Hypermethylation-Modulated Downregulation of Claudin-7 Expression Promotes the Progression of Colorectal Carcinoma

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Abstract

Objective: The expression of tight junction-related transmembrane protein claudin-7 (CLDN7) and its regulatory mechanism were investigated in colorectal carcinomas (CRCs).

Methods: Methylation specific-polymerase chain reaction and treatment with demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) were conducted to analyze the methylation status at the *CLDN7* promoter region in the Colo320 CRC cell line. We used a total of 26 stage 0 CRCs with an adenoma component and 90 invasive CRCs (stage I-IV), as well as their corresponding lymph node metastases, in an immunohistochemical study.

Results: In Colo320 (claudin-7-negative) cells, hypermethylation at the *CLDN7* promoter was detected and treatment with 5-aza-dC restored *CLDN7* expression. In CRC tissues, decreased claudin-7 expression was detected in 62% of stage 0 CRCs and 80% of stage I-IV CRCs, compared with their adjacent adenoma lesions and non-neoplastic epithelia, which had a close correlation with the incidence of vessel infiltration and clinicopathologic stage. Hypermethylation at the *CLDN7* promoter was detected in 20% of CRCs with low claudin-7 expression. However, claudin-7 expression tended to be re-expressed in their corresponding lymph node metastases.

Conclusion: These findings suggest that the *CLDN7* gene silencing by promoter hypermethylation and the resultant reduction of claudin-7 expression may play an important role in the progression of CRCs.

(200 words)

Introduction

Metastasis is the primary cause of death from colorectal carcinoma (CRC), and consists of a series of sequential steps, all of which must be successfully completed for metastasis to occur: 1) the shedding of cells from a primary tumor into the circulation; 2) survival of the cells in the circulation; 3) arrest in a new organ; 4) extravasation into the surrounding tissue; 5) initiation and maintenance of growth; and 6) vascularization of the metastatic tumor [1]. Among these steps in the process of metastasis, the loss of cell-to-cell adhesion in the neoplastic epithelium is the initial step [2]; therefore, understanding the mechanism(s) through which the infiltration and/or metastatic potential of cancer cells may increase is required if a novel strategy for controlling human malignancies is to be developed. The junctional complex of simple epithelial cells located at the most-apical part of the lateral membrane consists of three components: tight junctions (TJs), adherens junctions and desmosomes [3]. Although adherens junctions and desmosomes mechanically link adjacent cells, TJs are responsible for intercellular sealing, resulting in their barrier function and paracellular transport [4]. In addition, TJs have been shown to influence various signaling pathways that regulate epithelial differentiation, proliferation and migration [3, 4].

Claudins are a family of transmembrane proteins that contains four transmembrane domains and two extracellular loops, which form the backbone of TJ strands by binding the homophilic and/or heterophilic interactions [4, 5]. The intracellular component of claudins is responsible for protein-protein interactions with the other TJ molecules, such as ZO-1 and occuldin [6]. The modulation of claudin expression is closely associated with the oncogenic transformation of cells, and the loss of normal TJ functions is therefore believed to constitute a hallmark of human carcinomas [7]. Indeed, altered expression of claudins has been documented in CRC: Overexpression of claudin-1, claudin-8 and claudin-12 are frequently detected in primary CRCs and liver metastases

[8, 9], whereas claudin-4 expression is downregulated in a large fraction of CRCs [10]. Although the mechanism of these alterations of claudin expression has not yet been explored, these findings indicate that altered claudin expression may play a critical role in the activities of invading and metastasizing CRC cells.

Recent reports suggest that the disruption of TJ protein expression can represent the onset of the epithelial-mesenchymal transition (EMT), which promotes cell motility and invasiveness [11]. We have previously demonstrated that the loss of claudin-4 expression was detected in CRCs and the siRNA-mediated claudin-4 knockdown in SW480 claudin-4-positive CRC cells upregulated cell motility, suggesting that the disruption of claudin-4-mediated TJ construction enhances cancer cell invasion and metastasis in CRCs [10]. In this study, we examined claudin-7 (CLDN7) expression in CRCs to determine the relationship between their expression and clinicopathological findings. In particular, we focused on the incidence of lymph node metastasis, since the reduction of claudin-7 expression at the invasive front of esophageal cancer was significantly associated with the depth of invasion, lymphatic vessel infiltration and lymph node metastasis [12]. Furthermore, the methylation status of *CLDN7* promoter was investigated to reveal the mechanism which controls *CLDN7* expression levels during the progression and development of CRCs.

