

Selective Up-regulation of Claudin-1 and Claudin-2 in Colorectal Cancer

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Abstract. *Background: To understand the molecular and morphological alterations in the tight junction in colorectal cancer (CRC) tissues, the expression of eight tight junction proteins in normal and cancer colorectal tissues were compared. Patients and Methods: Adenocarcinoma tissues and paired normal mucosa were resected from surgical specimens of CRC patients. The expression of occludin, ZO-1, ZO-2, and claudin-1 ~ 5 was analyzed at the mRNA level by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and at the protein level by immunohistochemistry. Results: The expression of claudin-1 and claudin-2 in cancer tissues was up-regulated 40- and 49.2-fold, respectively, at the mRNA level, as compared with that in normal tissues. The up-regulation of these two claudins was also observed at the protein level and it appeared to depend on the depth of tumor invasion. Conclusion: Claudin-1 and claudin-2 were found to be overexpressed in CRC tissues. They may be useful as tumor markers and targets for the treatment of colorectal cancer.*

The luminal surface of the mammalian intestine is lined by a highly polarized and continuously renewing epithelium. The tightness and stability of this epithelium are established by the formation of different intercellular junctions such as tight junctions (TJs), adherence junctions and desmosomes (1-7). Tight junctions include occludin, the claudin family of proteins and junctional adhesion molecules. Occludin was identified as the first component of TJ strands (8). However, subsequent

studies, including gene knockout analyses, have shown that TJ strands can be formed without occludin (9, 10). Claudins comprise a multi-gene family consisting of more than 26 members (11), the first identified claudins were claudin-1 and claudin-2 (12). All claudins also bear four transmembrane domains but do not show any sequence similarity to occludin. The second region of the TJ structure is the plaque or peripheral region, which includes the molecules that anchor the transmembrane molecules to the TJ structure and link it to the cell cytoskeleton and signaling pathways, thereby controlling the TJ structure and function. The zonula occludens (ZO) family, such as ZO-1, ZO-2 and ZO-3, falls into this category. Both ZO-1 and ZO-2 are members of the membrane-associated guanylate kinase (MAGUK) protein superfamily, characterized by different molecular domain structures (1, 13, 14). Recent gene expression profiling analyses have shown that claudin gene (*CLDN*) expression is frequently altered in various carcinomas (7, 15). For example, *CLDN 3* and *CLDN 4* have been frequently found to be up-regulated in ovarian, breast, prostate and pancreatic tumors (16-19). *CLDN 1* is down-regulated in various carcinomas, but has also been reported to be up-regulated. These claudin family genes may therefore have a positive effect on tumorigenesis. The function of claudins may be highly tissue-specific and may depend on the exact molecular circuitry of the cell (15). Indeed, recent studies suggest that claudins may be involved in the survival and invasion of cancer cells (20-22).

The aim of this study was to investigate the expression of the TJ proteins (TJPs), including occludin, the ZO family and the claudin family in colorectal cancer (CRC) and to elucidate their possible value as tumor biomarkers.

Patients and Methods

Patients and tissue samples. A total of 15 patients with CRC underwent surgical treatment at Fukuoka University Hospital in

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Table I. Clinical characteristics of patients and their colorectal specimens (n=15).

Mean age (years)	65.7±12.5 (39-90)		
Gender (M:F)	12:3		
Location			
Cecum	2		
Ascending	2		
Transverse	1		
Descending	0		
Sigmoid	4		
Rectum	6		
Dukes' differentiation	A: 5	B: 5	C: 5
Pathology			
tub1	9		
tub2	5		
poor	1		
Lymph node metastasis (-/+)	11 / 4		
Liver metastasis (-/+)	14 / 1		

tub1: well-differentiated adenocarcinoma; tub2: moderately-differentiated adenocarcinoma; poor: poorly-differentiated adenocarcinoma.

