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Heterogeneous Expression of Claudin-4 in Human Colorectal Cancer: Decreased Claudin-4 Expression at the Invasive Front Correlates Cancer Invasion and Metastasis

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Key Words
Claudin-4 · Colorectal cancer · Invasion · Metastasis

Abstract
Objective: Claudin-4 plays a key role in constructing the tight junction (TJ), and altered claudin-4 expression has been documented in various human malignancies; however, little is known about the biological significance of claudin-4 in colorectal cancers (CRCs). The aim of this study is to investigate the significance of claudin-4 expression in CRC and its association with clinicopathological factors. ***Methods:*** The levels of claudin-4 expression in a total of 129 CRCs and 44 metastatic tumors were examined by immunohistochemistry. A small interfering RNA (siRNA)-mediated claudin-4 knockdown examination was also conducted to assess the biological role(s) of claudin-4 in cultured cells. ***Results:*** Expression of claudin-4 at the intercellular membrane was well preserved at the surface of the tumor; however, decreased claudin-4 expression was detected in 57% of CRCs, particularly at the invasive front. Interestingly, decreased claudin-4 expression was detected in metastatic lesions of CRC. The siRNA-mediated claudin-4 knockdown in SW480 claudin-4-positive CRC cells upregulated cell motility, whereas no significant change was detected in cell proliferation. ***Conclusions:*** These observations suggested that disruption of claudin-4-mediated TJ construction enhances cancer cell invasion and metastasis in human CRC. Claudin-4 might be a good biomarker for diagnosing the risk of distant metastasis.

Introduction

The invasive and metastatic activities of cancer cells are regulated by cell-to-cell adhesion and cell-to-extracellular matrix adhesion [1–5]. Among the numerous cell-to-cell adhesion apparatuses, tight junctions (TJs) have been revealed to regulate the invasive and metastatic potential of cancer cells [6–10]. TJs are located on the most apical side in the intercellular adherent structure of epithelial cells in the gastrointestinal tract [11]. Claudins are crucial components of TJs that play important roles in the formation of tightly connected cell sheets, the creation of physiologic barriers separating the apical and basolateral spaces, and the control of the permeability of electrolytes across a paracellular barrier via the formation of hetero- or homodimers [12–14]. Claudins bind to each other via two extracellular loops, and also bind to a

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PSD-95/Dscs-large/ZO-1 (PDZ)-domain-containing TJ protein, ZO-1, through their carboxyl terminus [15]. ZO-1 binds the TJ to the actin cytoskeleton and interacts with several cell signaling and transcriptional regulatory proteins; therefore, claudins are believed to play an important role in determining cell polarity through cytoskeleton rearrangement [16–19].

To date, about 24 claudins have been identified and many members of the claudin family show a distinct organ-specific distribution pattern within the human body [12, 16, 20]. Claudin-4 was initially identified as a transmembrane receptor for *Clostridium perfringens* enterotoxin which binds to claudin-4 in epithelial cells of the intestinal tract with high affinity. As a result, small pores are formed in the cell membrane, which increases the cellular permeability and disrupts the balance of osmosis, ultimately destroying the cells [21]. Recently, some claudins have been associated with carcinogenesis and the progression of human malignancies [22–26]. In particular, altered levels of claudin-4 at the mRNA and protein levels are likely to be associated with cancer development and progression; reduced claudin-4 expression exhibited a close correlation with invasion and metastasis of human pancreas cancers, and overexpression of *CLDN4* effectively diminished invasion and anchorage-independent growth in pancreas cancer cells [27]. These data suggested that decreased claudin-4 expression may promote cancer cell invasion and metastasis in vitro and in vivo, as has been reported for claudin-3 and claudin-7 in several malignancies [28]. However, *CLDN4* expression was frequently upregulated in ovarian tumors [22, 29]; since little is known about the relationship between claudin-4 expression and cancer progression, further investigation is required to understand the biological roles of claudin-4 in human cancers. In this study, we analyzed the correlation between the levels of claudin-4 expression and clinicopathological findings in human CRC tissues. A small interfering RNA (siRNA)-mediated *CLDN4* knockdown experiment was conducted to determine the biological significance of claudin-4 expression in the progression of CRC cells.