Materials and Methods

Cell Culture and Treatment

Human colon cancer cell lines (DLD-1, SW480, LoVo, TCO, Colo201 and Colo320) were obtained from American Type Culture Collection (Manassas, VA, USA), Cell Resource Center for Biomedical Research (Sendai, Japan) and Health Science Research Bank (Osaka, Japan). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37ºC in an atmosphere containing 5% CO_2. Colo320 cells

were seeded in a 100 mm plate at a density of 1 x 10^6 cells. After 24 h, cells were treated with DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich, St. Louis, MO, USA) and histone deacetylase inhibitor trichostatin A (TSA; Sigma-Aldrich), either alone or in combination. The medium was changed every day for 5 days. For the treatment combining 5-aza-dC and TSA, cells were cultured in the presence of 5-aza-dC (0–25 μM) for 4 days and were subsequently treated with TSA (50 ng/ml) for another 24 h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the OneStep RT-PCR kit (Qiagen, Hilden, Germany). Primers for RT-PCR amplification were as follows: *CLDN7*, 5'-CCA CTC GAG CCC TAA TGG TG-3' (forward)/5'-GGT ACC CAG CCT TGC TCT CA-3' (reverse); and *G3PDH*, 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward)/5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse) [13]. After initial step at 50°C for 30 min, cDNA was amplified for 25 cycles. RT-PCR products underwent electrophoresis.

Western Blot Analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 125 mM NaCl, 0.1% Triton-X and 5 mM EDTA) and a protease inhibitor (Sigma-Aldrich). Proteins form each cell line were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis, electrotransferred onto an Immunobilon-P membrane (Millipore, Billerica, MA, USA), and then immunoblotted with rabbit polyclonal antibodies against claudin-7 (1:1000 dilution; Zymed, San Francisco, CA, USA). Mouse monoclonal antibody to β-actin (1:1000 dilution; Sigma-Aldrich) was used for loading control. Horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG and sheep anti-rabbit IgG (GE Healthcare, Piscataway, NJ, USA) were used as secondary antibodies. Proteins

were visualized using enhanced chemiluminescence.

Immunofluorescence

Cells plated in eight-chamber slides were fixed in 100% methanol and were incubated with the following rabbit monoclonal claudin-7 antibody (1:1000 dilution). Cells were then incubated in RPMI-1640 medium with anti-rabbit fluorescein isothiocyanate-conjugated antibody (Jackson, West Grove, PA, USA). 4',6-diamidino-2-phenylindole (DAPI) was used for staining of the nuclei.

Methylation-Specific Polymerase Chain Reaction (MSP) Analysis

Genomic DNA (1 μg) extracted from tissue samples or cell lines was denatured by 0.3M NaOH for 10 min at 37ºC, and the samples were incubated at 50ºC for 16 h after adding hydroquinone (Sigma-Aldrich) and sodium bisulfate (Wako Pure Chemicals, Osaka, Japan). MSP was performed to analyze the methylation status at CpG-rich area in the *CLDN7* promoter (–831 ~ –330 bp) as described elsewhere [13]. Primers specific for unmethylated DNA and methylated DNA were as follows: unmethylated DNA, 5’-TGG GGA AAG GGT GGT GTT G-3’ (forward)/5’-TTA CCC AAT TTT AAC CAC CAC-3’ (reverse); and methylated DNA, 5’-GAC GTT AGG TTA TTT TCG GTC-3’ (forward)/5’-AAA CGC GTT TCT AAA CGC CG-3’ (reverse). Polymerase chain reaction (PCR) was carried out for 35 cycles at 95ºC for 30 s, 60ºC for 30 s, followed by a final extension at 72ºC for 5 min. PCR products were electrophoresed on 8% acrylamide gels. Genomic DNA isolated from DLD-1 cells methylated by SssI methylase (New England Biolabs, MA, USA) was used as a methylation-positive control, and that demethylated by 5-aza-dC was used as a demethylation-positive control.

Bisulfite Genomic Sequence Analysis

Bisulfite sequence analysis was performed to check the methylation status in cell lines. Genomic DNAs extracted were carried out bisulfite modification and amplified for the 5' region. The primers were designed from regions in which there are no CpG dinucleotides. The PCR products were gel-purified using the QIAquick Gel Extraction (Qiagen) according to the manufacturer's instructions. Each amplified DNA samples was subcloned and applied to the ABI 310 DNA analyzer using BigDye terminator (Applied Biosystems, Foster City, CA, USA).

Tissue Samples

Formalin-fixed and paraffin-embedded specimens from 26 stage 0 CRCs with an adenoma component and 90 invasive CRCs (stage I-IV) removed at Kobe University Hospital (Kobe, Japan) were employed. Histological subtypes and grade were assigned the criterion of *the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus* [14] along with *the International Union Against Cancer classification* [15]. Informed consent was obtained from all patients and the study was approved by the Kobe University Institutional Review Board. These samples were sliced into 4-μm-thick section for immunohistochemistry and DNA extraction. Also, non-neoplastic epithelia and adenoma lesions adjacent to these CRCs were also investigated, as well as 32 of corresponding lymph node metastases.

