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

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Key Words
Claudin-7  • Promoter hypermethylation  • 5-aza-2'-deoxycytidine  • Lymph node metastasis  • Colorectal cancer

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 the demethylating agent 5-aza-2'-deoxycytidine 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 (CLDN7-negative) cells, hypermethylation at the CLDN7 promoter was detected and treatment with 5-aza-2'-deoxycytidine restored CLDN7 expression. In CRC tissues, decreased CLDN7 expression was detected in 62% of stage 0 CRCs and 80% of stage I–IV CRCs, compared with their adjacent adenoma lesions and nonneoplastic 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 CLDN7 expression. However, CLDN7 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 CLDN7 expression may play an important role in the progression of CRCs.

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 occludin [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 is 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 CLDN7 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 37°C in an atmosphere containing 5% CO2. Colo320 cells were seeded in a 100-mm plate at a density of 1 × 106 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
Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the OneStep RT-PCR kit (Qiagen, Hilden, Germany). Primers for RT-PCR amplification were as follows: CLDN7, 5’-CCA GTC GAG 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 from each cell line were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis, electrotransferred onto an Immobilon-P membrane (Millipore, Billerica, Mass., USA), and then immunoblotted with rabbit polyclonal antibodies against CLDN7 (1:1,000 dilution; Zymed, San Francisco, Calif., USA). Mouse monoclonal antibody to β-actin (1:1,000 dilution; Sigma-Aldrich) was used for loading control. Horseradish peroxidase-conjugated donkey anti-mouse IgG and sheep anti-rabbit IgG (GE Healthcare, Piscataway, N.J., 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 CLDN7 antibody (1:1,000 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 PCR Analysis

Genomic DNA (1 μg) extracted from tissue samples or cell lines was denatured by 0.3 M NaOH for 10 min at 37°C, and the samples were incubated at 50°C for 16 h after adding hydroquinone (Sigma-Aldrich) and sodium bisulfate (Wako Pure Chemicals, Osaka, Japan). Methylation-specific PCR (MSP) was performed to analyze the methylation status at CpG-rich area in the CLDN7 promoter (~381 to ~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 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). PCR was carried out for 35 cycles at 95°C for 30 s, 60°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were electrophoresed on 8% acrylamide gels.

Results

Promoter Hypermethylation-Mediated CLDN7 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 CLDN7 expression were almost equivalent to those of CLDN7 in these CRC cell lines, and membranous distribution of CLDN7 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 CLDN7 in the mRNA or 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 CLDN7 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 CLDN7 Expression Is Associated with the Progression of CRCs

Next, we investigated altered CLDN7 expression in stage 0 CRCs with an adenoma component to clarify the significance of CLDN7 during multistep colorectal carcinogenesis. In normal colorectal epithelia, CLDN7 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 CLDN7 expression was relatively retained in adenoma lesions, the frequency of the downregulation of CLDN7 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 CLDN7 expression demonstrated CLDN7 promoter hypermethylation (fig. 3A).

The expression of CLDN7 was then examined to clarify its role during the progression of CRCs. Among 90 invasive CRCs (stage I–IV), decreased CLDN7 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 CLDN7 expression at the invasive front than in superficial areas (fig. 3B).
No statistical significance was detected in the clinico-pathologic findings; however, reduced CLDN7 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 CLDN7 expression. To investigate the relationship between the reduction of CLDN7 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 CLDN7 expression, 9 (20%) cases demonstrated methylated CLDN7 gene products, whereas no methylation of the CLDN7 promoter was present in 14 tumors with high CLDN7 (p = 0.069; data not shown).

Forty-two CRCs with lymph node metastasis were employed to compare CLDN7 expression in primary CRCs and matched lymph node metastatic lesions. In primary CRCs, 37 (88%) cases exhibited low CLDN7 expression; however, decreased CLDN7 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 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 CLDN7 expression in the transition from ad-
In these cases, CRCs are thought to have developed from adjacent adenomas, but the incidence of the reduction of CLDN7 in stage 0 CRCs was much higher than in adenomas, suggesting that the downregulation of CLDN7 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 CLDN7 reduction in the progression of CRCs. According to these results, we attempted to evaluate the alteration of CLDN7 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 to cancer.
mor 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), CLDN7 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 CLDN7 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 CLDN7 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 CLDN7-overexpressing SCC cells became more adhesive and less invasive along with increased E-cadherin expression.

Table 1. Expression of CLDN7 in CRCs: relationship with clinicopathologic findings