Materials and Methods

Cell Lines and Tissue Samples

Five colon cancer cell lines (LoVo, TCO, DLD-1, WiDr, SW480) were maintained in RPMI-1640 (Invitrogen, Grand Island, N.Y., USA) supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotic-antimycotic solution (Invitrogen). Cells were incubated at 37°C in humidified atmosphere of 95% air and 5% CO_2.

Table 1. Variation in metastatic lesions in CRC patients analyzed in the primary tumor study

Primary tumors	Cases
Total	129
No metastasis	74
Metastasis present	55
Lymph node[a]	38
Distant metastasis[b]	4
Only liver	2
Only lung	1
Lung and dissemination	1
Lymph node[a] and distant metastasis[b]	13
Lymph node and liver	9
Lymph node and dissemination	1
Lymph node, liver and lung	2
Lymph node, liver and dissemination	1

[a] Matched lymph node metastases were also confirmed by histological examination. Their claudin-4 protein expression was also investigated.

[b] Tumors were diagnosed pathologically in the cases with surgical resection of metastasis or on imaging in the cases without surgical treatment.

Table 2. Variation in metastatic lesions in patients with CRC analyzed in the metastatic tumor study

Metastatic tumors	Cases
Total	44
Only lymph node[a]	30
Lymph node and liver	6
Lymph node, liver and lung	3
Lymph node and dissemination	1
Only liver	3
Only liver and lung	1

[a] Intra-abdominal and intra-thoracic lymph node metastases were confirmed by histological examination. Their expression of claudin-4 protein was also investigated.

In total, 129 cases of sporadic human CRCs surgically removed at Kobe University Hospital were studied. Formalin-fixed and paraffin-embedded specimens from 129 cases of primary CRC and from 44 cases of metastatic CRC were collected. Variation in the metastatic lesions of the CRC patients analyzed in this study is shown in tables 1 and 2. None of these cases had received adjuvant chemotherapy or radiotherapy before surgery, and informed consent was obtained from all patients. Histologic examination was performed according to the general rules of the Japanese Society for Cancer of the Colon and Rectum [30] and the classification of the International Union against Cancer [31].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs from each CRC cell line were isolated using a RNeasy Mini kit (Qiagen, Hilden, Germany). RT-PCR analysis was performed with a OneStep RT-PCR assay Kit (Qiagen) [32]. According to the poly(A) cDNA-specific RT-PCR method [33], the primer sets were designed as follows; *CLDN4*, 5′-GGACAGCTTCACCCTTGG-3′/5′-TTTTTTTTTTTTTTTTCCTGTGCA-3′, and *β-actin,* 5′-CCACGAAACTACCTTCAACTCC-3′/5′-TCATACTCCT GCTGCTTGCTGATCC-3′. Each 25-μl reaction mixture containing 10 ng of total RNA, 1 μM of the primer pair and 0.75 units of reverse transcriptase and *Taq* DNA polymerase was amplified for 35 cycles using the following regimen: RT at 50°C for 30 min; denaturation at 94°C for 30 s; annealing at 58°C for 30 s, and extension at 72°C for 1 min. RT-PCR products were subjected to electrophoresis in 2% agarose gel.

Western Blotting

The cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100 and 5 mM EDTA containing both 1% protease inhibitor (Sigma-Aldrich, St. Louis, Mo., USA) and 1% phosphatase inhibitor cocktail II (Sigma-Aldrich) [32]. Proteins (40 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto polyvinylidene fluoride membranes (Millipore, Bedford, Mass., USA). Antisera against claudin-4 (Zymed, clone 3E2C1; San Francisco, Calif., USA) and anti-β-actin (Sigma-Aldrich) were used in the primary reaction. The specific reaction of anti-claudin-4 antibody has been confirmed in previous reports [22, 29]. Horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK) was used as a secondary antibody for an enhanced chemiluminescence reaction system (Immunostar Reagents; Wako, Osaka, Japan).

siRNA Transfection Assay

Human *CLDN4*-specific siRNA (5′-CGCACAGACAAGCCUUACUUU-3′/5′-AGUAAGGCUUGUCUGUGCGGG-3′) was designed and based on the coding sequence of human *CLDN4* [34]. Control (non-silence) siRNA (5′-UUCUCCGAACGUGUCACGUdTdT-3′/5′-ACGUGACACGUUCGGAGAAdTdT-3′) was also synthesized. All of the siRNA sequences were subjected to a basic local alignment search tool to confirm the absence of homology to any additional known coding sequences in the human genome. Each siRNA was added to the SW480 cells (4×10^4 cells/well) in 24-well plates at a final concentration of 10 nM using RNAiFect Transfection Reagent (Qiagen). Twenty-four hours after siRNA transfection, cells were incubated in RPMI-1640 plus 2% FBS. The medium was renewed every 48 h. We confirmed that the transfection of *CLND4* siRNA and control siRNA did not affect the levels of *β-actin*.

