Decreased Expression of Claudin-1 in Rectal Cancer: A Factor for Recurrence and Poor Prognosis

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Abstract. *Aim: To investigate the potential involvement of claudin-1 (CL-1) in the tumorigenesis of rectal cancer by analyzing the correlation between CL-1 expression, clinicopathological factors and prognosis. Patients and Methods: Rectal cancer tissue specimens from 306 patients that had undergone surgical treatment were evaluated using immunohistochemical analysis for expression of CL-1 and correlated with clinicopathological factors. Results: A reduced expression of CL-1 (less than 30% of tumor cells strongly, positively stained) correlated significantly with poor prognosis in stage II and III rectal cancer. Moreover, the expression levels of CL-1 correlated significantly with tumor differentiation and perineural invasion (p=0.037 and 0.009, respectively). However, no significant differences were detected between the expression levels of CL-1 and other clinicopathological factors. Conclusion: Loss of claudin-1 expression is a strong predictor of disease recurrence and poor patient survival in stage II and III rectal cancer.*

Claudin-1 (CL-1) and claudin-2 (Cl-2) are transmembrane proteins which are an integral component of tight junction strands (1). The altered expressions of some claudins (CLs) have been found in many types of human cancer, such as breast, ovary, prostate, liver, stomach and colon (2, 3). CLs have also been identified as potential targets in the development of molecular-based strategies for diagnosis (4), prediction of progression (5), disease recurrence (6), cell invasion and metastasis (7-11), as well as for the improvement of therapeutic strategies (12). Recent studies have implicated CLs in tumorigenesis, cancer cell invasion and migration (13).

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The up-regulated expression of CL-1 has been seen in colorectal cancer (CRC) (14, 15). The loss of function of the tight junction proteins (TJPs) in CRC (16) is believed to increase the access of tumor cells to nutrients and signaling peptides (17), down-regulate cell-to-cell adhesion and increase motility and metastasis (18, 19). It has also been seen that β-catenin and CL-1 co-localize in the nucleus of many metastatic colonic adenocarcinomas (20). The differential expression of genes encoding TJPs, which increases the metastatic potential of tumor cell lines (22), has also been reported in CRC (6, 21).

CL-1 expression has been reported to have a prognostic value in CRC (14, 15, 21). Resnick *et al*. (23) reported that CL-1 expression was a positive prognostic indicator and correlated with lower tumor grade, absence of lymphovascular invasion and increased patient survival in stage II colonic cancer. But the relationship between CL-1 expression and prognosis in rectal cancer has not yet been examined.

The aim of this study was to analyze the correlation between the expression of CL-1 in rectal cancer tissues and clinicopathological factors and to investigate the effectiveness of CL-1 as a possible prognostic marker in rectal cancer patients.

Patients and Methods

Patients and tissue samples. A total of 306 patients with rectal cancer who had undergone surgical treatment at Kurume University Hospital in Fukuoka, between January 1999 and December 2007 were included in the study. Informed consent was obtained from all the patients prior to surgical resection. The study was approved by the Institutional Review Committee for Research on Human Subjects, at Kurume University Hospital. Clinical follow-up was performed for the duration of patient survival. Patients were classified as having no evidence of disease or having evidence of relapse. Relapse was defined as local recurrence or the development of distant metastasis.

Histopathology. After surgery, tumor specimens, the resection margin and lymph nodes were fixed in formalin and embedded in paraffin. Hematoxylin and eosin-stained sections were reviewed to

Table I. *Patient demographics*.

Variable	TNM stage					Total
	0	I	II	III	IV	
Patients (n)	13	60	100	85	48	306
Mean age, years	61	64	65	64	61	64
(range)	(42-70)	(32-84)	(32-89)	(29-87)	(38-76)	(29-89)
Gender						
Female	7	29	30	27	14	107
Male	6	31	70	58	34	199
Localization of tumor						
RS	0	9	26	35	14	84
Ra, Ra > Rb	1	14	31	23	19	88
Rb, Rb > P	12	37	41	26	12	128
P	0	0	2	1	3	6
Primary tumor						
Tis	13	0	0	0	0	13
T1	0	20	0	4	1	25
T2	0	40	0	9	2	51
T3	0	0	72	44	21	137
T4	0	0	28	28	24	80
R0	13	60	100	85	0	258
R1,R2	0	0	0	0	48	48
Tumor type						
Tub 1, 2	13	59	96	79	40	287
Poor, muc, sig	0	1	4	6	8	19

