Original Article

Expression of Claudin18.2 in metastatic lesions in peritoneum of gastric cancer

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Background: Patients with advanced gastric cancer (GC) with peritoneal involvement have a dismal prognosis. Recent clinical trials have shown that anti-Claudin18.2 (CLDN18.2) antibody (zolbetuximab) enhances survival in patients with GC expressing high levels of CLDN18.2. However, the effectiveness of the zolbetuximab in patients with peritoneal metastases (PMs) remains unclear. In this study, we aimed to evaluate the expression of CLDN18.2 in disseminated lesions to assess the clinical utility of zolbetuximab in the treatment of GC with PM.

Methods: In 42 patients diagnosed with stage IV GC with PM, biopsy samples from the primary tumors and peritoneal metastatic nodules were collected and immunostained using the specific antibody (43-14A, Ventana). The expression of CLDN18.2 was comparatively evaluated based on staining intensity and the proportion of positive cells.

Results: Positive immunoreactivity of CLDN18.2 was observed in 37 (88%) of the primary tumors. Specifically, CLDN18.2 positivity was identified in 26 (62%) or 12 (29%) patients based on moderate to strong membrane staining in at least 40% or 75% of tumor cells, respectively. In comparison, the staining intensity in tumor cells was consistently reduced in PM across all patients. CLDN18.2 expression was absent in PM of 29 (69%) patients, while 3 (7.1%) cases were determined to be CLDN18.2-positive based on a cutoff value of 40% for high staining. This trend was particularly pronounced in cases with undifferentiated type and human epidermal growth factor receptor 2 (HER-2) negative primary tumors.

Conclusions: Although CLDN18.2 expression in PM mirrored that in primary lesions, the levels were generally reduced. When zolbetuximab is used for GC patients with peritoneal involvement, it is preferable to assess the expression of CLDN18.2 in the disseminated lesions.

Keywords: Claudin18.2 (CLDN18.2); gastric cancer (GC); peritoneal metastasis (PM); immunohistochemistry; zolbetuximab

Submitted Sep 26, 2024. Accepted for publication Dec 19, 2024. Published online Feb 26, 2025.
doi: 10.21037/jgo-24-743
View this article at: https://dx.doi.org/10.21037/jgo-24-743

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Introduction

Gastric cancer (GC) continues to be a significant cause of cancer-related mortality globally (1). Although platinum/fluoropyrimidine-based chemotherapy remains the standard first-line treatment for unresectable or metastatic GC, the median overall survival (MST) is approximately 1 year (2,3). The combination of trastuzumab (4) or nivolumab (5,6) with conventional chemotherapy can improve the outcome of some patients. However, only a limited number of patients benefit, highlighting the urgent need to develop additional targeted therapies to improve the outcome of patients with human epidermal growth factor receptor 2 (HER-2) negative or low programmed cell death ligand 1 (PD-L1) expression GC (7,8).

Claudins (CLDNs) are principal components of tight junctions, which physiologically mediate cell-cell adhesion and regulate selective permeability across epithelial cellular sheets (9). Various tissues express different types of claudins, and alterations in their expression and function have been linked to the development of cancer in these tissues (10,11). Claudin18.2 (CLDN18.2) is specifically expressed in gastric epithelial cells and is retained in a significant proportion of primary GCs, with ectopic activation observed in various human cancers (12-14). In normal gastric tissue, monoclonal antibodies to CLDN18.2 cannot access the binding epitope of CLDN18.2. However, in GC, disruptions in cell polarity associated with malignant transformation expose the epitopes of CLDN18.2, enabling the binding of targeted monoclonal antibodies. These distinctive characteristics of CLDN18.2 have attracted significant interest as potential therapeutic targets in GC, and a chimeric immunoglobulin G (IgG) monoclonal antibody, IMAB362 (zolbetuximab), which specifically binds to the first extracellular loop of CLDN18.2 molecule was developed for the treatment of the patients with unresectable advanced gastric or gastroesophageal junction (GEJ) adenocarcinoma (15).

