ANTICANCER RESEARCH *42*: 4119-4127 (2022)
doi:10.21873/anticanres.15910

Chitinase 3-like 1, Carcinoembryonic Antigen-related Cell Adhesion Molecule 6, and Ectopic Claudin-2 in the Carcinogenic Processes of Ulcerative Colitis

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Abstract. *Background/Aim: The cumulative cancerous rate of colitis-associated cancer (CAC) has increased exponentially in patients with ulcerative colitis (UC). We have investigated the factors involved in the carcinogenic processes of CAC among UC patients. Patients and Methods: A total of 42 UC patients who underwent surgical treatments between January 2001 and December 2010 at Kurume University Hospital (Fukuoka, Japan) were enrolled. We conducted this study using 3 cases of CAC out of 42 UC cases and 1 case of colorectal cancer. cDNA microarray analyses were performed using normal, inflamed, and cancerous tissues from surgical CAC specimens and protein expression was confirmed by immunohistochemical analyses. Results: cDNA microarray revealed 32 genes that were dominantly expressed in tumorous regions of CAC. Gene ontology analysis revealed that these genes were involved in inflammatory responses and cytokine–cytokine receptor interactions. Chitinase 3-like1 (CHI3L1), carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), and Claudin-2 (CLND2) were selected from CAC-related genes as candidate molecules. Immunostaining revealed strong expression of each protein in cancerous regions. Conclusion: In this study, we identified CAC-related genes and found that CHI3L1, CEACAM6, and CLND2 were expressed in patient samples. All the above genes were associated with adherent invasive Escherichia coli (AIEC), which suggested that these molecules are likely involved in AIEC infection. Further analyses would be required to reveal unknown mechanisms of CAC-related genes in the tumor microenvironment.*

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Key Words: Colitis-associated cancer, ulcerative colitis, CHI3L1, CEACAM6, CLND2.

Inflammatory bowel disease (IBD), with its two types Crohn's disease (CD) and ulcerative colitis (UC), is a widespread disease that shows a high incidence rate in developed countries, including Japan (1, 2). The etiology behind the chronic intestinal inflammation is still unknown. Furthermore, patients who suffer from long-term UC are at increased risk of developing colorectal cancer (CRC) compared with the general population. According to a previous meta-analysis, the cumulative cancerous rate of colitis-associated cancer (CAC) in UC was exponentially high (3). Additionally, recent population-based studies reported that 15%-20% of UC patients have CAC 10 years after the initial diagnosis (4, 5).

Regarding the pathology, colon cancer is one of the major factors that worsen the prognosis of UC patients, and it develops through the inflammation–dysplasia sequence. CAC also goes through multiple stages of tumorigenesis and carcinogenic processes, which differ from the sporadic adenoma–carcinoma sequence (6, 7).

As a result, IBD patients are significantly prone to develop CRC or small bowel cancer, and the risk is much higher in extensive and long-term cases (8). CAC is frequently discovered at an advanced stage with metastasis, and it is

Table I. *Background clinical characteristics of patients.*

	Colitis-associated cancer			Sporadic cancer
Age (years)	51	79	51	79
Sex (M/F)	M	M	M	M
Chief complaint	Abdominal pain	Diarrhea	Bloody stool	Anemia
BMI	24.3	23.3	22.0	
Type of UC	Total	Left	Total	Sigmoid colon cancer
Duration of disease (years)	15.5	11.7	15.4	-
Total steroids (g)	0	>10	>10	-
LCAP (yes/no)	No	No	No	-
Preoperative blood test				
WBC (/μl)	6,600	3,600	7,400	7,000
PLT ($\times 10^4$ /μl)	20.4	10.7	15.4	21.5
CRP (mg/dl)	0.18	0.04	1.39	0.38
Albumin (g/dl)	3.8	3.37	3.7	4

BMI: Body mass index; LCAP: leukocyte apheresis; WBC: white blood cell; PLT: platelet; CRP: C-reactive protein.

usually hard to identify its precancerous lesions, unlike sporadic CRC that develops from colonic polyps. The flat shape of precancerous lesions determines the entire extent of the dysplastic area, which impedes the endoscopic removal of the lesion. In general, characteristic features of CAC represent multiple tumors and signet ring-like morphology, which tend to be associated with poor prognoses.