Immunohistochemistry

Deparaffinized and rehydrated sections were autoclaved to retrieve antigenicity. After blocking endogenous peroxidase activity and non-specific binding, sections were incubated with rabbit polyclonal claudin-7 (1:100 dilution, Zymed) and were then incubated with biotinylated secondary antibody and HRP-conjugated streptavidin

reagent (LSAB2 Kit, Dako, CA, USA) [12]. Claudin-7 protein was visualized using 3,3'-diamino-benzidine and sections were then counterstained in hematoxylin. The degree of claudin-7 expression was evaluated according to the number of stained cells and the staining intensity in individual cells: low, almost no claudin-7-positive cells or < 30% of tumor cells showed weak immunoreactivity; and high, > 30% of tumor cells showed intense immunoreactivity. The results of immunohistochemistry were evaluated by three independent observers (F.N., S.S. and Y.U.), and all of the sections were scored twice to confirm the reproducibility of the results. The *chi*-square test was used to determine correlations between claudin-7 expression and clinicopathologic findings. *P* values less than 0.05 were regarded to be statistically significant.

Results

Promoter hypermethylation-mediated claudin-7 downregulation in CRC cells

To determine whether *CLDN7* mRNA levels reflect protein expression levels, we performed RT-PCR and Western blot analyses on a panel of 6 CRC cell lines. The levels of claudin-7 expression were almost equivalent to those of *CLDN7* in these CRC cell lines, and membranous distribution of claudin-7 was detected by immunofluorescence (fig. 1A-C). Since promoter hypermethylation impedes *CLDN7* expression in breast cancers [13], we analyzed the methylation status at the *CLDN7* promoter CpG sites. Colo320 cells did not express either claudin-7 in the mRNA nor protein levels in accord with hypermethylation at the *CLDN7* promoter CpG sites (fig. 1D). An identical result was obtained from MSP analysis using another MSP primer set (data not shown). In addition, we validated the results of MSP analysis by sequencing of the bisulfite-treated genomic DNAs (fig. 1E).

To investigate if hypermethylation-mediated silencing of claudin-7 expression is functional, treatment with 5-aza-dC demethylating agent in the presence or absence of

TSA DNA methyltransferase inhibitor was attempted in Colo320 cells. Treatment with 5-aza-dC promoted demethylation of the *CLDN7* promoter along with increased *CLDN7* mRNA expression in a dose-dependent manner, irrespective of additional TSA treatment (fig. 2).

Reduced claudin-7 expression is associated with the progression of CRCs

Next, we investigated altered claudin-7 expression in stage 0 CRCs with an adenoma component to clarify the significance of claudin-7 during multistep colorectal carcinogenesis. In normal colorectal epithelia, claudin-7 expression was clearly detected around the cell membrane of the basal crypt region as well as that of the superficial crypt region (fig. 3A), a result that was similar to that of the other claudin family members [9, 10, 16]. Although claudin-7 expression was relatively retained in adenoma lesions, the frequency of the downregulation of claudin-7 expression was significantly higher in the adjacent stage 0 CRC lesions (adenoma, 4 [15%] cases, and stage 0 CRC, 16 [62%] cases; $P = 0.004$); some CRC cases with decreased claudin-7 expression demonstrated *CLDN7* promoter hypermethylation (fig. 3A).

The expression of claudin-7 was then examined to clarify its role during the progression of CRCs. Among 90 invasive CRCs (stage I-IV), decreased claudin-7 expression was frequently (72 [80%] cases) detected, a frequency that was much higher than that in adjacent normal mucosa and adenomas. Interestingly, 18 (20%) of 90 invasive CRCs demonstrated remarkably lower claudin-7 expression at the invasive front than in superficial areas (fig. 3B). No statistical significance was detected in the clinicopathologic findings; however, reduced claudin-7 expression was correlated with the presence of infiltration into lymphatic ($P = 0.027$) and venous vessels ($P = 0.009$) as well as clinicopathologic stage ($P = 0.033$; Table 1). The incidence of lymph node metastasis and distant metastasis tended to be higher in CRCs with low levels of

claudin-7 expression. To investigate the relationship between the reduction of claudin-7 expression and DNA hypermethylation at the *CLDN7* promoter sites, we performed the MSP analysis in these invasive CRC cases. Among 45 informative tumors with low claudin-7 expression, 9 (20%) cases demonstrated methylated *CLDN7* gene products, whereas no methylation of the *CLDN7* promoter was present in 14 tumors with high claudin-7 ($P = 0.069$; data not shown).

Forty-two CRCs with lymph node metastasis were employed to compare claudin-7 expression in primary CRCs and matched lymph node metastatic lesions. In primary CRCs, 37 (88%) cases exhibited low claudin-7 expression; however, decreased claudin-7 expression in lymph node metastases was detected only in 21 (50%) cases ($P < 0.001$) (fig. 4). We confirmed demethylation of the *CLDN7* promoter in several metastatic lesions of the these lymph nodes (fig.4A).