Clinical trials with this antibody were promptly initiated, showing that zolbetuximab improves outcomes in patients with gastric or GEJ cancer who exhibit moderate-to-strong CLDN18.2 expression in at least 40% or 50% of tumor cells (16,17). In the phase 3 SPOTLIGHT trial, it has been shown that the inclusion of zolbetuximab with mFOLFOX6 chemotherapy significantly reduces the risk of disease progression or death in patients with CLDN18.2-positive (defined as ≥75% of tumor cells showing moderate-to-strong membranous CLDN18 staining), HER-2-negative, locally advanced unresectable or metastatic gastric or GEJ adenocarcinoma (18). Another phase 3 GLOW trial has revealed that the combination therapy of zolbetuximab plus CAPOX significantly extends both progression-free survival (PFS) and overall survival (OS) compared to the placebo plus CAPOX group in the same patient demographic (19). Consequently, zolbetuximab combined with chemotherapy emerges as a promising new first-line treatment for these patients, and now approved in Japan, Europe and US.

Although peritoneal metastases (PMs) represent the most life-threatening form of recurrence in GC, the prognosis for these patients remains exceedingly poor, necessitating the development of new therapeutic strategies (20-22). Given that CLDN18.2 expression is relatively high in undifferentiated tumors, which frequently develop PM, the potential effects of zolbetuximab for patients with PM are of significant clinical interest. However, the existing randomized clinical trials do not provide conclusive data on its efficacy for patients with PM. It is hypothesized that CLDN18.2 expression levels may undergo significant alterations during the metastatic process. The aim of this study is to assess and compare the expression levels of CLDN18.2 in biopsy samples from metastatic peritoneal nodules and their corresponding primary tumors using immunohistochemical staining. We present this article in

Highlight box

Key findings

- The expression levels of Claudin18.2 (CLDN18.2) in peritoneal metastases are generally reduced compared to those observed in corresponding primary lesions.

What is known and what is new?

- Recent clinical trials have demonstrated that the anti-CLDN18.2 antibody, zolbetuximab, improves survival outcomes in patients with gastric cancer (GC) who exhibit high levels of CLDN18.2 expression. However, its clinical efficacy in patients with peritoneal metastasis (PM) remains uncertain.
- While the expression pattern of CLDN18.2 in PMs partially resembles that of primary tumors, a notable reduction in expression is observed in a cohort of 42 GC patients, particularly those with undifferentiated primary tumors.

What is the implication, and what should change now?

- To better predict the therapeutic efficacy of zolbetuximab in GC patients with peritoneal involvement, it is preferable to evaluate CLDN18.2 expression not only in primary but also in metastatic lesions. This approach may improve patient stratification and treatment outcomes.

accordance with the REMARK reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-743/rc).

Methods

Patients and tissue specimens

This retrospective observational study was conducted using a single patient cohort to investigate the expression of CLDN18.2 in metastatic lesions and its relationship with expression at the corresponding primary sites. From May 2014 to May 2022, a total of 104 patients were diagnosed with stage IV GC with accompanying PM and underwent surgical and chemotherapeutic treatment at the Department of Surgery, Jichi Medical University Hospital. Among them, biopsy specimens from peritoneal metastatic nodules were collected from 42 of these patients during laparoscopic examinations or open surgeries. In those patients, the expression of CLDN18.2 in metastatic nodules was evaluated along with biopsy samples from the primary tumors. Demographic data including gender, age, date of operation, and clinical and pathological findings—such as histological subtype, HER-2 expression, peritoneal cytology, ascites, and surgical observations including the Peritoneal Cancer Index (PCI) score and P-classification—were retrieved from an electronic database after the acquisition of written informed consent and evaluated for the possible association with the CLDN18.2 expression. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Jichi University Hospital (approval No. Clinic A21-064) and informed consent was taken from all the patients.

MAbs and reagents

Monoclonal antibody to CLDN18.2 (790-7027, clone 43-14A, prediluted) was acquired from Ventana (Mannheim, Germany) and stored at –20 ℃ before usage. Although 43-14A recognizes CLDN18 and is not specific to the CLDN18.2 isoform, it has been used for biomarker testing in previous clinical trials. Additionally, the Signal Enhancer HIKARI for Immunostain Solution B (02375-34), antibody dilution buffer, and blocking solution One-Histo (06349-64) were sourced from Nacalai Tesque and stored at 4 ℃ (Kyoto, Japan).

