의학석사 학위논문

위암 환자에서의 claudin 18.2 발현 양상에 대한 병리학적 분석

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이 논문을 의학석사학위 논문으로 제출함

2024 년 2 월

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최유진의 의학석사학위 논문을 인준함

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Abstract

Claudin 18.2 is a tight junction protein expressed on the cellular surface of normal gastric epithelium, and its expression is frequently upregulated in gastric cancer. Due to the recent success of zolbetuximab – a monoclonal antibody agent targeting claudin 18.2 – in two phase 3 trials (SPOTLIGHT and GLOW), it has emerged as a promising therapeutic target in gastric cancer. In this systematic study, the same antibody clones and evaluation methods were utilized for assessing claudin 18.2 expression, to provide the consistency of the overall analysis.

Part 1 of this study focused on investigating the clinicopathologic features and survival outcomes of claudin 18.2 positive tumors in patients with stage I–III gastric cancer. This study aimed to provide insights for the potential application of claudin 18.2-targeted treatment in earlier stages of gastric cancer. Claudin 18.2 positivity was observed in 46.5% of the total 299 patients, with slightly higher rate among stage I patients (51.1%). Claudin 18.2 positivity was associated with a younger age (median, 61 vs 66 years, $p<0.001$), a shallower depth of invasion ($p=0.014$), Borrmann type 4 morphology ($p=0.008$) and diffuse histological type ($p=0.011$). However, it was not an independent prognostic factor in a localized setting. These findings aligned with previous research conducted in patients with advanced gastric cancer.

In part 2, the heterogeneity of claudin 18.2 expression was investigated in 166 patients with stage IV gastric cancer. paired tissue samples of primary and metastatic tumors from 135 of these patients were thoroughly analyzed, revealing a concordance rate over 50%. Notably, patients with peritoneal metastasis displayed the highest rate of claudin 18.2 positivity, suggesting that patients with peritoneal metastasis could potentially derive the greatest benefit from claudin 18.2-targeted therapy in terms of systemic disease control. Furthermore, this study provided specific cutoff values to maximize the efficacy of claudin 18.2-targeted treatment, recommending a threshold of 120 for H-score and 30% for the percentage of tumor

cells exhibiting moderate to strong intensity. Additionally, this study revealed a high prevalence of intratumoral heterogeneity, pointing out the limitations of endoscopic biopsy in representing the entire tumor characteristics. These findings may provide a deeper insight for the complexities of claudin 18.2 expression status in stage IV gastric cancer.

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Part 1. Clinicopathologic features and prognostic value of claudin 18.2 expression in resectable gastric cancer

Introduction

Gastric cancer is one of the most common and fatal solid tumors worldwide. Especially in Korea, it accounts for 2nd place in prevalence and 4th place in mortality[1,2]. Although the national health screening examination made remarkable improvement of the 5-year survival in Korean population by earlier detection, the prognosis of unresectable or metastatic gastric cancer remains poor.

Patients with gastric cancer are clinically categorized into two groups according to the feasibility of curative resection: resectable and unresectable[3]. As the patients with unresectable disease – locally advanced, recurrent, and metastatic – receive palliative managements including systemic chemotherapy instead of curative surgical resection, numerous studies have been focusing on exploring potential therapeutic target to prolong the survival outcomes in these patients. As a result, Trastuzumab have been contributed to the improved survival in case of human epidermal growth factor 2 (HER2)-positive tumors, and Nivolumab in the patients with combined positive score (CPS)≥5 by PD-L1 IHC 28-8 pharmDx. However, these regimens could not benefit the patients with HER2-negativity and low PD-L1 CPS.

Claudin 18.2 has emerged as a novel target in this context. It is a tight junction protein which is selectively expressed on the membrane surface of normal gastric epithelium, regulating intercellular signal transduction. During malignant transformation, the breakage of tight junction results in the increased exposure of these proteins in the cellular surface, which makes claudin 18.2 a promising target for treatment[4]. Recently, two phase 3 trials – SPOTLIGHT and GLOW – proved that zolbetuximab, a monoclonal antibody agent targeting claudin 18.2, improved overall survival (OS) in patients with unresectable or metastatic gastric and gastroesophageal junction cancer when combined to conventional chemotherapy regimen[5,6]. While claudin 18.2 is indeed expressed in normal gastric mucosa, it is noteworthy that there have been relatively few reported adverse events in patients

undergoing zolbetuximab treatment. The prevailing hypothesis suggests that normal gastric epithelial cells harbor claudin 18.2 within the tight junction complex, whereas cancer cells exhibit markedly elevated expression of claudin 18.2 on their cell membranes, potentially serving as competitive inhibitors[7].

Considering this efficacy and safety, it is not difficult to imagine the expanded application of zolbetuximab to earlier stages of gastric cancer in the near future. However, clinicopathologic characteristics of claudin 18.2-positive tumors have been either studied in the palliative setting [8] or in the localized setting without the antibody or scoring method used in the recent clinical trials[9-13].

This study aimed to find clinicopathological features associated with claudin 18.2 expression and evaluate the prognostic value of it in the localized setting.

Materials and Methods

Patient selection and grouping

This retrospective study included 299 Korean patients with histologically confirmed resectable stage I–III gastric cancer by either biopsy or surgical resection at Asan Medical Center (Seoul, Korea) from March 2018 to February 2019. Clinical data including patient age, sex, date of surgery, overall survival (OS) and recurrence free survival (RFS) and histological information including the location and gross type of tumor, histologic subtypes and findings associated with the prognosis such as lymphovascular invasion, perineural invasion and depth of invasion were obtained from previous medical records. The disease stage was defined by American Joint Committee on Cancer (AJCC) staging criteria 7th edition.

This study was approved by the Institutional Review Board (IRB) of Asan Medical Center (IRB No. 2023-0154), and the requirement for informed consent from patients was waived because of the following de-identification process: after de-identifying information of research subjects, random research subject numbers were assigned. Data were analyzed based on the de-identified patient information, and all related documents, such as research data, were be encrypted and stored in the researcher's private office so that only the researcher could access them, and the data were handled only by the researcher within the office. This study was conducted in accordance with the ethical standards of the latest Declaration of Helsinki.

Claudin 18.2 immunohistochemistry and scoring method

For the surgical specimens, representative sections from formalin-fixed paraffin-embedded (FFPE) blocks were selected by pathologists. Immunohistochemistry (IHC) was performed on 4μm thick FFPE sections, which were deparaffinized and re-hydrated using xylene and ethanol serially. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 10 minutes, followed by heat-induced antigen retrieval. IHC labeling was performed using a Claudin18.2 antibody (clone 43-14A, Ventana)

with an autostainer (Benchmark XT, Ventana Medical Systems) and the OptiView DAB Detection Kit (Ventana Medical Systems), following the manufacturer's protocol.

Considering the nature of claudin 18.2 as a tight junction protein expressed on cellular surface, only membranous and linear staining pattern was interpreted as positive. Any other immunoreactivity, such as granular expression in the cytoplasm or nucleus, was disregarded. The immunoreaction status was assessed using two well-established methods used in previous studies on claudin 18.2 expression in gastric cancer[5,6]. Claudin 18.2 expression status was categorized into two distinct groups – claudin 18.2-positive and claudin 18.2-negative. Claudin 18.2 positivity was defined as moderate-to-strong positivity in at least 75% of the tumor cells. Cases which did not meet this criterion were designated as claudin 18.2 negative. The intensity of expression was categorized into 4 tiers: absence of any expression (0), weak expression (+1), moderate expression (+2), or strong expression (+3). Then, the H-score was calculated by summing the product of this stratified intensity score multiplied by the percentage of positive tumor cells exhibiting the respective intensity. By employing this calculation method, the score varied between a minimum value of 0, indicating an absence of expression in any of the tumor cells, and a maximum value of 300, representing strong expression in all tumor cells.

HER2 IHC was performed using a Ventana anti-Her2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical System, Tucson, AZ), and HER2 positivity was determined using the gastric cancer consensus panel recommendations[14]. Silver-enhanced in situ hybridization (SISH) was used additionally in cases interpreted as equivocal (2+) by IHC staining. The presence of Epstein-Barr virus (EBV) in the cancer cells was evaluated by EBV-encoded RNA, detected by chromogenic in situ hybridization, which was performed using a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions.