RS: Rectosigmoid, Ra: rectum above peritoneal reflection, Rb: rectum below peritoneal reflection, P: anal canal, Ro: no residual tumor, R1: microscopic residual tumor, R2: macroscopic residual tumor, Tub: tubular adenocarcinoma, Poor: poorly differentiated adenocarcinoma, muc: mucinous adenocarcinoma, sig: signet-ring cell carcinoma, Tis: carcinoma *in situ*: intraepithelial or invasion of *lamina propria,* T1: tumor invades submucosa, T2: tumor invades *muscularis propria,* T3: tumor invades through the *muscularis propria* into the subserosa, or into non-peritonealized pericolonic or perirectal tissues, T4: Tumor directly invades other organs or structures and/or perforates visceral peritoneum.

establish the pathological diagnosis. Tumor differentiation and the degree of invasion were examined by pathologists and histopathological classification was performed according to the General Rules for Colorectal Cancer Study (24) and/or TNM. The patient clinical information is summarized in Table I.

Immunohistochemistry. Immunohistochemistry was performed as described in our previous studies (14, 25). The tissue sections were stained with monoclonal antibodies (diluted at 1:400) against CL-1 (Zymed Laboratories Inc., San Francisco, CA, USA). The sections were stained using a BenchMark XT device IHC automated system (Ventana medical systems, Tucson, Arizona, USA).

Quantification of immunostaining. To evaluate the cytoplasmic staining intensity, an intensity score (IS) was used as follows: zero staining intensity was scored as 0, marginal intensity as +1, medium intensity as +2 and strong intensity as +3 (Figure 1) (26). At the same time, the immunoreactivity of the membranous CL-1 expression was also scored by estimating the percentage of strongly positive tumor cells (PS), termed CL-1 PS (27) (Figure 2). All the immunohistochemical studies were evaluated by two experienced observers who were blinded to the condition of the patients.

Statistical analysis. Statistical analysis was performed using JMP version 8.0 (SAS Institute, Cary, NC, USA). Statistical comparisons were made using Fisher's exact test, the chi-square test or the Wilcoxon rank-sum test, depending on the type of data. Values of $p<0.05$ were considered to indicate statistical significance.

The relationships between CL-1 expression and overall (OS) and disease-free survival (DFS), as well as between other clinicopathological findings and molecular markers, were examined using the Kaplan-Meier method and the log-rank test. Hazard ratios were estimated by Cox regressions.

Results

Patient clinical course. For the rectal cancer patients, the median duration of follow-up after surgery was 38 months, with a range of 1-123 months over all stages. Relapse was diagnosed in 21.1% of the patients and the five-year survival rate was 86.2% in both stage II and III patients.

Expression of CL-1 protein in human rectal cancer tissue specimens. The CL-1 expression was correlated with the CL-1 PS as shown in Figure 3. Figure 4A shows the CL-1 expression in terms of the IS at all cancer stages. No significant correlation was detected. Surprisingly, positive cases (1+, 2+ and 3+) were not as common in stage IV as in