Immunohistochemistry and histologic evaluation of stained samples

All specimens were fixed in formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. IHC was conducted according to the standard protocol of CLDN18.2 staining (23) using the DAKO REAL™ Envision™ Detection system (Glostrup, Denmark). The process involved deparaffinization in xylene, followed by rehydration through a graded ethanol series, and a subsequent wash in distilled water for 10 minutes. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide for 30 minutes. Antigen retrieval was achieved by microwaving the sections in 10 mmol/L sodium citric buffer (pH 6.0) for 10 minutes. After a phosphate-buffered saline (PBS) wash, a non-specific staining blocking agent (Blocking One Histo) was applied for 10 minutes to prevent non-specific binding. The sections were then incubated with the primary antibody for 30 minutes at room temperature. Following extensive washing with PBS, the sections were treated with the Histofine Simple Stain PO(M) kit (Nichirei) appropriate for either rabbit or mouse, for 30 minutes at room temperature. The sections were then washed again with PBS and the primary antibody binding was visualized using the DAKO Envision kit according to the manufacturer's instructions. Because the antibody used recognizes CLDN18 and is not specific for the CLDN18.2 isoform, we will assess CLDN18 expression under the assumption that it is mainly CLDN18.2 that is expressed in the stomach and gastric tumors. To determine CLDN18 expression status, samples were evaluated using a two-component scoring method that considered both the intensity of membranous staining (including complete basolateral and lateral membrane staining) and the percentage of tumor cells stained at different intensities relative to all tumor cells present in the sample. Immunohistochemical evaluations were independently and blindly performed by three investigators, A.S., R.K. and J.K. A consensus between these investigators was necessary to finalize the decision.

As shown in *Figure 1*, the staining intensity was categorized as 0 (no reactivity), 1+ (weak reactivity), 2+ (moderate reactivity), and 3+ (strong reactivity). Specific staining with a minimum intensity of 1+ in any fraction of the tumor cells classified the samples as 'any positivity' (24). The frequency of cells exhibiting 2+ or 3+ staining was quantified, and the prevalence of CLDN18.2-positive