Cell Motility Assay

Cell motility and invasive activity were estimated using a modified two-chamber invasion assay with Transwell plates (6.4 mm in diameter; polyethylene terephthalate membrane, 8-μm pore size) as described previously [27, 34]. Twenty-four hours after siRNA transfection, an aliquot of 4×10^4 cells was placed in the upper chamber with 0.8 ml of serum-free medium, whereas the lower chamber was loaded with 0.5 ml of medium containing 10% FBS. Cells on the upper side of the membrane were wiped off, the membrane was fixed with 100% methanol and cells on the lower side of the membrane were counterstained with Mayer's hematoxylin. The cells that had migrated into the lower chamber were counted under a light microscope. Experiments were done in triplicate.

Immunohistochemical Analysis

Immunohistochemistry was performed using the streptavidin-biotin-peroxidase method with LSAB kit (DAKO, Carpinteria, Calif., USA) [32]. Briefly, deparaffinized and dehydrated 4-μm sections were autoclaved in a citrate buffer (pH 6.0) for 15 min at 121°C to retrieve antigenicity. After blocking endogenous peroxidase with 0.3% hydroxyperoxide and nonspecific binding sites with 3% BSA, anti-claudin-4 antibody (Zymed) was applied to the sections as the primary antibody and incubated. Subsequently, sections were incubated with biotinylated goat anti-mouse IgG and streptavidin conjugated to horseradish peroxidase. Chromogenic fixation was carried out by immersing the sections in a solution of 3,3-diaminobenzidine tetrahydrochloride. The sections were then counterstained with Mayer's hematoxylin.

Claudin-4 immunoreactivity was graded according to the number of stained cells and the staining intensity in individual cells as follows: – = almost no positive cells; + = 5–50% of tumor cells showed weak immunoreactivity; ++ = 5–50% of tumor cells showed moderate immunoreactivity or <30% of tumor cells showed intense immunoreactivity; +++ = >30% of tumor cells showed intense immunoreactivity [32]. Claudin-4 expression was evaluated independently by three independent observers (J.U., S.S., H.Y.) and all the sections were scored twice to confirm the reproducibility of the results.

Statistical Analysis

We used the χ^2 test and the Mann-Whitey U test to evaluate relationships between claudin-4 immunoreactivity and clincopathologic characters in 129 CRC cases . A p value <0.05 was considered statistically significant. Moreover, we used the log rank test to evaluate the relationship between claudin-4 immunoreactivity and recurrence after surgical operation in 40 stage II CRCs which received neither adjuvant chemotherapy nor radiotherapy after the initial resection. Multivariate Cox analysis of recurrence was also performed.

Results

Downregulation of Claudin-4 at the Invasive Front and Metastatic Lesions of CRC Tissues

Immunohistochemical analysis was performed to investigate the expression of claudin-4 in normal colorectal epithelia and CRC tissues. In normal colorectal epithelia, claudin-4 immunoreactivity was detected at the intercellular membrane of enterocytes and in the crypt epithelia specifically (fig. 1a, b). Table 3 summarizes the relationship between claudin-4 immunoreactivity at the invasive front and the clinicopathologic characteristics of the CRCs. Claudin-4 immunoreactivity tended to be well

preserved in well-differentiated adenocarcinomas, but it tended to be decreased in both moderately and poorly differentiated adenocarcinomas ($p = 0.00013$, fig. 1c–e). Interestingly, the expression of claudin-4 was heterogeneous; strong immunoreactivity of claudin-4 was detected on the surface of CRC tissue, while claudin-4 immunoreactivity was decreased at the invasive front and regions of vessel infiltration (fig. 1f–i). Furthermore, reduced claudin-4 expression was significantly correlated with depth of invasion ($p < 0.0001$), invasive pattern ($p < 0.0001$), lymphatic vessel invasion ($p < 0.0001$), venous vessel infiltration ($p = 0.00011$) and metastases [lymph nodes ($p = 0.00038$), liver ($p = 0.013$) and other distant organs ($p = 0.00079$)].

