

Original Article



Clinical Significance of CLDN18.2 Expression in Metastatic Diffuse-Type Gastric Cancer

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ABSTRACT

Purpose: Isoform 2 of tight junction protein claudin-18 (CLDN18.2) is a potential target for gastric cancer treatment. A treatment targeting CLDN18.2 has shown promising results in gastric cancer. We investigated the clinical significance of CLDN18.2 and other cell-adherens junction molecules (Rho GTPase-activating protein [RhoGAP] and E-cadherin) in metastatic diffuse-type gastric cancer (mDGC).

Materials and Methods: We evaluated CLDN18.2, RhoGAP, and E-cadherin expression using two-plex immunofluorescence and quantitative data analysis of H-scores of 77 consecutive mDGC patients who received first-line platinum-based chemotherapy between March 2015 and February 2017.

Results: CLDN18.2 and E-cadherin expression was significantly lower in patients with peritoneal metastasis (PM) than those without PM at the time of diagnosis ($P=0.010$ and 0.013 , respectively), whereas it was significantly higher in patients who never developed PM from diagnosis to death than in those who did ($P=0.001$ and 0.003 , respectively). Meanwhile, CLDN18.2 and E-cadherin expression levels were significantly higher in patients with bone metastasis than in those without bone metastasis ($P=0.010$ and 0.001 , respectively). Moreover, we identified a positive correlation between the expression of CLDN18.2 and E-cadherin ($P<0.001$), RhoGAP and CLDN18.2 ($P=0.004$), and RhoGAP and E-cadherin ($P=0.001$). Conversely, CLDN18.2, RhoGAP, and E-cadherin expression was not associated with chemotherapy response and survival.

Conclusions: CLDN18.2 expression was reduced in patients with PM but significantly intact

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Author Contributions

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Conflict of Interest

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

in those with bone metastasis. Furthermore, CLDN18.2 expression was positively correlated with other adherens junction molecules, which is clinically associated with mDGC and PM pathogenesis.

Keywords: Gastric cancer; Claudin; Cadherins; Chemotherapy

INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer-related death [1]. In South Korea, GC is the most common cancer in men and the fourth most frequently diagnosed cancer in women [2,3]. It has been categorized into two main histological subtypes based on Lauren's criteria: intestinal and diffuse [2,4]. Diffuse-type GC (DGC) is associated with a worse prognosis, occurrence at an earlier age, and the highest recurrence frequency [5]. DGCs are poorly differentiated histologically; they are characterized by a lack of intercellular adhesion molecules and disrupted tight junction molecules, and often have poorly cohesive and scattered signet-ring cell morphology, which predisposes to invasion and growth patterns [6]. DGC is more frequently associated with peritoneal metastasis (PM) than intestinal-type GC [7]. PM is the most frequent metastatic pattern of GC, although Paget's 'seed and soil' hypothesis has been considered the fundamental theory of metastasis; however, the mechanisms underlying peritoneal dissemination are yet to be elucidated [8]. According to this hypothesis, detachment of cancer cells from the primary tumor is the first step in peritoneal dissemination; thus, cancer cell detachment is considered important in PM [9]. The loss of cell-cell adhesion systems may result in the dissemination of single carcinoma cells from the primary tumor sites and trigger remodeling of the actin cytoskeleton, leading to the development of a mesenchymal phenotype and the dispersal of carcinoma cells [10]. The importance of the loss of cell-cell adhesion molecules such as E-cadherin in the context of DGC initiation and PM formation has been evaluated in several studies [11,12].

Claudin-18 (CLDN18), a member of the claudin family, is a component of tight junctions that regulates paracellular barrier functions. DGC is associated with an inter-chromosomal translocation between CLDN18 and *ARHGAP* (the gene encoding Rho GTPase-activating protein [RhoGAP], which contributes to the organization of actin and microtubule cytoskeletons), resulting in the generation of a RhoGAP domain-containing fusion protein with impaired function of CLDN18 and RhoGAP [13-17]. A previous in vitro study reported that CLDN18-ARHGAP26 fusion-positive cell lines showed impaired barrier properties, reduced cell-cell adhesion, and augmented invasiveness [18]. In younger patients with DGCs, this fusion status can act as a predictor of metastasis, such as peritoneal dissemination [15]. Moreover, gastric CLDN18 expression is related to not only size and invasiveness but also to potential metastatic ability and patient outcome [19]. Additionally, CLDN18-ARHGAP26/6 or the RhoGAP domain-containing fusion protein is associated with poor prognosis of DGC [14,16]. Yang et al. [14] performed western blot analysis and migration assay to confirm the functional effects of the CLDN18-ARHGAP fusion gene and revealed its poor prognostic role; its migration capacity was more enhanced compared to that of the control vector.

The expression of isoform 2 of CLDN18 (CLDN18.2) has been limited to differentiated epithelial cells in the gastric mucosa and primary gastric malignancies, emphasizing its potential as a candidate for targeted therapy [20]. Recently, a treatment strategy targeting CLDN18.2 has shown promising results in inoperable or recurrent GC patients [21,22].

