	49 (48.0)	7 (6.9)	0.58
	Positive	14 (13.7)	32 (31.4)	
Low (≥1%)	Negative	29 (28.4)	7 (6.9)	0.57
	Positive	14 (13.7)	52 (51.0)	

CLDN18.2 = claudin 18.2; PGC = primary gastric cancer; MGC = metastatic gastric cancer; IRS = immunoreactivity score.

CLDN18.2 positivity and clinicopathologic characteristics

We analyzed clinical factors that showed at least one statistically significant difference ($P < 0.05$) or marginal differences ($P < 0.1$) based on 5 cutoff points according to the scoring methods. In PGC, the positivity rate was high in cases with synchronous metastasis, the presence of peritoneal metastasis, poorly differentiated histologic grade, and biopsy

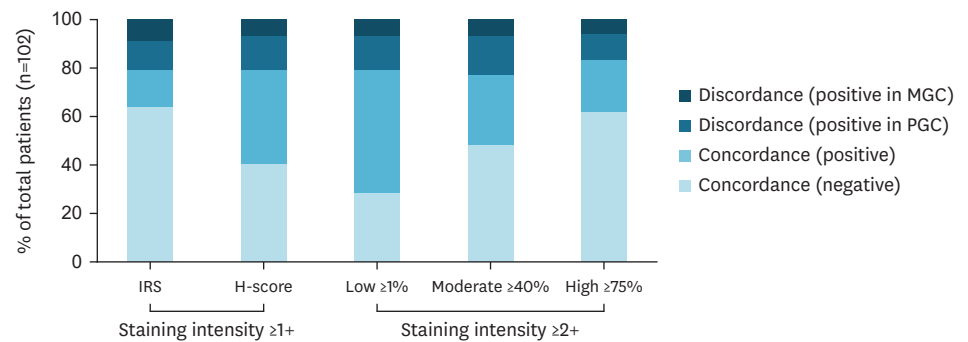


Fig. 3. Concordance of CLDN18.2-positivity between PGC and MGC according to scoring criteria. CLDN18.2 = claudin 18.2; IRS = immunoreactivity score; PGC = primary gastric cancer; MGC = metastatic gastric cancer.

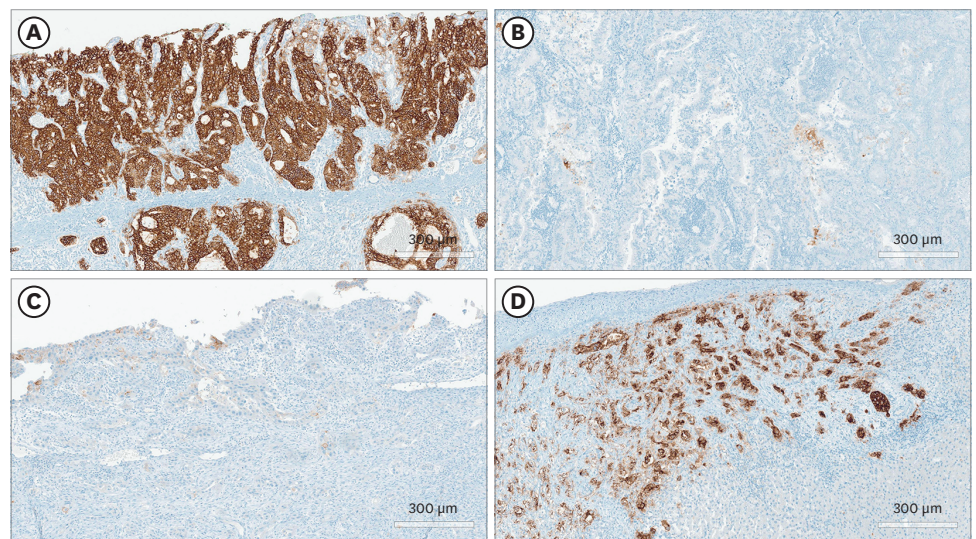


Fig. 4. Representative cases with discordant CLDN18.2 positivity between the PGC and MGC. A gastrectomy specimen from one patient showed strong membranous staining (A; 100% of tumor cells with a staining intensity of 3+; original magnification, $\times 100$; scale bar, 300 μm), while the resected specimen of a metastatic distant lymph node showed CLDN18.2 negativity (B; 0% of stained tumor cells; original magnification, $\times 100$; scale bar, 300 μm). In another patient, a gastrectomy specimen showed very weak membranous staining (C; 5% of tumor cells with a staining intensity of 1+; original magnification, $\times 100$; scale bar, 300 μm), while the resected specimen of a liver metastasis showed a strong CLDN18.2 expression (D; 90% of tumor cells with a staining intensity of 3+; original magnification, $\times 100$; scale bar, 300 μm). CLDN18.2 = claudin 18.2; PGC = primary gastric cancer; MGC = metastatic gastric cancer.

