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Clinical Implications of Claudin18.2 Expression in Patients With Gastric Cancer

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Abstract. *Background/Aim: Claudin18.2 (CLDN18.2) is a tight junction protein that has been identified as a promising target in gastric cancer. This study aimed to evaluate the clinical relevance of CLDN18.2 expression in gastric cancer. Patients and Methods: This study included 367 patients diagnosed with gastric cancer, who underwent curative surgical resection. Immunohistochemical staining for CLDN18.2 was carried out, and expression was scored semi-quantitatively, based on staining intensity and the percentage of staining. Results: CLDN18.2 expression was observed in 273 patients (74.4%), and 108 (29.4%) were classified as CLDN18.2-positive by predefined criteria. CLDN18.2 expression was not correlated with age, sex, tumor location, or stage. Expression rates were higher in diffuse-type and HER2-positive tumors. In multivariate survival analysis, CLDN18.2 expression was not associated with survival outcomes. Conclusion: Higher expression of CLDN18.2 was observed in diffuse-type and HER2-positive gastric cancers. Meanwhile, CLDN18.2 expression was not associated with survival in patients with gastric cancer.*

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Gastric cancer is the fifth most common malignancy and the third-leading cause of cancer-related mortality worldwide, even though rapid advances in treatment options have improved its prognosis (1). Recently, remarkable progress in tumor biology has led to the development of new therapeutics that target critical aspects of oncogenic pathways or the immune system. In advanced gastric cancer (AGC), various targeted agents have already been evaluated in randomized studies, where trastuzumab [anti-human epidermal growth factor receptor 2 (HER2) monoclonal antibody] exhibited anti-tumor activity against 15-20% of HER2-positive AGCs (2), while ramucirumab [anti-vascular endothelial growth factor receptor 2 (VEGFR2) monoclonal antibody] and nivolumab [anti-programmed cell death protein 1 (PD-1) monoclonal antibody] improved survival duration in a second- or third-line setting (3, 4). However, most responses to chemotherapy with targeted agents are limited and of short duration: median survival is 10-16 months, and overall survival (OS) at 2 years rarely exceeds 10% (5). Given these results, the development of new targeted agents with improved efficacy and reasonable toxicity, and which extend the treatment possibilities in AGC, is urgently needed.

The claudin (CLDN) family of transmembrane proteins has a crucial role in the formation of tight junctions and comprises at least 27 members (6). CLDNs are associated with multimolecular complexes and transduction of cell signaling pathways (7), and have also been reportedly associated with regulation of proliferation and differentiation through interactions with signaling proteins (8). Previous studies have reported that the expression levels of CLDNs are altered in various cancers compared to normal tissues (9). Among CLDNs, claudin18.2 (CLDN18.2) is not expressed

in any healthy tissues, except the stomach mucosa, but is broadly expressed in gastric cancers, especially of the diffuse type (10).

Zolbetuximab (formerly known as IMAB362) is a monoclonal antibody specific to CLDN18.2. A recent randomized phase II study demonstrated that the addition of zolbetuximab prolonged survival and improved objective response rate relative to chemotherapy alone. Therefore, CLDN18.2 was identified as a promising treatment target in patients with AGC or gastro-esophageal junction cancer and CLDN18.2 overexpression (11).

In previous studies, the expression rate of CLDN18.2 was found to be inconsistent. In a Japanese study, the positive rate of CLDN18.2 was observed in 52% of primary gastric tumors and 45% of lymph node metastases (12). However, positive rates of 42.2% and 48.0% were reported in Caucasian studies (11, 13). Moreover, CLDN18.2 expression was not associated with survival outcomes (13).

Accordingly, the present study investigated the clinical relevance and prognostic impact of CLDN18.2 expression in a large population of Korean patients with localized gastric cancer who underwent surgical resection.

