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Loss of claudin-1 expression induces epithelial-mesenchymal transition through nuclear factor-κB activation in colorectal cancer

Nae Yu Kim[a], Jung-Soo Pyo[b,*], Dong-Wook Kang[b], Seung-Min Yoo[c]

[a] Department of Internal Medicine, Eulji University Hospital, Eulji University School of Medicine, Daejeon, Republic of Korea
[b] Department of Pathology, Eulji University Hospital, Eulji University School of Medicine, Daejeon, Republic of Korea
[c] Department of Microbiology and Immunology, Eulji University School of Medicine, Daejeon, Republic of Korea

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ABSTRACT

Objective: The aim of this study was to elucidate the clinicopathological significance and prognostic role of loss of claudin-1 in colorectal cancer (CRC).
Methods: The correlations between claudin-1 expression and clinicopathological characteristics, including survival rates, were assessed using immunohistochemistry on 260 archival, paraffin-embedded CRC tissues. In addition, the correlations between cludin-1 and nuclear factor-kappa B (NF-κB), epithelial-mesenchymal transition markers and tumor-infiltrating lymphocytes were investigated.
Results: Claudin-1 expression was markedly lost in 42.7% of the 260 CRCs analyzed. Loss of claudin-1 expression significantly correlated with larger tumor size, vascular invasion, higher pT stage, and high metastatic lymph node ratio. In addition, loss of claudin-1 expression significantly correlated with NF-κB activation ($P < 0.001$), high SNAI ($P < 0.001$), and low E-cadherin ($P < 0.001$) expressions. Patients with high immunoscores showed significantly lower rates of claudin-1 expression loss ($P = 0.020$). In detail, loss of claudin-1 expression were frequently found in CRCs low CD3- and CD8-positive lymphocytes. There were significant correlations between claudin-1 expression loss and poor overall and recurrence-free survivals ($P < 0.001$ and $P < 0.001$, respectively).
Conclusion: Taken together, our results suggest that the loss of claudin-1 expression significantly correlates with aggressive tumor behaviors, high SNAI expression, lower immunoscore, and poor prognoses.

1. Introduction

Claudins are integral membrane proteins involved in the formation of tight junctions [1]. Additionally, the functions of claudins are regulation of the differentiation, proliferation, and migration of epithelial cells [1–3], and claudin expression is elevated in various malignant epithelial tumors [4–6]. Claudin-1 mRNA expression has been found to be increased in colorectal cancer (CRC), compared to in colonic mucosa [7,8]. In addition, claudin-1 has been correlated with colon cancer tumorigenesis [8]; however, other reports have shown loss of claudin-1 expression in CRCs significantly correlated with aggressive tumor behaviors [9–11], as observed for other malignant tumors, such as oral squamous cell carcinoma [12]. Therefore, the clinicopathological significance of claudin-1 expression has not been fully elucidated. The present study investigates the clinicopathological significance and prognostic implication of claudin-1 expression loss and its correlation with NF-κB activation and epithelial-mesenchymal transition markers and tumor-infiltrating lymphocytes, through immunohistochemistry in human CRC.

2. Materials and methods

2.1. Patients and tissue array methods

The files of 260 patients who had undergone surgical resections for CRC at the Eulji University Medical Center, between January 1, 2001 and December 31, 2010, were analyzed. We reviewed the medical charts, pathological records, and glass slides, in order to assess clinicopathological characteristics, such as age, sex, tumor size, tumor location, tumor differentiation, vascular, lymphatic, and perineural invasion, tumor depth, lymph node metastasis, metastatic lymph node ratio, distant metastasis, and pathologic tumor node metastatic (pTNM)

* Corresponding author at: Department of Pathology, Eulji University Hospital, Eulji University School of Medicine, 95 Dunsanseo-ro, Seo-gu, Daejeon, 35233, Republic of Korea.
E-mail address: jspyo@eulji.ac.kr (J.-S. Pyo).

