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Claudin-1 Expression at the Invasive Front of Human Colorectal Adenocarcinoma

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ABSTRACT

Cell adhesion includes tight junction, adherens junction and desmosome. The claudin family has been identified as the major protein of the tight junction, while the cadherin family has been revealed to be adherence junction proteins. We examined the expression of claudin-1 (CL-1), claudin-4 (CL-4) and E-cadherin (E-cad) in 92 cases of human colorectal adenocarcinoma, and analyzed their clinicopathological significance. Immunohistochemically, CL-1 exhibited low-grade expression in 39.1% (36/92) and high-grade expression in 60.9% (56/92), while E-cad revealed low-grade expression in 62.0% (57/92) and high-grade expression in 38.0% (35/92). Cases with low-grade CL-1 expression, i.e., decreased CL-1 expression, frequently showed lymph node metastasis (63.6%, $p<0.05$) and liver metastasis (27.3%, $p<0.05$). Digital quantitative evaluation demonstrated that CL-1 expression levels at the invasive front (98.3 ± 14.1 arbitrary unit, $p<0.05$) were significantly decreased in colorectal cancer cases with recurrence/metastasis, compared with cases without recurrence/metastasis (107 ± 18.3). In conclusion, decreased CL-1 expression at the invasive front is thought to be an important prognosis prediction factor.

Keywords: Colorectal cancer, Adenocarcinoma, Claudin, Cadherin, Metastasis

INTRODUCTION

Individual cells in epithelial sheets are attached to each other by cell adhesion factors, i.e., tight junction, adherens junction and desmosome [1-3]. The tight junction is located to the apical side of cells, and plays several roles, such as cell-to-cell adhesion, cell polarity and a physical barrier preventing solutes and water from passing freely through the paracellular space. Recent studies have identified a number of integral membrane proteins associated with the tight junction, including occludin, junctional adhesion molecule and claudin. The claudin family consists of 27 four-trans membrane domain proteins. On the other hand, the cadherin family has been discovered to be adherence junction proteins, including E-cadherin (E-cad) (epithelium origin), N-cadherin (nerve origin), P-cadherin (placenta origin), and VE-cadherin (vascular endothelial cell origin).

Colorectal cancer is the third most common cancer in the United States, as well as in Japan [4,5]. When it is discovered in its early stages, colon cancer is treated with surgery and is often cured; however, many patients with colon cancer have no remarkable symptoms until the disease reaches an advanced stage, such as metastasizing to other organs. Colorectal cancer is the second leading cause of cancer deaths in the United States. Recent advances in molecular biology have clarified multi-step carcinogenesis of colorectal cancer, i.e., adenoma-carcinoma sequence [6-8].

In a recent study, claudin-1 (CL-1) was shown to be involved in the β-catenin-Tcf/LEF signaling pathway, and to regulate cellular transformation and metastatic behavior in colon cancer. In addition, the expression of CL-1, claudin-3, and claudin-4 (CL-4) was up regulated in colorectal tumor tissues; however, the immune histochemical localization of

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CL-1, CL4 and E-cad have not yet been extensively analyzed. In this study, we examined the expression of CL-1, CL-4, and E-cad in human colorectal adenocarcinoma, and discuss their clinicopathological significance.

MATERIALS AND METHODS

Colorectal tissue specimens

Tissue samples were obtained from 92 patients with colorectal cancer lesions (59 men and 33 women; mean age 67.2, age: 41 to 88. The cases were surgically resected at Hirosaki University Hospital. The stages of colorectal cancer were classified according to the TNM classification [9]. Tumor budding (sprouting) was defined as a clump of cancer cells having less than five nuclei at the invasive front [10-12].

Histological Examination

Colorectal tissue specimens were rapidly fixed in 10% buffered formalin for 24-48 h for histological and immunohistochemical analyses, and routinely embedded in paraffin. Tumor invasion was examined in sections 4 μm thick stained with hematoxylin and eosin as described previously [13].

Immunohistochemical staining

Sections for immunostaining were routinely deparaffinized in xylene, dehydrated in graded ethanol solutions and washed with phosphate buffered saline (PBS) [14].

