Claudin-1 Protein is a Major Factor Involved in the Tumorigenesis of Colorectal Cancer

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Abstract. *Background: The molecular and morphological alterations of the tight junctions in colorectal cancer (CRC) are still poorly understood. The possible involvement of claudin-1 (CL-1), one of the major tight junctional proteins (TJPs), was investigated in the tumorigenesis of CRC. Patients and Methods: Adenocarcinoma tissue and paired normal mucosa specimens were resected from surgical specimens of CRC patients and analyzed to determine whether the expression of CL-1 correlated with the clinicopathological factors and to determine the role of CL-1 in the alteration of tight junctions during tumorigenesis. Results: The expression of CL-1 at the mRNA and protein levels was analyzed in 41 cases and was found to increase in the CRC tissue in comparison to that in the normal tissue specimens. The mRNA levels of CL-1 were correlated with tumor depth, but not with the preoperative carcinoembryonic antigen (CEA) serum level. When T84 cells, a human colon cancer cell line, were transfected with the CL-1 gene, the CL-1 overexpressing cells grew as aggregates in contrast to the monolayer formation of the parental cells. In addition, trypsin-treated CL-1 overexpressing cells aggregated more easily than did the parental cells. Conclusion: CL-1 plays a pivotal role in cell morphology and behavior in the colonic epithelium. CL-1 protein may therefore be one of the major factors involved in the tumorigenesis of CRC.*

The function of cells is closely correlated to their connections with other cells. For epithelial cells, tight and adherent junctions between the cells are very important for the integrity of the cell layers (1-7). Among these junctional complexes, tight junctions are the most apical structures and tight junction proteins (TJPs) are very important in the maintenance of normal epithelial physiology. Tight junctions play a critical role in establishing and maintaining epithelial cell polarity by acting as a diffusion barrier to the movement of proteins and lipids within the plasma membrane. Barrier, signaling and fence functions of tight junctions have been clearly illustrated (8, 9). Moreover, their pivotal role in organizing diverse processes such as morphogenesis, cell polarity, cell proliferation and differentiation are also well recognized (10).

Occludin (OC) was the first component of the tight junction strands to be identified (11). However, subsequent studies, including gene knockout analyses, have shown that tight junction strands can be formed without OC (12). Two other transmembrane proteins, claudin (CL)-1 and CL-2, have also been identified as integral components of tight junction strands (13). The CLs comprise a multi-gene family consisting of more than 24 members (8). All CLs bear four transmembrane domains, but do not show any sequence similarity to OC. CLs are functionally involved in the tight junction barrier and aqueous pore formation, and most of them have a binding site for the PDZ domains of proteins such as zonula occludens (ZO)-1, ZO-2 and ZO-3 (14), which are potentially involved in signaling (15, 16). To date, the CLs and OC have been identified as tight junction-specific integral membrane proteins.

Recent studies have shown that considerable changes in TJP expressions are associated with various carcinomas. Neoplastic cells frequently exhibit both structural and functional disorganization in their tight junctions. The altered expression of some CLs has also been found in many human carcinomas such as those of the breast, ovary, prostate, liver and stomach (17, 18). CL expression has been shown to have prognostic value: CL-1 in colon cancer (19), CL-18 in gastric cancer (20) and CL-10 in hepatocellular carcinoma (21).

Colorectal cancer (CRC) is one of the major causes of cancer death in the world (22, 23). The differential expression of genes encoding TJPs has been detected in CRC

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(19, 24). It is important to investigate the various TJPs in the CRC tissues for their potential role in the therapy and diagnosis of CRC. The up-regulated expression of CL-1 in CRC has also been reported (25-28). These studies have suggested that the CL family, especially CL-1, thus plays a causal role in the process of cellular transformation and invasion in CRC. The aim of this study was to analyze the correlation between the expression of CL-1 in CRC tissues from patients with clinicopathological factors and to investigate its possible functions in tumorigenesis and/or metastasis in CRC.