Statistical analysis

RFS was defined as the interval of time between the date of surgical resection (index date) and the date of recurrence or death. OS was defined as the interval of time between the index date and the date of death from any cause. Survival outcomes were analyzed using the Kaplan-Meier method, and then compared among subgroups by using the log-rank test. Categorical variables among subgroups were compared by using the chi-square test or Fisher's exact test. A p-value of < 0.05 was considered statistically significant. All statistical analysis was conducted by IBM SPSS Statistics ver. 28.0 for Windows (IBM Corp., Armonk, New York, USA) and R software ver. 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinical characteristics of the patients

Total of 266 patients were included in this study. The median age was 63 years, with male occupying 68.6% of the overall population (Table 1). The number of enrolled patients were 90 (30.1%) for stage I, 96 (32.1%) for stage II and 113 (37.8%) for stage III.

Rate of claudin 18.2 positivity

Patients with claudin 18.2 positivity occupied 46.5% of overall patients with stage I–III gastric cancer (n = 139) (Table 1). When divided into each stage, 51.1% of stage I patients, 47.9% of stage II patients and 41.6% of stage III patients were claudin 18.2-positive. The median H-score for claudin 18.2 expression was 180, 170 and 110 for patients with stage I, II and III disease, respectively.

Clinicopathological characteristics according to claudin 18.2 expression

Claudin 18.2 positivity was associated with a younger age (median, 61 vs. 66 years, $P < 0.001$) and fewer male proportion (62.6% vs. 73.8%, $P = 0.051$) (Table 1). Early gastric cancer (EGC) gross type was more frequently observed in claudin 18.2-positive tumors (30.2% vs. 17.5%, $P = 0.014$) (Table 1). Among patients with advanced gastric cancer (AGC), the proportion of Borrmann type 4 was higher in claudin 18.2-positive tumors (20.6% vs. 10.6%, $P = 0.008$) (Table 1). Diffuse histological type was more frequently observed in claudin 18.2-positive tumors (48.2% and 33.1%, $P = 0.011$) (Table 1).

Claudin 18.2-positive tumors tended to show fewer lymph node metastasis without statistical significance (55.4% vs. 66.7%, $P = 0.061$) (Table 1). Also, they were associated with a lower rate of lymphovascular invasion (56.1% vs. 75.5%, $P < 0.001$), but there was no difference in the proportion of patients with perineural invasion (37.4% vs. 40.0%, $P = 0.734$) (Table 1).

The rate of claudin 18.2 positivity was significantly higher in HER2-negative tumors than in HER2-positive tumors (48.7% vs. 15.8%, P = 0.011) (Table 2), whereas it was higher in Epstein-Barr virus (EBV)-positive tumors (72.2% vs. 45.3%, P = 0.049) (Table 3).

Survival outcomes according to claudin 18.2 expression

Claudin 18.2-positive patients tended to have favorable RFS (3-year RFS rate: 6.9% and 5.7%, P = 0.085) and OS (3-year OS rate: 85.6% vs. 81.3%, P = 0.062), respectively, as compared to claudin 18.2 negative patients (Figure 2).

When survival outcomes were analyzed in each tumor stage, RFS did not show significant difference according to claudin 18.2 expression (P = 0.1, P = 0.86, and P = 0.37 for stage I, II, and III disease, respectively). Neither OS showed difference between the two groups (P = 0.13, P = 0.68, and P = 0.35 for stage I, II, and III disease, respectively) (Figure 3).

Multivariate analysis revealed patient age (> 60 years) was an unfavorable independent factor for OS (P=0.008) and stage III for RFS (P=0.009) (Table 4). Perineural invasion was also an unfavorable independent factor for both OS and RFS (P<0.001). However, claudin 18.2 expression status was not an independent factor for RFS (hazard ratio [HR] 0.80, 95% confidence interval [CI] 0.48–1.32, P = 0.376) or OS (HR 0.77, 95% CI 0.47–1.25, P = 0.290).

Discussion

This study aimed to investigate the association of claudin 18.2 expression and clinicopathologic features using the same antibody clone and evaluation criteria for claudin 18.2 expression status as in the recent phase 3 trials[5,6]. The overall rate of claudin 18.2 positivity was 46.5% in resectable gastric cancer, with a slightly higher rate among stage I tumors (51.1%). Claudin 18.2-positive tumors were less likely to be aggressive, with depth of invasion limited to the mucosa and submucosa (EGC). There were fewer lymph node metastasis and less frequent lymphovascular invasion. On the other hand, claudin 18.2 positivity was associated with Borrmann type 4 as mentioned in a previous report in the metastatic setting[8], and with the diffuse histological type, as in previous reports [9,12]. These findings align with the hypothesis established in previous studies that claudin 18.2 expression might be associated with some pathological features.

In the overall study population of stage I–III gastric cancer, claudin 18.2-positive tumors tended to show favorable survival outcomes, possibly due to its association with limited invasion depth and fewer lymph node metastasis. However, when analyzed separately by each stage, change in claudin 18.2 expression status did not affect the survival outcomes. Therefore, these results indicate that claudin 18.2 expression status is not an independent prognostic factor in a localized setting, as previously confirmed in metastatic setting[8].

The rate of claudin 18.2 positivity in localized resectable gastric cancer (46.5%) in current study was comparable to those reported in the phase 3 trials based on the palliative setting (38.5% and 38.4% in the SPOTLIGHT and GLOW studies, respectively). The significant ratio of claudin 18.2 positivity suggests the potential for wide feasibility of claudin 18.2-targeted treatment in the peri-operative setting. Therefore, prospective adjuvant or neoadjuvant trials toward claudin 18.2 expression can be considered in patients with localized resectable gastric cancer.

As discussed by Ungureanu et al[15], previous studies concerning the clinicopathologic characteristics of claudin 18.2-positive tumors have used variable claudin 18.2 IHC clones[9,12,13] or adopted variable methods for the assessment of claudin 18.2 expression[10,11]. Considering that the sensitivity of claudin 18.2 detection may be dependent to the application of different IHC clones[10], choosing identical clone and methodology used in recent phase 3 trials is essential for the consistency of the analysis. Therefore, this study may provide additional practical insights for applying claudin 18.2-targeted treatments such as zolbetuximab to patients with localized gastric cancer.

In this study, the focus was on the analysis of claudin 18.2 expression, categorizing it into two distinct groups: claudin 18.2-positive and claudin 18.2-negative, rather than directly utilizing the H-score method. The purpose was to elucidate the contrast in pathological features and survival outcomes based on the expression status of claudin 18.2 among the patients with surgically resectable gastric cancer. The calculation of the H-score was conducted as a preliminary trial in preparation for the forthcoming analysis in the part 2.

Meanwhile, this study has several limitations to be considered. Its retrospective nature, the single center-based analysis, and the absence of a validation cohort may limit the interpretation and generalizability of this data. In addition, given that novel claudin 18.2-targeting agents other than zolbetuximab are currently under investigation[16-19], the scoring method for claudin 18.2 expression adopted in this analysis may not be universally applied to future analysis of other claudin 18.2-targeted agents.

In conclusion, claudin 18.2 positivity was observed in almost half of resectable gastric cancer patients. Although it was associated with some clinicopathological characteristics, it was not an independent prognostic factor in a localized setting. Considering the substantial rate of claudin 18.2 positivity in resectable gastric cancer

and the survival benefits of zolbetuximab-based treatments in a metastatic setting, further studies are warranted for claudin 18.2-directed perioperative treatments.

Part 2. Heterogeneity of claudin 18.2 expression in stage IV gastric cancer

Introduction

In part 1, the clinical and pathologic characteristics associated with claudin 18.2 expression were investigated within the context of surgically resectable gastric cancer. Notably, claudin 18.2-positive tumors exhibited a shallower invasion depth and a reduced occurrence of lymph node metastasis, suggesting a potential link to more favorable survival outcomes within the cohort. However, theses tumors also displayed a tendency to manifest as Borrmann type 4 tumors and exhibit a diffuse histological type, consistent with the findings from prior research[8,9,12].