Hereby, in this study, we aimed to investigate the clinical significance of CLDN18.2 and other adherens junction molecules (RhoGAP and E-cadherin) in metastatic DGC (mDGC).

MATERIALS AND METHODS

Study population and design

This study included a total of 77 patients with mDGC who were treated with first-line platinum-based chemotherapy between March 2015 and February 2017 at Seoul St. Mary's Hospital, the Catholic University of Korea. We performed surgical biopsy and gastric endoscopic biopsy of the primary gastric tumor lesion. Computed tomography (CT) and bone scans were performed for staging. If needed, additional imaging scans, such as magnetic resonance imaging and positron emission tomography/CT, were performed. Patients were subsequently evaluated for their response to chemotherapy after 6±2 weeks based on their radiological imaging results. Radiological changes were evaluated using the Response Evaluation Criteria in Solid Tumors version 1.1 [23]. The tumor location was classified as upper/middle/lower third. An immunohistochemical score of 3+ or 2+ was considered to determine human epidermal growth factor receptor 2 positivity by fluorescence in situ hybridization analysis. For patients with unclear diagnosis of PM using radiological examination, diagnostic laparoscopy was performed. If the patient showed definite PM on CT or other imaging modalities, diagnostic laparoscopy was not performed.

Ethical statement

This study was approved by the Institutional Review Board of Seoul St. Mary's Hospital [KC18SESI0521]. It conforms to the provisions of the Helsinki Declaration as revised in 2013. Written informed consent was obtained from all patients.

Assessment of CLDN18.2, RhoGAP, and E-cadherin expression using two-plex immunofluorescence

Sections of GC specimens (4 µm) were cut from formalin-fixed, paraffin-embedded blocks. Samples were heated for at least 1 hour in a dry oven at 60°C, dewaxed using xylene, dehydrated by sequential incubation in 100%, 95%, and 70% ethanol, and treated with hydrogen peroxide. Antigens were retrieved by microwave treatment for 15 minutes in citrate buffer (pH 6.0). Slides were washed twice with 1x Tris-buffered saline with Tween 20®, and blocking was performed using an antibody diluent (#ARD1001EA, PerkinElmer, Waltham, MA, USA) for 10 min. Samples were incubated with primary antibodies against CLDN18.2 (#700178, Invitrogen, Carlsbad, CA, USA; dilution 1:500), RhoGAP (#ab32328, Abcam, Cambridge, UK; dilution 1:1,000), and E-cadherin (#ab76055, Abcam; dilution 1:400) for 30 minutes in a humidified chamber at 23°C–25.5°C, followed by detection using Polymer HRP Ms+Rb (ARH1001EA, PerkinElmer) for 10 minutes. Visualization of CLDN18.2, RhoGAP, and E-cadherin was accomplished by incubation with Opal 690 TSA Plus (dilution 1:150) for 10 minutes after which the samples immobilized on the slides were immersed in citrate buffer (pH 6.0) and heated by microwave treatment. The nuclei were subsequently visualized by detecting nuclear spectral elements using DAPI, and the sections were mounted using HIGHDEF® immunohistochemical fluoromount (#ADI-950-260-0025, Enzo Life Sciences Inc., Seoul, Korea).

Image acquisition and quantitative data analysis

Slides were scanned using the PerkinElmer Vectra 3.0 Automated Quantitative Pathology Imaging System (PerkinElmer), and images were analyzed using the inForm software and TIBCO Spotfire (PerkinElmer). To acquire reliable unmixed images, representative slides exposed to each emission spectrum and unstained tissue slides were used. Each of the individually stained sections (E-cadherin, RhoGAP, and CLDN18.2; Opal690) were used to establish the spectral library of fluorophores required for multispectral analysis. This spectral library served as a reference for target quantitation; the intensity of each fluorescent target was extracted from the multispectral data by linear unmixing. Each cell was identified using DAPI. Our two pathologists selected areas of interest in the primary tumor section. The total number of CLDN18.2-, RhoGAP-, and E-cadherin-positive cells was considered the total number of cell infiltrations in the tissue. The fluorescence intensity of CLDN18.2, RhoGAP, and E-cadherin in the tissue samples was determined based on the H-score: strong (3+), intermediate (2+), weak (1+), or no (0) membranous staining. The H-score of each tissue sample was calculated using the following formula: $H\text{-score} = [3 \times \text{the percentage of strongly stained cells}] + [2 \times \text{the percentage of moderately stained cells}] + [\text{the percentage of weakly stained cells}]$. H-scores ranged from 0 to 300 [24].