Patients and Methods

Patient and tissue samples. A total of 41 patients with sporadic CRC underwent surgical treatment at Fukuoka University Hospital in Fukuoka. Informed consent was obtained from all of the patients before performing a surgical resection, while also receiving approval from the Institutional Review Committee for Research on Human Subjects in Fukuoka University Hospital. 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 (29). The tissue specimens were frozen in liquid nitrogen and kept at –80˚C until the experiments were performed. Normal colonic crypts were isolated as described previously (30) and then were used as normal tissue samples. The clinical information on the patients and their CRC samples is summarized in Table I.

Table I. *Clinical information on patients and their colorectal specimens (n=41).*

Mean age (years)	68.5±11.1 (48-90)			
Gender (M:F)	25:16			
Location				
Cecum	4			
Ascending	4			
Transverse	5			
Descending	1			
Sigmoid	12			
Rectum	15			
Dukes' differentiation	A: 2	B: 24	C: 12	D: 3
Pathology				
Tub1	18			
Tub2	22			
Poor	1			
Lymph node metastasis (–/+)	27/14			

Tub1: well-differentiated adenocarcinoma; Tub2: moderately differentiated adenocarcinoma; Poor: poorly differentiated adenocarcinoma.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses. The total RNA isolation from the samples and quantitative RT-PCR were performed as described previously (25). The expression of the TJPs, *OC, ZO-1, ZO-2* and *CL-1~5*, was examined at the transcriptional level by quantitative RT-PCR. The ratios of the TJP mRNA levels between the CRC tissue and the corresponding non-neoplastic mucosa were calculated.

Immunohistochemistry. Immunohistochemistry was performed as described previously (25). The tissue sections were stained with mono-/polyclonal antibodies (diluted at 1:100) against CL-1 (Zymed Laboratories Inc., San Francisco, CA, USA), E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Biovision, Mountain View, CA, USA).

Gel electrophoresis and Western blotting analysis. The expression of CL-1 at the protein level was confirmed by Western blot analysis. The total protein was isolated from the crushed samples using a PARISTM Kit (Ambion, Austin, TX, USA) containing Complete Mini (Roche, Indianapolis, IN, USA) according to the methods described by the manufacturers. The protein concentration of each sample was quantified by Bradford assay (Bio-Rad Laboratories, Inc., CA, USA). Gel electrophoresis and Western blotting analysis were performed as described previously (2).

Gene cloning and the construction of recombinant genes. To study the possible effects of CL-1 protein overexpression on the growth, morphology and adherence in a human colon cancer, a human colon cancer cell line, T84, obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), was transfected with a mammalian expression vector pcDNA3.1(–). Human *CL-1* cDNA was generated from the total RNA extracted from the T84 cells by RT-PCR. The resulting products were cloned into the pCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA). The plasmid was sequenced, using an Applied Biosystems Model 373A DNA sequencer with the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA). The *CL-1* cDNA was inserted into the corresponding sites of the pcDNA3.1(–) mammalian expression vector (Invitrogen).

Cell culture and transfection. The T84 cells were cultured as described previously (2) and were transfected with the pcDNA3.1(–)/*CL-1* construct using Lipofectamine 2000 (Invitrogen). CL-1 subunit stable transfectants, designated CL-1-overexpressing T84 cells, were established by selection in a medium supplemented with 500 μg/ml G418 (Roche, Mannheim, Germany) for over 5 weeks.

Assessment of the paracellular barrier functions of intestinal epithelial cells. For the transepithelial electrical resistance (TER) measurements, 1×10^6 cells were plated on 6-well Falcon cell culture inserts with a pore size of 0.4 μm (Becton Dickinson, Franklin Lakes, NJ, USA) in triplicate samples. A Millicell-ERS epithelial volt-ohmmeter (World Precision Instrument, Inc., Sarasota, FL, USA) was used to determine the TER value. All TER values were determined after background subtraction and multiplied by the surface area of the filter (9.6 cm^2) to yield the area of resistance in ohms × cm^2.