If a correlation between specific histologic traits and the expression of claudin 18.2 exists, it becomes plausible to consider that histological examination could aid in the identification of optimal candidates for zolbetuximab treatment. However, the effectiveness of this treatment may be compromised by the variability in claudin 18.2 expression within individual tumors. For instance, there is a possibility that liver metastasis, which originally displayed claudin 18.2 positivity in gastric lesion, might subsequently appear as claudin 18.2-negative, or vice versa. Similarly, a single endoscopic biopsy specimen from the primary tumor may indicate claudin 18.2-positivity, while the overall expression pattern in the entire surgical specimen could appear as claudin 18.2-negative. Hence, it becomes crucial to verify the representativeness of the biopsy specimen. This involves confirming whether the claudin 18.2 expression in the biopsy specimen can reliably predict the effectiveness of zolbetuximab treatment, both in the context of localized therapy and systemic disease control.

Part 2 focused on the heterogeneous profile of claudin 18.2 expression in stage IV gastric cancer, with comparative analysis of claudin 18.2 expression between primary and metastatic sites. The primary goal was to determine an optimal cutoff value for predicting the consistency of claudin 18.2 expression between primary and metastatic tumors. This was intended to serve as a valuable guideline for the selection

of the most suitable target population in the palliative setting.

Materials and Methods

Patient selection and grouping

This retrospective study included 166 patients diagnosed with stage IV gastric cancer by either biopsy or surgical resection at Asan Medical Center (Seoul, Korea) from January 2012 to December 2022. Paired cases refer to those with tissue samples of both the primary and metastatic sites. Overall cohort included 135 paired cases (including both surgical and biopsy specimen), 16 cases of single surgical specimen and 15 cases of single biopsy specimen.

Clinical data including patient age, sex, the date of 1st chemotherapy, Eastern Cooperative Oncology Group (ECOG) performance score (PS), overall survival (OS) and progression free survival (PFS) and histological information including the location and gross type of tumor, histologic subtypes, Human epidermal growth factor receptor 2 (HER2) and Ebstein-Barr virus (EBV) status were obtained from previous medical records.

This study was approved by the Institutional Review Board of Asan Medical Center (IRB No. 2023-0154), and the requirement for informed consent from patients was waived because of the following de-identification process: after de-identifying information of research subjects, random research subject numbers were assigned. Data were analyzed based on the de-identified patient information, and all related documents, such as research data, were be encrypted and stored in the researcher's private office so that only the researcher could access them, and the data were handled only by the researcher within the office. This study was conducted in accordance with the ethical standards of the latest Declaration of Helsinki.

Claudin 18.2 immunohistochemistry and scoring method

For the surgical specimens, representative sections from formalin-fixed paraffin-embedded (FFPE) blocks were selected by pathologists. Immunohistochemistry

(IHC) was performed on 4μm thick FFPE sections, which were deparaffinized and re-hydrated using xylene and ethanol serially. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 10 minutes, followed by heat-induced antigen retrieval. IHC labeling was performed using a Claudin18.2 antibody (clone 43-14A, Ventana) with an autostainer (Benchmark XT, Ventana Medical Systems) and the OptiView DAB Detection Kit (Ventana Medical Systems), following the manufacturer's protocol.

Immunostaining pattern was interpreted as positive only in case of membranous, linear staining. Granular expression in the cytoplasm or nucleus was disregarded, considering claudin 18.2 being expressed on cellular surface. Claudin 18.2 expression status was assessed using two methods used in previous studies on claudin 18.2 expression in gastric cancer[5,6]. First, claudin 18.2 positivity was defined as moderate-to-strong positivity in at least 75% of the tumor cells. Cases which did not meet this criterion were designated as claudin 18.2-negative. In paired cases, a patient was categorized as claudin 18.2-positive if at least one of the paired tissues exhibited claudin 18.2 positivity. Second, the H-score of each case was calculated by summing the product of 4-tiered stratified intensity score (0: absence of any expression, 1: weak expression, 2: moderate expression, 3: strong expression) multiplied by the percentage of positive tumor cells exhibiting the respective intensity. By employing this calculation method, the score varied between a minimum value of 0, indicating an absence of expression in any of the tumor cells, and a maximum value of 300, representing strong expression in all tumor cells. In addition, the percentage of tumor cells with moderate-to-strong positivity was calculated accordingly.

Among the patients with claudin 18.2 positivity (moderate-to-strong positivity in $\geq$75% of the tumor cells), the expression patterns of claudin 18.2 were classified based on the homogeneity and the pattern of expression within the tumor. The homogeneous pattern was defined as expressing more than 90% of the area with a

moderate-to-strong intensity. Any other heterogeneous pattern that did not fall into the category of the homogeneous pattern was further categorized into superficial, invasive-front, and random patterns: the superficial pattern was defined as expression primarily shown in the mucosa, which showed an apparent decrease in immunostaining intensity toward the depths of the tumor; the invasive-front pattern was characterized by prominent expression in the deep invasive components of the tumor, with a decrease in the expression of the protein toward the tumor edge; and the random pattern was defined as a pattern in which the distribution of expression was patchy with varying intensities that were evenly distributed (Figure 4).

HER2 IHC was performed using a Ventana anti-Her2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical System, Tucson, AZ), and HER2 positivity was determined using the gastric cancer consensus panel recommendations[14]. Silver-enhanced in situ hybridization (SISH) was used additionally in cases interpreted as equivocal (2+) by IHC staining. The presence of EBV in the cancer cells was evaluated by EBV-encoded RNA, detected by chromogenic in situ hybridization, which was performed using a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions.

Metastatic sites were categorized into following five groups: GI tract, peritoneum, liver, lymph node and others. When a metastatic lesion is confirmed by endoscopic biopsy displaying mucosal involvement, it is categorized as GI tract involvement. On the other hand, when a metastatic lesion within the gastrointestinal (GI) tract extends from the serosal to mucosal side, it is classified as peritoneal involvement. Remaining metastatic sites such as ovary, bone and soft tissue are included in "others" group.

Statistical analysis

PFS was defined as the interval of time between the date of 1st systemic chemotherapy and the date of progression or death. OS was defined as the interval

of time between the index date and the date of death from any cause. Survival outcomes were analyzed using the Kaplan-Meier method, and then compared among subgroups by using the log-rank test. In case of two-tiered group of claudin 18.2 expression (positive and negative), the relationship between claudin 18.2 expression and clinicopathologic characteristics were analyzed using the Chi-Square test or Fisher's exact test for categorical variables and the t-test or Mann-Whitney U test for numerical variables. In case of three-tiered group (positively concordant, negatively concordant, and discordant), the Chi-Square test or Fisher's exact test was used for the analysis of categorical variables and the one-way ANOVA or Kruskal-Wallis H test was used for the analysis of numerical variables. Wilcoxon signed rank test was used to compare the H-score and the percentage of tumor cells in the paired group. Receiver operating characteristics (ROC) curves and area under the curve (AUC) were applied to calculate the optimal cutoff values for the H-score and the percentage of tumor cells. All statistical analysis was conducted by IBM SPSS Statistics ver. 28.0 for Windows (IBM Corp., Armonk, New York, USA) and R software ver. 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinical characteristics and survival outcomes of overall patients

Among the overall stage IV patients, the age (P = 0.459), ECOG performance status (P = 0.463) and the number of metastatic organs (P = 0.877) showed no statistical difference (Table 5). Neither the rate of EBV positivity (P = 0.188) and HER2 positivity (P = 0.180) were statistically significant. Overall MSI status and PD-L1 CPS score showed no difference between two groups (Table 5).

Both PFS and OS did not show significant difference depending on claudin 18.2 positivity (P = 0.85 / 0.51).

Clinical characteristics and survival outcomes of paired cases

Among the stage IV cases, there were 135 patients from whom the tissues from both the primary and metastatic tumors were obtained. These cases were reclassified into three groups based on the concordance of claudin 18.2 expression status between primary and metastatic sites, resulting in the following groups: positively concordant (n = 34), negatively concordant (n = 67) and discordant (n = 34). Negatively concordant group were older (P = 0.439) with higher male proportion (P = 0.072) and frequent HER2 positivity (P=305) when compared to the other two groups, without statistical significance (Table 6). Positively concordant group showed better ECOG performance score (P = 0.118) with fewer number of metastasis (P = 0.084) and frequent EBV positivity (P = 0.125) than other two groups without statistical significance either.

