

Expression of *CLDN1* in colorectal cancer: A novel marker for prognosis

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Abstract. Claudin1 (*CLDN1*) plays an important role not only in the intercellular barrier function of tight junctions (TJs) but also in migration and invasiveness of cancer cells. Previous reports show that *CLDN1* overexpression is significantly related to the malignant behavior in several cancer types whereas its significance in colorectal cancer (CRC) is not fully understood. The present study comprised 119 patients who underwent surgery for CRC, as well as 3 cell lines derived from human CRC. The correlation of gene expression with clinical parameters in patients was assessed by knockdown experiments using 3 cell lines. Patients with high *CLDN1* expression were statistically shown to have a relatively better prognosis, and those with low *CLDN1* expression showed poorer overall survival and disease-free survival than those with high expression. The assessment of *CLDN1* knockdown in the 3 cell lines demonstrated that the siRNA inhibition resulted in a statistically significant increase in cell invasiveness. In conclusion, the present data strongly suggest that *CLDN1* expression is a prognostic factor in CRC patients.

Introduction

Many developed countries including the United States and Japan have an aging population, where cancer is one of the most prominent illnesses, in terms of public welfare and health measures. For example, one in four deaths in the United States is due to cancer (1). In the United States, the incidence of CRC has increased significantly in recent years, in concert with the changing lifestyle (2), and CRC is one of the most prominent causes of death from neoplastic disease in Japan (2). The major cause of death in CRC is due to distant metastases (3). Identification of genes responsible for the development and progression of CRC and comprehension of

the clinical significance are critical for the diagnosis and adequate treatment of the disease. Characterization of the key molecules is particularly promising for the development of novel approaches to the treatment of gastrointestinal tumors.

The claudin (*CLDN*) family were first discovered in 1998, and the *CLDN* family consists of at least 24 members, the expression of which depends on cell type and tissue (4,5). *CLDNs* encode proteins with 4 transmembrane domains, and the C-terminal domain serves as a binding site for interaction with a complex set of proteins including site for PDZ domain proteins that are involved in intracellular signaling (6-8). Recent reports showed that *CLDN* gene expression is frequently altered in several cancer types, and the members of *CLDN* family are reported as up- or down-regulated according to the organ (9,10).

The aim of this study was to analyze the correlation between the expressions of *CLDN1* in CRC tissues from patients with clinicopathological factors and to investigate its possible functions in tumorigenesis and metastasis in CRC.

Materials and methods

Clinical tissue samples. One hundred and nineteen patients (74 men, 45 women) with CRC were registered and underwent curative surgery including distant metastasis at the Medical Institute of Bioregulation at Kyushu University from 1993 to 2001. None of the patients received chemotherapy or radiotherapy prior to surgery. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after written, informed consent had been confirmed, in accordance with the institutional ethical guidelines. The surgical specimens were fixed in formalin, processed through graded ethanol, and embedded in paraffin. The sections were stained with hematoxylin and eosin, and Elastic van Gieson stain, and the degree of histological differentiation, lymphatic invasion and venous invasion was examined. The samples of all specimens were also frozen in liquid nitrogen immediately after resection and kept at -80°C until RNA extractions. None of the patients received chemotherapy or radiotherapy prior to surgery. After surgery, the patients were followed up with a blood examination including the tumor markers serum carcinoembryonic antigen (CEA) and cancer antigen (CA19-9),

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and imaging modalities such as abdominal ultrasonography, computed tomography, and chest X-ray every 3-6 months. Postoperatively, stage III and IV patients received 5-fluorouracil-based chemotherapy, whereas stage I and II patients principally received no chemotherapy. All these therapies were performed according to the Japanese guidelines (11). Clinico-pathological factors were assessed according to the tumor node metastasis (TNM) classification of the International Union Against Cancer (UICC) (12).