specimens. In MGC, high positivity rates were also noted in cases with synchronous metastasis, the presence of peritoneal metastasis, and metastatic peritoneal tissue specimens (**Fig. 5, Supplementary Tables 2-6**). With regard to the relationship with other predictive biomarkers, among CLDN18.2-positive samples, 9.1% of PGC and 7.1% of MGC samples were HER2-positive; 72.7% of PGC and 78.6% of MGC samples had PD-L1 CPS ≥ 1 ; and 36.7% of PGC and 50.0% of MGC samples had PD-L1 CPS ≥ 5 . Additionally, among the 3 patients with dMMR tumors in both PGC and MGC, one was CLDN18.2-positive (**Supplementary Table 7**).

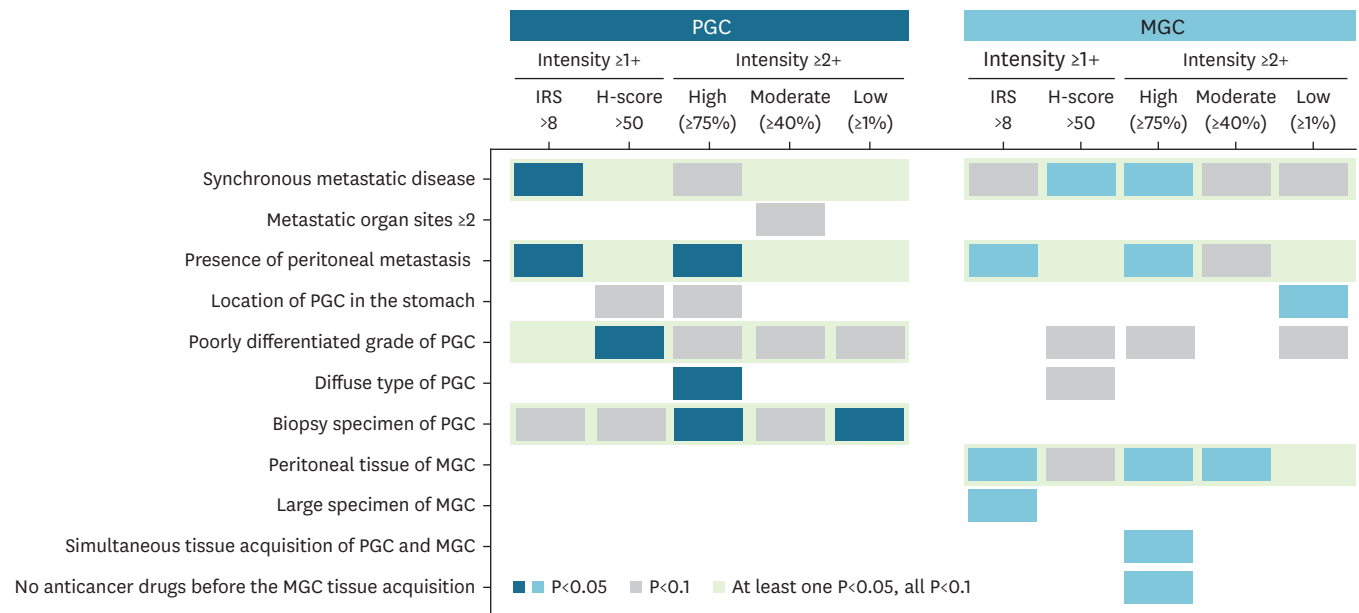


Fig. 5. Clinicopathological characteristics associated with CLDN18.2 positivity according to the scoring criteria. CLDN18.2 = claudin 18.2; PGC = primary gastric cancer; MGC = metastatic gastric cancer; IRS = immunoreactivity score.

DISCUSSION

In this study, we analyzed tumor heterogeneity of CLDN18.2 expression between PGC and MGC using various scoring methods, including the IRS, H-score, and the percentage of tumor cells with moderate-to-strong staining intensity. CLDN18.2 positivity varied from approximately 25% to 65%, depending on the scoring methods and cutoff levels employed. Notably, irrespective of the scoring method or cutoff level, the positivity rate in PGC was consistently higher than in MGC, with discordance in CLDN18.2 positivity between PGC and MGC observed in about 20% of cases.