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stage. These cases were evaluated according to the 8th Edition of the American Joint Cancer Committee TNM classification [13]. This protocol was reviewed and approved by the Institutional Review Board of the Eulji Univeristy Hospital (Approval No. EMC 2018-03-012). Five array blocks containing a total of 260 resected CRC tissue cores that were obtained from the patients were prepared. Briefly, tissue cores (2 mm in diameter) were taken from individual paraffin-embedded CRC tissues (donor blocks) and arranged in recipient paraffin blocks, using a trephine apparatus, as previously described [14]. The staining results of the different intratumoral areas in these tissue-array blocks showed excellent agreement. A core was chosen from each case for analysis. An adequate case was defined as a tumor that occupied more than 10% of the core area. Each block contained internal controls, consisting of non-neoplastic colon tissue. Sections that were 4 μm in thickness were cut from each tissue-array block, deparaffinized, and dehydrated. Overall survival and recurrence-free survival were respectively defined as the time from the date of surgery to the date of death and recurrence, and the follow-up periods ranged from 0 to 60 months.

2.2. Immunohistochemical staining

Sections were deparaffinized and hydrated using a routine xylene-alcohol series. For antigen retrieval, sections were treated with 0.01 M citrate buffer (pH 6.0) for 5 min in a microwave oven, followed by treatment with 3% H_2O_2, to quench endogenous peroxidase. Sections were treated with normal serum of the host animal of the secondary antibody, to block nonspecific binding. Sections were then incubated with anti-claudin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorlyated NF-κB (pNF-κB) p65 (Santa Cruz Biotechnology), anti-SNAI (Santa Cruz Biotechnology), anti-E-cadherin (Santa Cruz Biotechnology), anti-CD3 (Leica Biosystems, Newcastle Upon Tyne, UK), and anti-CD8 (Leica Biosystems). Immunohistochemical stainings were conducted following a compact polymer method, using a VENTANA benchmark XT autostainer (Ventana Medical Systems, Inc., Tucson, AZ). Visualization was performed by treatment with OPTIVIEW universal 3,3′-diaminobenzidine kit (Ventana Medical Systems, Inc.). To confirm the reaction specificity of the antibody, a negative control stain without primary antibody was performed. All immunostained sections were lightly counterstained with Mayer's hematoxylin.

2.3. Evaluation of immunohistochemistry

Immunoreactivity for claudin-1 was observed in the membrane and cytoplasm of tumor cells. Immunoreactivities for SNAI and E-cadherin were observed in the nucleus and cell membrane, respectively. The intensities of protein expression in the immunohistochemically-stained samples were scored from 0 to 3 (0 = negative; 1 = weak; 2 = moderate; and 3 = strong). The percentage of positively stained cells was categorized based on a scoring system from 0 to 4 (1 = 0–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100%). An immunoreactive score (IRS) was calculated by multiplying the staining intensities scores with the percentages of positively stained cells [15]. When the IRS was 0–4 or 6–12, claudin-1 expressions were classified as marked loss of expression and mild or no loss of claudin-1 expression, respectively. In the assessments of SNAI and E-cadherin expression, staining patterns were classified as low (IRS: 0–4) or high (IRS: 6–12). In addition, because NF-κB is constitutively expressed in the cytoplasm, tumor cells showing nuclear pNF-κB p65 staining, regardless of cytoplasmic staining, were considered to show NF-κB activation. NF-κB positivity was defined as unequivocal brown nuclear pNF-κB staining in ≥5% of tumor cells [14].

To determine the immunoscore (IS), all immunohistochemically-stained slides for CD3 and CD8 were scanned using Pannoramic MIDI II (3DHISTECH, Budapest, Hungary). Images were captured from two regions, the tumor core and the invasive margin, using CaseViewer 2.0 (3DHISTECH). From the captured images, CD3- and CD8-immunoreactive lymphocytes were qualified using NIH Image Analysis software (version 1.6.0, National Institute of Health, Bethesda, MD, USA), after setting one consistent intensity threshold. At each region, CD3- and CD8-immunoreactive lymphocytes were expressed as pixels. In the present study, the cut-offs used the median values of the pixels in each region. From the cut-off vales, patients were classified into two groups: high (score 1) and low (score 0). Immunoscore was defined as the sum of the scores of the two regions and was divided into high (IS 3–4) and low (IS 0–2) scores [16].