Immunostaining of CL-1, CL-4, and E-cad were preceded by digestion in citric acid 0.01 M for 10 min using an autoclave at 121ºC. After rinse in PBS, endogenous peroxidase activity was quenched by dipping the sections into 0.3% hydrogen peroxide and 100% methanol for 20 min. The sections were then incubated with normal goat serum for 15 min at room temperature. The sections were subsequently incubated with (a) the first antibodies of CL-1 (claudin-1 rabbit polyclonal antibody diluted 1:100 (Zymed, South San Francisco, CA)), CL-4 (claudin-4 mouse monoclonal antibody diluted 1:400 (Zymed)), and E-cad (E-cadherin mouse monoclonal antibody diluted 1:50 (Takara, Tokyo, Japan)) overnight at 4ºC; (b) secondary antibodies (goat-anti-rabbit antibody, SAB-PO (R) kit or rabbit-anti-mouse antibody, SAB-PO (M) kit; Nichirei, Tokyo, Japan) followed by incubation with for 20 min at room temperature; (c) peroxidase-conjugated avidin for 20 min at room temperature; and (d) a mixture of 0.1% hydrogen peroxidase and 0.05% diaminobenzidine for 30-60 sec. The sections were counterstained with hematoxylin.

Histological semiquantitative evaluation

Immuno reactivity was evaluated at the invasive front (vertical advanced margin), lateral front (lateral advanced margin) and central part of each colorectal cancer tissue. Immunohistochemical expression patterns of CL-1, CL-4, and E-cad were divided into four groups as follows: -, negative; +1, <30% positive at the cell membrane and weakly positive at the cell cytoplasm; +2, >30% positive at the cell membrane and weakly positive at the cell cytoplasm or <30% positive at the cell membrane and moderately positive at the cell cytoplasm; +3, >30% positive at the cell membrane and moderately positive at the cell cytoplasm.

Digital quantitative evaluation

Computer digital scanning was performed. Slice preparations of colorectal cancers were scanned at 1200 dpi (1200 pixels/inch). The color component of scanned preparation was divided for RGB (red-green-blue). The red component was (r), the green component was (g), and the blue component was (b), defined respectively. The intensity of the immunohistochemical expression was represented as 100 x r/b. These mean expression levels were quantitatively evaluated at the invasive front (200 x 50 pixels), and in the entire tumor. The expression level in non-neoplastic glands was defined as 100.

Statistical analyses

The expressions of CL-1, CL-4 and E-cad for histological evaluation and clinicopathological parameters were examined using Fisher's exact test and the Chi-squared test (software; SPSS for Windows, version 12.0 [Chicago, IL, USA]). The expression of CL-1 for digital evaluation and clinicopathological parameters were examined using Student's T test (software; SPSS version 12.0). P values were two-tailed, and a P value < 0.05 was statistically significant.

RESULTS

The invasive front of colorectal cancer cases ubiquitously expressed CL-1, i.e., +1 or +2 (low-grade expression) in 35.9% (33/92) and +3 (high-grade expression) in 64.1% (59/92) of the colorectal adenocarcinoma tissues examined (**Table 1 & Figure 1**). CL-1 exhibited low-grade expression in 39.1% (36/92) and high-grade expression in 60.9% (56/92), while E-cad revealed low-grade expression in 62.0% (57/92) and high-grade expression in 38.0% (35/92). Low-grade CL-1 expression cases, i.e., decreased CL-1 expression, frequently showed lymph node metastasis (63.6%, $p<0.05$) and liver metastasis (27.3%, $p<0.05$), compared with CL-1 high-grade expression (40.0% and 6.8%, respectively). At the lateral front and central part of colorectal cancer cases, there were no significant differences between CL-1 expression and clinicopathological factors, while the colorectal cancers ubiquitously expressed CL-1. In addition, there were no significant differences between CL-4/E-cad expression and the clinicopathological factors (**Table 1**).

Table 1. The expression of claudin-1, claudin-4 and E-cadherin at the invasive front of colorectal cancer.