The HER2 monoclonal antibody, trastuzumab, has proven effective in treating cancers with high HER2 expression, such as certain gastric and breast cancers. More recent HER2-targeting agents, such as HER2 antibody-drug conjugates (ADCs) like trastuzumab deruxtecan, have also shown efficacy in treating cancers with lower levels of HER2 expression [27]. In this context, the phase 3 SPOTLIGHT and GLOW studies demonstrated improved OS in patients with high CLDN18.2-expressing disease ($\geq 75\%$ of tumor cells with moderate-to-strong staining intensity) treated with a combination of zolbetuximab and chemotherapy compared to chemotherapy alone [12,13]. However, newly developed CLDN18.2-targeting agents, including CLDN18.2-specific chimeric antigen receptor (CAR)-T cells and CLDN18.2 ADCs with enhanced affinity and antitumor activity, have shown potential therapeutic efficacy in tumors with low CLDN18.2 expression [28,29]. A recent phase 1 clinical trial reported efficacy of CLDN18.2-specific CAR-T cells in patients with both high and low/medium CLDN18.2-expressing GCs [28]. Similarly, a phase 1 trial of a CLDN18.2-targeting ADC (CMG901) demonstrated objective responses in patients with low CLDN18.2 expression [29]. These findings suggest that newer CLDN18.2-targeting agents may be effective even in tumors with low levels of CLDN18.2 expression. In our study, the CLDN18.2 positivity rate was approximately 30% using the zolbetuximab cutoff of $\geq 75\%$ of tumor cells with moderate-

to-strong staining intensity. In comparison, the rates were about 50% and 60% when defined by an H-score >50 and $\geq 1\%$ of tumor cells with moderate-to-strong staining intensity, respectively, representing criteria for CLDN18.2 low expression.

Tumor heterogeneity, a well-documented characteristic of GC, manifests as intratumoral, intertumoral, and temporal heterogeneity [14]. Previous studies have highlighted substantial variability in key predictive biomarkers in GC, specifically HER2 and PD-L1, which complicates the application of HER2-targeted therapies and immunotherapies [15-21]. The uneven distribution of these markers both within a single tumor and between PGC and MGC can influence treatment responses. To effectively address this heterogeneity, obtaining 6 to 7 biopsy samples or analyzing all available tissue is recommended. Additionally, tumor evolution during treatment necessitates re-biopsy and adaptive strategies. For instance, resistance to HER2-targeted therapies such as trastuzumab can occur as tumor cells lose HER2 amplification or activate alternative signaling pathways [17,18]. Similarly, PD-L1 expression may fluctuate following chemotherapy or immunotherapy, impacting the sensitivity to subsequent treatments [21]. CLDN18.2 expression likely exhibits similar variability; hence, understanding and addressing its heterogeneity is crucial for optimizing treatment efficacy. In the current study, intratumoral heterogeneity, where a single specimen's tumor cells exhibited 2 or more staining intensities, was observed in 25.5% of PGC and 19.6% of MGC samples, with an overall occurrence of approximately 20%. Intertumoral heterogeneity also showed that CLDN18.2 expression was consistently higher in PGC compared to MGC, regardless of the scoring methods or cutoff levels used. These findings align with recent studies, where CLDN18.2 positivity was higher at primary sites than at metastatic sites [30]. Since CLDN18.2 is a tight junction molecule and the loss of adherens junction integrity is generally regarded as the initial step in metastasis formation, it follows that CLDN18.2 expression may be reduced in MGC [10]. The concordance of CLDN18.2 positivity between PGC and MGC was approximately 80%, with the number of cases only positive in PGC being approximately 1.5–2 times higher than those only positive in MGC. This approximately 20% discordance in both intratumoral and intertumoral CLDN18.2 expression between PGC and MGC reflects a level of heterogeneity similar to that observed with HER2 in patients with GC. Consistent with previous studies highlighting CLDN18.2 heterogeneity in resectable GC, intratumoral heterogeneous expression was noted in surgical specimens of PGC [25,31]. However, there is limited information on the intertumoral heterogeneity of CLDN18.2 expression, particularly in metastatic disease. A Japanese study reported an 18% discordance in CLDN18.2 positivity, using a cutoff of $\geq 40\%$ of tumor cells with $\geq 2+$ staining intensity, between PGC and regional lymph node lesions in localized resectable cases [32]. A recent analysis of intertumoral heterogeneity between PGC and MGC in metastatic disease revealed that approximately 25.2% of patients exhibited discordant CLDN18.2 positivity when using a cutoff of $\geq 75\%$ of tumor cells with $\geq 2+$ staining intensity, similar to our findings [30]. As with HER2 evaluation, due to the heterogeneity in CLDN18.2 expression, a comprehensive analysis of an adequate number of available specimens is essential for accurate predictive biomarker analysis in CLDN18.2-targeted therapy. A recent study reported that the sensitivity for detecting CLDN18.2 positivity increased from 2 to 9 biopsies; however, after 6 biopsies, the increase was more limited [33]. Therefore, obtaining 6 biopsies should be considered the standard practice for assessing CLDN18.2 expression. Additionally, since CLDN18.2-targeted systemic therapy aims to treat both PGC and MGC, it is crucial to include tumor tissue from metastatic sites in the analysis to effectively guide treatment decisions where available.