2.4. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 software (IBM Co., Chicago, IL, USA). The significance of the correlation between claudin-1 expression and clinicopathological characteristics, epithelial-mesenchymal transition markers, and immunoscore was determined by either χ^2 test or Fisher's exact test (two-sided). The comparisons between claudin-1 expression and age, tumor size, or metastatic lymph node ratio were analyzed using the two-tailed Student's *t*-test Survival curves were estimated using the Kaplan-Meier product-limit method, and differences between the survival curves were determined to be significant based on the log-rank test. Results with P values < 0.05 were considered statistically significant.

3. Results

3.1. The clinicopathological significance of claudin-1 expression loss in CRC

Loss of claudin-1 expression was found in 111 of 260 CRCs (42.7%). Fig. 1 shows representative figures of claudin-1, SANI, and E-cadherin expression in CRC. Loss of claudin-1 expression significantly correlated with larger tumor size, vascular invasion, higher pT stage, and high metastatic lymph node ratio (Table 1). There was a significant correlation between loss of claudin-1 expression and poor tumor differentiation ($P = 0.003$). CRCs with loss of claudin-1 expression were frequently found in left-sided colon and rectum ($P = 0.004$). However, there were no significant differences between loss of claudin-1 expression and other parameters, such as age, sex, lymphatic and perineural invasion, lymph node metastasis, distant metastasis, and pTNM stage.

3.2. The correlation between claudin-1 expression and NF-κB, epithelial-mesenchymal transition markers, and immunoscore

Nuclear pNF-κB expression was frequently found in CRCs with loss of claudin-1 expression than in CRCs without loss of claudin-1 expression ($P < 0.001$; Table 2). Because NF-κB activation can be significantly correlated with epithelial-mesenchymal transition, the correlations between claudin-1 expression and epithelial-mesenchymal transition markers, including SNAI and E-cadherin were investigated. Loss of claudin-1 expression significantly correlated with high SNAI expression and low E-cadherin expression ($P < 0.001$ and $P < 0.001$, respectively). In CRCs with loss of claudin-1 expression, high SNAI and low E-cadherin expression were found in 64.0% and 49.5%, respectively, of the 111 cases. In addition, nuclear pNF-κB expression was significantly correlated with SNAI expression, but not E-cadherin ($P = 0.001$ and $P = 0.332$, respectively). Next, claudin-1 expression was investigated according to tumor-infiltrating lymphocytes. Loss of claudin-1 expression was less frequently found in patients with high IS (score 3–4) than patients with low IS (score 0–2) ($P = 0.020$; Table 3). In detail, loss of claudin-1 expression was also correlated with CRCs low CD3- and CD8-positive lymphocytes.