Statistical analysis

The correlations between the H-scores of CLDN18.2, RhoGAP, and E-cadherin, and the clinicopathological factors and radiological responses were analyzed using the independent-sample t-test. The correlation between H-scores among each marker was assessed by Pearson's correlation analysis. Overall survival (OS) and progression-free survival (PFS) were calculated from the start date of first-line palliative chemotherapy until the date of death and disease progression or death, respectively. For survival analyses, data from living patients or those with no disease progression were censored from the last follow-up date. Cox proportional hazards regression analysis was performed to identify the risk factors for overall mortality. Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test. All analyses were performed using the SPSS software (version 24; IBM Corp., Armonk, NY, USA), and a two-sided P-value of <0.05 was considered significant.

RESULTS

Baseline patient characteristics

A total of 77 patients were included in the present study. Patient characteristics are listed in **Table 1**. The median age of patients was 52 years (range, 27–82 years). The patient cohort comprised 35 (45.5%) men and 42 (54.5%) women. PM was observed in 50 (64.9%) patients. Distant lymph node, liver, and bone metastases were observed in 34 (44.2%), 9 (11.7%), and 12 (15.6%) patients, respectively.

Evaluation of CLDN18.2, RhoGAP, and E-cadherin expression

CLDN18.2, RhoGAP, and E-cadherin expression was evaluated using whole tissue sections (**Fig. 1**). The mean H-scores of CLDN18.2, RhoGAP, and E-cadherin were 45 (min–max, 0–170), 17 (0–70), and 54 (0–150), respectively. The distribution of these markers is summarized in **Fig. 2**.

Table 1. Baseline characteristics of the patients (n=77)

Characteristics	Data
Mean age (yr)	52 (27–82)
<45	23 (29.9)
≥45	54 (70.1)
Sex	
Female	42 (54.5)
Male	35 (45.5)
Tumor location	
Upper third	14 (18.2)
Middle third	36 (46.8)
Lower third	27 (35.1)
cT stage	
1	2 (2.6)
2	6 (7.8)
3	19 (24.7)
4	50 (64.9)
cN stage	
0	14 (18.2)
1	9 (11.7)
2	30 (39.0)
3	24 (31.2)
HER2	
Negative	57 (74.0)
Positive	10 (13.0)
Unknown	10 (13.0)
Peritoneal metastasis*	
No	27 (35.1)
Yes	50 (64.9)
Distant LN metastasis*	
No	43 (55.8)
Yes	34 (44.2)
Liver metastasis*	
No	68 (88.3)
Yes	9 (11.7)
Bone metastasis*	
No	65 (84.4)
Yes	12 (15.6)

Data are expressed as median (range) or number (%).

cT = stage clinical T stage; cN stage = clinical N stage; HER2 = human epidermal growth factor receptor 2; LN = lymph node.

*Metastasis patterns were dichotomized.

Correlation between clinicopathological factors and H-scores of CLDN18.2, RhoGAP, and E-cadherin

We investigated the correlation between cell-adherens junction protein expression and clinicopathological factors (**Table 2** and **Fig. 3**). CLDN18.2 expression was significantly lower in patients with PM than those without PM (mean H-scores, 36.98 vs. 60.67, $P=0.010$). In contrast, CLDN18.2 expression levels were significantly higher in patients with bone metastasis than in those without bone metastasis (78.19 vs. 39.22, $P=0.010$). Additionally, CLDN18.2 expression was lower in patients who were younger (<45 years old) and had liver metastases. There were no other significant correlations between clinicopathological factors and CLDN18.2 expression. Furthermore, E-cadherin expression was lower in patients with PM than in those without PM (46.74 vs. 68.63, $P=0.013$). In contrast, E-cadherin expression was higher in patients with bone metastases (89.27 vs. 47.85, $P=0.001$). There were no other significant correlations between clinicopathological factors and E-cadherin expression. RhoGAP expression was not correlated with any of the clinicopathological factors.