Cell lines and culture. Three cell lines derived from human colorectal cancer (DLD-1, HCT116 and LoVo) (13-15) were obtained and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO₂ atmosphere. For siRNA inhibition, double-stranded RNA duplexes targeting human *CLDN1* were purchased from Invitrogen as Stealth RNAi (HSS113420, HSS189840, HSS189841; Invitrogen, Carlsbad, CA, USA). For comparison, a negative control siRNA (NC) was also purchased from Invitrogen. CRC cell lines were transfected with siRNA at a concentration of 20 µmol/l using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen) for the time indicated, and analyzed using a proliferation and invasion assay after 48 h. All siRNA duplexes were used together in a triple transfection. A significant reduction in *CLDN1* by siRNA was confirmed by quantitative real-time RT-PCR. The growth rate of the cultured CRC cell lines was measured by counting cells using CellTac (Nihonkoden, Tokyo, Japan). Each cell line with siRNA was compared to the wild-type and a negative control (NC). Values were expressed as standard error of mean (SEM) at least 3 independent experiments.

RNA preparation and expression analysis. Total RNA was prepared with DNase by a modified acid guanidium-phenol-chloroform procedure (16). Reverse transcription was performed with 2.5 µg of total RNA as described previously (17). A 155-bp *CLDN1* fragment was amplified. Two human *CLDN1* oligonucleotide primers for the PCR reaction were designed as follows: 5'-CCCTATGACCCCAGTCAATG-3' (forward); 5'-GTTTTGGATAGGGCCTTGGT-3' (reverse). The forward primer is located in exon 3 and the reverse primer in exon 4. A PCR Kit (Takara, Kyoto, Japan) on a GeneAMP PCR System 9600 (PE Applied Biosystems, Foster City, CA, USA) was used to perform 35 cycles of PCR reaction with the following parameters: 95°C for 40 sec, 45°C for 40 sec, and 72°C for 60 sec. An 8-µl aliquot of each reaction mixture was size-fractionated in a 1.5% agarose gel and visualized with ethidium bromide staining. To ensure that the RNA was not degraded, a PCR assay with primers specific for the glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) gene was performed for 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C for 30 cycles. The *GAPDH* primers 5'-TTGGTATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCATCATATTGGCAGGTT-3' (reverse) produced a 270-bp amplicon. Complimentary DNA from the Human Reference total RNA (Clontech, Palo Alto, CA, USA) was studied concurrently as a source of positive controls. For quantitative assessment, real-time quantitative reverse transcriptase (RT)-PCR was performed using a LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics,

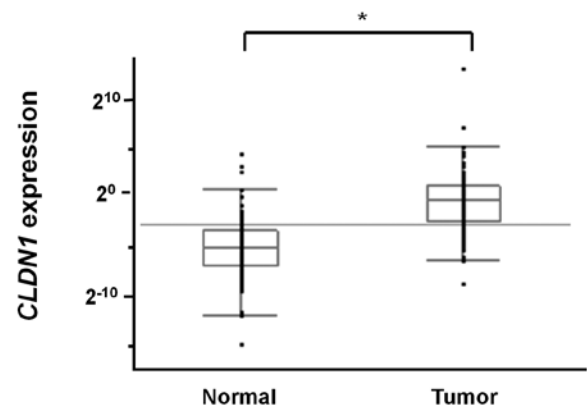


Figure 1. *CLDN1* expression in clinical tissue specimens. Quantitative real-time RT-PCR on 119 paired clinical samples showed the relative expressions of *CLDN1* mRNA in tumors and paired normal tissues (normalized by *GAPDH* mRNA expression) (**p*<0.001).

Tokyo, Japan) for cDNA amplification of *CLDN1* and *GAPDH*. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and elongation at 72°C for 10 sec. The products were then subjected to a temperature gradient from 55 to 95°C with continuous fluorescence monitoring to produce a melting curve of the products. The expression ratios of *CLDN1* mRNA copies in tumor and normal tissues were calculated, normalized against *GAPDH* mRNA expression.

Invasion assays. Cell invasion were assessed with CytoSelect Cell Invasion Assay according to the protocol of the manufacturer (Cell Biolabs, San Diego, CA) after 48 h of the transfection. Cells in DMEM (1.0x10⁵) were placed on each 8.0-µm pore size membrane insert in 96-well plates. DMEM with 10% FBS was placed in the bottom wells. After 24 h, cells that did not invade were removed from the top side of the membrane chamber and completely dislodge the cells from the underside of the membrane by tilting the membrane chamber in the Cell Detachment Solution (Cell Biolabs). Lysis Buffer/CyQuant GR dye solution (Cell Biolabs) was added to each well, the fluorescence of the mixture was read with a fluorescence plate reader at 480/520 nm. The values were expressed as a ratio with wild-type (every parental cell).