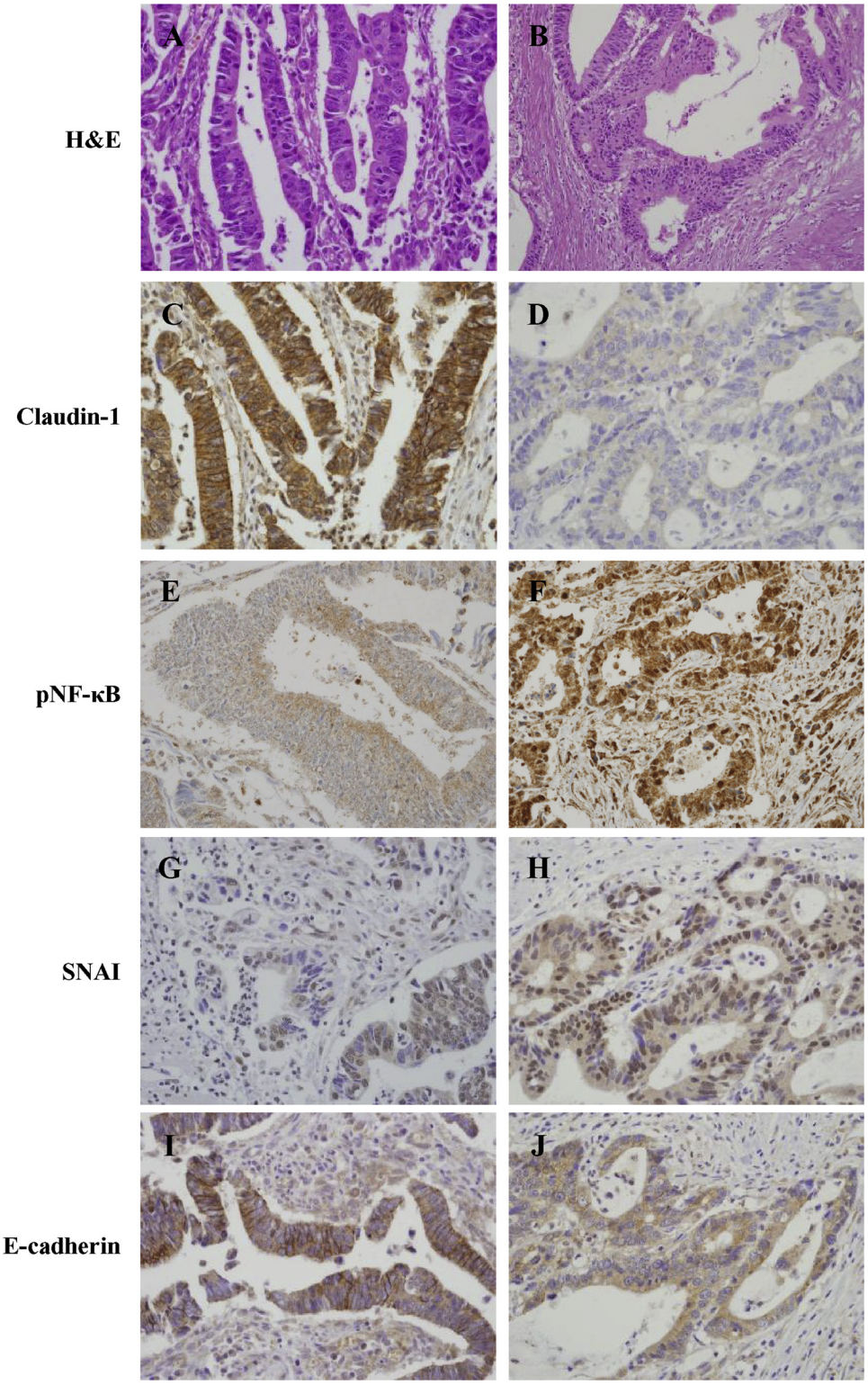

Fig. 1. Representative images showing the immunoreactivity for claudin-1, SNAI, and E-cadherin in colorectal cancer. (A and B) Hematoxylin and eosin staining. (C and D) Immunohistochemistry for claudin-1. (E and F) Immunohistochemistry for nuclear pNF-κB expression. (G and H). Immunohistochemistry for SNAI. (I and J) Immunohistochemistry for E-cadherin (×400). (pNF-κB, phosphorylated NF-κB).

3.3. The correlation between claudin-1 expression and survival

Patients with CRCs with loss of claudin-1 expression had worse overall and recurrence-free survivals compared to those with claudin-1 expression ($P < 0.001$ and $P < 0.001$, respectively; Fig. 2). However, there were no significant differences between SNAI and E-cadherin expression and worse survival (data not shown).

4. Discussion

In CRCs, the prognostic role of claudin-1 has been reported, like in other malignant tumors [9–11,17,18]. However, the correlations

Table 1
The correlation between claudin-1 expression and clinicopathological parameters in colorectal cancers.