Significance of CLDN18.2 Expression in mDGC

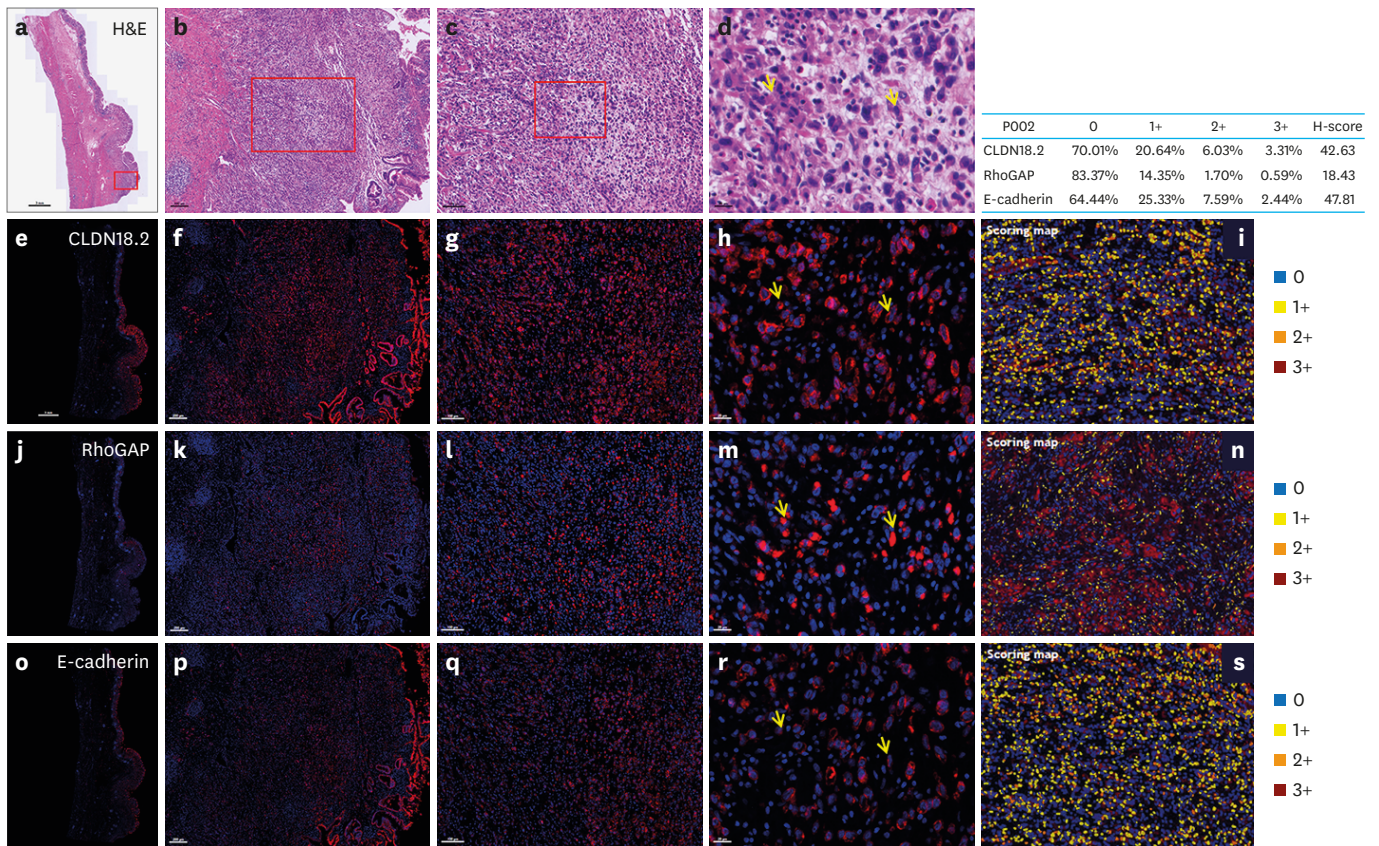


Fig. 1. Expression of CLDN18.2, RhoGAP, and E-cadherin using two-plex IHC. Formalin-fixed, paraffin-embedded sections of gastric cancer were stained using OPAL for CLDN18.2, RhoGAP, and E-cadherin and imaged on the Vectra 3.0 Automated Quantitative imaging system to obtain composite images. Using the lower power images, high-powered fields (g, h, l, m, q, r) were selected from specific tumor regions. (i,n,s) Scoring maps, composite images that displayed fluorescence intensity of CLDN18.2, RhoGAP, and E-cadherin in the tissue samples, were used to determine H-scores. The intensity was determined based on tumor cells showing no (0), weak (1+), intermediate (2+), or strong (3+) membranous staining of CLDN18.2, RhoGAP, and E-cadherin. (a, b, c, d) hematoxylin and eosin (×10, ×40, ×100, ×400); (e, j, o) two-plex IHC (×10); (f, k, p) two-plex IHC (×40); (g, l, q) two-plex IHC (×100); (h, m, r) two-plex IHC (×100); (d, h, m, r) Yellow arrows indicate poorly cohesive tumor cells. CLDN18.2 = isoform of claudin-18; RhoGAP = Rho GTPase-activating protein; IHC = immunofluorescence; H&E = hematoxylin and eosin.

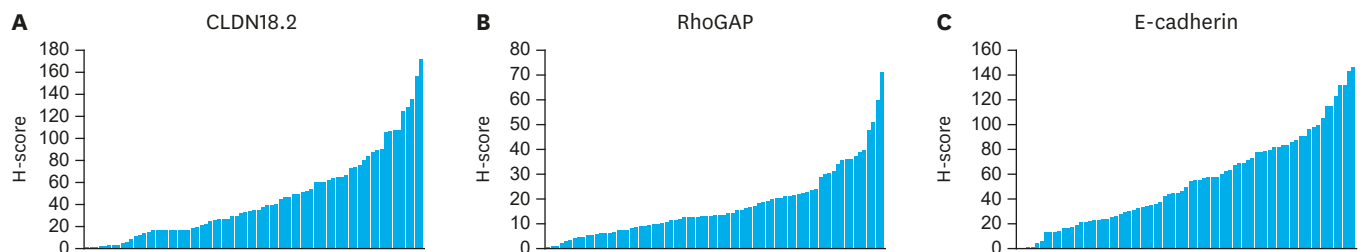


Fig. 2. H-score distribution of CLDN18.2 (A), RhoGAP (B), and E-cadherin (C). The mean H-score of CLDN18.2, RhoGAP, and E-cadherin was 45 (min-max, 0-170), 17 (0-70), and 54 (0-150), respectively. CLDN18.2 = isoform of claudin-18; RhoGAP = Rho GTPase-activating protein.