We next examined claudin-4 expression in 44 available cases of primary CRC at the invasive front and in their corresponding metastatic lesions. In comparison with the corresponding primary cancer tissues, claudin-4 immunoreactivity of metastatic lesions was decreased in 30 cases (68.1%, $p < 0.0001$; table 4). Representative illustrations of claudin-4 immunohistochemistry in metastatic carcinoma of the liver and lymph nodes are shown in figure 2.

Relationship between the Immunoreactivity of Claudin-4 and Recurrence in Stage II CRC

We further investigated the relationship between immunoreactivity of claudin-4 and recurrence after surgical resection in 40 cases with stage II CRCs to confirm whether low claudin-4 expression at the invasive front was a valuable prognostic indicator independent of clinical stage. The average observation period was 31 months. Although only 1 (9%) of the 14 cases with high claudin-4 expression showed recurrent carcinoma, there were as many as 7 (27%) recurrent cases among the 26 cases with decreased claudin-4 expression. The patients with tumors that showed low claudin-4 expression tended to have more frequent local recurrences ($p = 0.1795$; fig. 2g). Multivariate Cox analysis of local recurrence in stage II CRC revealed that cases with decreased claudin-4 expression tended to have more recurrent tumors ($p = 0.1573$) than those with high histological grade ($p = 0.2218$) or vessel invasion ($p = 0.8598$); however, no statistical significance was found.

Downregulation of Claudin-4 Expression Effectively Promoted Cancer Cell Motility

We investigated claudin-4 expression at the mRNA and protein levels in five CRC cell lines. Although *CLDN4* expression was almost equivalent in these lines, claudin-4 was expressed at a different level in each CRC