Statistical analysis. The relationship between *CLDN1* expression and clinico-pathological factors was analyzed with the Chi-square test. Kaplan-Meier survival curves were plotted and compared with the generalized log-rank test. Univariate and multivariate analyses to identify prognostic factors were performed using a Cox proportional hazard regression model. The values *in vitro* assays were analysed with Wilcoxon rank test. All tests were analyzed with JMP software (SAS Institute, Cary, NC, USA). *p*<0.05 was considered statistically significant.

Results

Expression of *CLDN1* in clinical tissue specimens and clinico-pathological characteristics. We performed quantitative real-time RT-PCR analysis with primary CRC and adjacent

Table I. Clinicopathological factors and CLDN1 mRNA expression in 119 CRC.

Factors	High expression n=59 (%)	Low expression n=60 (%)	p-value
Age			
<68	27 (45.7)	30 (50.0)	0.643
≥68	32 (54.2)	30 (50.0)	
Gender			
Female	26 (44.1)	19 (31.7)	0.163
Male	33 (55.9)	41 (68.3)	
Histological grade			
Others	5 (8.5)	4 (6.7)	0.709
Wel/mod.	54 (91.5)	56 (93.3)	
Tumor type			
0-1	5 (8.5)	11 (18.3)	0.115
2-4	54 (91.5)	49 (81.7)	
Tumor size			
<30 mm	49 (83.1)	49 (81.7)	0.843
≥30 mm	10 (16.9)	11 (18.3)	
Depth			
3<T	51 (86.4)	42 (70.0)	<u>0.030</u>
T<2	8 (13.6)	18 (30.0)	
Lymph node metastasis			
Absent	34 (57.6)	30 (50.0)	0.404
Present	25 (42.4)	30 (50.0)	
Lymphatic invasion			
Absent	34 (57.6)	36 (60.0)	0.793
Present	25 (42.4)	24 (40.0)	
Venous invasion			
Absent	49 (83.1)	46 (76.7)	0.386
Present	10 (16.9)	14 (23.3)	
Monochronous metastasis (UICC)			
Absent	43 (72.9)	38 (63.3)	0.264
Present	16 (27.1)	22 (36.7)	

Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

normal colorectal regions (Fig. 1). *CLDN1* expression was calculated by *CLDN1/GAPDH* expression. For the clinicopathological evaluation, experimental samples were divided into two groups according to the expression status. Patients with values more than the median *CLDN1* expression value (median, 0.700) were assigned to the high-expression group and the others were assigned to the low-expression group. Clinico-pathological factors related to *CLDN1* expression status of the 119 patients are summarized in Table I. The data indicated that *CLDN1* expression was correlated with tumor invasion ($p=0.030$). Other factors were not significantly correlated with *CLDN1* expression.

Relationship between *CLDN1* expression and prognosis. The data showed that the post-operative overall survival rate was

significantly higher in patients in the high-expression group than the low-expression group ($p=0.024$) (Fig. 2). The median follow-up was 3.9 years. The disease-free survival rate after curative surgery except for cases with monochronous metastasis was higher in the high-expression group than the low-expression group (Fig. 3). Table II shows the results of the univariate and multivariate analyses for factors related to overall survival. The univariate analysis showed that grade of differentiation ($p=0.045$), morphological type ($p=0.030$), tumor size ($p=0.005$), tumor invasion ($p<0.001$), lymph node metastasis ($p<0.001$), lymphatic invasion ($p<0.001$), venous invasion ($p<0.001$) and metastasis ($p<0.001$) and *CLDN1* expression ($p=0.025$) were significantly correlated with overall survival. The multivariate regression analysis indicated that *CLDN1* high-expression group (relative risk,

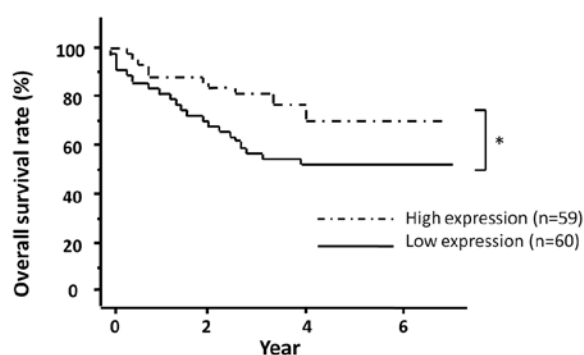


Figure 2. Overall survival rates of patients with CRC based on *CLDN1* mRNA expression status. The overall survival rate was higher in the *CLDN1* high-expression group than the low-expression group ($p=0.024$). *CLDN1* expression in tumor normalized by *GAPDH* mRNA expression is presented as relative expression to the paired normal colorectal mucosa.