The three groups did not demonstrate any significant differences in PFS (Figure 6). The 3-year PFS rates were 6.5%, 5.5% and 3.5% in the discordant group, negatively concordant group, and positively concordant group, respectively (P = 0.84). Likewise, there were no significant differences in OS among these groups, with 3-year OS rates of 12.4% in the discordant group, 17.6% in the negatively

concordant group, and 4.6% in the positively concordant group (P = 0.83).

Heterogeneity of claudin 18.2 expression between primary and metastatic sites

In this analysis, the concordance of claudin 18.2 expression between primary and metastatic sites was over 50% in total, with liver metastasis exhibiting the highest concordance (82.0%, n = 32), followed by GI tract (80.0%, n = 12) and peritoneum (71.2%, n = 32) (Figure 7).

In discordant group (n = 34), there were 20 cases with claudin 18.2-positive primary tumor, and 14 cases with claudin 18.2-negative primary tumor (Figure 8). Primary tumors exhibited higher H-score (median, 205 vs. 140, P = 0.034) and higher percentage of tumor cells with moderate-to-strong positivity (median, 80% vs. 55%, P = 0.025) when compared to the metastatic tumors.

In positively concordant group (n=34), metastatic tumors displayed a higher H-score than primary tumors (median, 300 vs. 270, P = 0.032), but there was no significant difference in the percentage of tumor cells with moderate-to-strong positivity (median, 100% vs. 100%, P = 0.178).

Negatively concordant group did not exhibit any significant difference in either H-score (median, 0 vs. 0, P = 0.505) or the percentage (0% vs. 0%, P = 0.567).

Then, ROC curves to calculate the optimal cutoff value for both H-score and the percentage of tumor cells with moderate-to-strong positivity was calculated. Paired cases were further subdivided into three groups based on the concordance of claudin 18.2 expression between the primary and metastatic sites. “Concordantly positive” referred to patients with claudin 18.2 positivity in both primary and metastatic tumors, “discordantly positive” included patients with claudin 18.2 positivity in either one of primary or metastatic tumor, and “negative” included cases in which both tumors were claudin 18.2-negative.

In case of detecting concordantly positive group, H-score was more efficient than the percentage (AUC, 0.913 vs. 0.909) with the optimal cutoff value being 180. In case of screening any positivity (concordantly positive and discordantly positive), both H-score and the percentage showed same AUC of 0.976. The optimal cutoff value for H-score was 120 and the percentage was 30% (Figure 9).

Heterogeneity of claudin 18.2 expression within tumor

Intratumoral heterogeneity of claudin 18.2 expression was further evaluated in 39 patients with stage IV gastric cancer who went through surgical resection. Seven patients were excluded as they did not exhibit any expression at all. The homogeneous expression pattern was observed in 53.1% of analyzed 32 patients (n =17). Among the remaining heterogeneous pattern, the mucosal type accounted for 15.6% (n = 5), the invasive front type accounted for 9.4% (n = 3) and the random type accounted for 21.9% (n = 7).

The patients with homogeneous pattern exhibited higher H-score (median, 290 vs. 60, $P < 0.001$) and larger percentage of tumor cells with moderate to strong positivity than those with heterogeneous pattern (median, 100% vs. 20%, $P < 0.001$) (Figure 10). After the heterogenous pattern was divided into three subgroups, homogeneous pattern still represented the highest H-score and percentage, while there was no significant difference among each subgroup of heterogenous pattern.

Claudin 18.2 expression and lymphovascular invasion

Some novel findings were observed in this study. In one case, tumor cells within the lymphatic space exhibited more intense expression of claudin 18.2 (Figure 11A). In another case, tumor cells seemed to acquire claudin 18.2 expression after invading the vascular wall, as the primary lesion had a total absence of claudin 18.2 expression

(Figure 11B). Further investigation is needed to determine the potential association between claudin 18.2 expression and the process of lymphovascular invasion.

Discussion

This study aimed to investigate the heterogeneous profile of claudin 18.2 expression among stage IV gastric cancer and provide an optimal cutoff value for clinical decision by adopting the same protocol and criteria as recent phase 3 trials[5,6].

The rate of claudin 18.2 positivity was 47.0% in overall stage IV gastric cancer, which was comparable to those reported in previous studies[2,9,13]. Claudin 18.2-positive tumors were frequently observed in patients with younger age, better ECOG PS status, and fewer metastasis, without statistical significance. However, claudin 18.2 positivity did not exhibit any discernible prognostic influence on the survival outcomes, as noted in previous studies[8,20]. Within current study cohort, there were no significant variations in biomarkers including EBV, HER2, MSI status and PD-L1 CPS score, in relation to claudin 18.2 expression. Given the conflicting results from previous studies regarding the correlation between these biomarkers and claudin 18.2 expression[2,8,9,21], additional research with larger cohort is warranted for a more comprehensive understanding.

When the paired cases of primary and metastatic tumors from same 135 patients were categorized based on the concordance of claudin 18.2 expression status, there were no significant differences observed in patient characteristics and survival outcomes among each group (positively concordant, negatively concordant, and discordant groups). Within the discordant group, primary tumors displayed a higher H-score and a larger percentage of tumor cells exhibiting moderate-to-strong intensity compared to metastatic tumors. While the H-score was elevated in the metastatic tumors of the positively concordant group, the percentage did not show a remarkable difference. In the negatively concordant group, both the H-score and the percentage were similar between primary and metastatic tumors.

The overall cohort of this study had over 50% of concordance between primary and metastatic sites. Overall concordance was the highest in cases with liver metastasis (82.0%). However, the proportion of positively concordant pairs was highest in peritoneum. Additionally, cases with peritoneal metastasis exhibited the highest rate of claudin 18.2 positivity in metastatic site (17.9%), suggesting that patients with peritoneal metastasis might derive the greatest benefit from claudin 18.2-targeted therapy in terms of systemic disease control.

While recent phase 3 trials[5,6] have categorized claudin 18.2 positivity as "moderate to strong positivity in over 75% of the tumor cells", precise numerical criteria defining claudin 18.2 positivity have not yet been standardized. In this study, the optimal cutoff values for both the H-score and the percentage of tumor cells were proposed to predict the concordance of claudin 18.2 expression between primary and metastatic tumors, offering a solution for cases where obtaining biopsy specimens from metastatic tumors is challenging, as is often the case for patients with compromised health or limited accessibility. Positively concordant groups were the most efficiently detected when the cutoff values were 180 for the H-score and 60% for the percentage. However, from a perspective of overall disease control, the discordant group might also benefit from claudin 18.2-targeted therapy as they also have claudin 18.2-positive tumor. Thus, cutoff values of 120 for the H-score and 30% for the tumor cell percentage are recommended to expand the target population that could potentially benefit from the treatment.

Heterogeneity within single primary tumor was investigated in surgical specimens. The H-score was significantly higher in homogeneous group, probably due to the definition of homogeneous group as those showing moderate to strong positivity in more than 75% of the tumor cells. Of note, near half of the cases displayed heterogeneous pattern. High prevalence of intratumoral heterogeneity observed in this study points out that endoscopic biopsy has limitations in representing the entire tumor.

There were some intriguing observations in this study. The initial hypothesis was that the primary tumors with a homogeneous pattern would consistently exhibit claudin 18.2 positivity when metastasized. Surprisingly, among the 11 cases with homogeneous primary tumors and paired biopsies from metastatic tumors, three cases turned out to be claudin 18.2-negative. The second hypothesis was that metastatic tumors arising from primary tumors with an invasive front type would express claudin 18.2. However, two out of three cases with invasive front type primary tumors exhibited relatively low H-scores (0 and 60). These results suggest that a change in claudin 18.2 expression status might happen during the metastatic process.

This study has certain limitations. Being a single-institutional retrospective study with a limited sample size and a restricted racial representation, these results may not be fully representative of claudin 18.2 expression in general population. Also, despite employing the same antibody clone and evaluation method as recent phase 3 trials, interobserver variation could have affected the interpretation of claudin 18.2 expression. For instance, in cases of poorly cohesive carcinoma or signet ring cell carcinoma, there might have been an underestimation of the expression of scattered tumor cells.