The biological changes behind CAC development have not been completely understood yet. Our current data suggest that different genetic sequences initiate CAC compared to sporadic CRC. According to some animal models, the inflammatory events in IBD patients can lead to genomic changes and can also promote the initiation and progression of neoplastic changes in the affected area (9, 10). *TP53* mutations are early events of CAC and are often detected in non-dysplastic or dysplastic regions in patients with IBD (11, 12). However, while *TP53* is a late mutation in sporadic CRC, WNT pathway activation, especially by *APC* inactivation, is often the initiation event in sporadic colorectal adenocarcinomas (13, 14). However, it is not clear whether the subsequent genomic changes in CAC are similar to those in sporadic CRC or not.

This study investigated whether there is a difference in the mechanisms of CAC and CRC. We performed cDNA microarray analyses using normal, inflamed, and neoplastic CAC samples from UC patients to compare them with those from CRC patients. Furthermore, we confirmed candidate protein expression by immunohistochemistry.

Patients and Methods

Patient and tissue samples. This prospective, single-center study included a total of 42 UC patients who underwent surgical treatment and gave written informed consent between January 2001 and December 2010 at Kurume University Hospital (Fukuoka, Japan). The study was conducted in accordance with good clinical practice and the Helsinki Declaration after receiving the ethical approval from the Ethics Committee in Human Subjects at Kurume University School of Medicine (approval no. 09315). We conducted this study using 3 cases of CAC out of 42 UC cases and 1 case of CRC. The patients' characteristics are shown in Table I. All UC patients underwent total colectomy, ileo-anal anastomosis, and diverting ileostomy. However, ileostomy was closed after 3 to 6 months of the surgical procedure. Cancerous lesions were evaluated for performing appropriate surgery. A medical oncologist (TK, TS, or YA) reviewed the clinical history of each patient with IBD. Sigmoidectomy and lymph node dissection were performed in patients with sigmoid colon cancer.

Total RNA isolation. TRIzol reagent was used to isolate total RNA from colon tissues. Then, according to the manufacturer's instructions, the samples were purified using SV Total RNA Isolation System (Promega, Madison, WI, USA). ND-1000 spectrophotometry was used to quantify RNA samples (NanoDrop Technologies, Wilmington, DE, USA). However, the quality was confirmed with a 2200 Tape Station (Agilent Technologies, Santa Clara, CA, USA).

Gene expression microarrays. According to the manufacturer's instructions, the cRNA was amplified, labelled, and hybridized to a 60K Agilent 60-mer oligomicroarray. However, all hybridized microarray slides were scanned using an Agilent scanner. Agilent Feature Extraction Software calculated relative hybridization intensities and background hybridization values (9.5.1.1). We normalized the gene expression values (GEVs) observed in neoplastic or inflamed regions by the respective GEVs observed in normal tissue. Then, the normalized GEVs from the neoplastic region were statistically compared to the inflamed one.

Gene ontology (GO) analysis. Gene ontology (GO) term enrichment was analyzed by Metascape (15), including biological process, molecular function, and cellular component.