Discussion

Much is known regarding the function of claudins in the maintenance of normal glandular epithelial cell homeostasis; however, their role in the process of tumorigenesis is less elucidated. The exact biological significance of altered claudin expression in cancer remains unknown. For example, a decrease or loss of claudin-4 expression has been documented in various human malignancies, including those of the breast [17], stomach [18], colon [10], and urinary bladder [19]; however, claudins are overexpressed in uterine [20] and ovarian cancers [21]. In the present study, we analyzed stage 0 CRCs and their corresponding adenoma lesions to evaluate changes of claudin-7 expression in the transition from adenoma to cancer. In these cases, CRCs are thought to have developed from adjacent adenomas, but the incidence of the reduction of claudin-7 in stage 0 CRCs was much higher than in adenomas, suggesting that the downregulation of claudin-7 is a key event during the malignant transformation of CRCs. In addition, this

downregulation at the invasive front of CRCs was closely correlated with tumor infiltration into the lymphatic and venous vessels, and with clinicopathologic stage, strongly supporting the biological significance of claudin-7 reduction in the progression of CRCs. According to these results, we attempted to evaluate the alteration of claudin-7 expression on the process of vessel infiltration; however, no significant change was found in cancer cells between inside and outside of the lymphatic or venous vessels (data not shown). Several hypotheses have been suggested to explain the impact of the disruption of TJs, consequently resulting in the increased accessibility of the tumor cells to nutrients [22], the increased cell proliferation [23], and the increased motility associated with metastasis [24, 25]. Therefore, claudin-mediated TJs are an impediment to the unrestricted cell growth and invasion phenotype in cancer.

Epithelial carcinogenesis is associated with the loss of tissue architecture characterized by the loss of organized cell-cell and cell-matrix adhesions, which allows the migration of malignant cells from their immediate environmental niche. In esophageal squamous cell carcinomas (SCC), claudin-7 expression is often lost or localized to the cytoplasm, with this loss closely correlated with the grade of malignancy [12, 26], suggesting that the dysregulation of membranous claudin-7 expression may be one mechanism for the loss of epithelial architecture and the invasion observed in esophageal SCC. Lioni *et al.* [26] have demonstrated that knockdown of claudin-7 in SCC cells led not only to a decrease in E-cadherin expression but also to increased cell growth and enhanced invasion into a three-dimensional matrix; conversely, the claudin-7-overexpressing SCC cells became more adhesive and less invasive along with increased E-cadherin expression. The immunohistochemical study of the expression of claudin-7 in CRC performed in the present study was compatible with the previous reports of Lioni *et al.* [26]. Increased claudin-7 expression was reported in gastric adenocarcinomas, particularly in their

intestinal-type tumors, whereas claudin-7 was localized mainly in the cytoplasm but not in the cellular membrane in gastric adenocarcinomas [27]. Taken together, these results suggest that the mislocalization of claudin-7 may play an important role in the malignant transformation of epithelial cells. The complex consists of epithelial cell adhesion molecule (EpCAM), claudin-7, tetraspanin CO-029, and CD44 variant isoform v6 (CD44v6) recruited into tetraspanin-enriched membrane microdomains. Claudin-7 is a key molecule for the EpCAM/claudin-7/CO-029/CD44v6 complex formation [28]. Further investigation will be required to clarify the biological significance of claudin-7 during the progression of CRCs.

In addition to the effect of nonsteroidal anti-inflammatory drug-modulated induction of *CLDN4* expression [29], the *CLDN4* promoter is controlled by epigenetic modifications of the Sp1-containing critical promoter region [30]. In the present study, we detected the methylation-mediated *CLDN7* gene silencing in 20% of the claudin-7-negative CRCs, which was similar to the previous report on invasive breast cancer [13]. Interestingly, suppression of claudin-7 expression by hypermethylation was observed in stage 0 CRCs but not in either adjacent non-neoplastic mucosa nor adenoma lesions. To our knowledge, this is the first report that demonstrated downregulation of claudin-7 expression by promoter hypermethylation in CRCs. However, only 20% of invasive CRCs with decreased claudin-7 expression showed hypermethylation in the *CLDN7* promoter CpG sites. This can result from the fact that we only evaluated methylation status of the predicted promoter region (–851 ~ –330 bp). Since the *CLDN7* regulatory CpG islands may possibly spans the promoter region and exon 1 (–851~ +279 bp), we should have analyzed methylation status of the other CpG island around predicted promoter region. Furthermore, the levels of claudin-7 expression in metastatic lymph node CRCs were much higher than those in the primary tumors. This discrepancy supports the idea that the loss of claudin-7 expression in the invasive front

of primary CRCs was negatively regulated by such epigenetic alterations at the *CLDN7* promoter CpG sites. However, claudin-7 expression might be suppressed by other mechanism(s) in 80% of the claudin-7-negative CRCs. As shown in fig. 2, since claudin-7 mRNA level was slightly elevated after treatmentment with TSA and without 5-az-dC, histone acetylation might be involved in the regulation of claudin-7 expression. Ikenouchi *et al.* [11] reported that transcription repressor Snail induced EMT with concomitant repression of the expression of claudins and occuldin; Snail binds directly to the E-box of the promoters of the *CLDN* and *occuldin* genes, resulting in complete reexpression of their promoter activity. Further investigation is necessary to clarify the other mechanisms by which claudin-7 expression is tightly regulated.