	CL-1			CL-4			E-cad		
	1+, 2+	3+		1+, 2+	3+		1+, 2+	3+	
Site			p=0.632			p=1.000			p=0.476
Right	8	18		10	16		18	8	
Left	25	41		26	40		39	27	
Histology			p=0.274			p=0.153			p=0.221
wel	4	15		4	15		10	9	
mod	26	41		28	39		41	26	
por	1	0		1	0		1	0	
muc	2	3		3	2		5	0	
Primary tumor (T)			p=0.033*			p=0.391			p=0.572
T1, T2	4	13		7	10		11	6	
T3	11	30		13	28		23	18	
T4	18	16		16	18		23	11	
Venous invasion			p=0.186			p=0.384			p=1.000
negative	10	27		12	35		23	14	
positive	23	32		24	31		34	21	
Lymphoduct invasion			p=1.000			p=1.000			p=0.633
negative	1	3		1	3		2	2	
positive	31	56		35	53		55	33	
Regional lymph nodes (N)			p=0.03*			p=0.287			p=0.286
No	12	36		16	32		27	21	
N1, N2	21	23		20	24		30	14	
Liver metastasis			p=0.01*			p=0.358			p=0.357
negative	24	55		29	50		47	32	
positive	9	4		7	6		10	3	
Distant metastasis (M)			p=0.13			p=0.53			p=0.706
M0	28	56		30	54		51	33	
M1	5	3		6	2		6	2	
TNM Classification grade			p=0.034*			p=0.255			p=0.212
I, II	10	32		13	29		25	17	
III	12	19		13	18		17	14	
IV	11	8		10	9		15	4	
Budding grade (sprouting)			p=0.24			p=0.197			p=0.98
1	12	24		10	26		22	14	
2	4	14		8	10		11	7	
3	17	21		18	20		24	14	

**p<0.05: Statistical significance; Chi-squared test and Fisher's exact test; wel: Tubular Adenocarcinoma, Well-Differentiated Type; mod: Tubular Adenocarcinoma, Moderately Differentiated Type; por: Poorly Differentiated Adenocarcinoma; muc: Mucinous Adenocarcinoma*

Tumor budding (sprouting) is defined as a clump of tumor cells having < five nuclei at the invasive front.

Budding grade (sprouting)

1. *number of tumor buds was 0-4 at x200 magnification*
2. *number of tumor buds was 5-9 at x200 magnification*
3. *number of tumor buds was > 10 at x200 magnification*