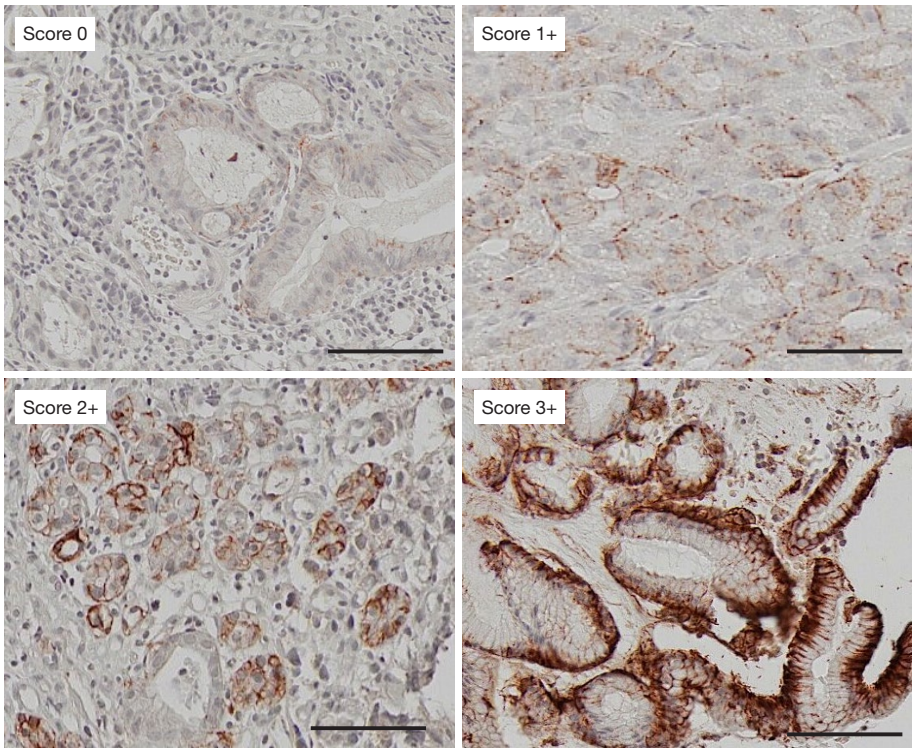

Figure 1 The scoring of staining intensity for CLDN18.2 was conducted on primary gastric cancer tissues using the anti-Claudin18.2 monoclonal antibody (43-14A, Ventana). Representative micrographs illustrate cases with varying staining intensities, categorized as score 0 (no reactivity), score 1+ (weak staining), score 2+ (moderate staining), and score 3+ (strong staining). Scale bar: 50 micrometers (CLDN18.2 immunohistochemistry, 200×). CLDN18.2, Claudin18.2.

samples was assessed using two threshold values: ≥40% (17) and ≥75% (18,19), which were chosen based on outcomes from recent clinical trials.

Statistical analysis

Statistical analyses were performed by using Prism 9 (GraphPad Software, San Diego, CA, USA). Differences in the expression of CLDN18.2 between groups were assessed using the Wilcoxon signed-rank test or the Chi-squared test for continuous or nominal valuables, respectively. A significance threshold was established at $P<0.05$.

Results

CLDN18.2 expression in primary gastric tumor in patients with PMs

The expression of CLDN18.2 in biopsy samples of primary gastric tumors from patients with PM was evaluated in a cohort of 42 patients. As presented in *Table 1*, positive CLDN18.2 staining signal was detected in 37 patients (88%), with 26 patients (62%) showing moderate to strong membrane staining in ≥40% of tumor cells, and 12 patients (29%) showing such staining in ≥75% of tumor cells. The expression of CLDN18.2 tended to be higher in tumors of younger patients and those with undifferentiated histology, although this difference did not reach statistical significance. However, CLDN18.2 expression did not correlate with any factors indicative of PM severity, such as the PCI score, P classification, cytology status, and the presence of ascites.

CLDN18.2 expression in corresponding PMs

In the cohort, the expression of CLDN18.2 in disseminated peritoneal nodules was evaluated using the same staining protocol as for primary tumors. As illustrated in *Figure 2A*, the staining intensity of CLDN18.2 was notably reduced in peritoneal nodules. In all patients with primary tumors exhibiting staining intensities of 0–3, the intensity of

Table 1 Claudin18.2 expression in primary gastric tumor (n=42)