In conclusion, claudin 18.2 positivity was identified in nearly half of the patients with stage IV gastric cancer, yet it did not exhibit a discernible prognostic impact. Notably, there was a significant spatial heterogeneity in claudin 18.2 expression, not only between primary and metastatic tumors but also within individual tumors. Moreover, this study has contributed significant insights by suggesting optimal cutoff values for patient selection in claudin 18.2-targeted treatment, by employing the identical methods utilized in recent phase 3 trials. These findings might serve as a guidance for future treatment strategies targeting gastric cancer.

Table 1. Clinicopathologic characteristics according to claudin 18.2 expression

Variables	Total (n = 299)	Claudin 18.2 negative (n = 160)	Claudin 18.2 positive (n = 139)	p-value
Median age (years) (range)	63 (27–95)	66 (33–95)	61 (27–83)	<0.001
Male sex	205 (68.6%)	118 (73.8%)	87 (62.6%)	0.051
Gross type				0.014
EGC	70 (23.4%)	28 (17.5%)	42 (30.2%)	
AGC	229 (76.6%)	132 (82.5%)	97 (69.8%)	
AGC subtype	(n = 229)	(n = 132)	(n = 97)	0.008
Borrmann type 1	9 (3.9%)	8 (6.1%)	1 (1.0%)	
Borrmann type 2	49 (21.4%)	36 (27.3%)	13 (13.4%)	
Borrmann type 3	108 (47.2%)	57 (43.2%)	51 (52.6%)	
Borrmann type 4	34 (14.8%)	14 (10.6%)	20 (20.6%)	
EGC-like or unclassifiable	29 (12.7%)	17 (12.9%)	12 (12.4%)	
Location				0.537
Lower/middle	237 (79.3%)	129 (80.6%)	108 (77.7%)	
Upper	58 (19.4%)	30 (18.8%)	28 (20.1%)	
Entire	4 (1.3%)	1 (0.6%)	3 (2.2%)	
WHO classification				0.089
WD/MD/papillary	110 (36.8%)	68 (42.5%)	42 (30.2%)	
PD/PD with SRC/SRCa	166 (55.5%)	81 (50.6%)	85 (61.2%)	
Others	23 (7.7%)	11 (6.9%)	12 (8.6%)	
Lauren classification				0.011
Intestinal type	113 (37.8%)	72 (45.0%)	41 (29.5%)	
Diffuse type	120 (40.1%)	53 (33.1%)	67 (48.2%)	
Mixed /indeterminate type	66 (22.1%)	35 (21.9%)	31 (22.3%)	
Invasion depth				0.035
Mucosa/submucosa	70 (23.4%)	28 (17.5%)	42 (30.2%)	
Proper muscle	62 (20.7%)	40 (25.0%)	22 (15.8%)	
Subserosa	73 (24.4%)	42 (26.2%)	31 (22.3%)	
Serosa /adjacent organ	94 (31.4%)	50 (31.2%)	44 (31.7%)	
LN metastasis	183 (61.4%)	106 (66.7%)	77 (55.4%)	0.061

Lymphovascular invasion	198 (66.4%)	120 (75.5%)	78 (56.1%)	<0.001
Perineural invasion	116 (38.8%)	64 (40.0%)	52 (37.4%)	0.734
Overall pathologic stage (7^{th})				0.379
I	90 (30.1%)	44 (27.5%)	46 (33.1%)	
II	96 (32.1%)	50 (31.2%)	46 (33.1%)	
III	113 (37.8%)	66 (41.2%)	47 (33.8%)	

* Abbreviations : EGC, early gastric cancer; AGC, advanced gastric cancer; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; SRC, signet ring cell; SRCa, signet ring cell carcinoma; LN, lymph node.

Table 2. Claudin 18.2 expression according to HER2 status

	HER2 positive (n = 19)	**HER2 negative (n = 271)**	**p-value**
Claudin 18.2-negative	16 (84.2)	139 (51.3)	0.011
Claudin 18.2-positive	3 (15.8)	132 (48.7)	

Table 3. Claudin 18.2 expression according to EBV status

	EBV positive (n = 18)	**EBV negative (n = 267)**	**p-value**
Claudin 18.2-negative	5 (27.8)	146 (54.7)	0.049
Claudin 18.2-positive	13 (72.2)	121 (45.3)	

Table 4. Factors associated with recurrence-free survival (RFS) and overall survival (OS)

Variables	Recurrence-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Age > 60 years	1.38 (0.84–2.27)	0.197	1.75 (1.05–2.93)	0.032	1.65 (1.02–2.68)	0.043	1.97 (1.20–3.26)	0.008
Male sex	1.03 (0.61–1.72)	0.917	1.25 (0.73–2.15)	0.415	0.92 (0.57–1.49)	0.734	1.07 (0.65–1.76)	0.799
Lymphovascular invasion	1.63 (0.94–2.83)	0.081	0.90 (0.48–1.68)	0.745	1.75 (1.03–2.98)	0.039	1.00 (0.55–1.83)	0.993
Perineural invasion	4.20 (2.52–6.99)	<0.001	3.35 (1.86–6.01)	<0.001	2.92 (1.84–4.65)	<0.001	2.74 (1.58–4.75)	<0.001
Lauren (reference: intestinal)								
Diffuse subtype	0.96 (0.56–1.65)	0.890			0.85 (0.51–1.44)	0.551		
Mixed+indeterminate subtype	0.97 (0.51–1.83)	0.919			1.03 (0.58–1.85)	0.911		
Stage (reference: stage I)								
II	2.25 (0.97–5.22)	0.059	1.62 (0.65–4.06)	0.304	1.09 (0.54–2.21)	0.813	0.78 (0.37–1.68)	0.530
III	5.57 (2.61–11.87)	<0.001	3.36 (1.36–8.35)	0.009	3.06 (1.70–5.53)	<0.001	1.87 (0.90–3.87)	0.094
Claudin 18.2 expression status	0.65 (0.40–1.06)	0.087	0.80 (0.48–1.32)	0.376	0.64 (0.40–1.03)	0.064	0.77 (0.47–1.25)	0.290

Table 5. Clinicopathologic characteristics according to claudin 18.2 expression in stage IV gastric cancer

Variables	Claudin 18.2 negative (n = 88)	Claudin 18.2 positive (n = 78)	p-value
Median age (years) (range)	62 (19–84)	58 (20–82)	0.459
Sex			0.117
Female	28 (31.8%)	35 (44.9%)	
Male	60 (68.2%)	43 (55.1%)	
ECOG PS			0.463
0–1	72 (81.8%)	68 (87.0%)	
≥2	16 (18.2%)	10 (13.0%)	
Number of metastatic organs			0.877
0–1	38 (43.2%)	45 (57.7%)	
≥2	50 (56.8%)	33 (42.3%)	
Initial status			0.319
Initially metastatic	75 (85.2%)	59 (75.6%)	
Recurrent	12 (13.6%)	17 (21.8%)	
Locally advanced	1 (1.1%)	2 (2.6%)	
EBV expression status	(n = 80)	(n = 71)	0.188
Negative	79 (98.8%)	67 (94.4%)	
Positive	1 (1.2%)	4 (5.6%)	
HER2 expression status			0.180
Negative	66 (75.0%)	66 (84.6%)	
Positive	22 (25.0%)	12 (15.4%)	
MSI status	(n = 60)	(n = 54)	0.999
MSS	59 (98.3)	53 (98.1)	
MSI high	1 (1.7)	1 (1.9)	
PD-L1 CPS	(n = 30)	(n = 26)	0.521
≥1	14 (46.7)	9 (34.6)	

* Abbreviations : ECOG PS, Eastern Cooperative Oncology Group performance score; LN, lymph node; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; EBV, Ebstein-Barr virus; HER2, human epidermal growth factor receptor 2; MSI, microsatellite instability; MSS, microsatellite stable.