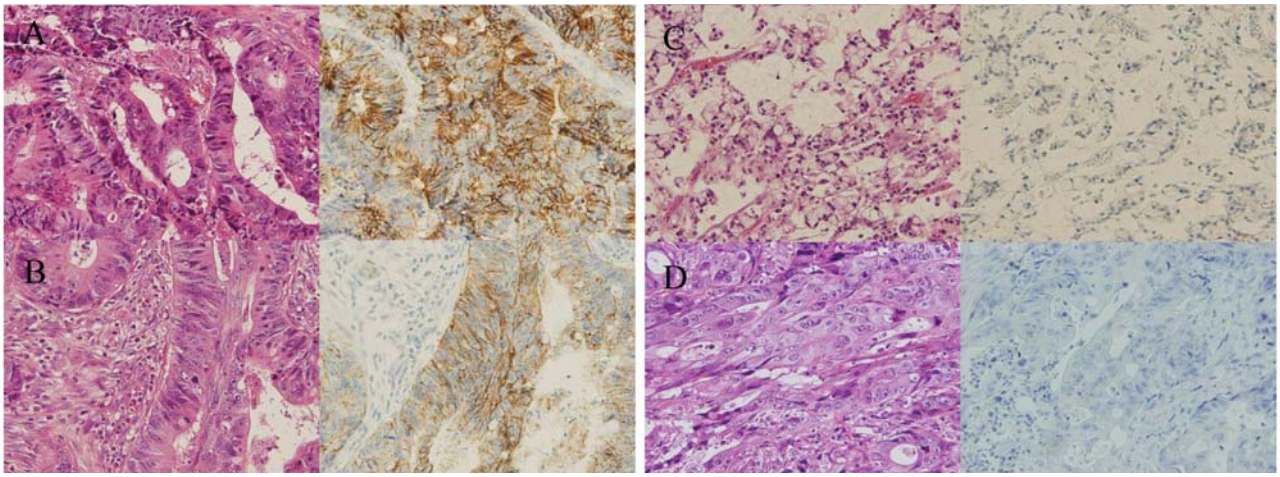

Figure 1. *Representative examples of histological and immunohistological staining. Left panel: HE staining. Right panel: CL-1. A: Well-differentiated adenocarcinoma, CL-1 IS=3. B: Moderately differentiated adenocarcinoma, CL-1 IS=2. C: Signet-ring cell adenocarcinoma, CL-1 IS=1. D: Moderately differentiated adenocarcinoma, CL-1 IS=0. Magnification: ×200. IS: Intensity score.*

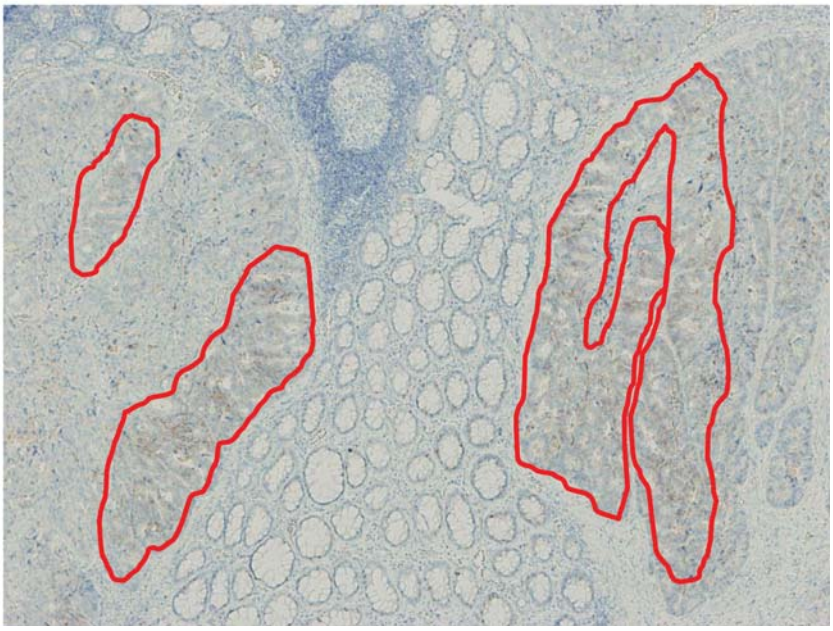

Figure 2. *CL-1 PS scoring system. Red frames indicate strongly positive staining regions. Magnification: ×20.*

the other stages. On the other hand, in the group where PS was <30%, CL-1 PS showed increased correlation with disease stage in the same cases, while correlation decreased in the 30%<PS<60% group (Figure 4B).

Using these results, the cut-off value of the CL-1 PS was determined by means of a receiver operating characteristic (ROC) curve, fitted using JMP software. The cut-off value was determined to be at 30%.