Reduced intercellular adhesion is implicated in the establishment of metastasis, as it allows the detachment of the malignant cells from the primary tumors. Indeed, we frequently detected decreased claudin-7 expression in primary CRCs with a close correlation with infiltration into vessels and clinicopathologic findings; however, in lymph node metastases, about a half of the metastatic lesions re-expressed claudin-7. Park *et al.* [31] examined the expression of the intercellular adhesion molecules, E-cadherin, α-catenin, β-catenin, γ-catenin and claudin-7, in primary breast carcinoma and their corresponding axillary lymph node metastasis and found a significant reduction of these adhesion proteins in primary tumors; nevertheless, re-expression in their axillary lymph node metastases was frequently observed. Although the role of the mechanism of re-expression of these proteins in metastatic sites remains unclear, re-expression of the proteins enables the circulating tumor cells to resettle at the metastatic organs by facilitating the intercellular adhesion process for metastatic tumor progression. In fact, claudin-7 overexpression increases the homotypic adhesion of the TE-8 SCC cells and subsequently leads to the enhancement of spheroid formation, which is likely to be performed by E-cadherin [26]. Since the epithelial differentiation,

but not EMT, of cells is characterized by the gaining of cell-cell adhesion and polarity followed by the formation of cytoskeletal organization, the re-expression of claudin-7 may help circulating CRC cells to differentiate into epithelium, after which arrest in a new organ, extravasation into the surrounding tissue, and the initiation and maintenance of growth at metastatic sites take place. It is hoped that a more in-depth understanding of these processes will shed light on how to impede the progression and development of CRCs.

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Figure Legends

Fig. 1

Suppression of claudin-7 *(CLDN7)* expression is associated with the *CLDN7* promoter hypermethylation in Colo320 CRC cells. **A** Expression of *CLDN7* in CRC cell lines determined by RT-PCR analysis. *β-actin* was used as loading control. **B** Expression of claudin-7 in CRC cell lines determined by Western blot analysis. β-actin was used as loading control. **C** Results of immunofluorescence of claudin-7 expression in CRC cell lines. Membranous localization of claudin-7 was confirmed. Nuclei were stained with DAPI. **D** The *CLDN7* promoter region methylation status in CRC cell lines. DNAs extracted from each CRC cell line were treated with sodium bisulfate. U, unmethylated; M, methylated. **E** Results of bisulfite genomic sequence analysis. (**a**) Methylation status of the CLDN7 promoter in DLD-1 and Colo320 cells. Filled boxes indicate methylation; open boxes indicate absence of methylation. (**b**) Representative results of bisulfite genomic sequence analysis. Asterisks indicate the CpG islands.

Fig. 2

Restoration of *CLDN7* expression by treatment with 5-aza-dC in Colo320 CRC cells. **A** Results of MSP assay after treatment with 5-aza-dC and TSA. Treatment with 5-aza-dC demethylated at the *CLDN7* promoter region. **B** Treatment with 5-aza-dC increased *CLDN7* expression levels in a dose-dependent manner. Results of RT-PCR analysis (*upper panel*) were numerated by the intensity of each band (*lower panel*).

Fig. 3

Reduced expression of claudin-7 in CRC tissue samples. **A** Expression of claudin-7 in non-neoplastic mucosa (**a**), adenoma lesion (**b**) and stage 0 CRC (**c**). The *CLDN7* methylation status in each component is demonstrated. **B** Heterogenous expression of

claudin-7 in invasive CRC and adjacent adenoma lesion (**a**). At the surface of invasive CRC, claudin-7 expression was slightly decreased (**b**), which was significant at the invasive front (**c**).

Fig. 4

Re-expression of claudin-7 in lymph node metastases. **A** Representative case of primary CRC with decreased claudin-7 expression and its corresponding lymph node metastatic site with high claudin-7 expression was illustrated. Note that re-expression of claudin-7 in lymph node metastases was accompanied with demethylation of the *CLDN7* promoter CpG sites. U, unmethylated; M, methylated. **B** A comparative study of expression of claudin-7 in primary CRCs and their corresponding lymph node metastases.