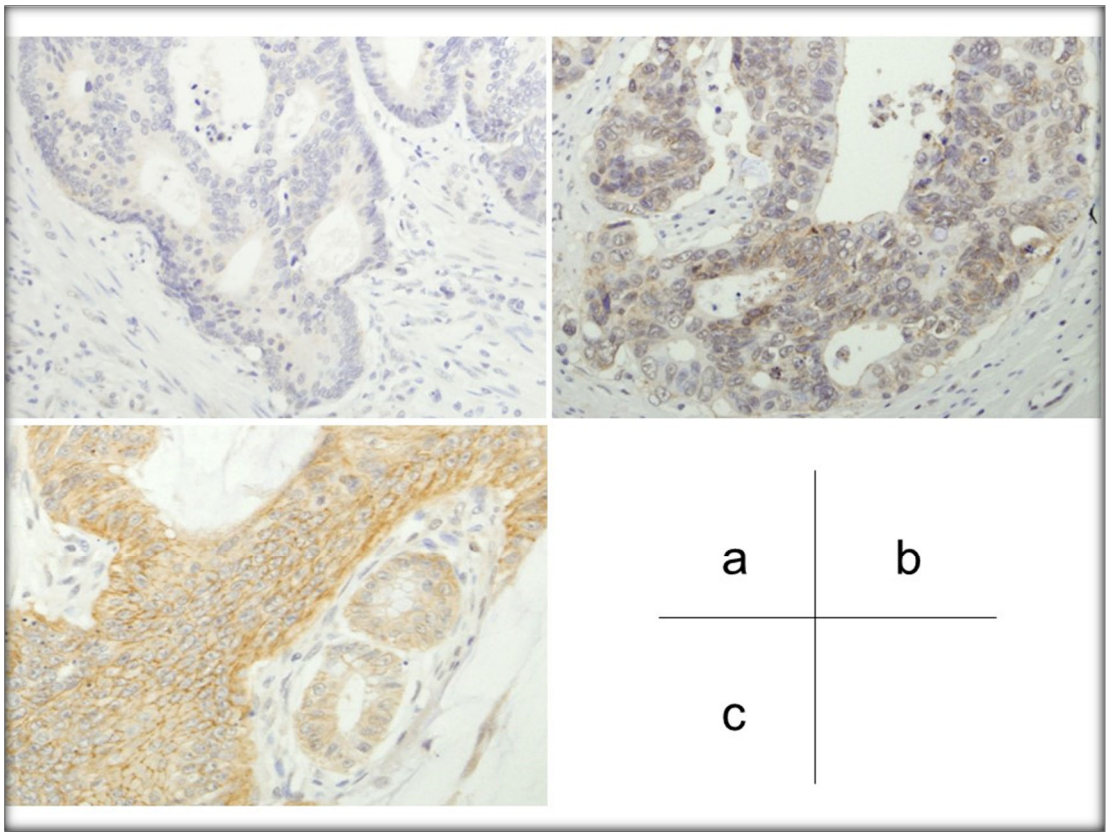

Figure 1. Immunohistochemical expression patterns of claudin-1 (CL-1). Expression of +1, <30% positive at cell membrane and weakly positive at cell cytoplasm (a: top, left); Expression of +2, >30% positive at cell membrane and weakly positive at cell cytoplasm or <30% positive at cell membrane and moderately positive at cell cytoplasm (b: top, right), and expression of +3, >30% positive at cell membrane and moderately positive at cell cytoplasm (c: bottom, left).

Digital quantitative evaluation of CL-1 expression demonstrated 117.3 ± 14.6 in colorectal cancer cases with recurrence/metastasis and 118.7 ± 17.7 in cases without recurrence/metastasis (mean ± standard deviation) (**Table 2 & Figure 2**). CL-1 expression levels at the invasive front (98.3 ± 14.1, $p<0.05$) were significantly decreased in colorectal cancer cases with recurrence/metastasis, compared with cases without recurrence/metastasis (107.0 ± 18.3).

Table 2. Digital quantitative evaluation of CL-1 expression.

	Total	Whole (mean±SD)	Invasive front (mean±SD)
Recurrence/metastasis group	46	117.3±14.6	98.3±14.1
No recurrence/metastasis group	43	118.7±17.7	107.0±18.3
		p=0.69	p=0.013*

Comparing the quantity of expression of non-neoplastic glands as 100

**p<0.05: statistical significance, Student's T test; SD: Standard deviation*

Recurrence/metastasis group: lymph node metastasis, liver metastasis, distant metastasis, peritoneal dissemination, and recurrence after operation