Correlation between CL-1 expression and clinicopathological features. The patients were divided into two groups: those with a CL-1 PS of 30% or less and those with a CL-1 PS >30%. As shown in Table II, using multivariate analysis, the expression of CL-1 significantly correlated with tumor differentiation and perineural invasion (PNI) (p=0.037 and 0.009, respectively). Surprisingly, no significant differences were detected between the expression

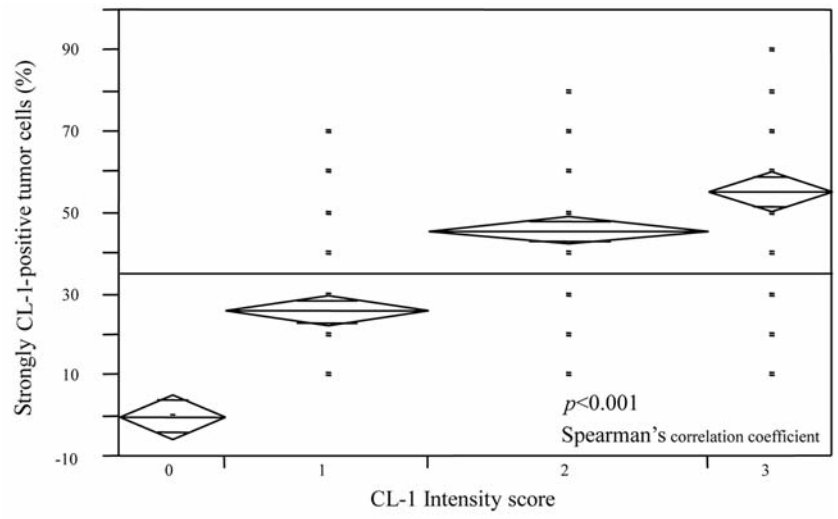

Figure 3. *Correlation between the percentage of cells positive for membranous CL-1 (CL1-PS) and the intensity of cytoplasmic CL-1 expression in tumor tissue (p<.001, Spearman rank correlation analysis).*

Table II. *Analysis of patient characteristics and CL-1 expression.*

Variable	CL-1		Chi-square	Odds ratio	95% CI	*p*-Value	
	PS ≤30%	PS >30%				Multivariate analysis	Univariate analysis
Mean age and (range in years)	64.5 (29-89)	62.7 (33-89)	-	-	-	-	0.155
Gender							
Female/male	61/102	46/97	-	-	-	-	0.400
TNM stage							
0, I, II	81	92	0.00	1.037	–1.141-1.021	0.972	0.011
III, IV	82	51					
Tumor location							
RS, Ra, Ra > Rb	91	81	-	-	-	-	0.909
Rb, Rb > P, P	72	62					
T Category							
Tis, T1, T2	48	41	-	-	-	-	0.900
T3, T4	115	102					
Tumor type							
Tub1, 2	147	140	5.38	3.940	–1.435-0.100	0.037	0.007
Poor, muc, sig	16	3					
Lymph node metastasis							
N –	84	94	0.02	1.139	–1.068-1.029	0.899	0.015
N +	79	49					
Lymphatic invasion							
–	64	78	1.74	1.429	–0.445-0.087	0.187	0.011
+	98	65					
Venous invasion							
–	29	32	0.22	0.858	–0.241-0.394	0.636	0.320
+	134	111					
Perineural invasion							
–	132	136	6.82	3.453	–1.120-0.177	0.009	<0.001
+	31	7					
CEA (ng/ml)							
<5	78	68	-		-	-	0.724
≥5	72	69					

T: Primary tumor, N: regional lymph nodes, CEA: carcinoembryonic antigen; other abbreviations as Table I.