Cell proliferation assay. Both transfected and parental T84 cells were seeded into 96-well plates at a density of 5×10^3 cells/well. Cell proliferation was assessed in triplicate using a Cell Titer 96 Aqueous kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Aggregation assay. The cells were released from the dish with trypsin (0.125%)-EDTA (0.01%) treatment at 37˚C for 5-10 min. Two million cells suspended in 2 ml of medium were rotated in a

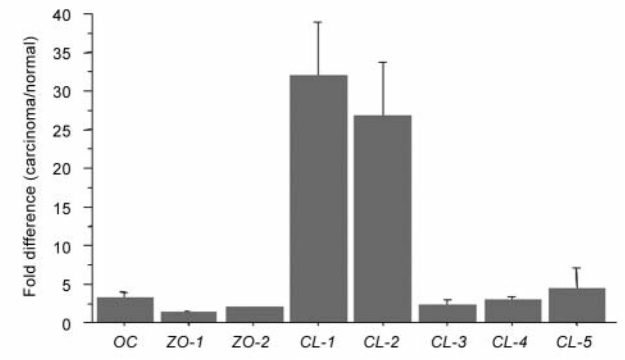

Figure 1. *Quantitative RT-PCR analysis of tight junction proteins in CRC tissue and corresponding normal mucosa. The fold-difference indicates the ratio of each protein mRNA level in CRC tissue specimens to that in the corresponding normal mucosa.*

35 mm dish on a rotary shaker (80 rpm) for 30 min at 37˚C. The extent of cell aggregation was viewed under a microscope and photographed.

Data analysis. Statistical comparisons were made using either Student's *t*-test or Welch's *t*-test. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Expression of CL-1 mRNA and protein in human normal and neoplastic colonic tissue specimens. Figure 1 shows the expression of all of the TJPs mRNA, especially *CL-1*, to be up-regulated in the CRC tissue specimens in comparison to the normal mucosa in all 41 of the cases, with a 31.8-fold increase. Figure 2 demonstrates that the expression of CL-1 protein was up-regulated in carcinoma tissue in comparison to the normal mucosa. An increase in the expression of the CL-1 protein was observed in 38 out of the 41 adenocarcinomas (92.6%) in comparison to the normal mucosa. These results were closely consistent with the mRNA expression levels.

Immunostaining was used to confirm the changes in the distribution pattern of CL-1 (Figure 3). In the normal colon mucosa, all of the epithelial cells expressed CL-1 along the cell membrane, but not in the cytoplasm. The immunostaining pattern for CL-1 showed much stronger and more diffuse staining, even in the cytoplasm of the CRC tissues in comparison to the normal colonic mucosa. The subsequent experiments focused on the CL-1 protein expression. The expression of E-cadherin was negative or down-regulated in all of the CRC tissue specimens.

Correlation between the mRNA levels of CL-1 and the clinicopathological factors. Table II shows that the mRNA levels of *CL-1* were higher in advanced cancer (deeper than muscularis propria (mp)) than in early cancer (mucosa (m)