Factors	Staining intensity			Staining intensity ≥2+ in ≥40% tumor cells			Staining intensity ≥2+ in ≥75% tumor cells		
	No staining [5 (12%)]	Any positivity [37 (88%)]	P value	Negative [16 (38%)]	Positive [26 (62%)]	P value	Negative [30 (71%)]	Positive [12 (29%)]	P value
Age (years)	71 [60–82]	65 [40–83]	0.09	69 [41–82]	59 [40–83]	0.04	67 [40–83]	59 [40–81]	0.34
Gender			0.48			0.82			0.46
Male (n=28)	4 [14]	24 [86]		11 [39]	17 [61]		19 [68]	9 [32]	
Female (n=14)	1 [7]	13 [93]		5 [36]	9 [64]		11 [79]	3 [21]	
Location			0.37			0.29			0.85
GEJ (n=3)	0 [0]	3 [100]		2 [67]	1 [33]		2 [67]	1 [33]	
Gastric (n=39)	5 [13]	34 [87]		25 [64]	14 [36]		28 [72]	11 [28]	
Macroscopic type			0.49			0.82			>0.99
Non-type 4 (n=14)	1 [7]	13 [93]		5 [36]	9 [64]		10 [71]	4 [29]	
Type 4 (n=28)	4 [14]	24 [86]		11 [39]	17 [61]		20 [71]	8 [29]	
Histological type			0.32			0.23			0.16
Differentiated (n=9)	2 [22]	7 [78]		5 [56]	4 [44]		8 [89]	1 [11]	
Undifferentiated (n=33)	3 [9]	30 [91]		11 [33]	22 [67]		22 [67]	11 [33]	
P Classification			0.93			0.44			0.73
P1a (n=17)	2 [12]	15 [88]		5 [29]	12 [71]		11 [65]	6 [35]	
P1b, P1c (n=25)	3 [12]	22 [88]	0.83	11 [44]	14 [56]	0.90	19 [76]	6 [24]	0.25
PCI score	19 [2–30]	16 [1–39]		19 [1–39]	11 [2–39]		21 [1–39]	6 [2–39]	
Cytology			0.57			0.76			0.67
CY0 (n=12)	2 [17]	10 [83]		5 [42]	7 [58]		8 [67]	4 [33]	
CY1 (n=30)	3 [10]	27 [90]		11 [37]	19 [63]		22 [73]	8 [27]	
Ascites			0.72			0.81			0.38
Absent (n=22)	3 [14]	19 [86]		8 [36]	14 [64]		17 [77]	5 [23]	
Present (n=20)	2 [10]	18 [90]		8 [40]	12 [60]		13 [65]	7 [35]	
HER-2 expression			0.243			0.93			0.12
Negative (n=37)	5 [14]	32 [86]		14 [38]	23 [62]		28 [76]	9 [24]	
Positive (n=5)	0 [0]	5 [100]		2 [40]	3 [60]		2 [40]	3 [60]	
PD-L1 expression			0.55			0.71			0.21
Negative (CPS <1) (n=27)	2 [7]	25 [93]		5 [19]	22 [81]		17 [63]	10 [27]	
Positive (CPS >1) (n=15)	3 [20]	12 [80]		11 [73]	4 [27]		13 [87]	2 [13]	

Age and PCI score are shown as median [Min–Max]. The other data are presented as n [%]. GEJ, gastroesophageal junction; PCI, Peritoneal Cancer Index; Ascites: absent or present was determined by CT image; CY1, peritoneal lavage cytology positive; CY0, peritoneal lavage cytology positive. P classification: P1a, peritoneal metastasis is limited to the stomach, greater omentum, lesser omentum, anterior lobe of the transverse colon mesentery, pancreatic capsule, and spleen; P1b, metastasis is found in the peritoneum of the upper abdomen; P1c, metastasis is found in the peritoneum of the middle and lower abdomen; CPS, combined positive score for PD-L1 expression.