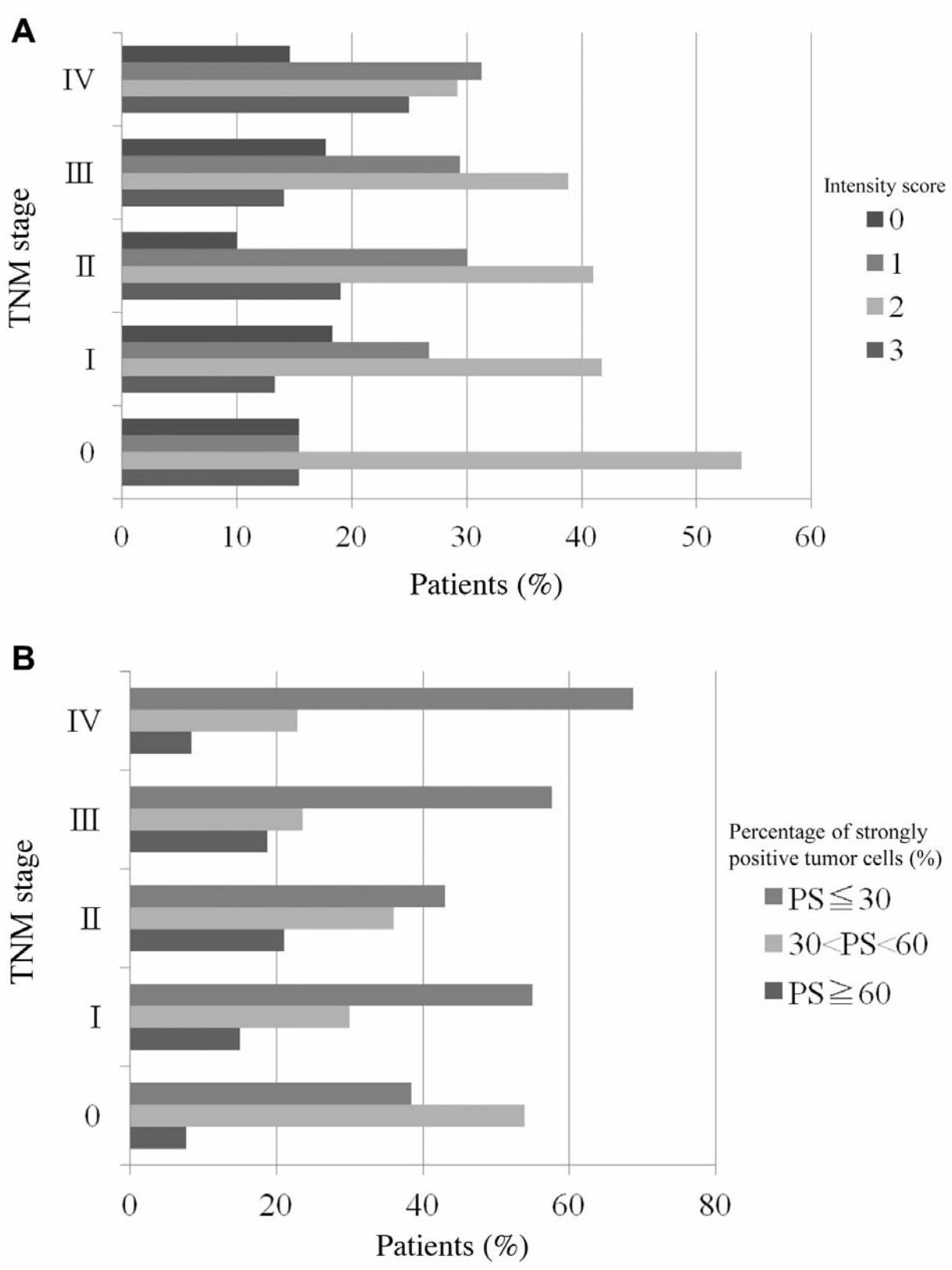

Figure 4. *CL-1 expression at each cancer stage. A: Staining intensity score (IS) of cytoplasm: no staining=0; marginal staining=1; medium staining=2 and strong staining=3. B: Percentage of strongly CL-1-positive membrane-stained tumor cells.*