Table II. *Gene up-regulation in colitis-associated cancer (CAC).*

Probe ID	Gene symbol	Fold change (T/I)	*t*-Test (T/N *vs*. I/N)	Fold change (I/N)	Fold change in sporadic CRC (T/N)	T/I ratio/ T/N ratio
A_23_P324754	*CEMIP*	101.8675	0.008133	0.384573908	1.025973	99.28871
A_33_P3402868	*GRIN2D*	21.73701	0.032707	0.461017014	0.32348	67.19737
A_19_P00318645	*CRNDE*	20.6203	0.000451	1.003280126	0.316896	65.06968
A_33_P3249046	*CLDN2*	333.0855	0.038317	1.399229302	6.241631	53.36513
A_23_P137665	*CHI3L1*	120.1304	0.000259	0.676563336	2.275657	52.78932
A_23_P218646	*TNFRSF6B*	11.19635	0.006639	0.991858589	0.249593	44.85839
A_33_P3243887	*IL11*	42.26457	0.022547	1.619516073	0.997097	42.38761
A_23_P369343	*KLK8*	50.04781	0.001696	0.957357072	1.190291	42.0467
A_33_P3408918	*SAA2*	45.62166	0.048164	1.016304377	1.158616	39.376
A_19_P00322533	*CRNDE*	18.99334	0.024259	1.447601378	0.521559	36.41646
A_24_P203328	*TPRXL*	32.97538	0.045185	1.590182343	1.014675	32.49845
A_33_P3305571	*TNFRSF6B*	14.08314	0.025599	0.99289072	0.466972	30.15844
A_33_P3411628	*CDKN2A*	10.08713	0.004754	0.832811491	0.414212	24.35259
A_23_P57658	*HRASLS*	22.77732	0.008945	1.062804361	0.991599	22.97029
A_23_P52067	*GRHL3*	10.69101	0.000993	0.671741572	0.486671	21.96764
A_23_P154688	*SLC4A11*	21.7991	0.010206	1.808567651	0.9941	21.92848
A_23_P127288	*IL2RA*	7.885232	0.029235	0.577062291	0.378389	20.83894
A_33_P3279629	*UCN2*	8.90368	0.029704	2.715812579	0.428079	20.79916
A_33_P3345534	*KRT14*	21.37476	0.000657	1.479677681	1.028442	20.78363
A_33_P3233784	*TMEM211*	5.41202	0.016577	0.698337632	0.263547	20.53529
A_23_P92860	*CCNO*	11.7167	0.01712	0.980809329	0.616717	18.99849
A_33_P3246418	*MDFI*	19.20304	0.034458	1.044171116	1.042159	18.42621
A_23_P218442	*CEACAM6*	4.921751	0.022815	0.571773936	0.272485	18.06249
A_23_P115261	*AGT*	7.716972	0.007079	0.626361946	0.4628	16.67452
A_23_P85209	*IL13RA2*	26.82207	0.022801	0.349319685	1.630988	16.44529
A_33_P3290239	*DUOXA1*	4.355018	0.010078	1.027224073	0.278531	15.63565
A_24_P335092	*SAA1*	4.351727	0.009158	2.235082019	0.292321	14.88682
A_33_P3232692	*IL24*	15.75963	0.001001	0.888806812	1.082312	14.56108
A_23_P58228	*ODAM*	38.0082	0.004611	0.63854174	2.699579	14.07931
A_24_P882732		15.09746	0.02903	0.838086788	1.122437	13.45061
A_33_P3593719	*LINC00114*	14.9315	0.01068	0.419699295	1.115306	13.3878
A_24_P887857		10.78025	0.000291	1.583315414	0.822748	13.10274
A_21_P0011578		10.56534	0.005435	0.823079223	0.823237	12.8339
A_33_P3319886	*C19orf45*	4.311201	0.026355	0.628641524	0.342473	12.58843
A_23_P79302	*LYPD6B*	4.402524	0.031385	1.170156623	0.377021	11.67714
A_23_P6066	*CPXM1*	4.649111	0.0452	1.520347468	0.440527	10.55352
A_33_P3298810	*FFAR3*	26.24005	0.003855	0.62074026	2.511385	10.44844

Histopathology. Formalin (37%) was used to fix neoplastic specimens, resection margins, and lymph nodes; then, they were embedded in paraffin. Hematoxylin- and eosin-stained sections were diagnosed pathologically. However, tumor differentiation and the degree of invasion were examined blindly by three pathologists (J.A., A.K., and M.O). Additionally, the General Rules for Colorectal Cancer Study and/or/or TNM were used as a reference for histopathological classification. The patients' clinical data are shown in Table I.

Immunohistochemistry. Immunohistochemistry was conducted as described in our previous studies (16). The tissue sections were stained with monoclonal or polyclonal antibodies against CHI3L1 (Bioss Antibodies Inc. Woburn, MA, USA), CEACAM6, CLND2, and lipopolysaccharide (LPS) (Abcam, Cambridge, UK).