In the present study, several clinicopathologic features were found to be associated with increased CLDN18.2 positivity. In PGC, synchronous metastasis, the presence of peritoneal metastasis, and poorly differentiated histological grade were associated with high CLDN18.2 positivity, a result consistent with those of previous studies [12,13,30,33]. Moreover, the positivity rate was significantly higher in biopsy specimens than in resected specimens in PGC. This higher positivity rate in biopsy specimens may reflect a sampling bias, as biopsies tend to capture superficial regions of the tumor, potentially providing a poor representation of heterogeneity under the $\geq 75\%$ 2+ criterion. In MGC, higher positivity was observed in cases with synchronous metastasis, the presence of peritoneal metastasis, and peritoneal tissue compared to non-peritoneal sites. These results align with prior findings that indicated a high CLDN18.2 positivity rate in Borrmann type 4 or diffuse histologic subtypes, where peritoneal metastasis is common [12,13,30,33]. A recent study analyzing CLDN18.2 expression in metastatic sites also reported a higher positivity rate (44.3%) in peritoneal metastatic tissue compared to other organs [30]. Similarly, our study revealed a 46.5% positivity rate in peritoneal tissue, significantly higher than the 13.6% observed in non-peritoneal tissue. Given the poor prognosis and suboptimal response to current standard systemic therapies such as chemotherapy, HER2-targeted therapy, and immunotherapy in patients with GC and peritoneal metastasis, CLDN18.2-targeted therapy may offer a promising treatment option for these patients. Additionally, we observed potential temporal tumor heterogeneity in CLDN18.2 expression, with lower positivity in cases of metachronous metastasis or MGC tissue when anticancer drugs were administered prior to MGC tissue acquisition, using the current zolbetuximab cutoff level of $\geq 75\%$ of tumor cells with $\geq 2+$ staining intensity. This suggests a possible loss of CLDN18.2 expression over time or after treatments. In a retrospective study of a cohort of Japanese patients including 17 patients with samples collected before and after first-line chemotherapy, cases of negative conversion of CLDN18.2 ($n=3$) were more common than cases of positive conversion ($n=1$) after chemotherapy [34]. Therefore, if the tissue being analyzed is from an older tumor or a post-treatment sample, if feasible, additional tissue should be obtained from either the primary gastric tumor or metastatic sites to reassess CLDN18.2 status before initiating CLDN18.2-targeted therapy.

This study has several limitations that could impact the validity and generalizability of the findings. Firstly, the retrospective, single-center design limits the generalizability and introduces potential biases, such as selection bias. Secondly, the relatively small sample size may reduce the robustness of subgroup analyses. Thirdly, as archived tissue samples were used, the results may not accurately reflect temporal changes in CLDN18.2 expression during disease progression or treatment. Fourthly, the study lacks a direct correlation between CLDN18.2 expression and treatment response.

In conclusion, approximately 20% of the observed tumor heterogeneity was seen in intratumoral heterogeneity within a single tumor and intertumoral heterogeneity between PGC and MGC, consistent with the heterogeneity reported for other biomarkers such as HER2 and PD-L1. Comprehensive tissue sampling and reassessment of CLDN18.2 status, especially before initiating CLDN18.2-targeting therapy, are essential to optimize treatment strategies. Future prospective, multi-center studies with standardized scoring criteria and larger patient cohorts are needed to confirm these findings and assess their clinical significance.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

HER2, PD-L1, and MMR status and concordance in the PGC and MGC

Supplementary Table 2

Clinicopathological characteristics based on CLDN18.2 positivity as defined by IRS

Supplementary Table 3

Clinicopathological characteristics based on CLDN18.2 positivity as defined by H-score

Supplementary Table 4

Clinicopathological characteristics according to the CLDN18.2 positivity, defined as $\geq 75\%$ of tumor cells with moderate to strong intensity

Supplementary Table 5

Clinicopathological characteristics according to the CLDN18.2 positivity, defined as $\geq 40\%$ of tumor cells with moderate to strong intensity

Supplementary Table 6

Clinicopathological characteristics according to the CLDN18.2 positivity, defined as $\geq 1\%$ of tumor cells with moderate to strong intensity

Supplementary Table 7

The relationship between HER2, PD-L1, MMR status, and CLDN18.2 positivity

Supplementary Fig. 1

CLDN18.2 expression in PGC and MGC according to different levels of scoring criteria.

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