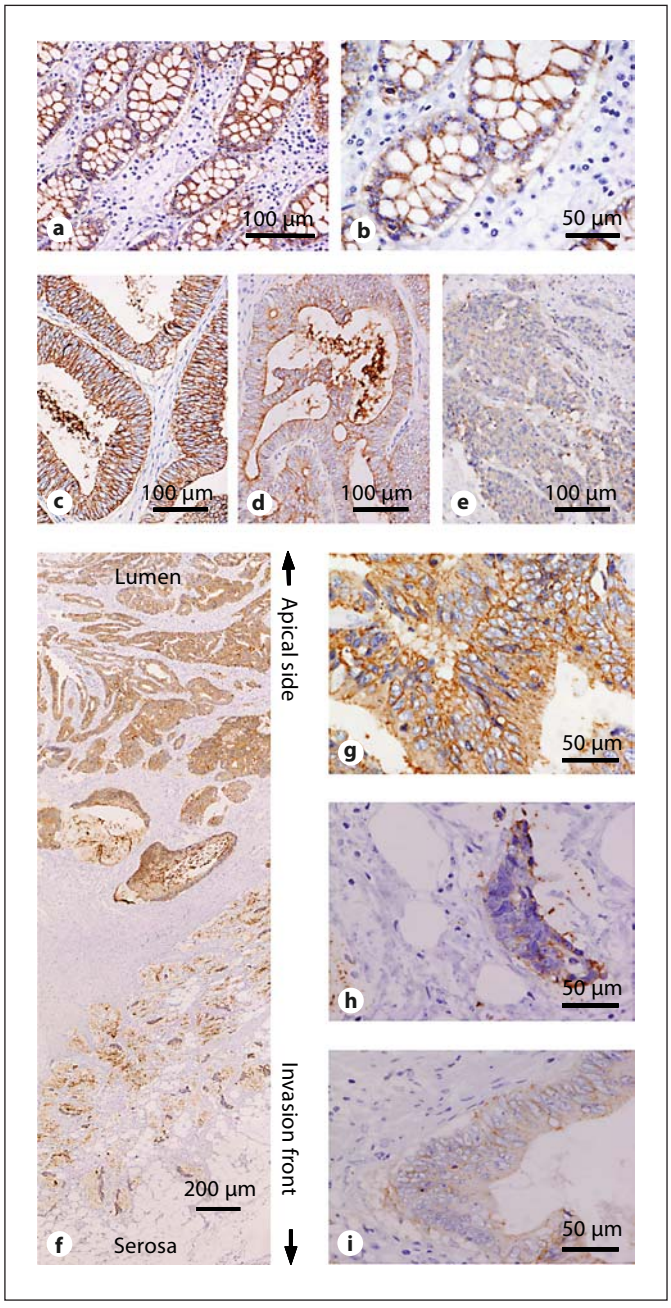

Fig. 1. Reduced expression of claudin-4 at the invasive front of human colon cancer tissues. **a**, **b** Expression of claudin-4 in normal colon epithelia. Membranous localization of claudin-4 was detected. **a** ×200. **b** ×400. **c–e** Claudin-4 immunoreactivity was correlated with histological type. **c** Well-differentiated type (×200). **d** Moderately differentiated type (×200). **e** Poorly differentiated type (×200). **f–i** Representative illustration of heterogenous expression of claudin-4 in colon cancer tissue sample. Expression of claudin-4 was well-preserved at the tumor surface but decreased at the invasive front of colon cancer cells. **f** Low magnification of the tumor (×40). **g** Cancer cells at the surface (×400). **h** Cancer cells at the invasive front (×400). **i** Cancer cells showing lymphatic vessel infiltration (×400).

Table 3. Relationship between claudin-4 immunoreactivity and clinicopathologic characters of CRC

	Cases		Claudin-4 expression[a]				p value[b]
			+++		+++ to –		
	n	%	n	%	n	%	
Total	129	100	55	43	74	57	
Sex							
Male	85	66	33	26	52	40	
Female	44	34	22	17	22	17	0.22
Location							
Right side of the colon	42	33	18	14	24	19	
Left side of the colon	87	67	37	29	50	38	0.97
Histology[c]							
Well differentiated	43	33	30	23	13	10	
Moderate	82	64	25	20	57	44	
Poor or mucinous	4	3	0	0	4	3	0.00013
Depth of invasion							
m	11	9	11	9	0	0	
sm or mp	30	23	20	15	10	8	
ss, se, a1 or a2	83	64	24	19	59	45	
si	5	4	0	0	5	4	<0.0001
Invasion pattern							
α (expansive)	17	13	15	12	2	1	
β (intermediate)	51	40	30	23	21	17	
γ (infiltrative)	61	47	10	8	51	39	<0.0001
Vessel infiltration							
Lymphatic vessels							
Positive	99	77	32	25	67	52	
Negative	30	23	23	18	7	5	<0.0001
Venous vessels							
Positive	83	64	25	19	58	45	
Negative	46	36	30	24	16	12	0.00011
Metastasis							
Lymph node							
Positive	51	40	12	10	39	30	
Negative	78	60	43	33	35	27	0.00038
Liver							
Positive	23	18	4	3	19	15	
Negative	106	82	51	40	55	42	0.013
Metastatic organs							
Positive	29	22	4	3	25	19	
Negative	100	78	51	40	49	38	0.00079

The mean age of the cases was 68.1 years (73.3 and 57.8 for cases of +++ and ++ to – claudin expression; $p < 0.018$).

[a] The expression of claudin-4 was evaluated as described in Materials and Methods.

[b] Statistical analyses were performed by χ^2 test and Mann-Whitney U test. A p value < 0.05 was considered statistically significant.

[c] Histological classification and clinicopathological staging were performed according to previously established rules [30].

line (fig. 3a, b). We confirmed that TCO cells showed prominent membrane immunoreactivity for claudin-4, while the membranous immunoreactivity was faint in SW480 cells (data not shown). To clarify the biological role of claudin-4 in the invasive properties of CRC cells, we carried out an siRNA-mediated knockdown of *CLDN4* expression in SW480 cells. The siRNA knockdown examination specifically suppressed claudin-4 protein expression at a concentration of 10 nM, but there was no significant difference in cell proliferation in the presence