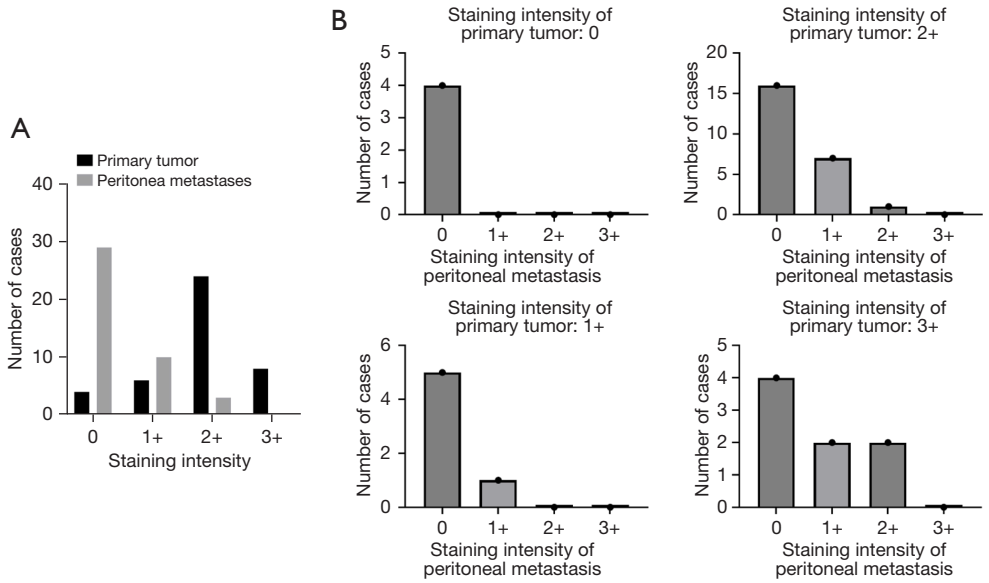

Figure 2 Comparison of staining intensity of CLDN18.2 in primary tumors and metastatic nodules in the peritoneum (PM) from the same patients is presented. The y-axis represents the total number of cases (A) as well as the number of cases in each group categorized by the staining intensity of CLDN18.2 in the primary tumor, ranging from 0 to 3+ (B). CLDN18.2, Claudin18.2; PM, peritoneal metastasis.

Table 2 Comparison of Claudin18.2 expression in primary tumor and peritoneal metastasis (n=42)

Factor	Staining intensity				Any positive		Intensity ≥2+ in ≥40% tumor cells		Intensity ≥2+ in ≥75% tumor cells	
	0	1+	2+	3+	n [%]	P value	n [%]	P value	n [%]	P value
All samples (n=42)						<0.001		<0.001		<0.001
Primary tumor	5	5	24	8	37 [88]		26 [62]		12 [29]	
Peritoneal metastasis	29	10	3	0	13 [31]		3 [7]		0 [0]	
Differentiated type (n=9)						0.64		0.314		0.23
Primary tumor	2	1	5	1	7 [77]		4 [44]		1 [11]	
Peritoneal metastasis	5	2	2	0	4 [44]		2 [22]		0 [0]	
Undifferentiated type (n=33)						<0.001		<0.001		<0.001
Primary tumor	3	4	19	7	30 [91]		22 [67]		11 [33]	
Peritoneal metastasis	24	8	1	0	9 [23]		1 [3]		0 [0]	
HER-2 expression positive (n=5)						>0.99		>0.99		>0.99
Primary tumor	0	2	1	2	5 [100]		2 [40]		1 [25]	
Peritoneal metastasis	1	3	1	0	4 [80]		2 [40]		0 [0]	
HER-2 expression negative (n=37)						<0.001		<0.001		<0.001
Primary tumor	5	3	23	6	32 [89]		24 [73]		11 [30]	
Peritoneal metastasis	28	7	2	0	9 [24]		1 [3]		0 [0]	

HER-2 positive status was defined as IHC 3+ or IHC 2+ with + by ISH. HER-2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization.