At the time of progression after receiving first-line chemotherapy, 12 out of 27 patients who did not show PM initially developed PM whereas 15 patients did not. Expression of CLDN18.2 and E-cadherin was significantly higher in these 15 patients than in those with PM (CLDN18.2, 73.35 vs. 39.05, $P=0.002$; E-cadherin, 81.04 vs. 48.36, $P=0.002$). Among the 15 patients who did not develop PM at the time of progression after receiving first-line chemotherapy, 5 did not develop PM until death. Expression of CLDN18.2 and E-cadherin

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Table 2. Correlation between cell-adherens junction proteins and clinicopathologic factors (n=77)

Variables	CLDN18.2			RhoGAP			E-cadherin		
	Mean±standard deviation	t	P	Mean±standard deviation	t	P	Mean±standard deviation	t	P
Age		-2.222	0.030*		0.056	0.956		-1.206	0.232
<45 (n=23)	32.75±27.11			17.67±12.43			46.56±36.55		
≥45 (n=54)	50.63±42.04			17.48±14.43			57.60±36.89		
Peritoneal metastasis		2.648	0.010*		0.436	0.664		2.532	0.013*
No (n=27)	60.67±44.19			18.48±14.48			68.63±40.40		
Yes (n=50)	36.98±33.32			17.03±13.51			46.74±32.85		
Liver metastasis		2.784	0.012*		0.191	0.849		0.937	0.352
No (n=68)	47.91±40.16			17.65±11.54			55.74±37.60		
Yes (n=9)	25.52±19.19			16.71±37.60			43.46±30.76		
Bone metastasis		-2.975	0.010*		0.128	0.899		-3.89	0.001*
No (n=65)	39.22±35.24			17.62±14.23			47.85±33.57		
Yes (n=12)	78.19±42.78			17.07±11.54			89.27±35.63		

CLDN18.2 = claudin-18.2; RhoGAP = Rho GTPase-activating protein. *Significant.

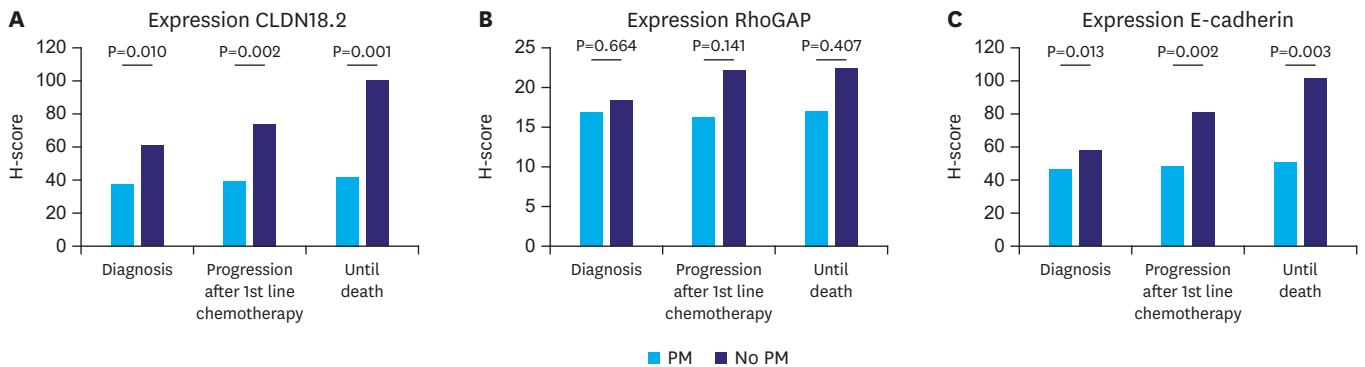


Fig. 3. Expression of CLDN18.2 (A), RhoGAP (B), and E-cadherin (C) based on the presence of PM during disease course. Patients who never developed PM until death had significantly higher expression of CLDN18.2 and E-cadherin. CLDN18.2 = isoform of claudin-18; RhoGAP = Rho GTPase-activating protein; PM = peritoneal metastasis.

was significantly higher in these 5 patients than the 72 patients who developed PM (CLDN18.2, 99.89 vs. 41.50, P=0.001; E-cadherin, 101.31 vs. 51.04, P=0.003) (Fig. 3).