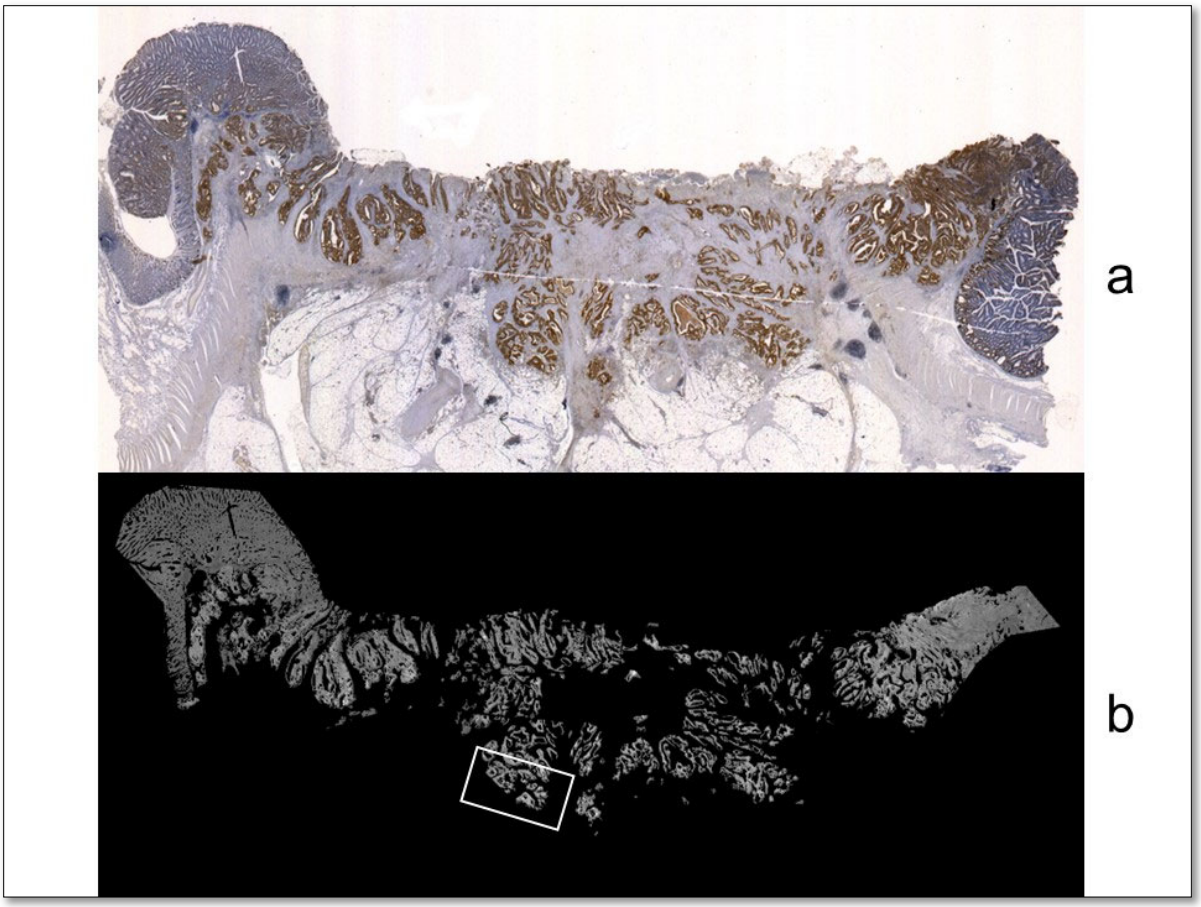

Figure 2. Digital quantitative evaluation of claudin-1 (CL-1). CL-1 immunostaining sections of colorectal cancer were scanned at 1200dpi (1200 pixels/inch) (a: top). Color components of the scanned preparation were separated into RGB (red-green-blue) (b: bottom). The intensity of immunohistochemical expression was represented as 100 x red / blue component. Mean expression levels were quantitative evaluated at the invasive front (200 x 50 pixels: square frame), as well as the entire tumor.

DISCUSSION

We examined the immune histochemical expression of CL1, CL4 and E-cad in human colorectal adenocarcinomas and analyzed their clinicopathological significance. This study examined the relationship between low-grade CL-1 expression at the invasive front and metastatic potentials (lymph node and liver metastases) in colorectal cancer patients. In addition, this is the first study demonstrating decreased CL-1 expression in colorectal cancers with metastasis/recurrence using digital quantitative evaluation. There were no apparent statistical correlations between clinicopathological features and the expressions of CL4 and E-cad.

Cell adhesion is crucial for the assembly of individual cells into three-dimensional tissues [1-3]. The functional units of cell adhesion are typically multi protein complexes consisting of three general classes, i.e., cell adhesion molecules/adhesion receptors, extracellular matrix proteins and cytoplasmic plaque/peripheral membrane proteins. Recent advances in molecular biology have clarified the structures and functional regulations of cell adhesion including the tight junction, adherens junction and desmosome. The claudin family and occludin have been identified as the major proteins of the tight junction, while the cadherin family has been discovered as adherence junction proteins [2].

On the other hand, oncological studies have focused on the relations between cancer invasion and the expression of cell adhesion molecules because decreased molecules may affect cancer invasion (cell migration) and metastasis, which reflect cancer outcome. Previous studies reported the up regulation of claudin expression including CL-1 and CL-4 in colorectal cancer [15,16]; however, these studies did not demonstrate the histological localization of the claudins. In our results, CL-1 expression was significantly decreased at the invasive front (advanced margin) of colorectal cancer, while cancer tissues generally showed high-grade CL-1 expression, i.e., no significant decrease of CL-1 expression was detected in the central parts of colorectal cancer [17,18].

Several studies reported the decreased claudin expression correlated with aggressive behaviors of colorectal cancer [19], and supported our results. We speculated that the low-grade CL-1 expression at the invasive front plays a role in cancer invasion and metastasis, and that metastasis/recurrence is correlated with decreased CL-1 expression using digital quantitative evaluation. Previous reports described the altered expression of E-cad in colorectal cancer [20-24]; however, our results showed that the expression pattern of E-cad was not significantly correlated with clinicopathological factors. The discrepancy between the previous reports and our study may result from the different fixation/staining conditions or different evaluations (entire tumor or part of cancer tissues such as the invasive front). Based on the present study, we speculated that CL-1 at the invasive front may affect cancer invasion and metastasis more effectively than the expressions of CL-4 and E-cad. In conclusion, decreased CL-1 expression at the invasive front is thought to be an important prognosis prediction factor.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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