Patients and Methods

Patients and treatment. This study retrospectively reviewed 938 patients diagnosed with gastric cancer and who underwent curative surgical resection at Ulsan University Hospital between January 2012 and December 2017. The patients were enrolled according to the following criteria: 1) pathologically diagnosed with primary gastric adenocarcinoma; and stage II or III gastric cancer classified by the American Joint Committee on Cancer staging (7th edition) (14). A total of 268 patients met these criteria and were included in the study. Another 99 patients with stage I gastric cancer were included for comparing the expression status of CLDN18.2 by stage. Patient records were also reviewed for data regarding medical history, age, sex, adjuvant chemotherapy, surgical methods, and pathologic results.

The adjuvant chemotherapy for stage II or III patients started 4-6 weeks after surgery with tegafur/gimeracil/oteracil (15) or capecitabine/oxaliplatin (16). Observation without adjuvant therapy was also an option in the elderly population or patients with Eastern Cooperative Oncology Group (ECOG) performance status ≥3.

Immunohistochemical staining of CLDN18.2 and CLDN11. An immunohistochemical (IHC) study for CLDN18.2 (Abcam, Cambridge, UK; 1:75) was carried out on formalin-fixed paraffin, with a 4-μm thick serial section of tissue embedded using the BOND-MAX system (Leica Biosystems, Wetzlar, Germany), with a bond polymer refine red detection kit, according to the manufacturer's recommended protocol.

The expression of CLDN18.2 was scored semi-quantitatively, based on staining intensity and the percentage of staining. Staining intensity was subclassified as: 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion of staining was scored as: 0, negative; 1, 1-10%; 2, 11-50%; and 3, 51-100%. Cases with a percentage of staining score of 3 (51-100%), and with moderate to strong staining intensity (2 or 3), were defined as positive. The pathologist, without prior clinical or pathologic information, scored expression at 100× magnification under light microscopy. All available areas in the section were evaluated. In the original study design, we also planned to evaluate CLND11 expression, but this expression was very weak in a preliminary study. Therefore, we did not evaluate CLND11 expression.

Statistical analysis. Descriptive statistics are reported as incidences and percentages. Associations between categoric variables were evaluated using the Chi-squared test. Disease-free survival (DFS) was calculated from the date of surgery to the date of tumor recurrence or death from any cause. OS was measured from the date of surgery to death from any cause. In event-free subjects, data were censored at the last follow-up. Survival curves were calculated by the Kaplan–Meier method and were compared by the log-rank test. Multivariate analysis of prognostic factors was carried out using Cox's proportional hazard regression model. The hazard ratio (HR) and 95% confidence interval (CI) were estimated for each factor. A p-value <0.05 was considered statistically significant. The statistical analyses were performed using SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA).

Results

Patient and tumor characteristics. Patient and tumor characteristics are summarized in Table I. The median age was 60 years (range=30-93 years) at the time of surgery, and 253 patients (69.8%) were male. Primary tumors were located in the upper stomach in 52 patients (14.2%), middle stomach in 124 (33.8%), and lower stomach in 191 (52.0%). According to tumor staging, 123 cases (33.5%) were stage III, 145 (39.5%) were stage II, and 99 (27.0%) were stage I. According to Lauren phenotype, 265 patients (72.2%) had intestinal-type, and 96 (26.2%) had diffuse-type. Two hundred and seventy-two patients (74.1%) were HER2 0 or 1+, and 95 (25.9%) were HER2 2+ or 3+, by IHC. Additional fluorescence *in situ* hybridization (FISH) results were not available. We defined HER2 2+ or 3+ cases as positive. Forty-two (29.0%) and 67 patients (46.2%) in stage II, and 63 (51.2%) and 31 (25.2%) in stage III, received capecitabine/oxaliplatin and tegafur/gimeracil/oteracil as adjuvant chemotherapy, respectively.

CLDN18.2 expression status. Overall, CLDN18.2 expression was observed in 273 patients (74.4%), and 108 (29.4%) were classified as positive by predefined criteria. In 74 cases (20.2%), weak (1) staining intensity was observed; and 153 (41.7%) and 46 cases (12.5%) were moderate (2) and strong (3), respectively. The cases of proportion of staining were as follows: 1, 1-10% in 49 cases (13.4%); 2, 11-50% in 102 cases (27.8%); and 3, 51-100% in 122 cases (33.2%).