Fukuoka and Fukuoka University Chikushi Hospital in Chikushino. Informed consent was obtained from all of the patients before the surgical resection, and after approval from The Institutional Review Committee for Research on Human Subjects in Fukuoka University Hospital or Fukuoka University Chikushi Hospital. CRC samples and adjacent normal mucosa at least 10 cm away from the tumor were obtained from the surgical specimens of the 15 patients. Immediately after the resection, the tissue specimens were frozen in liquid nitrogen and kept at -80°C until RNA extraction. A part of each tissue was promptly fixed in a 10% formaldehyde solution, embedded in paraffin and stained with hematoxylin and eosin. Tumor differentiation and the degree of invasion were examined by pathologists and histopathological classification was performed according to The General Rules for Colorectal Cancer Study (23). Clinical information on the patients and their CRC samples is summarized in Table I.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses. The frozen tissues were placed in 2-ml microtubes (Eppendorf, Hamburg, Germany) and pulverized into powder with a crusher (SK-100, Funakoshi, Tokyo, Japan). Total RNA was isolated from the pulverized samples by the conventional extraction method using TRIZOL reagent (Invitrogen; Carlsbad, CA, USA). The expression of the TJPs, occludin, ZO-1, ZO-2 and CLDN-1~5, at the transcriptional level was examined by quantitative RT-PCR. RT was performed using 1 µg of total RNA with a cDNA synthesis kit (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Primers for real-time PCR were purchased from TaKaRa BIO INC. (Otsu, Japan). PCR was performed with the SYBR Green Core Regents Kit (Applied Biosystems, Forster City, CA, USA) using an ABI GeneAmp 5700 Sequence Detection System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydro-genase (GAPDH) products were amplified

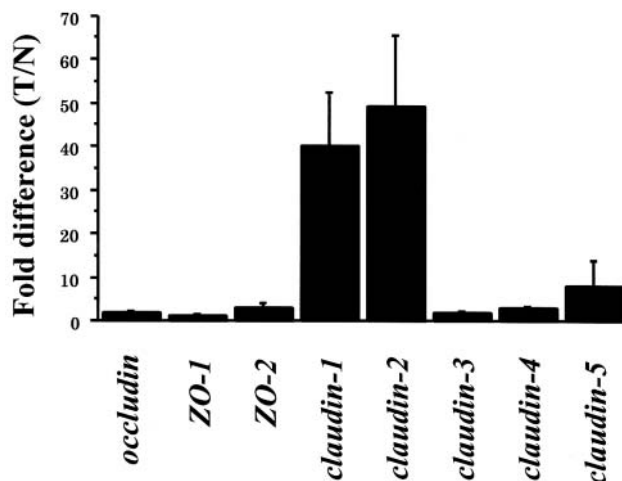


Figure 1. Quantitative RT-PCR analysis of tight junction proteins in CRC tissue and corresponding normal mucosa. The fold-difference indicates the ratio of each protein mRNA level in CRC (T) to that in the corresponding normal mucosa (N).

from the same RNA samples and served as the internal controls. The ratios of the TJPs mRNA levels between the CRC tissue (T) and the corresponding non-neoplastic mucosa (N) were calculated. T/N ratios of more than 10 were considered to indicate an up-regulation.

Immunohistochemistry. The formalin-fixed paraffin-embedded tissues were sectioned at a thickness of 3 µm, deparaffinized in xylene and rehydrated in a graded ethanol series followed by a rinse in water. Antigen retrieval was performed by microwaving at 98°C for 10 min, followed by washing for 15 min in 0.05 M Tris-buffered saline, pH 7.4. After incubation in 0.02 M phosphate-buffered saline, pH 7.4, containing 10% normal goat serum for 30 min to block non-specific protein binding, the tissue sections were stained with mono/poly-clonal antibodies against occludin, ZO-1 and each of the claudins (Zymed Laboratories Inc., San Francisco, CA, USA) and ZO-2 and E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). These antibodies, diluted at 1:100, were applied to each section for 1 hour at room temperature. The antigenic sites were then immunostained by using polyvalent antibody coupled to horseradish peroxidase (HRP; Dako Cytomation, Carpinteria, CA, USA). The HRP sites were visualized by incubation in a freshly prepared chromogen solution containing 0.1% 3,3'-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide in 0.05 M Tris-HCl, pH 7.6. The negative control included the replacement of specific antibodies with normal rabbit or goat IgG. The sections were counterstained with haematoxylin and mounted with glass coverslips for photomicrography. The immunostaining was assessed as follows; -, no immunostaining present; +, <25% of cells positive; 2+, 25-50% of cells positive; 3+, 50-100% of cells positive. In the evaluation, only membrane-bound positivity was considered significant. The results were analyzed by a pathologist who had no knowledge of the diagnosis at the time of the analysis.