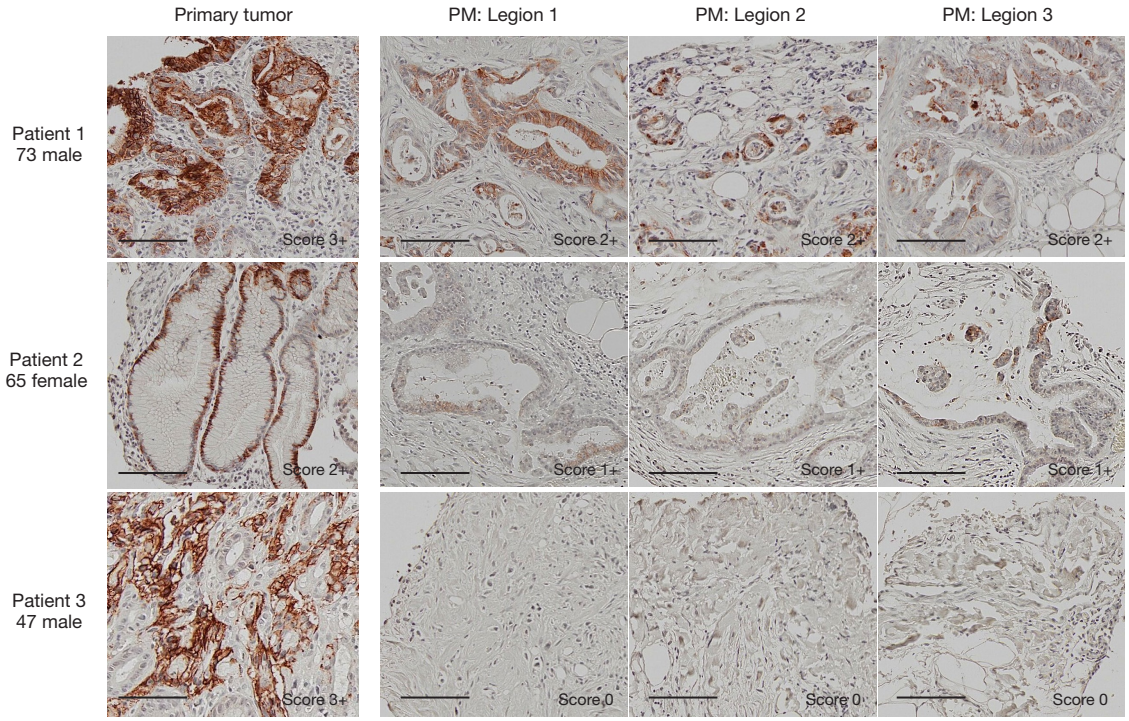

Figure 3 Expression of CLDN18.2 in primary tumors and 3 metastatic nodules in the peritoneum (PM) is demonstrated in 3 representative cases. The bar represents a length of 50 micrometers (CLDN18.2 immunohistochemistry, 200×). CLDN18.2, Claudin18.2; PM, peritoneal metastasis.

CLDN18.2 expression was consistently lower in PM (*Figure 2B*). Furthermore, the frequency of tumor cells with moderate to strong staining in PM was significantly reduced compared to that observed in the corresponding primary tumors. As summarized in *Table 2*, a positive CLDN18.2 signal was detected in 13 patients (31%), with only 3 (7.1%) lesions defined as positive based on moderate to strong staining in ≥40% of tumor cells, a significantly lower prevalence than in primary tumors (P<0.001). No lesions demonstrated high staining levels at the ≥75% threshold (P<0.001).

Among the 42 cases, multiple peritoneal lesions were sampled in 5 patients. *Figure 3* highlights the expression of CLDN18.2 in three disseminated lesions from 3 representative patients, demonstrating a high degree of consistency in staining patterns. In 2 other patients, two disseminated lesions were obtained, both of which entirely lacked CLDN18.2 expression (Figure S1).

As demonstrated in *Table 2*, among the 33 patients with undifferentiated primary tumors, 9 (27%) displayed some positivity for CLDN18.2 expression in PM, with only 1 patient (3.0%) meeting the criteria for CLDN18.2 positivity at the 40% cut-off. This rate was markedly lower than that observed in the corresponding primary tumors (67%, P<0.001). In contrast, among the 9 patients with differentiated primary tumors, 4 (44%) exhibited some positivity, with 2 (22%) reaching the CLDN18.2-positive threshold (40% cut-off) in PM. These rates did not differ statistically significantly from those observed in primary tumors. Furthermore, the reduction of CLDN18.2 expression in PM was more pronounced in 37 patients with HER-2-negative primary tumors, whereas this trend was not detected in the 5 patients with HER-2-positive tumors.

Discussion

Recent randomized clinical trials have verified the clinical efficacy of an anti-CLDN mAb, zolbetuximab, in patients with gastric or GEJ cancers exhibiting moderate-to-high CLDN18.2 expression in 40% to 75% of tumor cells within the primary tumor (17-19). However, those studies have focused on patients with high expression of CLDN18.2 in gastric tumor, and the specific patient populations that would benefit most from zolbetuximab treatment remain to

be determined. A meta-analysis indicated that the survival benefits of zolbetuximab are not significant in patients with three or more metastatic sites (25). However, the efficacy of zolbetuximab for patients with PM is still uncertain.

The therapeutic response to this anti-CLDN18.2 antibody primarily depends on the expression levels of CLDN18.2 in the tumor cells of the targeted lesions, as the antibody mediates effector functions against tumor cells through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (15,26). In light of this, we employed the monoclonal antibody 43-14A from Ventana and examined the CLDN18.2 expression in metastatic lesions in the peritoneum. Although 43-14A antibody recognizes both isoforms of CLDN18.1 and CLDN18.2, staining performed with this mAb has been reported as CLDN18.2 expression in most of the previous studies, based on the assumption that CLDN18 in GC predominantly corresponds to the CLDN18.2 isoform.