Table 6. Clinicopathologic characteristics according to concordance of claudin 18.2 expression in paired cases

Variables	Discordant (n = 34)	Negatively Concordant (n = 67)	Positively Concordant (n = 34)	p-value
Median age (years) (range)	58 (34–81)	62 (19–84)	58 (20–82)	0.439
Sex				0.072
Female	15 (44.1%)	19 (28.4%)	17 (50.0%)	
Male	19 (55.9%)	48 (71.6%)	17 (50.0%)	
ECOG PS				0.118
0–1	26 (76.5%)	54 (80.6%)	32 (94.1%)	
≥2	8 (23.5%)	13 (19.4%)	2 (5.9%)	
Number of metastatic organs				0.084
0–1	16 (47.1%)	24 (35.8%)	20 (58.8%)	
≥2	18 (52.9%)	43 (64.2%)	14 (41.2%)	
Initial status				0.163
Initially metastatic	29 (85.3%)	57 (85.1%)	24 (70.6%)	
Recurrent	5 (14.7%)	10 (14.9%)	8 (23.5%)	
Locally advanced	0 (0.0%)	0 (0.0%)	2 (5.9%)	
EBV expression status	(n = 29)	(n = 59)	(n = 32)	0.125
Negative	29 (100.0%)	58 (98.3%)	29 (90.6%)	
Positive	0 (0.0%)	1 (1.7%)	3 (9.4%)	
HER2 expression status				0.305
Negative	29 (85.3%)	49 (73.1%)	28 (82.4%)	
Positive	5 (14.7%)	18 (26.9%)	6 (17.6%)	

Figure 1. The median H-score in patients with resectable gastric cancer.

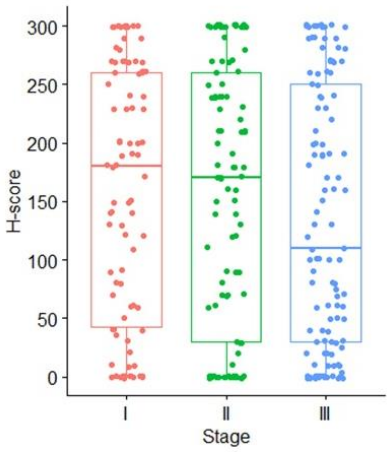


Figure 2. Kaplan–Meier curves of survival outcomes of patients with resectable gastric cancer according to claudin 18.2 expression. (A) Recurrence-free survival and (B) overall survival.

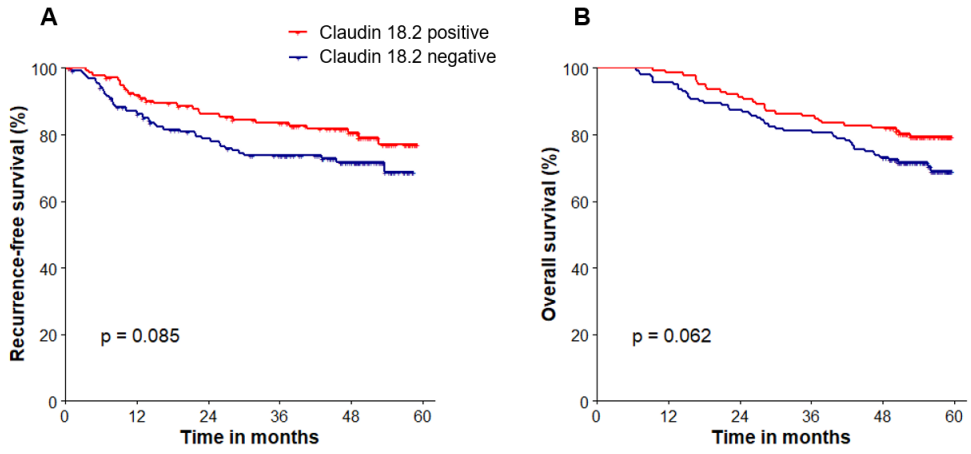


Figure 3. Kaplan–Meier curves of survival outcomes in patients with resectable gastric cancer in each clinical stage according to claudin 18.2 expression. (A) stage I, (B) stage II and (C) stage III.

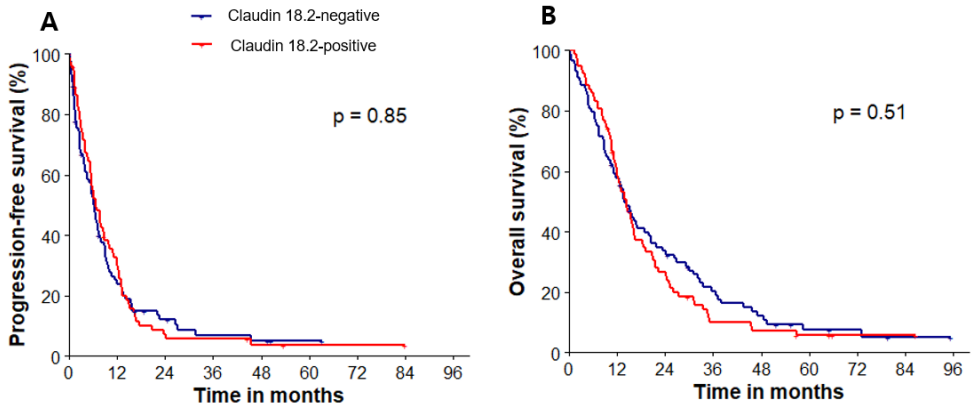


Figure 4. Representative immunohistochemical staining pattern of claudin 18.2. (A) Homogeneous pattern, (B) heterogeneous – superficial pattern, (C) heterogeneous – random pattern and (D) heterogeneous – invasive-front pattern.

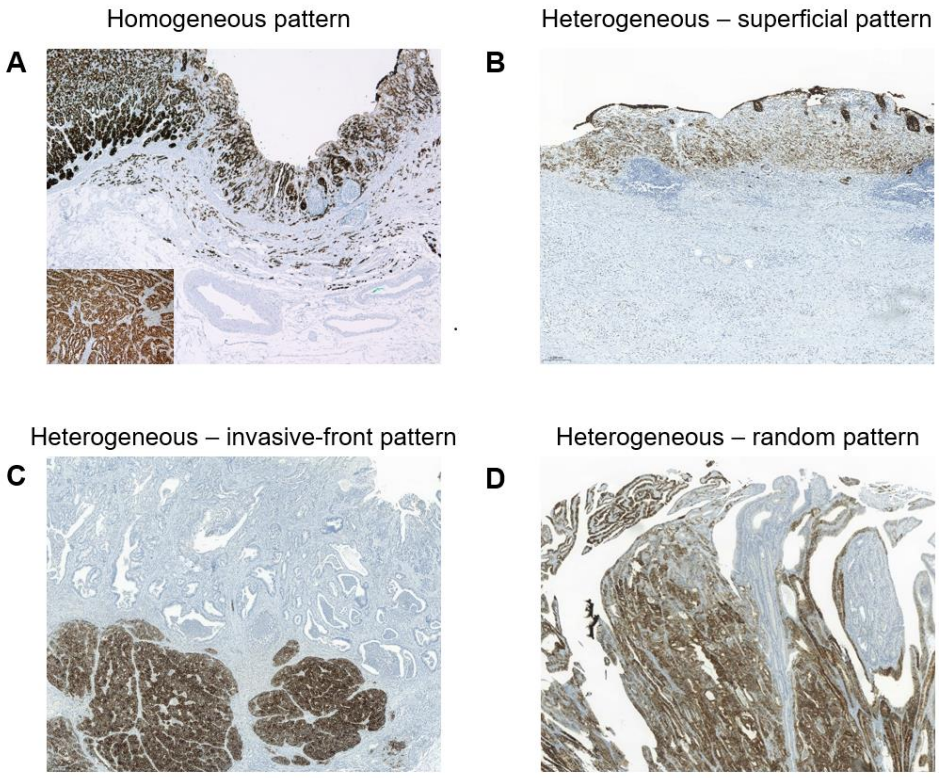


Figure 5. Kaplan–Meier curves of survival outcomes in patients with stage IV gastric cancer according to claudin 18.2 expression status. (A) Progression-free survival and (B) overall survival.