Table II. Expression of tight junction proteins in normal and CRC tissues analyzed by immunohistochemistry (n=15).

	Normal	CRC
Occludin	+	2+ ~ 3+
ZO-1	+	2+
ZO-2	+	2+ ~ 3+
Claudin-1	+	+ ~ 3+
Claudin-2	+	+ ~ 3+
Claudin-3	+	2+
Claudin-4	+	2+ ~ 3+
Claudin-5	+	2+

Results

Identification of up-regulated TJP genes in colorectal cancer tissue specimens. As shown in Figure 1, the expressions of all of the TJP genes, especially *CLND-1* and *CLND-2*, were up-regulated in the CRC tissue specimens in comparison to the normal mucosa in all 15 of the cases.

Expression of TJPs in human normal and neoplastic colonic tissue specimens. In the normal colon mucosa, all of the epithelial cells expressed each type of TJP along the cell membrane, but not in the cytoplasm. The expressions of all eight TJPs were up-regulated in the CRC tissues in comparison to the normal mucosa where low levels of staining were observed. The up-regulation of the TJPs was observed in all 15 CRC cases (1+, 2 cases; 2+, 6 cases; 3+, 7 cases) (Table II). As shown in Figure 2, in both the normal and CRC tissues from a patient whose clinical stage was Dukes B, all of the epithelial cells expressed claudin-1, claudin-2, and claudin-4 not only along the cell membrane but also in the cytoplasm. Some CRC tissue specimens showed heterogeneous levels of immunostaining, but the invasive front of the CRC generally revealed the up-regulated expression of the TJPs, as shown in Figure 3. Different staining patterns of the TJPs in the CRC tissues were also revealed: occludin, ZO-1, ZO-2, claudin-4, and claudin-5 were detected throughout in the CRC tissues, whereas claudin-1 ~ 3 were dotted in the same area (data not shown).

The relationship of the TJP up-regulation to the clinicopathological characteristics (Table I) was analyzed. The up-regulation of TJP was not correlated with the location, N grade, tumor size, or histological type (data not shown). However, patients in Dukes B and C stages showed a notably higher expression of claudin-1 and -2 at the transcriptional and protein level than patients in Dukes A (Figure 1 and Table III). The up-regulation of claudin-4 was also confirmed at the protein level in the

Table III. Relationship between the expression of claudin-1, -2, and -4 and Dukes classification in CRC by immunohistochemistry (n=15) claudin.

Dukes	Positivity	Claudin		
		1	2	4
A (n=5)	+	1	1	0
	2+	4	4	5
	3+	0	0	0
B (n=5)	+	1	1	0
	2+	2	3	3
	3+	2	1	2
C (n=5)	+	0	0	0
	2+	2	3	1
	3+	3	2	4

different invasion stages of CRC patients (Table III). The other five proteins were also detected in proportion to their transcriptional levels. The down-regulated expression of epithelial cadherin (E-cadherin) was also detected by immunostaining in all CRC tissue specimens (data not shown).

Discussion

Claudins are TJPs present in the endothelial and epithelial cells. They serve as barrier proteins and regulate the permeability of blood vessels and epithelium in various types of tissue. There are at least 26 different claudin family members known so far and their distribution in different tissues and cells may vary (24). The cellular organization observed in normal differentiated tissues is often lost in cancer, where tumor cells frequently exhibit reduced differentiation and cell polarity (25, 26). These features are important for the development of invasive phenotypes, and consequently for metastasis (27). The claudin family members interact with each other through both homo- and heterophilic interactions. In addition, the C-terminal domain of the claudins also serves as a binding site for interaction with a complex set of proteins including a number of PDZ domain proteins- ZO-1 ~ 3 and multi-PDZ domain protein1 (MUPP1), which are potentially involved in signaling (28, 29). The nuclear localization of several cell junction proteins (β -catenin, ZO-1 and ZO-2) is known to be correlated with oncogenic transformation and cell proliferation (30, 31). In the present study the expression of the claudin genes at the mRNA level was investigated, but it was obviously essential to validate these findings at the protein level, as posttranslational mechanisms have been shown to regulate claudin protein levels and localization (15).