In primary tumors, immunoreactivity was detected in 88% of patients and defined as CLDN18.2-positive in 62% (≥40% cut off value) and 29% (≥75% cut off value), with a tendency for expression to persist in undifferentiated tumors. This is mostly consistent with previous findings (13,24,27-30). In our series, CLDN18.2 expression in PM mirrored that in primary lesions in all cases. Within the same patients, CLDN18.2 expression demonstrated a high degree of consistency across multiple metastatic nodules within the peritoneum. However, the expression levels of CLDN18.2 in PM were consistently lower compared to those in primary gastric tumors across all patients. This trend was particularly pronounced in cases where the primary tumors exhibited undifferentiated histology and lacked HER-2 expression.

Previous studies have suggested that CLDN18.2 expression in primary tumors is largely maintained in metastatic lesions such as regional lymph nodes and ovary (24,29,31). During the preparation of this paper, two studies have additionally reported that CLDN18.2 expression shows a high concordance rate between primary and metastatic lesions in peritoneum (32,33). However, in the study by Ogawa *et al.* (32), CLDN18.2 expression in PM is significantly reduced compared to primary tumors, although the degree of downregulation is not as marked as in the present study. This discrepancy is likely attributable to differences in the evaluation of positivity, but both studies consistently suggest the reduced expression of CLDN18.2 in PM. Based on these findings, it is hypothesized that CLDN18.2 may be specifically downregulated during the metastatic process to the peritoneal cavity.

Since this retrospective study was conducted at a single institution with a small sample size, it has inherent limitations and further research is required to draw definitive conclusions. However, the finding that CLDN18.2 is scarcely expressed in peritoneal lesions in many patients suggests a possibility that zolbetuximab treatment may be less effective in some patients with peritoneal involvement. Given the increasing social issue of financial toxicity associated with expensive molecular targeted drugs, our findings suggest that it is preferable to assess the expression of CLDN18.2 not only in primary tumors but also in metastatic lesions to select good responders to zolbetuximab treatment. For patients with low levels of CLDN18.2 expression in PM, improving drug delivery efficiency could potentially enhance the therapeutic outcomes. Intraperitoneal (IP) administration might be considered as a viable option, as IP administration of high-molecular-weight antibody preparations has been shown to result in significantly higher accumulation in peritoneal nodules than systemic administration (34). Another strategy involves conjugating cytotoxic agents with anti-CLDN18.2 monoclonal antibodies to enhance antitumor efficacy. These innovative therapeutics have been already evaluated in preclinical studies (35,36) and may demonstrate potential for improved effectiveness in patients with PM exhibiting low CLDN18.2 expression.

Conclusions

Although CLDN18.2 expression in PM reflects that in primary tumors, the levels may be notably downregulated during the metastatic process to peritoneum. Evaluation of CLDN18.2 expression in metastatic lesions is preferable to predict the clinical efficacy of zolbetuximab in patients with PM of GC.

Acknowledgments

We appreciate Prof. T. Niki for his proper advice about the evaluation of immunohistochemistry. We also thank Ms. N. Nishiaki, J. Shinohara, H. Hatakeyama and I. Nieda for excellent technical and clerical contribution.

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-743/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-743/dss

Peer Review File: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-743/prf

Funding: This study was supported by the Japan Society for the Promotion of Science (Nos. 23H02985, 24K11874 and 23K15503).

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-743/coif). H.Y. reports payment from Astellas Pharma Inc. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Jichi University Hospital (approval No. Clinic A21-064) and informed consent was taken from all the patients.

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Cite this article as: Saito A, Ohzawa H, Kawashima R, Matsumoto S, Kurashina K, Saito S, Miyato H, Hosoya Y, Sata N, Kitayama J, Yamaguchi H. Expression of Claudin18.2 in metastatic lesions in peritoneum of gastric cancer. J Gastrointest Oncol 2025;16(1):67-76. doi: 10.21037/jgo-24-743