	Claudin-1 expression		*P-value*
	Marked loss	Mild or no loss	
Total (n = 260)	111 (42.7)	149 (57.3)	
Age (years)	64.60 ± 13.18	62.68 ± 12.70	0.237
Sex			
Male	57 (51.4)	73 (49.0)	0.707
Female	54 (48.6)	76 (51.0)	
Tumor size			
≤5 cm	34 (30.6)	69 (46.3)	0.011
> 5 cm	77 (69.4)	80 (53.7)	
Tumor size (cm)	5.97 ± 2.33	5.13 ± 1.80	0.002
Location of tumor			
right colon	41 (36.9)	82 (55.0)	0.004
left colon	70 (63.1)	67 (45.0)	
Tumor differentiation			
Well or Moderate	80 (70.3)	127 (85.2)	0.003
Poorly	33 (29.7)	22 (14.8)	
Vascular invasion			
Present	16 (14.4)	7 (4.7)	0.006
Absent	95 (85.6)	142 (95.3)	
Lymphatic invasion			
Present	34 (30.6)	34 (22.8)	0.156
Absent	77 (69.4)	115 (77.2)	
Perineural invasion			
Present	22 (19.8)	20 (13.4)	0.166
Absent	89 (80.2)	129 (86.6)	
pT stage			
pT1-2	11 (9.9)	28 (18.8)	0.047
pT3-4	100 (90.1)	121 (81.2)	
Lymph node metastasis			
Present	65 (58.6)	80 (53.7)	0.434
Absent	46 (41.4)	69 (46.3)	
Metastatic lymph node ratio	0.18 ± 0.26	0.10 ± 0.19	0.016
Distant metastasis			
Present	13 (11.7)	16 (10.7)	0.805
Absent	98 (88.3)	133 (89.3)	
pTNM stage			
I–II	46 (41.4)	66 (44.3)	0.646
III–IV	65 (58.6)	83 (55.7)	

Numbers in parentheses represent percentage.

Table 2
The correlation between claudin-1 expression and nuclear factor-kappa B (NF-κB) and epithelial-mesenchymal transition markers in human colorectal cancers.

	Claudin-1 expression		*P-value*
	Marked loss	Mild or no loss	
Total (n = 260)	111 (42.7)	149 (57.3)	
NF-κB activation			
Positive	84 (75.7)	79 (53.0)	< 0.001
Negative	27 (24.3)	70 (47.0)	
SNAI expression			
Positive	71 (64.0)	61 (40.9)	< 0.001
Negative	40 (36.0)	88 (59.1)	
E-cadherin expression			
Positive	55 (49.5)	118 (79.2)	< 0.001
Negative	56 (50.5)	31 (20.8)	

Numbers in parentheses represent percentage.

between claudin-1 expression and clinicopathological characteristics are controversial [9–11,17,18]. The present study aims to obtain confirmative information for the clinicopathological significance and prognostic role of claudin-1 expression, through an immunohistochemical analysis of human colorectal cancer tissue.

Claudin-1 expression could differ between colorectal adenocarcinoma, adenoma, and normal mucosa [19]. Also, there was a significant

Table 3
The correlation between claudin-1 expression and tumor-infiltrating lymphocytes in human colorectal cancers.

	Claudin-1 expression		*P-value*
	Marked loss	Mild or no loss	
Total (n = 260)	111 (42.7)	149 (57.3)	
Immunoscore			
High (3–4)	37 (33.3)	71 (47.7)	0.020
Low (0–2)	74 (66.7)	78 (52.3)	
CD3-positive lymphoctes			
High	24 (21.6)	65 (43.6)	< 0.001
Low	87 (78.4)	84 (56.4)	
CD3-positive lymphoctes			
High	23 (20.7)	67 (45.0)	< 0.001
Low	88 (79.3)	82 (55.0)	

Numbers in parentheses represent percentage.