Table 1. Expression of claudin-7 in CRCs: relationship with clinicopathologic findings

			Claudin-7 expression[1)]				*P* value[2)]
			Low		High		
Total			72	(80%)	18	(20%)	
Location[3)]	Proximal		42	(75%)	14	(25%)	0.128
	Distal		30	(88%)	4	(12%)	
Tumor size	≤ 25 mm		19	(68%)	9	(32%)	0.098
	> 25 mm		53	(85%)	9	(15%)	
Histologic type[4)]	W		23	(70%)	10	(30%)	0.063
	P		49	(86%)	8	(14%)	
Depth of invasion	T1/T2		24	(75%)	8	(25%)	0.378
	T3/T4		48	(82%)	10	(18%)	
Vessel infiltration							
Lymphatic vessels		Present	55	(86%)	9	(14%)	0.027*
		Absent	17	(65%)	9	(35%)	
Venous vessels		Present	48	(89%)	6	(11%)	0.009*
		Absent	24	(67%)	12	(33%)	
Distant metastasis							
Lymph node		Present	37	(88%)	5	(12%)	0.073
		Absent	35	(73%)	13	(27%)	
Other organs		Present	13	(93%)	1	(7%)	0.191
		Absent	59	(78%)	17	(22%)	
Stage	I/II		28	(70%)	12	(30%)	0.033*
	III/IV		44	(88%)	6	(12%)	

1) Immunoreactivity of claudin-7 was determined as described in the text.
2) Statistical analysis was performed by *chi*-square test. *P* values less than 0.05 were regarded to be statistically significant. *$P < 0.05$
3) Tumor location was divided into the proximal (cecum–ascending/transverse colon) and distal (descending/sigmoid colon–rectum) colons.
4) Histologic types were determined according to the morphological features of each CRC. W, well-differentiated type; P, poorly differentiated type.