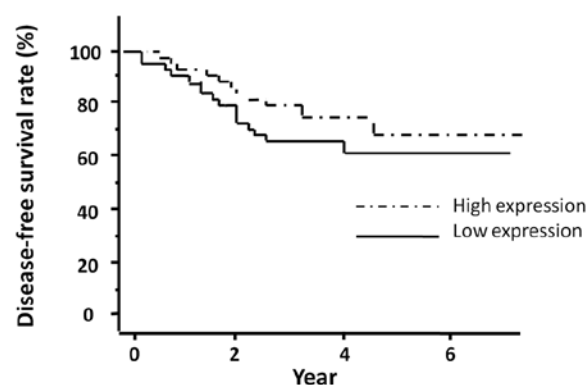


Figure 3. Disease-free survival rates of patients with CRC based on *CLDN1* mRNA expression status. The disease-free survival rate was higher in the *CLDN1* high-expression group than the low-expression group ($p=0.246$). *CLDN1* expression in tumor was normalized by *GAPDH* mRNA expression.

Table II. Univariate and multivariate analyses for overall survival (Cox proportional hazards regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	p-value	RR	95% CI	p-value
Age ($<68/\geq 68$)	1.58	0.83-3.08	0.159			
Gender (Male/female)	1.48	0.76-3.03	0.245			
Histological grade (Others/wel-mod)	1.73	1.01-2.67	<u>0.045</u>	1.64	0.93-2.66	0.082
Tumor type (2-4/0-1)	3.64	1.10-22.39	<u>0.038</u>	0.78	0.18-5.50	0.774
Tumor size ($\geq 30/<30$)	2.19	1.21-5.44	<u>0.005</u>	1.08	0.56-2.79	0.839
Depth ($3<T/T<2$)	3.73	1.73-15.72	<u>≤ 0.001</u>	2.38	0.90-11.19	0.085
Lymph node metastasis (Present/absent)	4.28	2.15-9.27	<u>≤ 0.001</u>	1.39	0.63-3.30	0.420
Lymphatic invasion (Present/absent)	2.97	1.57-5.75	<u>≤ 0.001</u>	1.48	0.73-3.07	0.277
Venous invasion (Present/absent)	2.92	1.47-5.56	<u>0.003</u>	1.37	0.64-2.79	0.393
Metastasis(UICC) (Present/absent)	10.38	5.16-22.71	<u>≤ 0.001</u>	5.46	2.42-13.14	<u>≤ 0.001</u>
<i>CLDN1</i> expression (Low/high)	2.09	1.09-4.20	<u>0.025</u>	1.99	1.01-4.12	<u>0.046</u>

Statistically significant values are underlined. RR, relative risk; CI, confidence interval; Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

1.99; 95% confidence interval, 1.01-4.12; $p=0.046$) and metastasis (relative risk, 5.46; 95% confidence interval, 2.42-13.14; $p<0.001$) were independent predictors of overall survival.

In vitro assessment of *CLDN1* expression knockdown. Three CRC cell lines were chosen for the proliferation and invasion study. In these cell lines, *CLDN1* expression correlated comparatively in clinical samples. The proliferation study was