difference in the subcellular localization of claudin-1 [19]. Compared to normal colonic mucosa, membranous claudin-1 expression was frequently lost in adenocarcinoma. According to previous studies, loss of claudin-1 expression was found in up to 79.4% of CRC [9–11,17,18]. However, other studies also reported that claudin-1 was up-regulated in CRC [4,7,8,20,21]. Kinugasa et al. reported that there was no significant correlation between claudin-1 expression and clinicopathological characteristics [22]. Oliveira et al. showed that claudin-1 protein expression increased in the membrane/cytoskeleton fraction of colon cancer cells [7]. However, this result was obtained from colon cancer cells, not human tissue. In the present study, significant loss of claudin-1 expression was found in 42.7% of 260 CRCs. In addition, loss of claudin-1 expression significantly correlated with aggressive tumor behavior and worse prognosis. Although loss of claudin-1 expression did not correlate with lymphatic invasion and lymph node metastasis, patients with loss of claudin-1 expression had higher metastatic lymph node ratios. Based on our data, loss of claudin-1 expression can be useful for predicting the prognosis of patients with CRC. In the previous meta-analysis, low expression of claudin-1 was significantly correlated with poor overall survival [23]. However, in all eligible studies, no significantly correlation between low expression of claudin-1 and poor overall survival from estimated hazard ratio and 95% confidence intervals [9–11,18,24]. Unlike our data, Resnick et al. reported that loss of claudin-1 expression did not correlate with clinicopathological characteristics [18]. Additionally, some studies reported that loss of claudin-1 expression did not correlate with worse survival [23]. Various factors, including different population and cut-offs, could affect on controversial results. In the previous studies, the cut-offs for loss of claudin-1 expression varied [9–11,18,24]. The large range of claudin-1 expression loss (12.5–79.4%) could be caused by variable cut-offs. In addition, Nakagawa's study was not evaluated claudin-1 protein expression [24]. From the present and previous studies, loss of claudin-1 expression, rather than claudin-1 overexpression was predictive of worse survival.

In the present study, loss of claudin-1 expression was significantly with depth of tumor. Therefore, the correlation between claudin-1 expression and epithelial-mesenchymal transition marker is important in understanding claudin-1-associated mechanism in CRC. However, in human CRC tissue, the impact of claudin-1 on epithelial-mesenchymal transition has not been fully elucidated. In our data, CRCs with loss of claudin-1 expression showed higher expression of SNAI and lower expression of E-cadherin than CRCs with no or mild loss of claudin-1 expression. However, significant correlations between SNAI and E-cadherin expression and prognoses were not found. Low-adherent cancer cells showed higher expression of epithelial-mesenchymal markers, such as SNAI and SLUG [24]. In addition, cluadin-1 and E-cadherin expressions in low-adherent cancer cells was lower than in highly-adherent cancer cells [22]. Colon cancer cells showed downregulated