Results

Gene expression microarrays and GO analysis. To determine the genes associated with CAC, we performed gene expression microarrays. After normalizing GEVs in tumors or inflammatory tissues, we selected 131 genes that exhibited normalized-GEVs (N-GEVs) in tumorigenic tissues that were 4-fold larger than those in inflammatory tissues [Ratio of Tumor (T)/Inflammation (I): T/I ratio >4)]. As a result of the T/I ratio, the microarray results showed that CLDN2, CHI3L1, and CEACAM6 gene expression levels were 333-fold, 120-fold, and 4.9-fold, respectively, up-regulated in tumorigenic tissues as compared to

Table III. *Results of ontology analysis*.

Category	Description	LogP	Log(q-value)	InTerm_ InList	Symbols
GO Biological Processes	Inflammatory response	–4.882096772	–0.810645325	6/504	CHI3L1, FFAR3, IL2RA, SAA1, SAA2, ODAM
KEGG Pathway	Cytokine-cytokine receptor interaction	–4.856300745	–0.810645325	5/295	IL2RA, IL11, IL13RA2, TNFRSF6B, IL24, SAA1, CHI3L1
GO Biological Processes	Regulation of inflammatory response	–4.256303566	–0.511678142	5/394	AGT, FFAR3, IL2RA, SAA1, DUOXA1, IL11, IL13RA2, UCN2, CHI3L1
GO Biological Processes	Response to wounding	–4.077191889	–0.510358695	5/430	SAA1, IL24, KLK8, ODAM, GRHL3
GO Biological Processes	Positive regulation of protein phosphorylation	–4.01194607	–0.510358695	6/722	AGT, CHI3L1, IL11, IL24, ODAM, CEMIP
GO Biological Processes	Positive regulation of cytosolic calcium ion concentration	–3.668432323	–0.275989418	4/281	AGT, GRIN2D, SAA1, CEMIP, KLK8, SLC4A11, UCN2, LYPD6B, SAA2
GO Biological Processes	Regulation of protein kinase activity	–3.263139765	–0.186074436	5/645	AGT, CDKN2A, CHI3L1, CCNO, CEMIP, KRT14
GO Biological Processes	Regulation of cell adhesion	–2.087999505	0	4/760	CDKN2A, IL2RA, CEACAM6, SAA1

inflammatory tissues without any tumors (Table II). Furthermore, we selected 32 genes with a T/I ratio that was 10-fold larger than the ratio of T/normal tissues (N) in sporadic CRC specimens (T/I ratio/T/N ratio >10) (Table II). GO analysis revealed that the selected 32 genes were involved in inflammatory responses, cytokine–cytokine receptor interactions, regulation of inflammatory responses, wound responses, and regulation of protein phosphorylation (Table III), suggesting that CAC is closely associated with the immune-mediated responses around the tumor microenvironment. Of note, CHI3L1 has been demonstrated as a critical protein involved in several tumorigenic processes such as in the colon and lungs. In addition, there are many reports about CEACAM6, showing that it has diverse functions in cell adhesion, intracellular and intercellular signaling, and during complex biological processes such as cancer progression, inflammation, angiogenesis, and metastasis.

Immunohistochemistry. To confirm the results of the microarray analysis, we performed immunohistochemical analyses for CHI3L1, CEACAM6, and CLND2. All the above proteins were clearly expressed in CAC tissues (Figure 1). In normal colonic mucosa, CEACAM6 and CLND2 were expressed in the cell membrane, but in CAC their expression was observed not only in the cell membrane but also in the cytoplasm. In CAC-derived samples but not normal colonic mucosa, CHI3L1 was also faintly but diffusely expressed in the cytoplasm (Figure 1).

To further confirm the protein expression levels of each detected gene, we performed immunohistochemical analyses using CAC surgical samples. The expression of CEACAM6, (a receptor for AIEC) and LPS (the principal component of the outer membrane of Gram-negative bacteria including AIEC) was almost co-localized in CAC tissues (Figure 2).

Discussion

We performed cDNA microarray analyses using surgically dissected normal, inflamed, and cancerous specimens from CAC patients with UC as well as CRC patients.