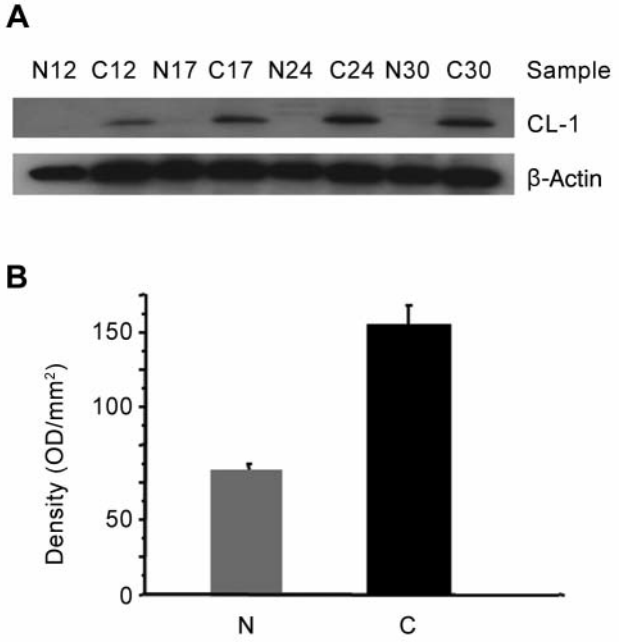

Figure 2. *A, Western blotting analysis of CL-1 in paired normal mucosa (N) and adenocarcinoma (C) obtained from 4 CRC patients. Typical blots are shown. B, The level of CL-1 expression in the adenocarcinoma (C) in comparison to that of the normal mucosa (N) using densitometry (n=41).*

Table II. *Correlation between the mRNA levels of claudin-1 and the clinicopathological factors.*

Clinicopathological factor	*p*-Value
Age (years)	
>60	0.355
<60	
Gender	
Male	0.780
Female	
Location	
Proximal (C, A, T)	0.039*
Distal (D, S, R)	
Tumor size	
>30 mm	0.294
<30 mm	
Depth	
<sm	0.010*
>mp	
N	
Negative	0.578
Positive	
CEA	
<2.5 ng/ml	0.493
>2.5 ng/ml	

CEA: Preoperative serum carcinoembryonic antigen level, C: cecum, A: ascending, T: transverse, D: descending, S: sigmoid, R: rectum, N: lymph node metastasis, sm: submucosa, mp: muscularis propria. *Statistically significant.