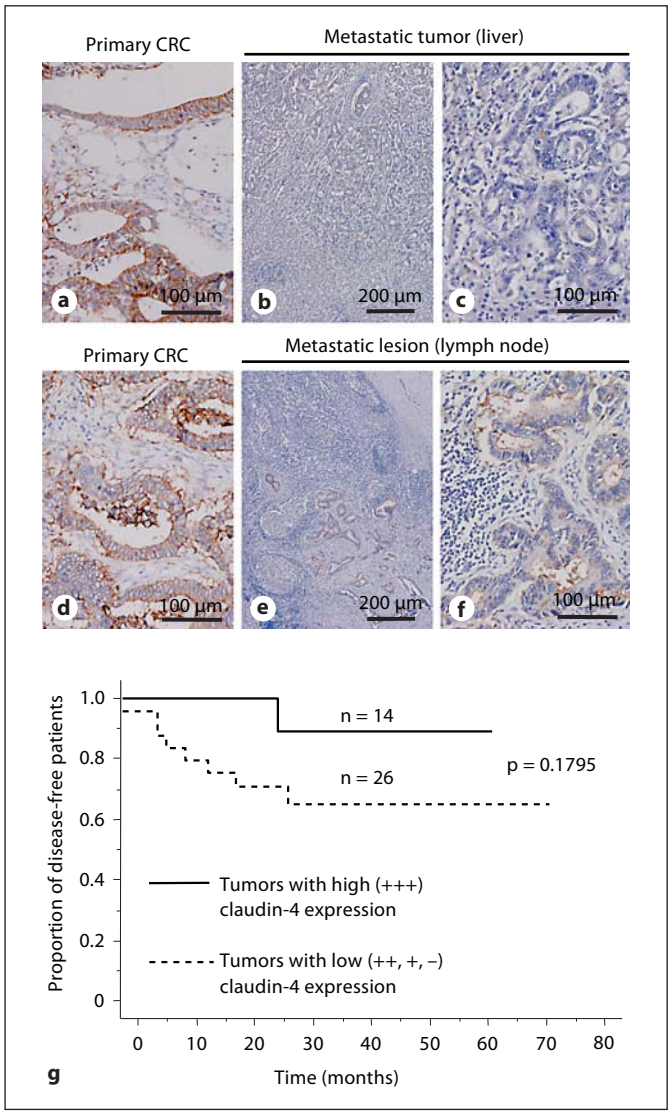

Fig. 2. Expression of claudin-4 in metastatic lesions of human colon cancer. Representative results of immunohistochemical analysis. **a–c** Primary colon cancer (**a**, ×200) and liver metastasis (**b**, ×40; **c**, ×200). **d–f** Primary colon cancer (**d**, ×200) and lymph node metastasis (**e**, ×40; **f**, ×200). **g** Kaplan-Meier disease-free analysis curves in stage II CRC for claudin-4 expression. Although no statistical difference was detected (p = 0.01796), the patients with tumors that showed a low level of claudin-4 tended to have a poorer prognosis.

or absence of *CLDN4* siRNA transfection (fig. 3c). However, *CLDN4* knockdown in SW480 cells resulted in significantly greater cell motility than was seen in the cells transfected with control siRNA (p = 0.0037; fig. 3d).

Discussion

Altered expression of claudins has been reported in several malignant tumors, including those of the ovary [22, 35, 36], prostate [23], breast [24, 37], pancreas [25], colorectum [26, 38] and stomach [39] in comparison with the corresponding normal tissues; however, little is known about the mechanism through which claudins control cancer cell behavior. A previous study reported that 75% of the stage II CRCs exhibited elevated levels of claudin-1 expression, while decreased claudin-1 expression was directly associated with higher tumor grade, recurrence and poor survival [26]. In addition, frequent nuclear localization of claudin-1 was reported in colon carcinoma tissues and cancer cell lines [40]; overexpression of claudin-1 in CRC cell lines induced structural and functional changes in markers of epithelial-mesenchymal transition and had significant effects on the growth of xenografted tumors and metastases in mice [40]. E-cadherin and β-catenin/Tcf signaling is considered to be a possible mechanism underlying claudin-1-dependent changes [41]. Furthermore, it has been documented that loss of claudin-7 expression correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast, occurring predominantly in high-grade (Nuclear and Elston grade 3) lesions [24]. In esophageal cancer, reduced expression of claudin-7 at the invasive front was significantly associated with the depth of invasion, clinicopathological stage, lymphatic vessel invasion and lymph node metastasis, suggesting that downregulation of claudin-7 at the invasive front of esophageal cancer may lead to tumor progression and subsequent metastatic events [42]. However, further investigation is required for the clarification of the biological role of claudins in human cancer.