Correlation between CLDN18.2, RhoGAP, and E-cadherin expression

We investigated the correlation between CLDN18.2, RhoGAP, and E-cadherin expression (Fig. 4). CLDN18.2 expression was positively correlated with that of E-cadherin (r=0.765, P<0.001). Similarly, RhoGAP expression was positively correlated with that of CLDN18.2 (r=0.325, P=0.004) and E-cadherin (r=0.373, P=0.001).

Survival and chemotherapy response based on CLDN18.2, RhoGAP, and E-cadherin expression

We investigated the tumor response after first-line chemotherapy treatment based on CLDN18.2, RhoGAP, and E-cadherin expression (Table 3). Of the 77 patients, 54 had a measurable lesion. CLDN18.2 levels were not significantly different between the objective response (complete response [CR]/partial response [PR]) and other (stable disease [SD]/progressive disease [PD]) groups (51.75 vs. 32.01, P=0.052). Similarly, RhoGAP and E-cadherin expression levels were also not statistically different between the CR/PR and SD/PD groups. Moreover, CLDN18.2, RhoGAP, and E-cadherin expression levels were not different between the disease control (CR/PR/SD) and PD groups. OS and PFS in all patients were investigated based on CLDN18.2, RhoGAP, and E-cadherin expression. CLDN18.2,

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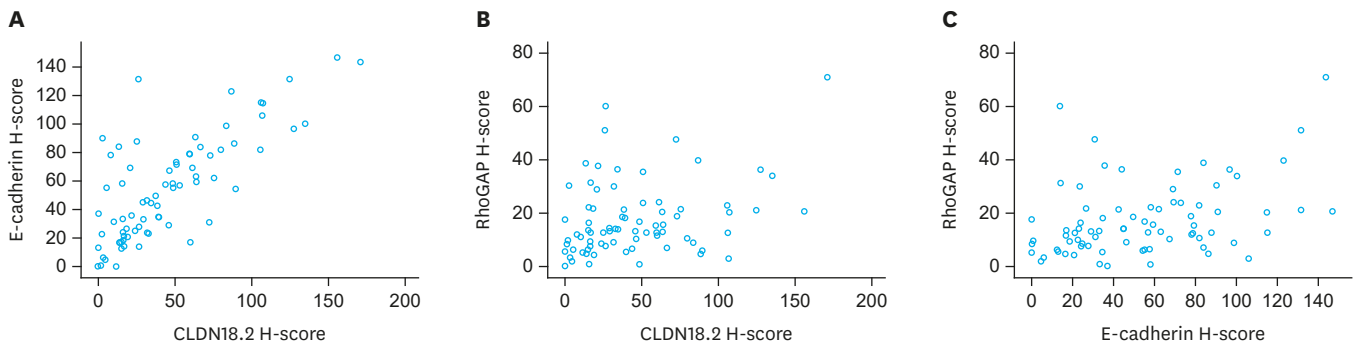


Fig. 4. Correlation between CLDN18.2, RhoGAP, and E-cadherin. The expression of CLDN18.2 was positively associated with that of E-cadherin ($r=0.765$, $P<0.001$) (A). RhoGAP expression was positively associated with CLDN18.2 ($r=0.325$, $P=0.004$) (B) and E-cadherin ($r=0.373$, $P=0.001$) (C) expression. CLDN18.2 = isoform of claudin-18; RhoGAP = Rho GTPase-activating protein.

Table 3. First-line chemotherapy response based on CLDN18.2, RhoGAP and E-cadherin expression (n=77)

Variables	CLDN18.2			RhoGAP			E-cadherin		
	Mean±standard deviation	t	P	Mean±standard deviation	t	P	Mean±SD	t	P
Chemotherapy response		1.987	0.052		1.546	0.131		1.68	0.099
CR/PR (n=25)	51.75±43.28			22.08±17.63			60.37±37.83		
SD/PD (n=29)	32.01±29.24			15.95±9.75			43.98±33.87		
Peritoneal metastasis		0.925	0.359		1.602	0.119		1.451	0.153
CR/PR/SD (n=43)	43.53±38.6			19.86±15.27			55.16±36.19		
PD (n=11)	31.82±32.17			14.06±7.67			37.52±35.14		

CLDN18.2 = claudin-18.2; RhoGAP = Rho GTPase-activating protein; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.

RhoGAP, and E-cadherin positivity was determined based on the median value (Fig. 5). CLDN18.2, RhoGAP, and E-cadherin positivity were not associated with OS and PFS as evidenced by multivariate analyses (Supplementary Table 1).

DISCUSSION

The aim of the present study was to evaluate the clinical significance of CLDN18.2 expression in mDGC. To the best of our knowledge, this is the first study to investigate the clinical significance of CLDN18.2 expression by immunofluorescence in mDGCs.