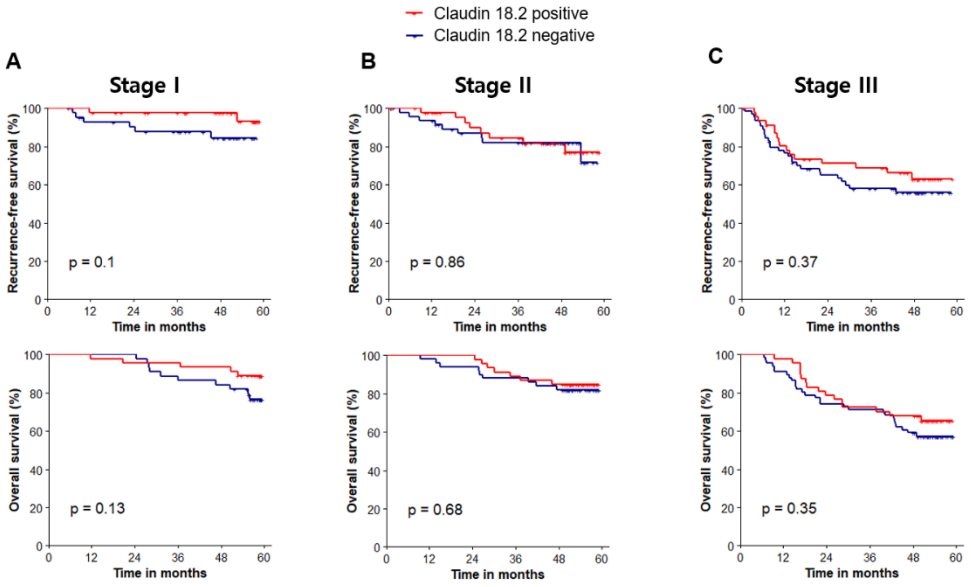


Figure 6. Kaplan–Meier curves of survival outcomes in patients with stage IV gastric cancer according to concordance of claudin 18.2 expression in paired cases. (A) Progression-free survival and (B) overall survival.

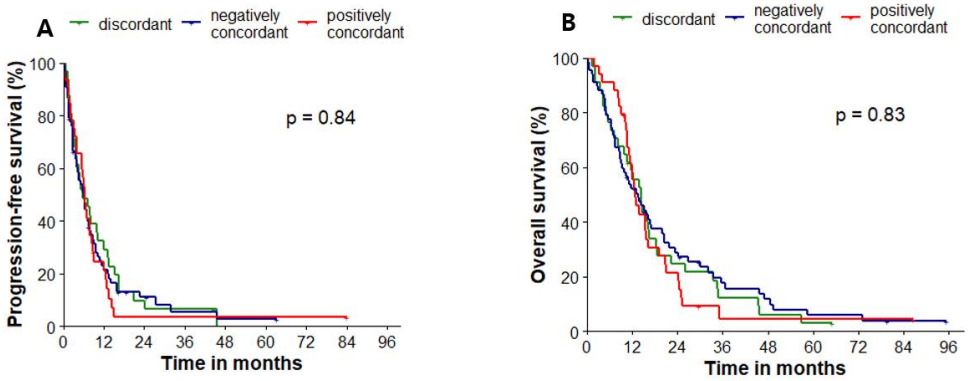
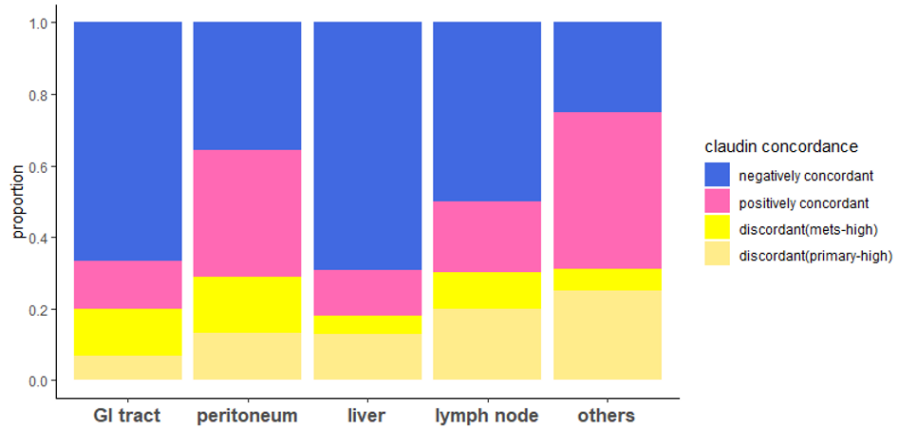


Figure 7. Concordance of claudin 18.2 expression in various metastatic sites.



* Abbreviations : mets-high, claudin 18.2-positivity in metastatic tumors; primary-high, claudin 18.2-positivity in primary tumors

Figure 8. Variations in claudin 18.2 expression status across primary and metastatic sites. (A) H-score and (B) the percentage of tumor cells showing moderate to strong positivity.

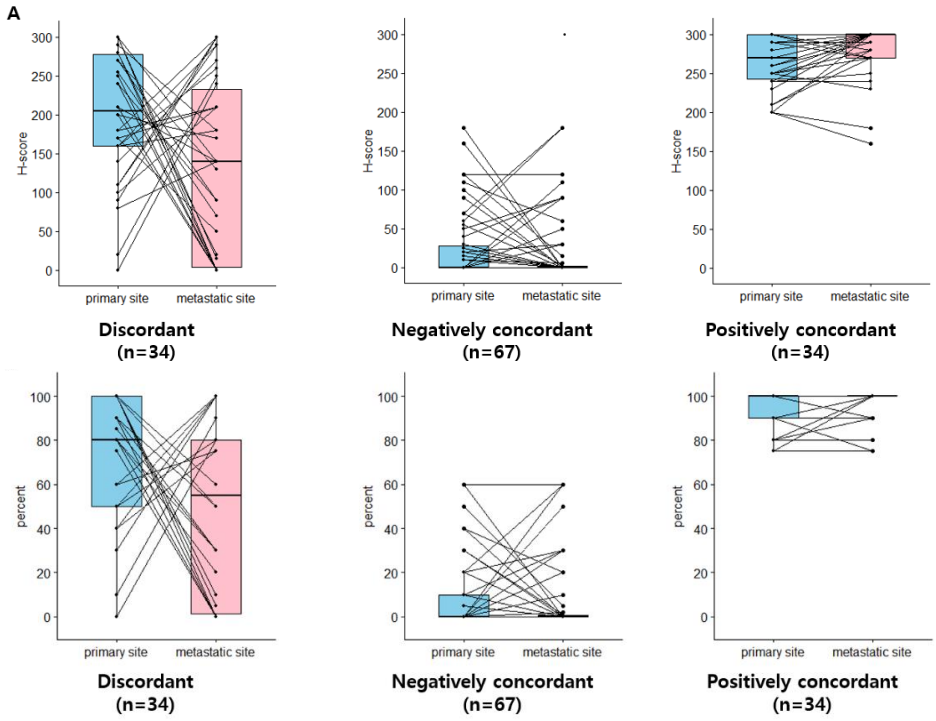


Figure 9. ROC curves for H-score and the percentage of tumor cells exhibiting moderate to strong positivity. (A) H-score and (B) the percentage.