Figure 1

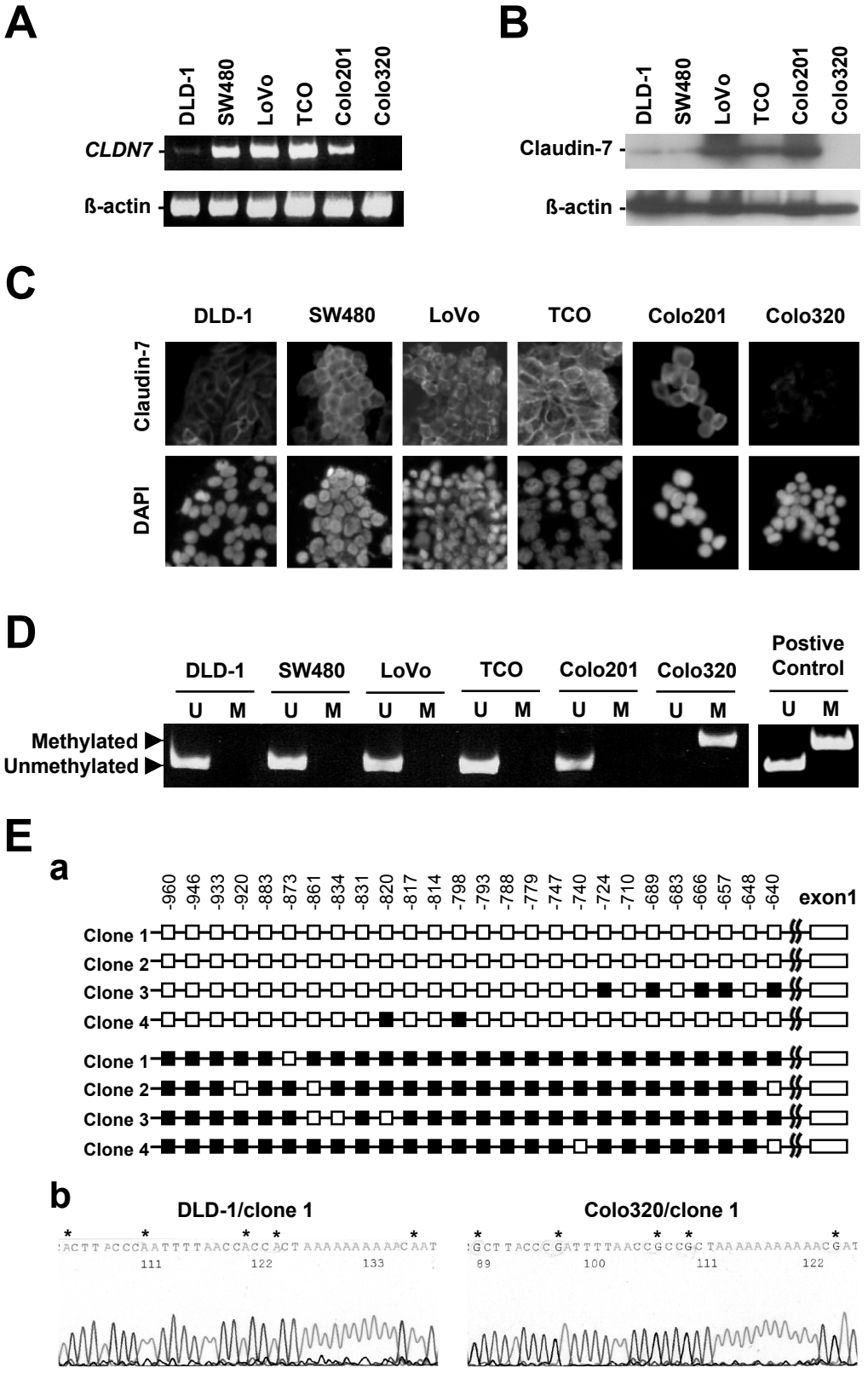

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Figure 2

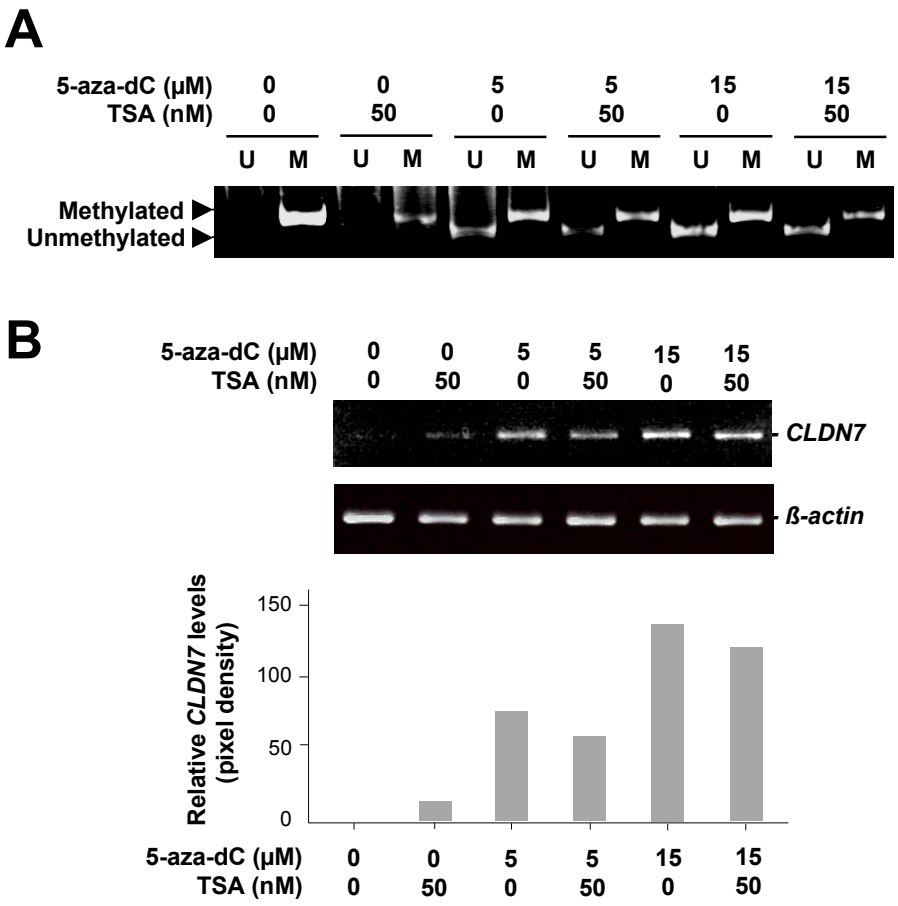

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Figure 3

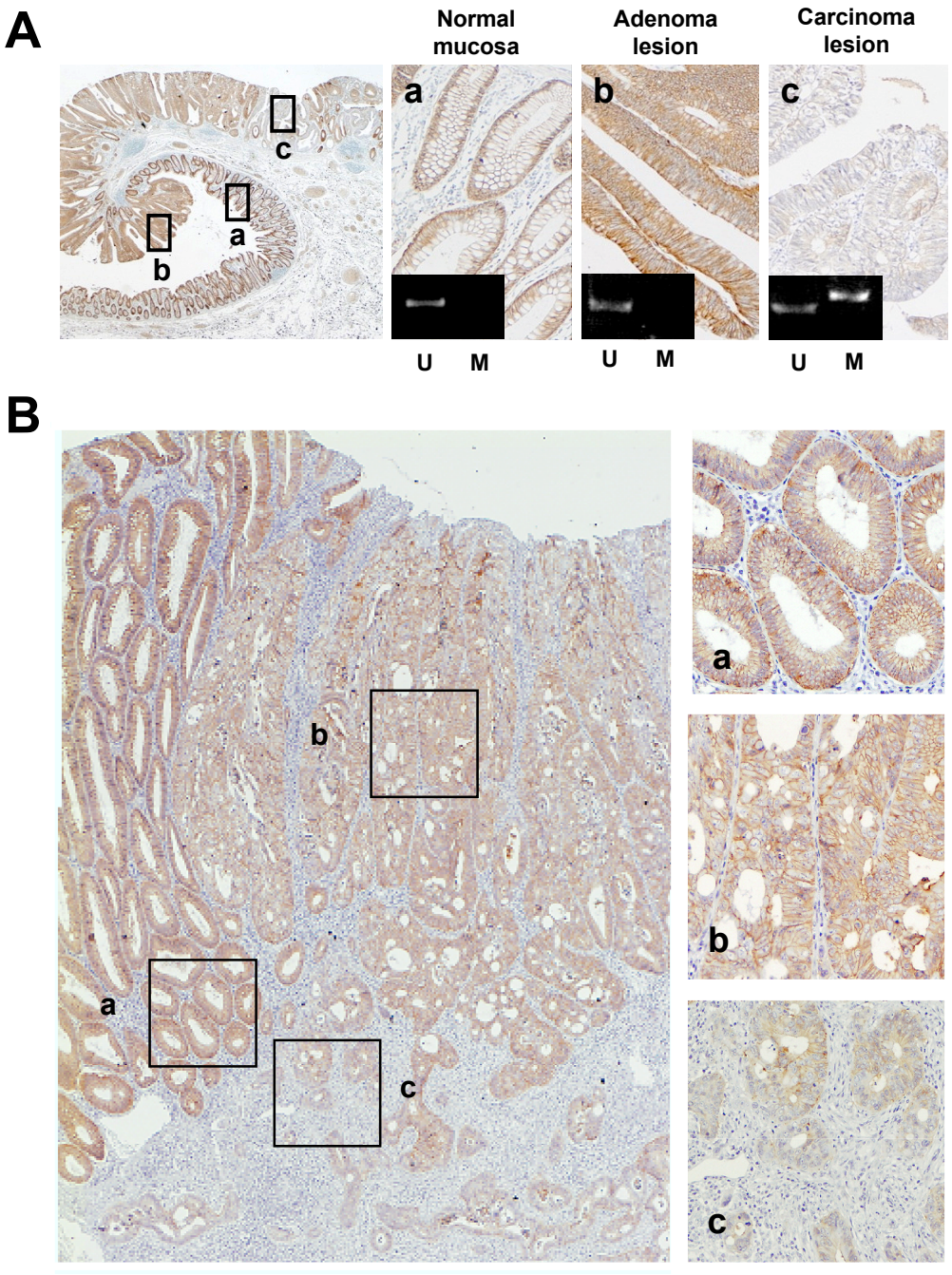