We investigated the association between the expression of adherens junction molecules and PM as several previous studies have suggested that the loss of adherens junction structure stability is associated with PM [12,25]. Yonemura et al. [12] demonstrated that reduced expression of E-cadherin and high expression of S100A4 promote PM, serosal involvement, and infiltrative tumor growth. Togano et al. [25] suggested that loss of E-cadherin expression is a critical step for PM of GC with sub-serosal invasion. To date, little is known about the clinical impact of CLDN18.2 expression in metastatic GC. Loss of CLDN18.2 expression is associated with an increased proliferative and invasive potential of GC [26,27]. We found that CLDN18.2 and E-cadherin expression was lower in patients with PM than in those without PM. Notably, CLDN18.2 and E-cadherin expression was significantly higher in patients who never developed PM until death than in those who developed PM. Our results suggest that adherens junction instability may be involved in the progression and formation of PM during the course of the disease.

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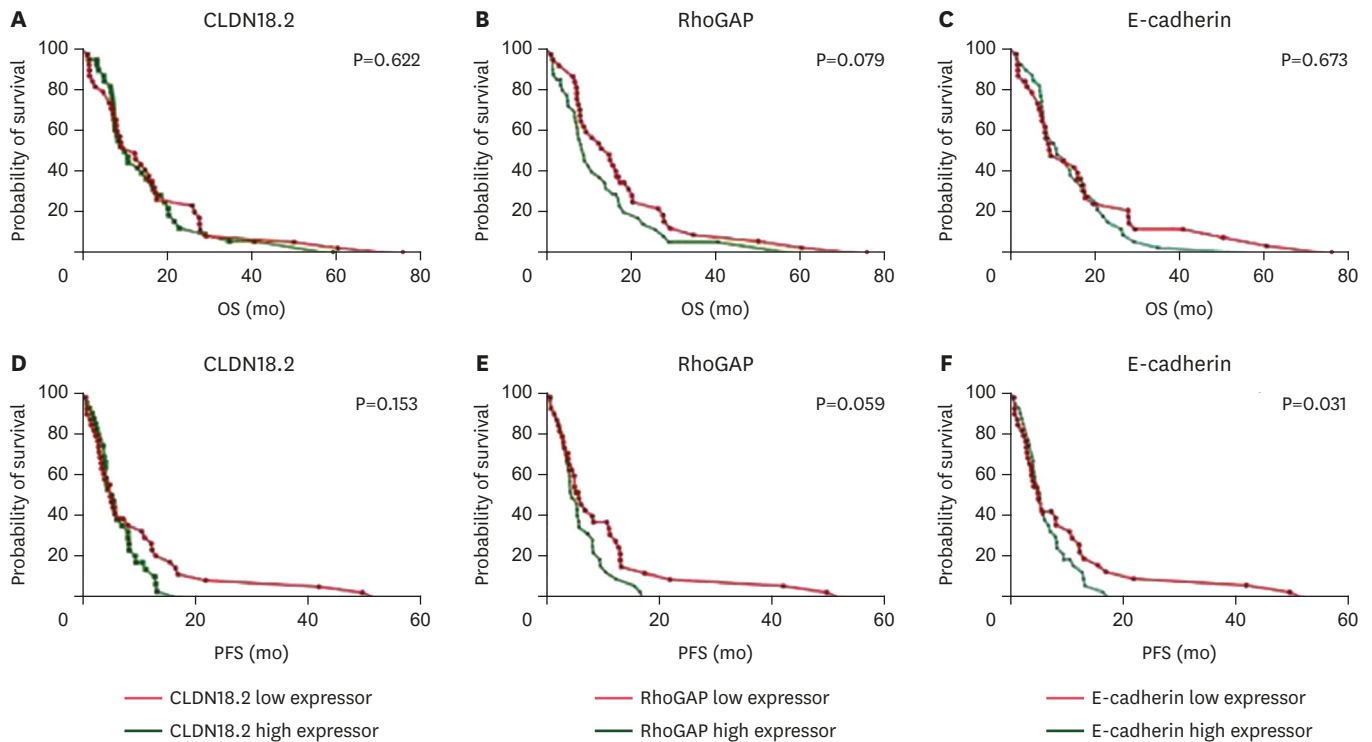


Fig. 5. OS (A-C) and PFS (D-F) according to CLDN18.2, RhoGAP, and E-cadherin expression. CLDN18.2, RhoGAP, and E-cadherin positivity was not associated with OS and PFS. OS = overall survival; PFS = progression-free survival; CLDN18.2 = isoform of claudin-18; RhoGAP = Rho GTPase-activating protein.

Remarkably, CLDN18.2 and E-cadherin expression was significantly higher in patients with bone metastases in the present study. In fact, bone metastasis and PM showed a nearly exclusive pattern (PM rates: 17% [2/12] and 74% [48/65] in patients with and without bone metastasis, respectively). Furthermore, among the five patients who never developed PM until death, three patients had initial bone metastasis. These results are in line with those reported in previous studies, which showed that bone metastasis is not frequently synchronous with PM [28], indicating that the mechanisms of organotropic metastasis are clearly different. Furthermore, our results reveal that loss of adherens junction integrity, which is generally accepted as the first step in PM formation, is more important in PM than in metastases to other sites such as bone, which occurs mostly through hematogenous spreading [29].