Our study identified CAC-related genes, *CHI3L1*, *CEACAM6*, and *CLND2*, and validated that the protein expression levels of the three genes were specifically up-regulated in CAC patient samples. All three genes were associated with AIEC, which suggested that these genes may be involved in AIEC infection. This is the first report to describe the relationship between these three genes and CAC surgical specimens from patients with UC.

Chitin is a polymer of N-acetylglucosamine and the second most common polysaccharide in nature. In contrast, chitinases are hydrolytic enzymes that can digest glycosidic bonds in chitin. CHI3L1 has chitin-binding ability without enzymatic activity and regulates many cellular and biological events such as oxidative stress, apoptosis, Th1/Th2 inflammatory balance, M2 macrophage differentiation, dendric cell accumulation, TGF-β expression, extracellular matrix regulation, and parenchymal scarring (17, 18). Thus,

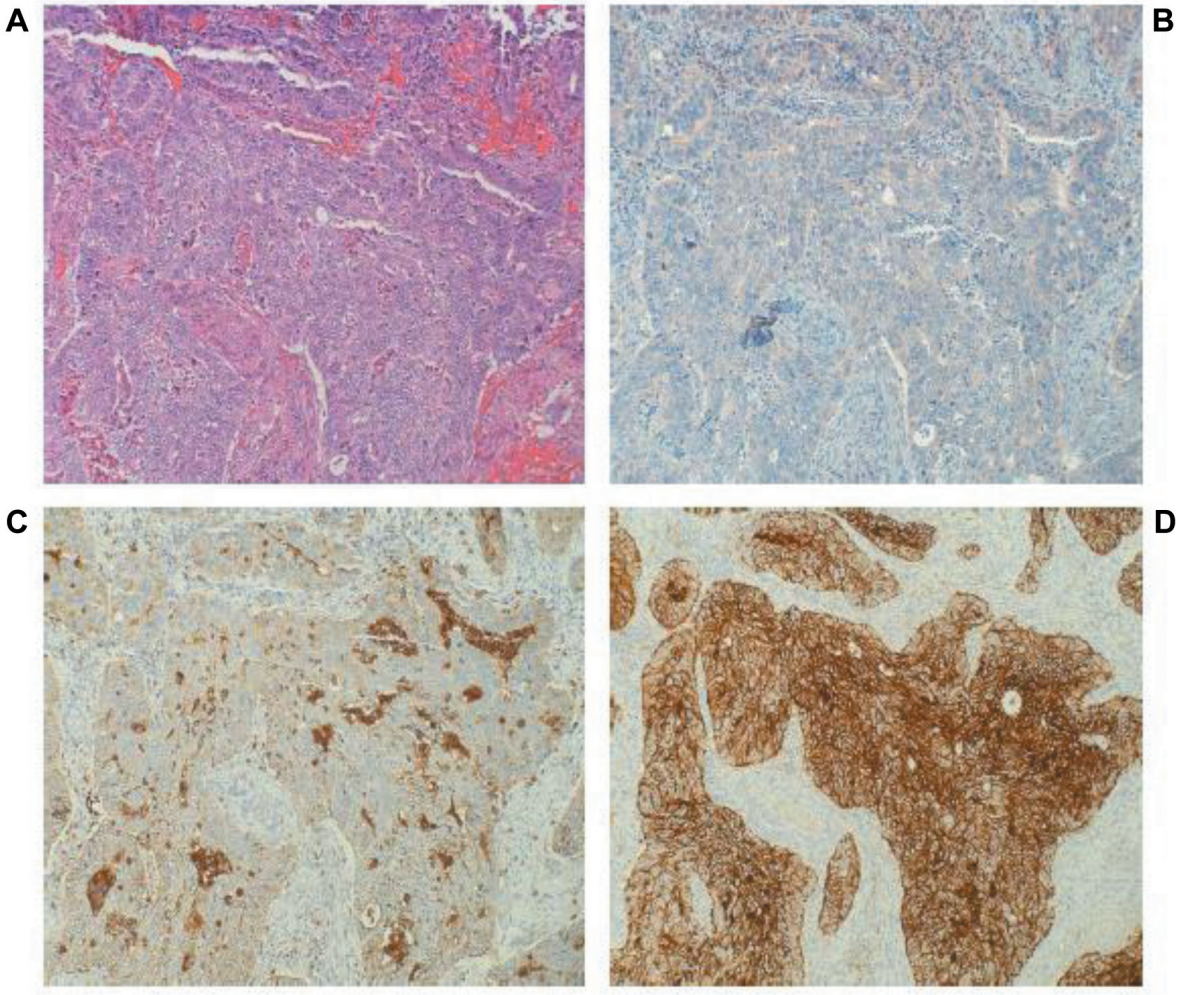

Figure 1. *Tumor sections of colitis-associated cancer (CAC) were analyzed by (A) hematoxylin-eosin staining (×200) and immunohistochemistry for the detection of (B) chitinase 3-like 1 (CHI3L1) (×200), (C) carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) (×200), and (D) claudin 2 (CLDN2) (×200).*

CHI3L1 plays a pivotal role in protection against several pathogens, antigen-induced and oxidant-induced injury responses, inflammation, and tissue repair and remodeling. Furthermore, many cancer types show CHI3L1 over-expression, including human or animal tumor models and CRC. Moreover, CHI3L1 regulates the expression of epithelial-mesenchymal transition markers (Twist, SNAIL, SLUG, N-cadherin, vimentin, and E-cadherin) (19). Moreover, CHI3L1 is also involved in developing CAC (20) and in tumorigenesis by secreting IL-8 and MCP-1 *via* MAPK in the intestinal epithelia *in vitro* (21). Low *et al*. and Mizoguchi *et al*. demonstrated that expression of CHI3L1 on inflamed colonic epithelial cells significantly enhanced the adhesion and invasion of bacteria in normal flora such as AIEC (22, 23). In particular, N-glycosylated CHI3L1 specifically facilitated AIEC adhesion to intestinal epithelial cells by interacting with bacterial chitinase (ChiA) *via* the specific chitin-binding domain, which is involved in host-microbial interactions during inflammation (23).