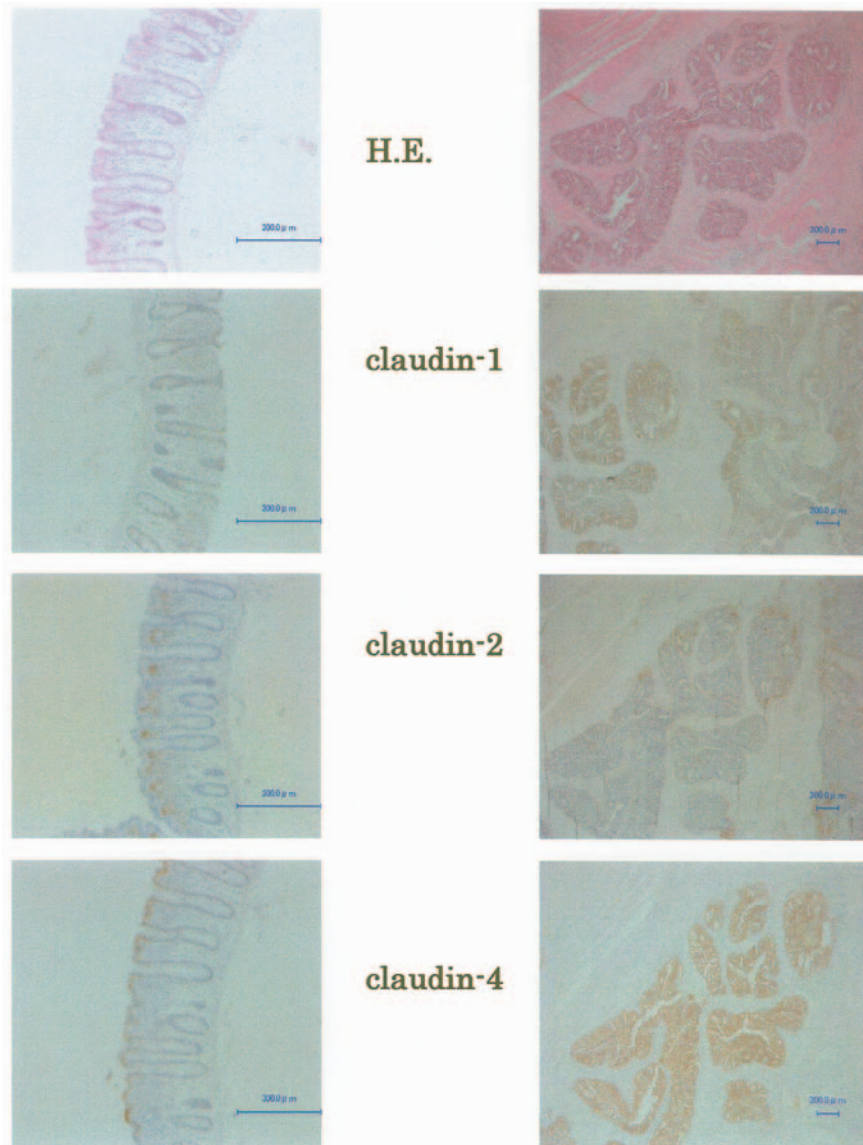


Figure 2. Hematoxylin-eosin (top) and immunostaining of the tight junction proteins in normal colorectal mucosa (left) and in the CRC tissues (right) of a CRC patient with a Dukes B classification. Scale bars =300 μ m.

Claudin protein expression may have significant clinical relevance. For example, claudin-1 expression has been shown to have a prognostic value in colon cancer (32), claudin-18 in gastric cancer (33), and claudin-10 in hepatocellular carcinoma (34).