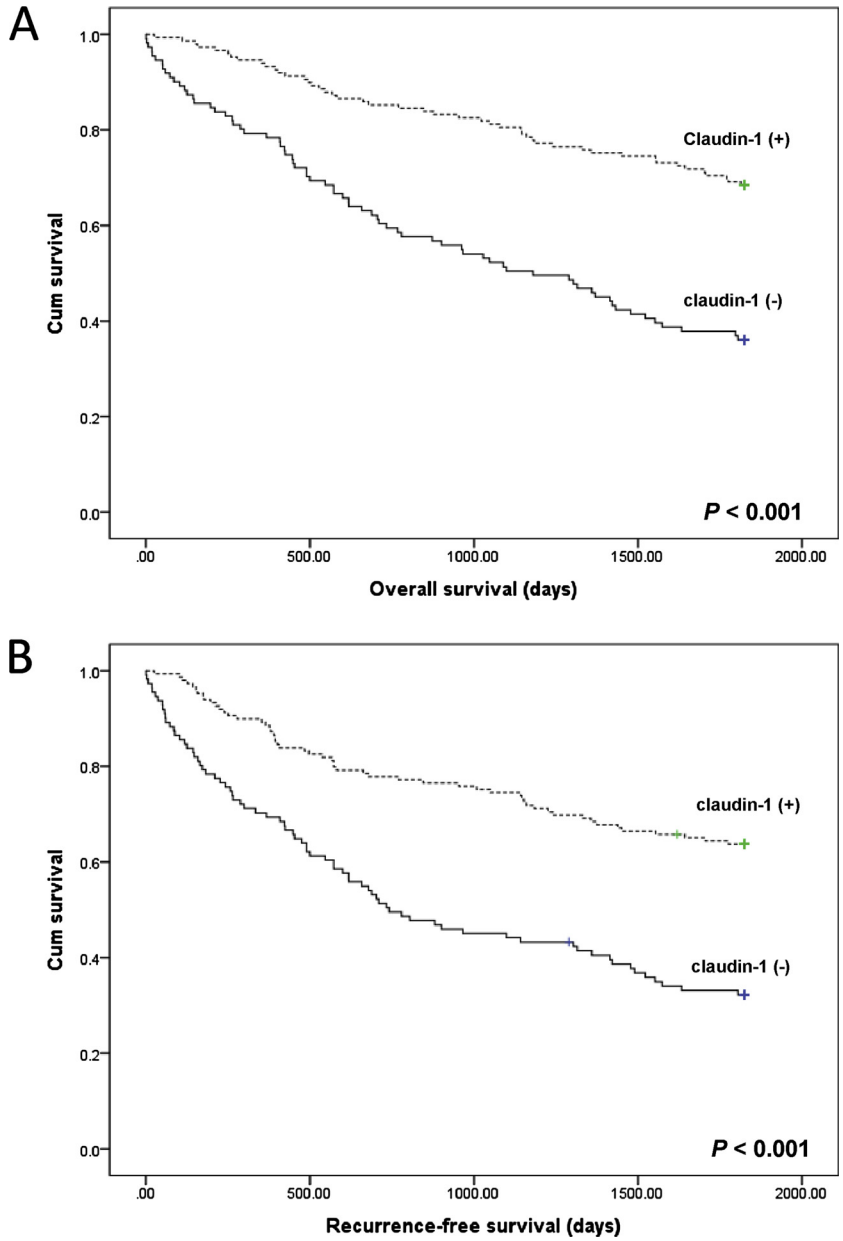

Fig. 2. Kaplan-Meier curves for patient survival according to claudin-1 immunoreactivity. Patients with loss of claudin-1 expression (solid line) and with claudin-1 expression (dotted line) showed significant differences in overall (**A**) and recurrence-free (**B**) survival.

claudin-1 and E-cadherin and upregulated SNAI mRNA levels, compared to normal colonic mucosa [25]. In colon cancer cells, increased claudin-1 expression and decreased E-cadherin expression were found [7]. These results were opposite to ours, and the discrepancies could be caused by difference between human cancer tissue and colon cancer cells. Certainly, in the present study, some patients showed increased claudin-1 expression and decreased E-cadherin expression; however, loss of claudin-1 expression significantly correlated with low E-cadherin expression. From Singh's report, claudin-1 might downregulate E-cadherin expression through ZEB-1 [1]. In addition, the current study investigated the correlations between NF-κB activation and expressions of claudin-1 and epithelial-mesenchymal transition markers in CRC. NF-κB activation was significantly correlated with increased SNAI and decreased E-cadherin expressions. Interestingly, CRCs with loss of claudin-1 expression showed frequent activation of NF-κB compared to CRCs without loss of claudin-1 expression. Although the correlation between loss of claudin-1 expression and NF-κB activation was found in the current study, further evaluation of detailed pathway is needed in CRC.

In breast cancer, low claudin expressing tumors had increased tumor-infiltrating lymphocytes [26]. However, in CRC, the correlations between claudin-1 expression and tumor-infiltrating lymphocyte remains unclear. This study is the first, to the best of our knowledge, to show the correlation between claudin-1 expression and tumor-infiltrating lymphocytes using human CRC tissue. The loss of claudin-1 expression significantly correlated with low IS ($P = 0.020$). That is, CRCs with increased tumor-infiltrating lymphocytes had significantly lower rates of claudin-1 expression loss. Because low IS significantly correlated with higher tumor depth (pT3–4) (data not shown), both lower tumor-infiltrating lymphocytes and loss of claudin-1 expression could be involved in tumor invasion. Further cumulative studies of the pathway details are required.

In conclusion, the present study showed that loss of claudin-1 expression significantly correlated with aggressive tumor behaviors, high SNAI expression, and low immunoscore in CRC. In addition, patients with loss of claudin-1 expression had worse prognoses than those with

claudin-1 expression. Loss of claudin-1 expression can be useful for predicting tumor behaviors and prognosis in CRC.

Conflicts of interest

None.

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None.

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