Table 4. Claudin-4 expression of primary and metastatic lesions in CRC cases

	Total	Claudin-4 expression				p value
		–	+	++	+++	
Primary lesion	44 (100%)	2 (4.5)	8 (18.1)	22 (50)	12 (27.2)	
Metastatic lesion	44 (100%)	9 (20.4)	25 (56.8)	7 (15.9)	3 (6.8)	<0.0001

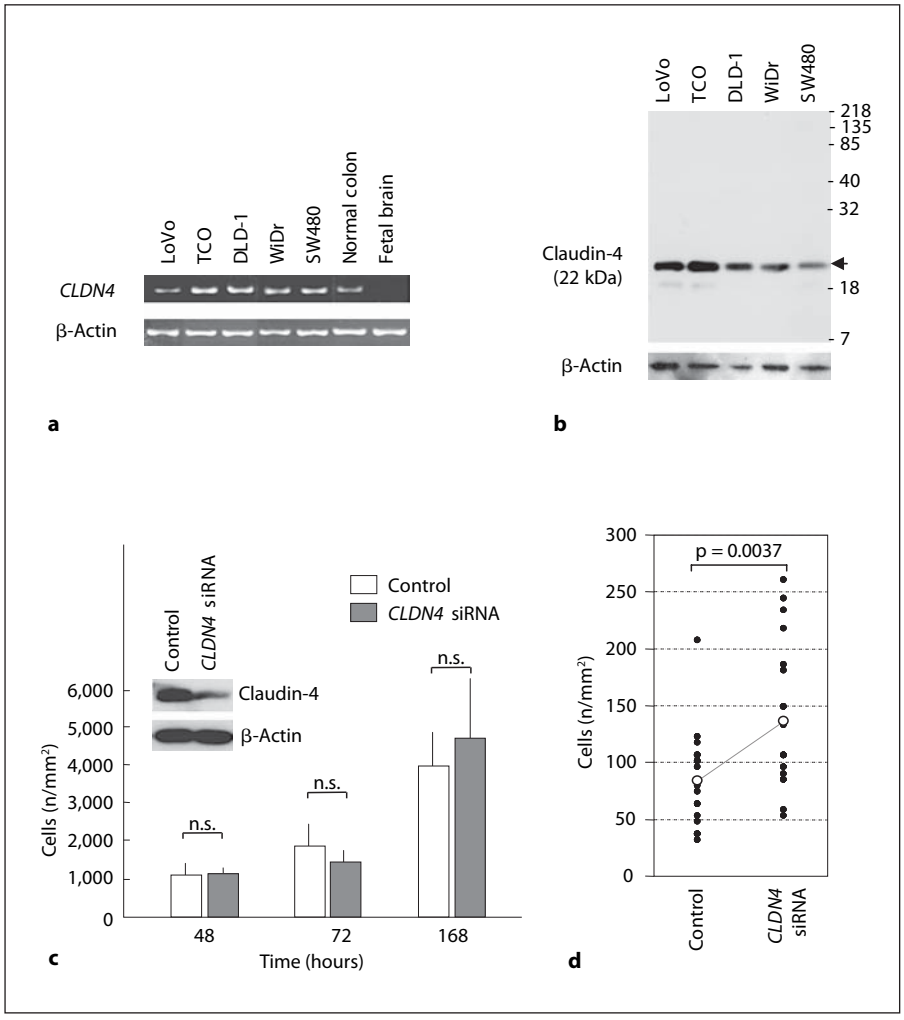

Fig. 3. Downregulation of claudin-4 expression promotes cancer cell motility. **a**, **b** Expression of claudin-4 in cultured CRC cells at mRNA (**a**) and protein level (**b**). **c**, **d** siRNA-mediated knockdown of *CLDN4* promoted cell motility. **c** The effect of *CLDN4* siRNA on SW480 cell growth. No significant change in cell growth was detected in the presence or absence of *CLDN4* siRNA transfection. *CLDN4* siRNA specifically suppressed claudin-4 expression (left upper corner). **d** Results of cell migration assay. Downregulation of *CLDN4* effectively promoted cell migration in SW480 cells ($p = 0.0037$). ● = Number of cancer cells migrating in a unit area; ○ = mean of migrating cells.