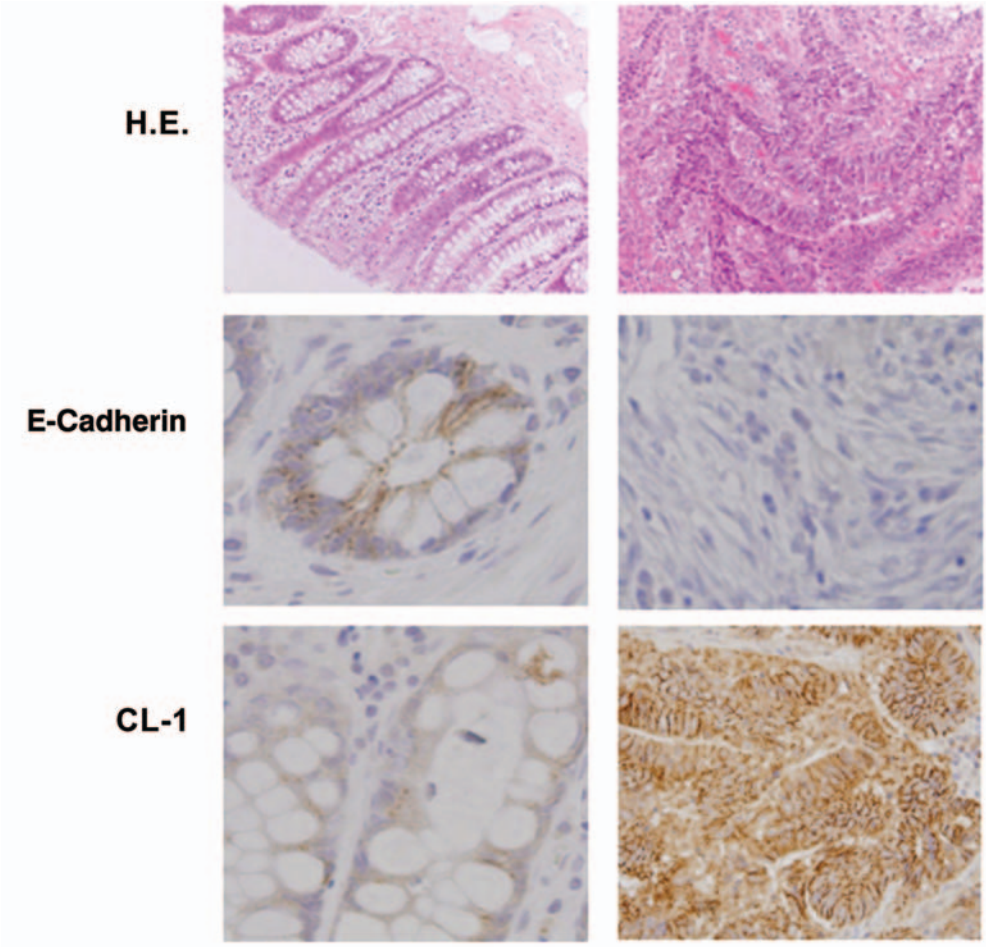

Figure 3. *Hematoxylin-eosin (H.E.: magnification: ×100) and immunostaining of CL-1 and E-cadherin in normal colorectal mucosa (left) and CRC tissue (right) of a CRC patient with a Dukes' B classification (Magnification: ×400).*

and submucosa (sm); $p<0.01$) and higher in the distal than in the proximal colon ($p<0.05$). No significant differences were detected between the mRNA levels of *CL-1* and the other clinicopathological factors (age, sex, tumor size, lymph node metastasis and preoperative serum carcinoembryonic antigen (CEA) level).

Effects of CL-1 overexpression in T84 cells on cell proliferation ability and TER. Two stable clones, T84 CL1-15 and T84 CL1-7, were selected that expressed high levels of *CL-1* mRNA (22-fold and 5-fold expression, respectively) in comparison to that in the control cells. Figure 4 shows that they expressed high levels of CL-1 protein consistent with the levels of mRNA. The T84 CL-1-15 (transfected T84) cells were used for further experiments. The proliferation of transfected T84 cells and parent T84 cells were compared using the MTT assay and TER. Figure 5A shows the growth rate of the *CL-1* transfected cells to be similar to that of the parental T84 cells. The parental T84 cell monolayers increased in TER from 411.2 to 10,528.47 $\Omega \times cm^2$ from day 1 to 11. In contrast, the transfected T84 cells displayed an increase in TER only from 432.2 to 2,741 $\Omega \times cm^2$ over the 11-day observation period (Figure 5B).

Aggregation activity of CL-1-overexpressing T84 cells and normal T84 cells. When the dissociated single cell suspensions were gently rotated, the transfected T84 cells tended to aggregate easily, while the parental T84 cells had a reduced tendency to aggregate (Figure 6).

Discussion

This study demonstrated that *CL-1* was significantly up-regulated in the CRC tissues. The mRNA level of *CL-1* expression significantly correlated with the depth of cancer in these specimens (the invasive front). It is also noteworthy that the *CL-1* mRNA level was higher in the distal site of colon than in the proximal site since the incidence of colon