Association between CLDN18.2 expression and clinico-pathologic features. CLDN18.2 staining intensity, staining proportion, and expression status were not correlated with age, sex, and primary tumor location. T stage, N stage, and tumor,

Table I. *Patient characteristics and CLDN18.2 expression.*

Variables	Total (n=367)	Staining intensity					Staining proportion					CLND18.2 expression		
		0	1	2	3	*p*-Value	0	1	2	3	*p*-Value	Negative (n=259, 70.6%)	Positive (n=108, 29.4%)	*p*-Value
Age														
Median (range)	60 (30-93)											61 (30-93)	60 (32-87)	0.388
Gender						0.728					0.306			0.719
Male	253	70	50	95	38		70	31	71	81		180	73	
Female	114	24	24	58	8		24	18	31	41		79	35	
Primary tumor location						0.097					0.314			0.303
Upper	52	11	7	20	14		11	6	14	21		32	20	
Middle	124	34	28	49	13		34	22	27	41		89	35	
Lower	191	49	39	84	19		49	21	61	60		138	53	
T stage						0.488					0.033			0.192
T1	107	16	34	48	9		16	17	33	41		71	36	
T2	45	13	6	20	6		13	4	11	17		29	16	
T3	107	35	15	38	19		35	13	31	28		81	26	
T4	108	30	19	47	12		30	15	27	36		78	30	
N stage						0.700					0.108			0.497
N0	156	33	42	68	13		33	24	44	55		108	48	
N1	80	19	12	36	13		19	11	23	27		55	25	
N2	66	22	9	23	12		22	3	21	20		49	17	
N3	65	20	11	26	8		20	11	14	20		47	18	
TNM stage						0.318					0.008			0.181
I	99	17	29	48	5		17	15	28	39		65	34	
II	145	34	29	56	26		34	19	46	46		103	42	
III	123	43	16	49	15		43	15	28	37		91	32	
Lauren phenotype						0.063					<0.001			0.001
Intestinal	265	83	41	105	36		83	34	76	72		199	66	
Diffuse	96	10	33	44	9		10	15	26	45		59	37	
Unclassified	6	1	0	4	1		1	0	0	5		1	5	
HER2 status						<0.001					0.003			0.009
0 or 1+	272	79	60	108	25		79	38	74	81		202	70	
2+ or 3+	95	15	14	45	21		15	11	28	41		57	38	

CLND18.2: Claudin18.2; TNM stage: tumor, node, metastasis stage; HER2: human epidermal growth factor receptor 2.

node, metastasis (TNM) stage were not correlated with CLDN18.2 staining intensity or expression status, but staining percentages showed a decreasing relationship with advanced T stage and TNM stage. CLDN18.2 expression by stage was as follows: 34.3% in stage I, 29.0% in stage II, and 26.0% in stage III. A slightly decreasing tendency was detected, but there was no statistically significant correlation between CLDN18.2 expression and TNM stage. Lauren phenotype and HER2 status were correlated with CLDN18.2 expression status. Lauren diffuse-type showed an increasing association with staining percentage and higher positive expression rate. CLDN18.2 staining intensities and percentage showed increasing trends in HER2-positive (2+ or 3+) cases, and the CLDN18.2 expression rate was significantly higher in HER2-positive than HER2-negative (0 or 1+) patients.