To our knowledge, this is the first report to show heterogeneous expression of claudin-4 at the surface and invasive front of the same CRC tissue sample. The results demonstrated that low claudin-4 expression contributed to CRC progression, histological grade, metastasis and poor prognosis, which is consistent with the results of examinations for pancreas cancer cells [25, 27]. In a previous study using tissue microarray (TMA) technology [26], no significant association was found between claudin-4 expression and any of the clinicopathological factors examined. The TMA method is valuable for comprehensive experiments that include many tissue samples. However, in the TMA, only a small region of each tissue sample is evaluated; TMA may thus be unsuitable for detailed evaluations of individual samples, such as heterogeneous expression patterns. However, intense positive claudin-4 immunolabeling is noted within virtually all primary and metastatic pancreatic cancers and pancreatic intraepithelial neoplasias with a membranous distribution [25], and overexpression of claudin-4 has also been reported to suppress the growth, invasion and metastasis of pancreas cancer cells [27]. Conversely, increased levels of claudin-4 expression have also been reported in the majority of ovarian carcinomas and shown to be closely associated with the grade of malignancy [22]. These observations suggest that claudin-4 may play different roles in cancers originating from different organs. In the present study, mislocalization or weak membranous expression of claudin-4 was observed in CRC cell lines and tissue samples containing precancerous lesions (data not shown). Such expression was not detected in normal epithelial tissues, suggesting that an alteration in the distribution of the claudin-4 protein may be the key to CRC progression. The *CLDN4* knockdown experiment

performed in this study confirmed that suppression of membranous claudin-4 expression in SW480 cells promoted the invasion of CRC cells. While SW480 cells transfected with siRNA *CLDN4* showed migration to various degrees, the mean of them was significantly increased compared to control. Further investigation using site-directed mutagenesis within the crucial domain for membranous claudin-4 distribution including the changes in TJ structure and functionality is required to clarify the biological significance of claudin-4 distribution in cancer cell progression.

Since claudin family members are crucial components of TJ, alterations in claudin-4 expression may affect permeability at TJs, possibly increasing the diffusion of nutrients and other extracellular growth factors to promote cell growth and survival [17]. Thus, dysfunction of cell-to-cell contact at the TJ may activate cell proliferation and motility even in cancer cells [18, 19, 43]. In this study, poorly differentiated adenocarcinomas exhibited lower levels of claudin-4 than well-differentiated adenocarcinomas, suggesting that disruption of claudin-4-mediated TJ may allow increased diffusion of nutrients and other extracellular signaling stimulators in poorly differentiated carcinomas [44]. *CLDN4* gene silencing by promoter hypermethylation has been found in bladder cancer [45] and ovarian cancer [46], and *CLDN7* gene silencing by promoter methylation has been found in breast cancer cells [24]. To evaluate the mechanism by which *CLDN4* expression is downregulated during the process of colorectal carcinogenesis, further investigations are needed on the status of promoter methylation of the *CLDN4* gene or allelic deletion at the *CLDN4* locus (7q11.23).

It has been proposed that inhibition of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-OH kinase (PI3K) signaling by specific inhibitors results in a decrease in claudin-4 expression [27, 47]. Since the MAPK pathway has been reported to activate cell migration and motility by activating Rho and integrin family members [48, 49], claudin-4 may affect MAPK-Rho-modulated cell motility. The PI3K-Akt signaling pathway plays a pivotal role not only in cancer cell survival but also in cell polarity or differentiation [50–52], and activation of PI3K-Akt signaling is a common event in almost all human malignancies [53–55]. The concentrations of epidermal growth factor and Ca^{2+} have been reported to regulate claudin-4 expression in MDCK normal dog kidney cells [56]. Escape from these claudin-4 regulators may allow the cells to come into contact with extracellular nutrients and growth factors, resulting in the promotion of cell transformation in the multistep process of carcinogenesis of the colorectum.

The present results suggest that decreased claudin-4 expression at the invasive front of CRC may be one of the important predictors of disease recurrence, and thus it may be necessary to strictly follow up not only stage II CRCs but all cases of low claudin-4 expression at the invasive front after a curative operation. Although the mechanism responsible for low claudin-4 expression in CRCs is still under investigation, this report unequivocally identifies claudin-4 as a biomarker in CRCs. Further investigation into the regulation of claudin-4 expression might characterize particular groups of patients who could benefit from a possible *C. perfringens* enterotoxin-based anticancer therapy targeting claudin-4.

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