CEACAM6 is a CEA-related antigen that is expressed on granulocytes and monocytes (24). CEACAM6 is expressed on extracellular vesicles or intestinal epithelial cells (IECs) either as a soluble factor or bounded to the membrane by a GPI anchor (25). Furthermore, its expression is high in gastric cancer and CRC (26). Interestingly, both CEACAM6 and CHI3L1 have been reported to be expressed in the ileum of CD that is in a state of worsening inflammatory condition (22, 23, 27). One study on CD patients found that CEACAM6 is an AIEC receptor and helps AIEC to be established in the ileal mucosa (27). However, more investigations are needed to find out whether the presence of AIEC leads to CEACAM6 over-expression and whether

CEACAM6 up-regulation leads to AIEC colonization. Patients who have basal levels of CEACAM6 would be genetically prone to develop ileal CD. Additionally, AIEC and cytokine secretion, including IFN-γ and TNF-α, may amplify bacterial colonization and consecutive chronic infection-based inflammation. These results suggest that CAC could be detected in UC patients by measuring CEACAM6 expression, which represents a potential diagnostic marker.

Claudin is a tight junction-specific protein discovered in 1998 that significantly maintains homeostasis in living organisms (28). It also represents an intercellular barrier at tight junctions. CLND2 is present in the tight junctions of normal colorectal mucosa, but previous studies have reported an increase in ectopic expression in sporadic CRC tissue (16). Similar findings were confirmed in CAC surgical specimens from UC patients in this study. According to previous findings, ectopic expression of CLND2 may be involved in neoplastic changes of epithelial cells because MAPK regulates CLND2 expression *via* IL-17 (29), and cell proliferation is enhanced *via* epidermal growth factor receptor (30). CHI3L1 has been reported to activate signaling such as MAPK/ERK, AKT/PKB, and WNT/β-catenin *via* IL-13Rα2 in the intestinal epithelium *in vitro* (18). CHI3L1 may be involved in the ectopic expression of CLND2 in CAC. Furthermore, AIEC could increase CLND2 expression in mice and humans (31), and therefore, it is likely that CLND2 could serve as an entry gateway for AIEC into IECs. As shown in Figure 3, over-expression of CLND2 increases epithelial barrier permeability (*in vitro* and *in vivo*) (32, 33).