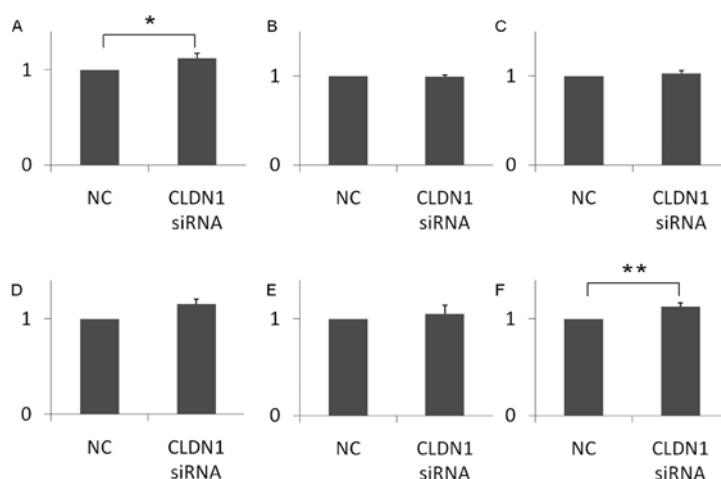


Figure 4. *In vitro* assays with siRNA inhibition in the 3 CRC cell lines. Proliferation assays were performed in 3 CRC cell lines (A, DLD-1; B, HCT116; C, LoVo). There was significant differences between NC and CLDN1 siRNA in DLD-1 (n=3, *p<0.01). These assays showed differences in the ratio with control (with no treatment). Invasion assays were also performed in them (D, DLD-1; E, HCT116; F, LoVo). There was significant differences between NC and CLDN1 siRNA in LoVo (n=5, **p<0.01). Invasion assays showed differences in the ratio with wild-type (every parental cell). Values are means and SEM. NC, negative control.

confirmed by seeding the cells (1.0×10^5) in 6-well dishes and culturing them for 48 h to determine the cell growth. The results showed significant differences in DLD-1 cell numbers between NC and *CLDN1* siRNA (n=3, p<0.05, Fig. 4A-C). In the invasion study, the results showed differences between NC and *CLDN1* siRNA (Fig. 4D-F) including statistical significance in LoVo (n=5, p<0.01).

Discussion

Regarding the function of Tjs, there are reports on the maintenance of normal epithelium (4,5,18,19). Their role in the neoplastic process, it is also important because of their reported malignant behavior (20). The role of CLDN in cancer has not been clearly defined, the expression of which are different in various organs (21-24). *CLDN1* is a capital component of Tjs and reported to be necessary for Tjs formation (25-27). However, the biological analysis is still to be performed.

The present study showed that *CLDN1* expression is an independent prognostic factor for CRC. The siRNA inhibition experiment in the CRC cell lines demonstrated the functional relevance of *CLDN1*. It suggests that the tumor malignancy correlates with *CLDN1* expression and it might affect the values of other prognostic factors in multivariate analysis such as distant metastasis which was significant in univariate analysis. *CLDN1* expression correlated with tumor invasiveness and was a significant prognostic factor similarly to distant metastasis. To the best of our knowledge, the present study is the first report to show *CLDN1* as a statistically significant predictive marker for CRC prognosis after curative resection, such as other well-known important predictors (28). Furthermore, the results indicated *CLDN1* plays a role as tumor suppressor, supported by a functional relevance to cell invasiveness. According to previous reports, our results suggest that *CLDN1*-dependent pathway might be involved in the suppression of CRC, which vary among tissue types, such as skin, breast, liver and prostate (22-24,29,30).

CLDN1 is useful to determine the necessity for intensive follow-up and adjuvant CRC therapy by predicting recurrence and metastases after curative surgical resection (31-33). In the present study, the clinico-pathological analysis revealed that CRC patients with high expression of *CLDN1* showed a better prognosis for overall and disease-free survival than the low-expression group. The data indicate that *CLDN1* is a presumptive novel predictor of CRC prognosis, especially relating to tumor invasiveness. Furthermore, it may be a therapeutic target in CRC based on the results from biological experiment in which CRC cell lines showed lower proliferation and invasiveness than those transfected with siRNA.

Several adjuvant chemotherapies are helpful in certain disease stages, especially in CRC, and indicate the usefulness of less invasive surgery for CRC (28,31-40). For these cases, an informative prognostic marker, which is independent of traditional TNM classification and contributes to diagnoses and treatments, is very important. The present data indicate the candidacy of *CLDN1*. While improved preoperative and postoperative treatments, such as chemotherapy and radiotherapy combined with surgery for CRC, have contributed to the reduction of recurrences, eventually half of the cases metastasize despite systemic chemotherapy followed by surgery (41). Adjuvant chemotherapy for CRC is desirable in highly suspicious recurrent cases. In these cases, *CLDN1* analysis may be useful to predict and treat patients with a poor prognosis.

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