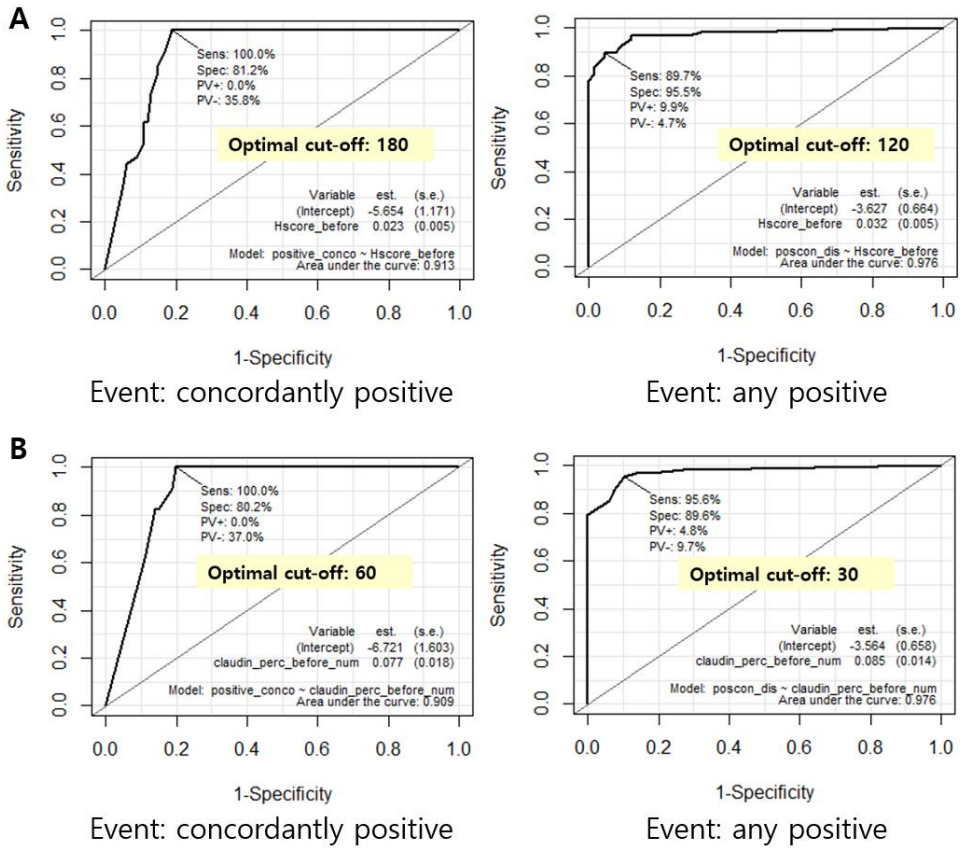


Figure 10. The median H-score of each pattern of claudin 18.2 expression.

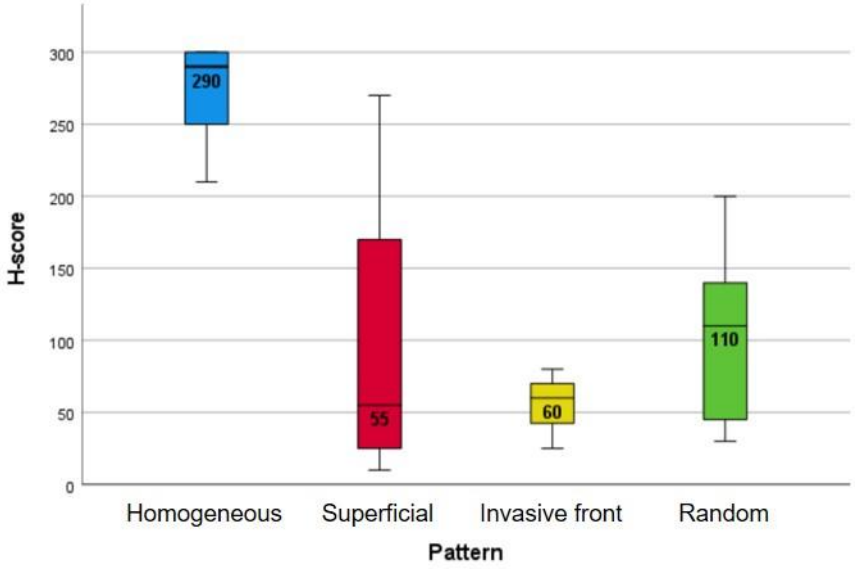
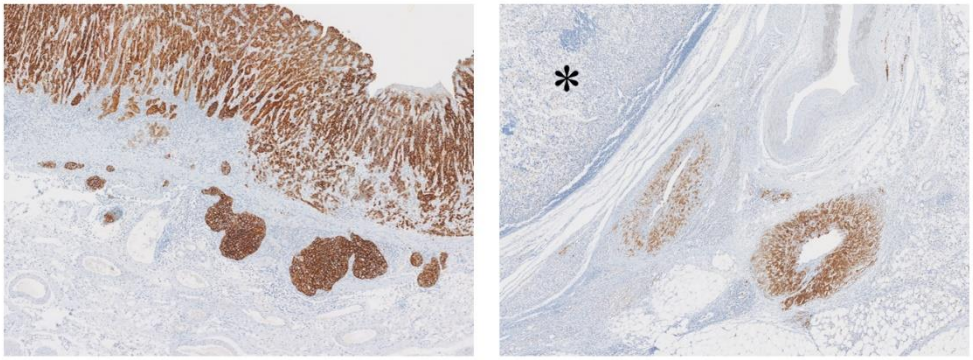


Figure 11. Claudin 18.2 expression and lymphovascular invasion. (A) Tumor cells within lymphatic space and (B) tumor cells invading the vascular wall (asterisk, main mass).



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국문요약

Claudin 18.2는 정상적인 위의 상피세포 표면에서 발현되는 밀착연접 (tight junction) 단백질로, 종종 위암세포에서도 강한 발현을 보이는 것으로 밝혀져 있다. 최근 claudin 18.2에 대한 표적 단일클론항체 제제인 Zolbetuximab이 두 건의 3상 임상시험 (SPOTLIGHT, GLOW)에서 치료 효능을 입증하면서 claudin 18.2에 대한 관심이 높아지고 있다. 본 연구에서 저자들은 상기 임상시험에서 사용된 것과 동일한 항체 클론과 claudin 18.2 발현 평가 방법을 차용하여 향후 임상적으로 적용하기 용이한 일관성을 제공하고자 했다.

본 연구는 2부로 구성되어 있다. 1부는 1-3기 위암 환자들을 대상으로 하며, 이는 상기 임상시험의 성공적인 결과를 고려할 때 가까운 미래에 절제 가능한 단계의 위암 환자들에게도 술후항암요법 등의 방식으로 claudin 18.2 표적치료가 적용될 가능성이 높다고 판단되기 때문이다. 이전까지 절제 불가능한 진행성 위암 환자에서 주로 조사되었던 claudin 18.2 발현 여부와 환자의 역학적·병리학적 특성 및 생존 지표의 관련성을 1-3기 환자에서 동일한 방식을 사용하여 탐색하였다. 그 결과 전체 299명의 환자 중 46.5%에서 claudin 18.2 양성을 확인하였으며, 1기 환자에서 51.1%로 소폭 높은 비율을 보였다. 또한 claudin 18.2 양성은 비교적 젊은 나이 (중간값 61세 vs. 66세, $p<0.001$) 및 얕은 침습 깊이 ($p=0.014$)와 연관되어 있었으며, 육안 상 Borrmann type 4 ($p=0.008$) 를 자주 보였고 조직학적으로 diffuse type ($p=0.011$)과 관련되어 있었다. 그러나 claudin 18.2 양성이 생존 지표에 대한 독립적인 예후 인자는 아닌 것으로 확인되었으며, 이는 절제 불가능한 진행성 위암 환자들을 대상으로 진행된 선행 연구들과 일치하는 결과였다.

2부에서는 4기 위암 환자 166명을 대상으로 claudin 18.2 발현의 이질성에 주목하였다. 전체 환자들 중 원발 병소와 전이 부위에 대한 생검 조직이 모두 확보된 135명을 대상으로 분석한 결과, 원발 병소와 전이 부위 간 claudin 18.2 발현 양상은 50% 이상의 일치율을 보였다. 특히, 복막 전이가 있는 환자에서 원발 병소 및 전이 부위를 포함하여 claudin 18.2 양성 비율이 가장 높았으며, 이를 통해 복막 전이가 있는 환자군에서 claudin 18.2 표적치료의 효과가 가장 높을 가능성이 있음을 시사한다. 또한, ROC 곡선을 사용하여 claudin 18.2 표적치료의 대상 환자를 선별하기 위한 H-score 및 claudin 18.2 발현 세포 비율의 기준값을 계산하였으며, 잠정적으로 효과를 볼 수 있을 것으로 생각되는 최대한의 환자들을 포함시키기 위해 H-score의 경우 120 이상, claudin 18.2 발현 세포 비율은 30% 이상으로 도출하였다. 마지막으로, 수술 검체의 대표 단면을 통해 단일 종양 내에서의 claudin 18.2 발현의 이질성이 비교적 높은 점을 확인하였고, 이를 통해 내시경적 조직 검사는 전체 종양의 claudin 18.2 발현 양상을 대변함에 있어 한계가 있다는 점을 지적하였다.