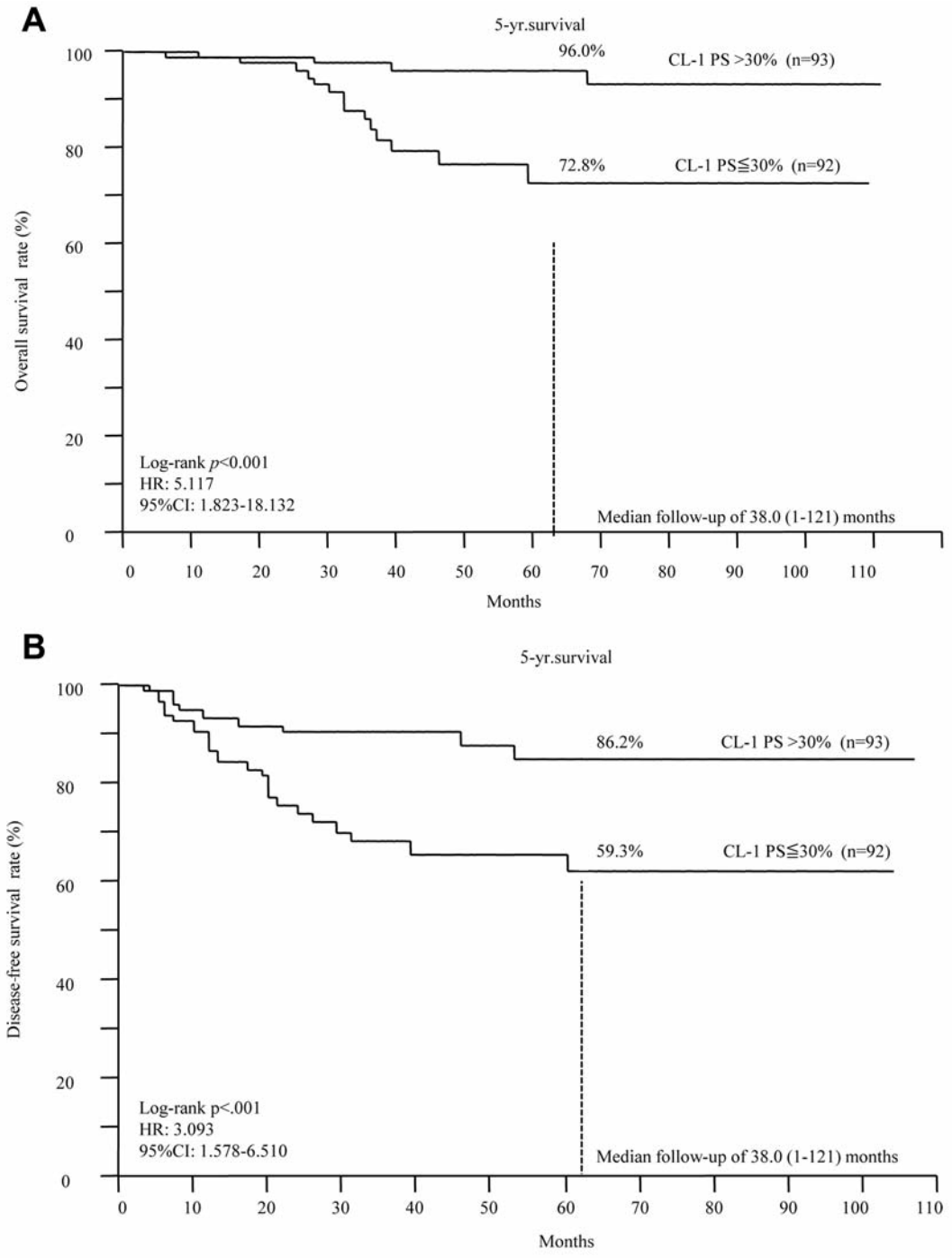

Figure 5. *A: Overall survival rates at stages II and III. B: Disease-free survival rates at stages II and III.*

levels of CL-1 and other clinicopathological factors, such as age, sex, tumor location, T category, lymph node metastasis and preoperative serum carcinoembryonic antigen (CEA) level. Univariate analysis of recurrence revealed a significant association with tumor differentiation (p=0.007), lymphatic invasion (p=0.048), PNI (p=0.001), preoperative CEA level (p=0.01) and low levels of CL-1 expression (CL-1 PS) (p=0.002) in stage II and III patients (Table III). Multivariate Cox analysis revealed that tumor location (p=0.005), venous invasion (p=0.038), PNI (p=0.033), preoperative CEA level (p=0.027) and low level of CL-1 expression (CL-1 PS) (p=0.046) were associated with