Furthermore, we found a positive correlation between CLDN18.2, RhoGAP, and E-cadherin expression. Several studies have proposed expression-related interactions between different adherens junction proteins. Lu et al. [30] investigated the function of claudin-7 with respect to the regulation of cell proliferation and maintenance of epithelial cell attachment via the engagement of integrin $\beta 1$. Wu et al. [31] reported that EpCAM modulates adhesion and tight junction function by regulating the intracellular localization and degradation of selected claudins. To date, the precise mechanism of interaction between adherens junction proteins is not well understood. A previous study suggested that intercellular junctions not only come apart but also undergo regulatory changes, including a phenomenon known as a cadherin switch, in which epithelial cells lose E-cadherin expression and start expressing N-cadherin during epithelial-mesenchymal transition [32]. However, our findings cannot explain the mechanism of interaction between CLDN18.2, RhoGAP, and E-cadherin. The positive

correlation between the expression of these adherens junction molecules may result in their ability to disrupt cellular cohesiveness synergistically. Further studies investigating these mechanisms are warranted.

We found no difference in chemotherapy response and survival with respect to the expression of adherens junction molecules. Cell-adherens junction molecules play an important role in the epithelial-mesenchymal transition and influence chemosensitivity of cells [33]. Skalova et al. [34] proposed that claudin-1 and claudin-3 play a role in the response to chemotherapy in breast cancer. Yang et al. [14] demonstrated that patients with in-frame fusion genes containing the RhoGAP domain have aggressive DGCs. Moreover, Wang et al. [35] revealed that loss of E-cadherin is associated with poor prognosis. However, there is no clear consensus regarding the chemosensitivity and prognosis of cell-adherens junction molecules in mDGCs. In addition, none of the patients had a measurable lesion, a characteristic feature of metastatic GC, causing a small subset size (54/77, 70%) and incomplete investigation of the chemotherapy response. Furthermore, the present study is retrospective in nature and is therefore not appropriate for investigating survival. Further studies are needed to confirm this.

Our study has several limitations. First, the association between cell-adherens junction molecules was not confirmed by other experimental methods. Recent studies have investigated these markers using several methods, such as whole genome sequencing and sequencing [13,14,16]. A previous study found that the fusion of CLDN18.2 and ARHGAP26, which includes the RhoGAP domain, was frequently observed in mDGC [15]. However, immunostaining is a practical method for expression analysis. In fact, a recent clinical trial investigating an anti-CLDN18.2 antibody for treating GC used immunohistochemistry as a predictive marker; thus, we believe our study has clinical implications. Furthermore, digital pathology has several advantages with respect to accuracy. As there is currently no optimal cut-off or standard for evaluating these markers, our results can provide valuable clinical information to clinicians evaluating specimens by immunostaining. Additionally, we evaluated the positivity of CLDN18.2, RhoGAP, and E-cadherin in only tumor cells. However, previous studies have shown that CLDN18.2, RhoGAP, and E-cadherin are not only expressed in tumor cells but also in normal gastric mucosa [20,36-38]. A previous study showed that CLDN18.2 expression level is significantly lower in GC than in the surrounding normal gastric mucosa and that downregulation of CLDN18.2 is associated with the proliferative potential of GC, suggesting its role in GC progression [26]. In our study, the expression of cell-adherens junction molecules in normal gastric mucosa was not assessed owing to tumor heterogeneity in slide imaging and the absence of normal control tissue in every patient. Our study did not show the clinical significance of CLDN18.2 and other molecules in terms of survival and prognosis; therefore, analysis of correlation of these proteins between cancer and normal tissues, such as the ratio of CLDN18.2 expression in tumor to normal mucosa, may provide meaningful results. Further studies are required to evaluate the patterns of cell-adherens molecules between tumor and normal mucosa. Second, our cohort included a limited number of patients. Therefore, the results should be interpreted with caution. An extensive study with a larger sample size is needed to confirm our findings. Finally, our study is retrospective, and a prospective study is also required.

Collectively, we evaluated the clinical significance of cell-adherens junction molecules, including CLDN18.2, which are being investigated as targets in GC treatment. We found that CLDN18.2 expression was significantly lower in patients with PM but intact in those with bone metastasis, which may be associated with the first step of the “seed and soil”

hypothesis, rather than with hematogenous metastasis. We also found a positive correlation between the expression of CLDN18.2 and other adherens junction molecules, which has clinical implications for DGC and PM pathogenesis. However, further studies are needed to confirm our results.

SUPPLEMENTARY MATERIAL

Supplementary Table 1

Univariate and multivariate analyses for CLDN18.2, RhoGAP, and E-cadherin positivity according to OS and PFS

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