AIEC may play a role in the pathogenesis of IBD. Despite important progress in the genomic and immune characterization of AIEC since it was first discovered in 1998 (34), many questions need to be answered, such as whether AIEC exists as a natural reservoir or whether it moves from other places due to inflammation. Over the last two decades, researchers have focused on the relationship between AIEC and CD etiology (35). Separate groups have reported a subpopulation of E. coli called AIEC that has adhesive in addition to invasive abilities to intestinal epithelial cells and macrophages (36-38).

The main characteristics of AIEC are (i) the ability to adhere to and invade intestinal epithelial cells; (ii) the ability to survive and replicate within macrophages without triggering host cell death; and (iii) the absence of influential invasive determinants (39).

AIEC can also survive within macrophages inducing high TNF-α secretion (31). A recent study showed that CHI3L1, CLDN2, and CEACAM6 were associated with AIEC infection (40). Our current study has suggested that these three AIEC-associated molecules are involved in tumorigenic processes not only in CD but also UC, and a schematic representation of this study is shown in Figure 3.

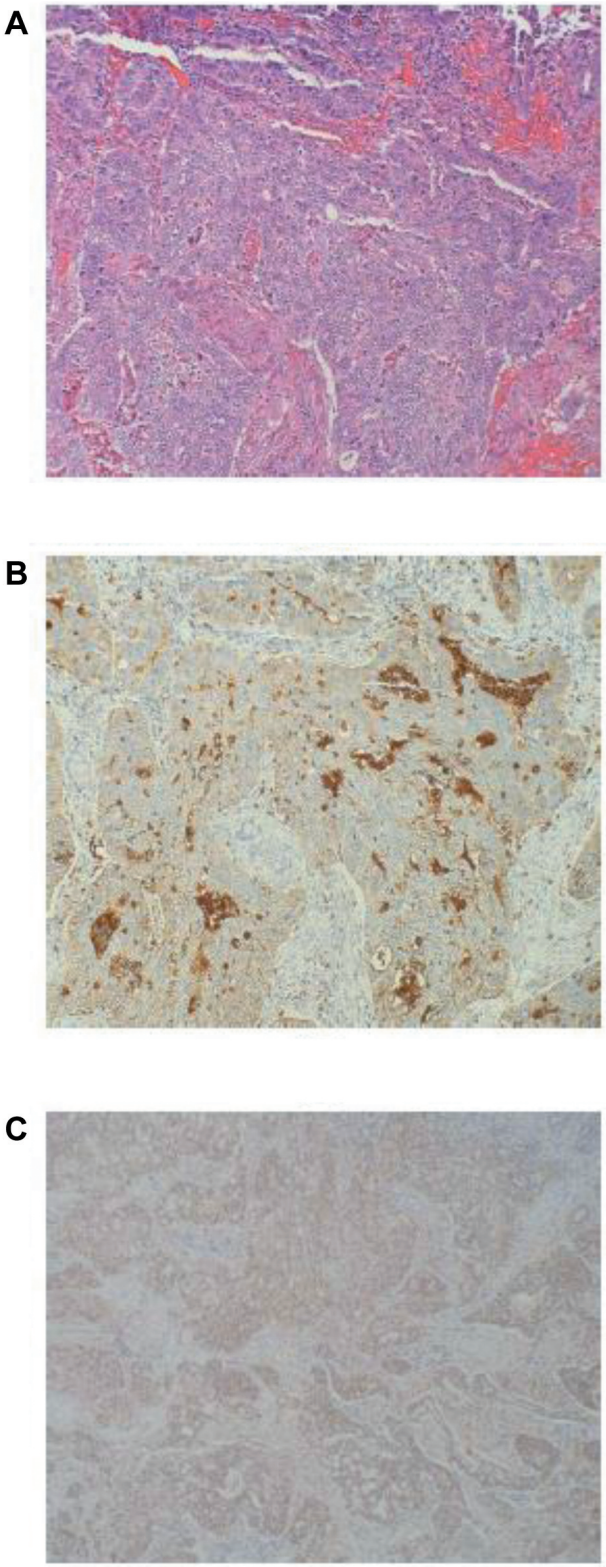

Figure 2. *Histological and immunohistochemical findings of colitis-associated cancer (CAC) with ulcerative colitis (UC). (A) Hematoxylin–eosin staining (×200). Immunohistochemistry for the detection of (B) CEACAM6 (×200) and (C) lipopolysaccharide (LPS) (×200).*