CLDN18.2 expression and survival outcomes. With a median follow-up of 917 (115-2571) days, estimated 5-year DFS and OS rates were 73.7% and 74.7%, respectively. Patients aged ≥60 years had significantly lower OS (p=0.044), and statistically insignificantly lower DFS (p=0.127), than patients aged <60 years. Female patients had significantly lower DFS (p=0.018), and statistically insignificantly lower OS (p=0.080), than male patients. There were statistically significant differences in DFS (p<0.001) and OS (p<0.001) according to TMN stage. However, there were no statistically significant differences in DFS and OS according to primary tumor location, Lauren phenotype, or HER2 status. Also, there were no statistically significant differences in DFS (p=0.878) and OS (p=0.914) according to CLDN18.2 expression status (Table II). In the multivariate analysis,

Table II. *Kaplan–Meier cumulative disease-free survival and overall survival.*

Variables	No. of patients	5-yrs DFS (%)	*p*-Value	5-yrs OS (%)	*p*-Value
Overall	367	73.7		74.7	
Age			0.127		0.044
<60	172	79.1		82.1	
≥60	195	68.6		68.1	
Gender			0.018		0.080
Male	253	77.9		78.1	
Female	114	64.2		67.9	
Primary tumor location			0.174		0.103
Upper	52	68.6		63.5	
Middle	124	74.7		76.4	
Lower	191	74.7		76.7	
TNM stage			<0.001		<0.001
I	99				
(3-yrs DFS)		97.0			
(3-yrs OS)				98.0	
II	145	85.7		87.0	
III	123	47.1		52.5	
Lauren phenotype			0.639		0.471
Intestinal	265	73.6		76.2	
Diffuse	96	71.0		70.6	
HER2 status			0.062		0.088
0 or 1+	272	71.0		72.4	
2+ or 3+	95	84.3		83.2	
CLDN18.2 expression			0.878		0.914
Negative	259	75.1		75.6	
Positive	108	68.0		69.6	

DFS: Disease-free survival; OS: overall survival; TNM stage: tumor, node, metastasis stage; HER2: human epidermal growth factor receptor 2; CLND18.2: claudin18.2.

female sex and TNM were identified as independent prognostic factors for DFS (p=0.002 and p<0.001, respectively) and OS (p=0.022 and p<0.001, respectively). In multivariate survival analysis, CLDN18.2 expression status was not an independent prognostic factor for DFS or OS (Table III).

Discussion

In the current study, we investigated the clinical significance of CLDN18.2 expression in localized gastric cancer patients who underwent gastrectomy. Our results showed that CLDN18.2 expression rates were higher in diffuse-type (p=0.001) and HER2-positive (2+/3+; p=0.007) cancers, but CLDN18.2 expression was not correlated with TNM stage. Meanwhile, there were no statistically significant differences in survival outcomes according to CLDN18.2 expression status.

The positive expression rate of CLDN18.2 was 29.4% in the present study, which was lower than that in previous studies. In the randomized, phase II, FAST study, 48% of patients were considered to have a positive expression (11). The CLAUDETECT™ 18.2 kit (Ganymed Pharmaceuticals, Mainz, Germany) was used for IHC, and moderate to strong CLDN18.2 expression and membrane staining intensity ≥2+ in ≥40% of cancer cells were defined as positive expression. Rohde *et al.* (12) reported that 52% of primary gastric tumors, and 45% of lymph node metastases, showed moderate to strong CLDN18.2 expression in Japanese patients with the CLAUDETECT™ 18.2 kit. Dottermusch *et al.* (13) also reported CLDN18.2 expression in 42.2% of gastric cancer cases in a Caucasian cohort using an anti-CLDN18.2 antibody from Abcam; these researchers used histoscore for expression scoring. In the current study, we used a semi-quantitative scoring method for staining intensity and percentage of staining. We defined cases with a percentage of staining score of 3 (51-100%) and with moderate to strong staining intensity (2 or 3) as positive. Moreover, an Abcam monoclonal antibody against CLDN18.2 was used due to unavailability of the CLAUDETECT™ 18.2 kit. Given these results, some differences in expression rates may be due to differences in monoclonal antibodies used for detection, stages, and predefined criteria. Plus, ethnic differences need to be further evaluated.