Both the protein and mRNA levels of claudin-1 and claudin-2 were highly elevated in the present study. De Oliveira *et al.* have reported that claudin up-regulation was associated with a significant disorganization of the TJ strands as observed in freeze fracture replicas and increased paracellular permeability by the ruthenium red technique (35). *Clostridium perfringens* enterotoxin (CPE)

is a natural ligand for claudin-3 and claudin-4 proteins, and binding of the toxin to these claudins leads to a rapid cytolysis of the cells (36). However, the therapeutic index of this compound will depend on the level of up-regulation in the various tumors under study and the mode of administration. In ovarian cancer, in which both *CLDN3* and *CLDN4* are highly up-regulated and for which intraperitoneal therapy is possible, CPE treatment is certainly an interesting possibility (10). Recent preclinical studies have suggested that CPE may be effective against claudin-3 and claudin-4 expressing malignancies (16, 18, 21, 37).

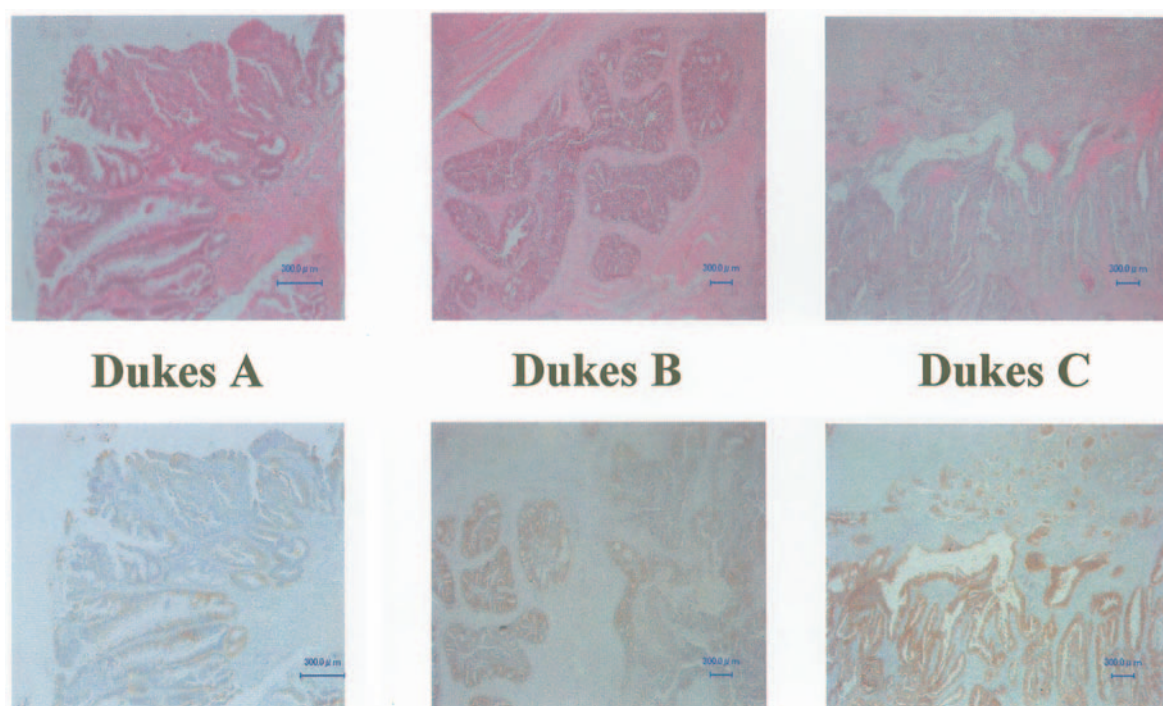


Figure 3. Hematoxylin-eosin (top) and immunostaining (bottom) of claudin-1 in CRC tissues. Samples were taken from three different Dukes classification CRC patients. Patients in Dukes B and C stages had a significantly higher expression of claudin-1 than those in Dukes A and normal colon mucosa. Scale bars =300 μ m

E-cadherin is the main cell-cell adhesion molecule in all epithelia. Aberrant expression of this molecule has been implicated in tumor invasion and metastasis (38). In this study, the expression of E-cadherin was down-regulated in the CRC tissues, thus indicating that the expression of the claudin family and E-cadherin were differently mediated in the CRC tissue specimens (39).

It will be important to investigate the various claudins studied here for their potential clinical use in CRC therapy and diagnosis. The claudin family of membrane proteins, especially claudin-1 and claudin-2, may represent ideal targets for both cancer diagnosis and therapy.

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