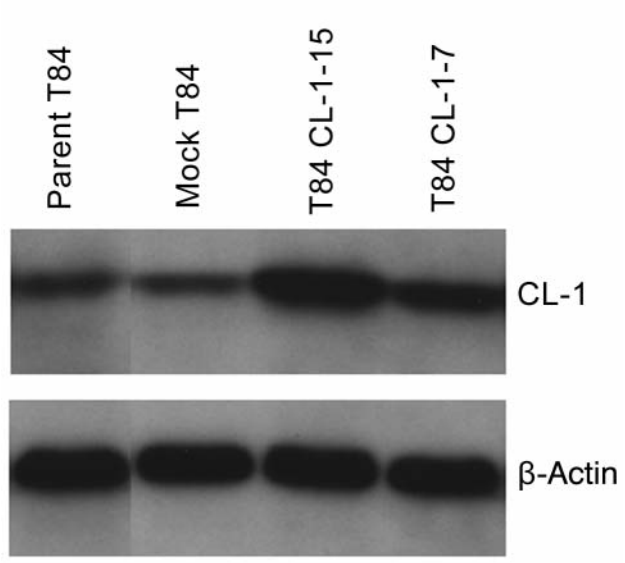

Figure 4. *Western blotting analysis of CL-1 expressed in stably transfected T84 cells using anti-CL-1 antibody. Mock T84 cells were transfected with the empty vector, pcDNA3.1(–).*

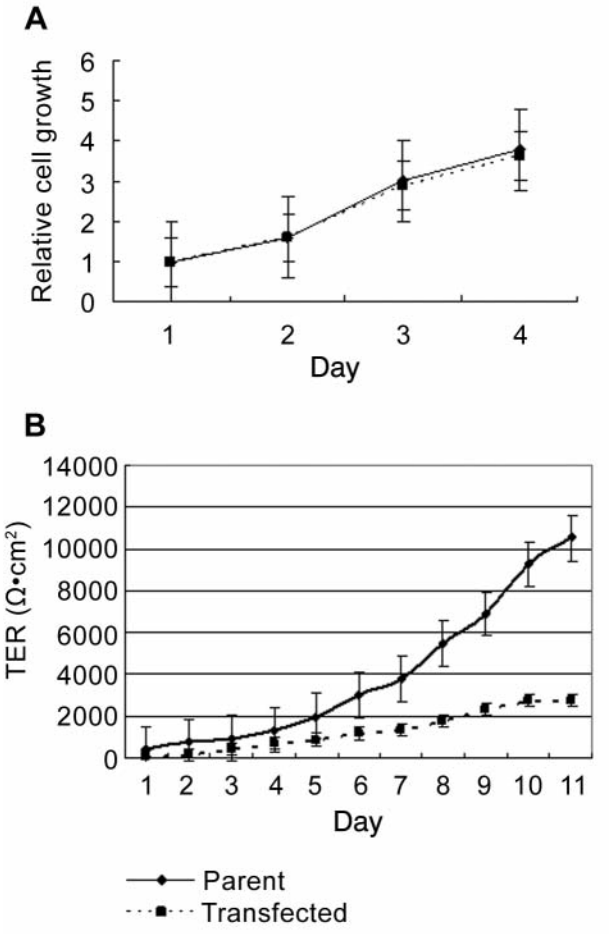

Figure 5. *Effect of CL-1 overexpression on the proliferation and monolayer formation of T84 cells. A, Proliferation of parent T84 cells and transfected T84 cells. B, Development of TER in parent and transfected T84 cell monolayers. TER was determined in triplicate.*

cancer is much higher in the former site. However, no relationship was observed between the expression level of *CL-1* mRNA and the preoperative serum CEA level in regard to the other clinicopathological factors tested. In addition, the *CL-1*-transfected cells grew in aggregate forms in contrast to the monolayer formation of the parental cells *in vitro*, and they revealed a tendency to aggregate more easily than the parental cells. Therefore, CL-1 protein may be one of the major factors involved in the tumorigenesis of CRC.

The loss of function of tight junctions has been observed in CRC (31) and other tumors (32). These changes are believed to increase access to nutrients and signaling peptides (33) and increase motility and metastasis (34). In general, the overall down-regulation of cell-cell adhesion molecules occurs during carcinoma development (35). Evidence of either a reduced expression or a loss of expression of the CL family members has been found to promote cell invasion and metastasis in malignant tumors, including cancer of the gastrointestinal tract (36, 37), pancreas (38) and breast (39). However, in the present study, *CL-1* was up-regulated in sporadic human CRC in comparison to the paired normal mucosa, at both the mRNA and protein level. In addition, a notable difference of distribution patterns in the cancer and normal mucosa was seen by immunohistochemistry (Figure 3), indicating that a great amount of CL-1 is membrane/cytoskeleton-associated in CRC tissues.