In our study, the expression rate of CLDN18.2 was higher in diffuse-type (p=0.001), but not correlated with age, sex, primary tumor location, or TNM stage. Associations between CLDN18.2 expression and clinicopathologic features were reported in some studies. Rohde *et al.* (12) reported that CLDN18.2 expression was significantly higher in diffuse-type and high-grade (G3) tumors than in other tumors. These investigators also reported that the fraction of tumor cells expressing CLDN18.2 at any staining intensity correlated strongly with the primary tumor and lymph node metastases, but T stage and N stage were not associated with CLDN18.2 expression. CLDN18-ARHGAP26 fusions have been identified in gastric cancers, with a predominance in diffuse-type gastric cancers (17, 18). Tanaka *et al.* (19) reported that almost all CLDN18-ARHGAP26 fusion-positive cancers showed positive CLDN18 immunostaining and CLDN18 translocation, and are significantly characterized by CLND18 overexpression.

The expression rate of CLDN18.2 was also higher in HER2 positive (2+/3+; p=0.007) tumors in the current study. However, Dottermusch *et al.* (13) reported that CLDN18.2 expression was not correlated with HER2 status or MET status. Sahin *et al.* (10) demonstrated that activation of human CLDN18.2 depends on binding of the transcription factor cAMP-responsive element-binding protein (CREB) to its unmethylated consensus site. An important role for HER-2/neu has been suggested for CREB expression and activity. Steven *et al.* (20) also reported a positive correlation of HER-2/neu and pCREB in breast cancer lesions by IHC

Table III. *Multivariate analysis of disease-free survival and overall survival.*

	DFS			OS		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age (<60 *vs*. ≥60)	1.204	0.754-1.924	0.436	1.534	0.890-2.645	0.124
Gender (Male *vs*. Female)	2.115	1.328-3.367	0.002	1.863	1.094-3.172	0.022
TNM stage			<0.001			<0.001
I *vs*. II	4.787	1.090-21.024	0.038	5.236	0.669-40.949	0.115
I *vs*. III	30.288	7.365-124.567	<0.001	32.721	4.479-239.026	0.001
CLDN18.2 expression (Negative *vs*. Positive)	1.221	0.733-2.033	0.443	1.146	0.621-2.113	0.663

DFS: Disease-free survival; OS: overall survival; HR: hazard ratio; CI: confidence interval; TNM stage: tumor, node, metastasis stage; CLND18.2: claudin18.2.

staining. Accordingly, further studies are warranted to validate the associations between CLDN18.2 and HER2 in gastric cancer. The co-expression of CLDN18.2 and HER2 was observed in 25.9% of patients in the present study and in 13.8% in the FAST study (21). Moran *et al*. (22) have also reported that 12% of cases showed CLDN18.2 and HER2 co-expression. Accordingly, dual targeting strategy (anti-HER2 and anti-CLDN18.2 monoclonal antibodies) could be a possible treatment option for these patients with co-expression of CLDN18.2 and HER2.

Some reports suggest that CLDN18.2 expression decreases as cancer progresses, contributing to the invasive potential of tumor cells and to metastasis (23, 24). Although, there were no correlations between CLDN18.2 expression and TNM stage or survival in our study, Jun *et al*. reported that reduced CLDN18 expression correlated with perineural invasion and poor overall survival (23). Therefore, further studies are needed to clarify the role of CLDN18.2 in gastric cancer progression and survival.

In summary, CLDN18.2 expression was positive in 29.4% of patients by predefined criteria in the present population. Although, CLDN18.2 expression was not associated with survival outcomes, CLDN18.2 expression rates were higher in diffuse-type and HER2 positive (2+/3+). Accordingly, further studies are warranted to standardize detection methods and to validate the clinical significance of CLDN18.2 in gastric cancer.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

JH Baek contributed to data acquisition, analysis, drafting and revising the manuscript. DJ Park and GY Kim performed surgical treatments and contributed to critically revising and approving the final version of the manuscript. J Cheon contributed to data acquisition and critically revising and approving the final version of the manuscript. BW Kang contributed to data analysis and critically revising and approving the final version of the manuscript. HJ Cha examined pathological findings and contributed to analysis, drafting and revising the manuscript. JG Kim conceived and designed the study, interpreted the data, and contributed to drafting, critically revising and approving the final version of the manuscript.

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