Table III. *Analysis of patient characteristics and disease-free survival.*

Variable	Recurrence (%)	No recurrence (%)	Hazard ratio	95% CI	*p*-Value Multivariate analysis	*p*-Value Univariate analysis
Gender						
Female	26	74	-	-	-	0.248
Male	19	81				
CL-1 PS (%)						
>30	12	88	2.19	1.013-4.959	0.046	0.002
≤30	30	70				
TNM stage						
II	18	82	-	-	-	0.283
III	25	75				
Tumor location						
RS, Ra, Ra > Rb	17	83	2.55	1.343-4.840	0.005	0.066
Rb, Rb > P	26	74				
P	67	33				
Tumor type						
tub1, 2	19	81	2.63	0.860-6.958	0.087	0.007
poor, muc, sig	60	40				
T categories						
Tis, 1, 2, 3	19	81	1.78	0.779-4.091	0.171	0.241
T4	27	73				
Lymph node metastasis						
N –	18	82	-	-	-	0.283
N +	25	75				
Lymphatic invasion						
–	15	85	1.09	0.507-2.429	0.834	0.048
ly +	27	73				
Venous invasion						
0, 1	19	81	2.82	1.063-6.854	0.038	0.064
2, 3	36	64				
Perineural invasion						
–	16	84	2.53	1.080-5.696	0.033	0.001
+	44	56				
CEA (ng/ml)						
<5	13	87	2.25	1.095-4.873	0.027	0.010
≥5	30	70				

CL-1 PS: strongly claudin-1-positive tumor cells, venous invasion 0: none, 1: minimal, 2: moderate, 3: maximum; other abbreviations as Tables I and II.

recurrent disease in the same group (Table III). Kaplan-Meier plots illustrating the association of CL-1 expression with recurrence and survival are shown in Figures 5A and 5B. A clearly reduced expression of CL-1 (less than 30% of CL-1 PS) correlated significantly with poor prognosis.

Discussion

Surgical resection is the primary treatment modality for CRC. The most powerful tool for assessing prognosis following surgery is pathological analysis of the resected specimen. Although the parameters that determine the pathological stage are the strongest predictors of postoperative outcome, such as histological grade, lymphatic or vessel invasion and PNI which have prognostic significance independent of stage (28). While tumor extent, lymph node status, tumor grade and the assessment of lymphatic and venous invasion remain the most important additional histological parameters, they are not regarded as essential in prognosis.

While molecular analysis and gene expression profiling as a means of improving identification of patients likely to have a poor clinical outcome and therefore more likely to benefit from adjuvant treatment have been reported (29-31), reliable prognostic markers identified by immunohistochemical protein profiling are yet to be established.

We have previously reported that CL-1 plays a pivotal role in the regulation of cellular morphology and behavior in the colonic epithelium using a CL-1 overexpressing CRC cell line (14, 15). While it is unclear why reduced expression of

CL-1 correlated significantly with poor prognosis, in the present study, this finding suggested that the loss of certain CLs has a greater impact on tumor aggression than others, and CL-1 loss may lead to tumor progression.

Interestingly, the present study showed that reduced expression of CL-1 was significantly associated with PNI. PNI is the process of neoplastic invasion of nerves and is an under-recognized route of metastatic spread. PNI has emerged as a key pathological feature of many other malignancies, including those of the pancreas, colorectal, prostate, biliary tract and stomach. For many of these malignancies, PNI is a marker of poor outcome and a harbinger of decreased survival (32-37). Shirouzu *et al*. reported that PNI was an important factor in influencing the prognosis of rectal cancer patients with stage III disease (38).

Taken together, these data suggest that CL-1 protein expression may have significant clinical relevance and it may therefore become a potentially useful immunohistochemical and prognostic marker in rectal cancer. This study was the first to examine comprehensively the expression of CL-1 in rectal cancer and to correlate CL-1 expression with disease progression. A reduced expression of CL-1 was found to be significantly associated with poorly differentiated tumors and PNI. It was concluded that the loss of CL-1 expression was a strong predictor of disease recurrence and poor survival in stage II and III rectal cancer patients. The use of CL-1 as a novel prognostic factor in rectal cancer is therefore proposed.

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