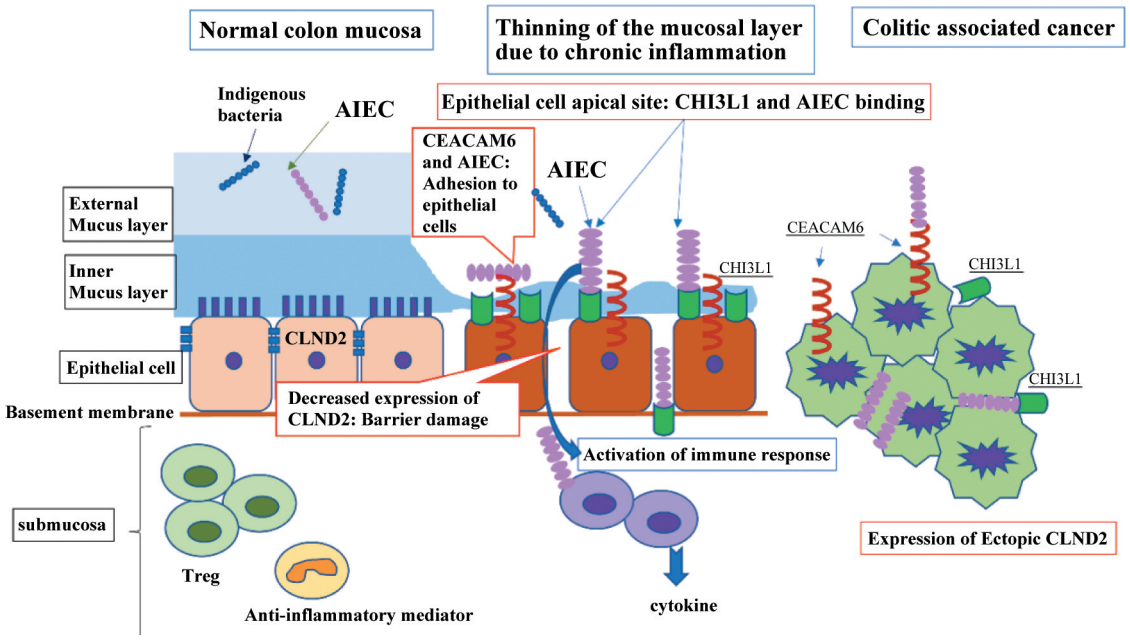

Figure 3. *Schematic of the mechanism of colitis-associated cancer (CAC) in ulcerative colitis (UC) patients according to the results of this study. The mechanism of CAC in UC patients is thought to be associated with several genes including Chitinase3-like1 (CHI3L1), carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), and Claudin-2 (CLND2). AIEC: Adherent invasive Escherichia coli.*

In conclusion, we have identified up-regulated expression of three key genes, *CHI3L1, CEACAM6*, and *CLDN2*, in CAC surgical samples from patients with UC. These genes may be involved in the perpetuation of chronic infection with AIEC in colonic epithelial cells. Further analyses will reveal the unknown mechanisms of CAC-related genes in the tumor microenvironment under chronic inflammation.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

TK participated in the study conception and design. TT, EM, TO, and TS participated in the acquisition and interpretation of data. TK and TS reviewed the patients' medical records for their clinical history of IBD. JA and AK established the pathological diagnosis. TK wrote the initial draft of the manuscript. EM, TT, TO, TS, and YA contributed to the preparation of the manuscript. TT, EM, TS, TO, and YA participated in the critical revision of the manuscript. The Authors read and approved the final manuscript.

Acknowledgements

This work was supported by JSPS KAKENHI (Grant-in-Aid for Science Research) grant number JP20K09114. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The Authors thank H. Nikki March, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

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Received May 20, 2022
Revised June 11, 2022
Accepted June 14, 2022