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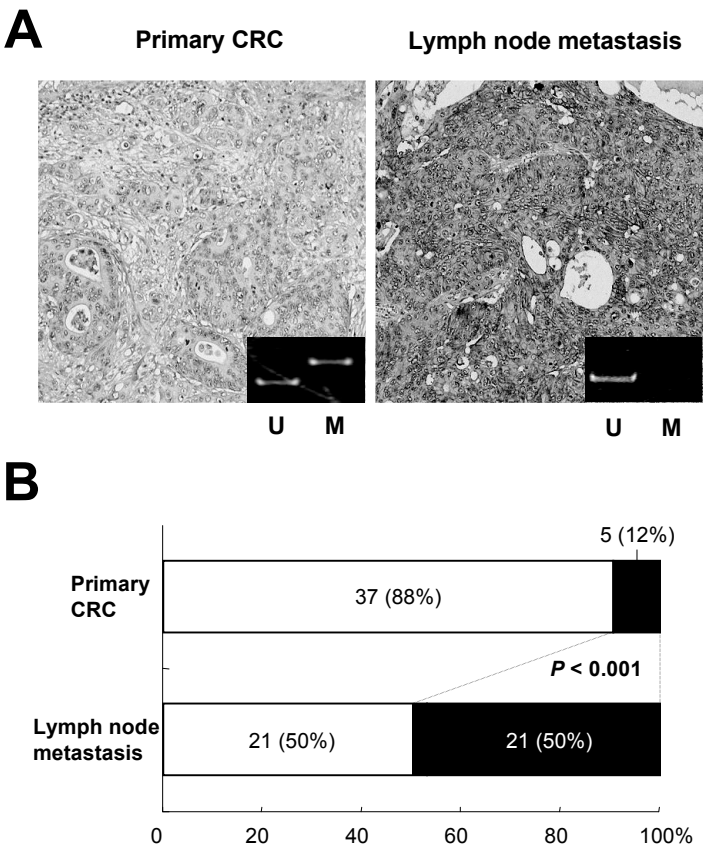
Figure 4


A
Primary CRC
Lymph node metastasis
U
M
U
M
B
5 (12%)
Primary CRC
37 (88%)
P < 0.001
Lymph node metastasis
21 (50%)
21 (50%)
0
20
40
60
80
100%