<table>
<thead>
<tr>
<th></th>
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<tr>
<td></td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Total</td>
<td>72 (80%)</td>
<td>18 (20%)</td>
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<tr>
<td>Location3</td>
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<tr>
<td>Proximal</td>
<td>42 (75%)</td>
<td>14 (25%)</td>
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<tr>
<td>Distal</td>
<td>30 (88%)</td>
<td>4 (12%)</td>
</tr>
<tr>
<td>Tumor size</td>
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<td></td>
</tr>
<tr>
<td>≤25 mm</td>
<td>19 (68%)</td>
<td>9 (32%)</td>
</tr>
<tr>
<td>&gt;25 mm</td>
<td>53 (85%)</td>
<td>9 (15%)</td>
</tr>
<tr>
<td>Histologic type4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>23 (70%)</td>
<td>10 (30%)</td>
</tr>
<tr>
<td>P</td>
<td>49 (86%)</td>
<td>8 (14%)</td>
</tr>
<tr>
<td>Depth of invasion</td>
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<td></td>
</tr>
<tr>
<td>T1/T2</td>
<td>24 (75%)</td>
<td>8 (25%)</td>
</tr>
<tr>
<td>T3/T4</td>
<td>48 (82%)</td>
<td>10 (18%)</td>
</tr>
<tr>
<td>Vessel infiltration</td>
<td></td>
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</tr>
<tr>
<td>Lymphatic vessels</td>
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</tr>
<tr>
<td>Present</td>
<td>55 (86%)</td>
<td>9 (14%)</td>
</tr>
<tr>
<td>Absent</td>
<td>17 (65%)</td>
<td>9 (35%)</td>
</tr>
<tr>
<td>Venous vessels</td>
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<tr>
<td>Present</td>
<td>48 (89%)</td>
<td>6 (11%)</td>
</tr>
<tr>
<td>Absent</td>
<td>24 (67%)</td>
<td>12 (33%)</td>
</tr>
<tr>
<td>Distant metastasis</td>
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<tr>
<td>Lymph node</td>
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<tr>
<td>Present</td>
<td>37 (88%)</td>
<td>5 (12%)</td>
</tr>
<tr>
<td>Absent</td>
<td>35 (73%)</td>
<td>13 (27%)</td>
</tr>
<tr>
<td>Other organs</td>
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<tr>
<td>Present</td>
<td>13 (93%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Absent</td>
<td>59 (78%)</td>
<td>17 (22%)</td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
</tr>
<tr>
<td>I/II</td>
<td>28 (70%)</td>
<td>12 (30%)</td>
</tr>
<tr>
<td>III/IV</td>
<td>44 (88%)</td>
<td>6 (12%)</td>
</tr>
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</table>

1 Immunoreactivity of CLDN7 was determined as described in the text.

2 Statistical analysis was performed by χ² test. * p < 0.05 (statistically significant).

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.

Fig. 4. Re-expression of CLDN7 in lymph node metastases. A Representative case of primary CRC with decreased CLDN7 expression and its corresponding lymph node metastatic site with high CLDN7 expression was illustrated. Note that re-expression of CLDN7 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 CLDN7 in primary CRCs and their corresponding lymph node metastases. White bar = Low CLDN7 expression; black bar = high CLDN7 expression.
The immunohistochemical study of the expression of CLDN7 in CRC performed in the present study was compatible with the previous reports of Lioni et al. [26]. Increased CLDN7 expression was reported in gastric adenocarcinomas, particularly in their intestinal-type tumors, whereas CLDN7 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 CLDN7 may play an important role in the malignant transformation of epithelial cells. The complex consists of epithelial cell adhesion molecule (EpCAM), CLDN7, tetraspanin CO-029, and CD44 variant isoform v6 (CD44v6) recruited into tetraspanin-enriched membrane microdomains. CLDN7 is a key molecule for the EpCAM/CLDN7/CO-029/CD44v6 complex formation [28]. Further investigation will be required to clarify the biological significance of CLDN7 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 CLDN7-negative CRCs, which was similar to the previous report on invasive breast cancer [13]. Interestingly, suppression of CLDN7 expression by hypermethylation was observed in stage 0 CRCs but not in either adjacent nonneoplastic mucosa or adenoma lesions. To our knowledge, this is the first report that demonstrated downregulation of CLDN7 expression by promoter hypermethylation in CRCs. However, only 20% of invasive CRCs with decreased CLDN7 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 to −330 bp). Since the CLDN7 regulatory CpG islands may possibly span the promoter region and exon 1 (−851 to 279 bp), we should have analyzed the methylation status of the other CpG island around the predicted promoter region. Furthermore, the levels of CLDN7 expression in metastatic lymph node CRCs were much higher than those in the primary tumors. This discrepancy supports the idea that the loss of CLDN7 expression in the invasive front of primary CRCs was negatively regulated by such epigenetic alterations at the CLDN7 promoter CpG sites. However, CLDN7 expression might be suppressed by other mechanism(s) in 80% of the CLDN7-negative CRCs. As shown in figure 2, since CLDN7 mRNA level was slightly elevated after treatment with TSA and without 5-aza-dC, histone acetylation might be involved in the regulation of CLDN7 expression. Ikenouchi et al. [11] reported that the transcription repressor Snail induced EMT with concomitant repression of the expression of claudins and occludin; Snail binds directly to the E-box of the promoters of the CLDN and occludin genes, resulting in complete re-expression of their promoter activity. Further investigation is necessary to clarify the other mechanisms by which CLDN7 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 CLDN7 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 CLDN7. Park et al. [31] examined the expression of the intercellular adhesion molecules, E-cadherin, α-catenin, β-catenin, γ-catenin and CLDN7 in primary breast carcinoma and their corresponding axillary lymph node metastases 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, CLDN7 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 gain of cell–cell adhesion and polarity followed by the formation of cytoskeletal organization, the re-expression of CLDN7 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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References