An aberrant expression of E-cadherin, one of the key cell-cell adhesion molecules in all epithelia, has been implicated in tumor invasion and metastasis (40). As shown in Figure 3, E-cadherin expression was markedly reduced in the tumor samples, indicating that the expression of the CL family and E-cadherin was differently mediated in the CRC tissue specimens (41). These results suggest that distinct signaling pathways regulate tight and adherence junctions differently under the same conditions.

Usually, diseases of the colon are characterized by increased tight junction permeability. De Oliveira *et al*. have reported CL up-regulation to be associated with a significant disorganization of the tight junction strands in CRC as observed in freeze fracture replicas and increased paracellular permeability by the ruthenium red technique (26). An increase in tight junction permeability may therefore constitute a critical change for determining the development of epithelial cells into tumors (31). As mentioned previously (42), some CL overexpression in the cell lines is not associated with the ability of these cells to form tight junctions. Because the CL

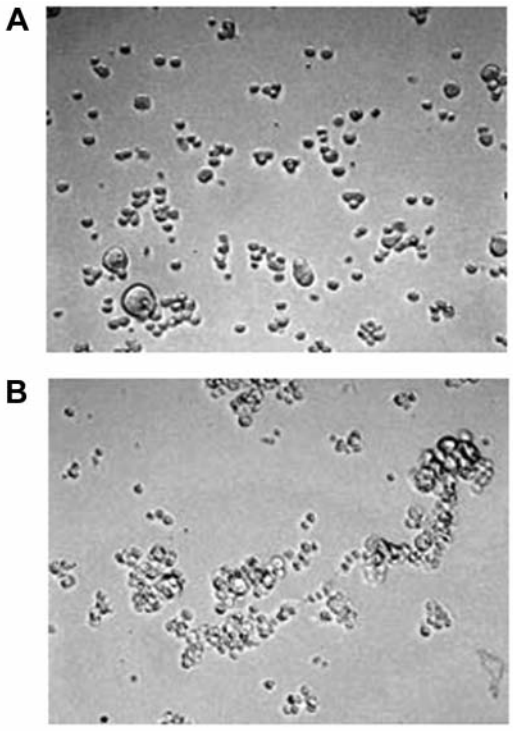

Figure 6. *Effect of CL-1 overexpression on the aggregation of T84 cells. A, Parent T84 cells; B, transfected T84 cells (Magnification: ×200).*

family members are crucial components of the tight junctions, the alteration of CL expression may thus affect the permeability and extracellular growth factor signaling to promote cancer cell growth.

The biological functions of CL-1 are still poorly understood. In this study, the proliferative ability of the T84 cells did not increase after transfection with *CL-1*. Moreover, as shown in the TER experiment, the transfected T84 cells formed multilayers with increased tight junction permeability, thus suggesting that the cells could not form tight junctions as the parent T84 cells did. The transfected T84 cells tended to aggregate and form multilayers more easily than did the parent T84 cells. In addition, they tended to stick together more tightly than did the control cells during culture. The cellular organization observed in normal differentiated tissues is often lost in cancer, where tumor cells frequently exhibit lower differentiation and cell polarity (31, 43). These features are important for the development of invasive phenotypes, and consequently for metastasis (34). The CL family members interact with each other through both homo- and heterophilic interactions. Among all the TJPs, the CL family is considered to be increasingly more important in the progression from normal colorectal epithelium to carcinoma. In further studies, it will therefore be important to investigate the various CLs, especially CL-1, for their potential clinical use in CRC therapy and diagnosis.

Taken together, these data suggest that the CL-1 protein expression may have significant clinical relevance and it may therefore become a potentially useful marker and therapeutic target in CRC.

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