DNA hypomethylation at the CpG island is involved in aberrant expression of the L1 cell adhesion molecule gene in colorectal cancer

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Abstract. The L1 cell adhesion molecule (L1CAM) has been identified as a target gene of ß-catenin-TCF signaling in colorectal cancer (CRC) and associated with aggressive tumor behavior such as invasion and metastasis. We investigated the methylation status at the L1CAM gene promoter and/or L1CAM mRNA/protein expression in 4 CRC cell lines and 71 primary CRCs. Aberrant L1CAM expression was immunohistochemically observed in 31 (43.7%) of 71 cases, and correlated with advanced stage and presence of lymph node and distant metastases ($P<0.05$). Treatment with a demethylating agent induced L1CAM mRNA/protein expression in two cell lines lacking L1CAM expression. Bisulfite-modified genome sequencing suggested that DNA methylation status at core promoter and putative TCF-binding sites within the L1CAM promoter was correlated with L1CAM mRNA/protein expression in 4 CRC cell lines. Using the crypt isolation followed by bisulfite-modified genome sequencing and methylation-specific PCR methods, we confirmed that the DNA hypomethylation at core promoter and putative TCF-binding sites was well correlated with the aberrant L1CAM protein expression in primary CRC samples. These results suggest that DNA hypomethylation at the L1CAM CpG islands might induce L1CAM aberrant expression and contribute to the acquisition of aggressive tumor behavior in CRC.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death throughout the world and 50% of the cases are advanced at the time of diagnosis (1). Advanced CRC is often accompanied by metastasis to peritoneum, lymph nodes or other organs. For the management of these patients, genetic alterations accumulated in colorectal carcinogenesis have been anticipated as good biomarkers to evaluate their disease outcome. A number of genetic and epigenetic alterations including allelic losses on specific chromosomal arms, mutations of oncogenes, tumor suppressor genes and mismatch repair genes, microsatellite instability in coding repeat sequences of target genes and methylation defects in gene promoters have been described in the tumorigenesis of CRCs and a stepwise model of colorectal carcinogenesis has been proposed (2). However, the molecular mechanisms underlying the progression and the formation of metastasis of CRC are still largely unknown.

A recent study suggests that the L1 cell adhesion molecule (L1CAM) is a new target gene of ß-catenin-TCF signaling in CRC cells, which is the most evident genetic alteration involved in CRC carcinogenesis (3). The L1CAM is one of a subgroup of structurally related integral membrane glycoproteins belonging to a large class of immunoglobulin superfamily cell adhesion molecules (CAMs) that mediate cell-to-cell adhesion at the cell surface (4). Functionally, the molecule plays a role in development of the nervous system, such as guidance of neurite outgrowth in development, neuronal cell migration, axon bundling, synaptogenesis, myelination, neuronal cell survival and long-term potentiation (4-6). The L1CAM-immunoreactivity is observed in not only the nervous system but also the collecting tubule of the kidney and blood cells (7-10); however, it has never been identified in colonic epithelium. The aberrant expression of L1CAM protein is found in several human malignancies, and correlated with aggressive tumor behavior such as invasion and metastasis (11-13). In CRCs, *in vitro* and *in vivo* studies suggested that L1CAM was localized on the cell surface in the invasive front of the tumor tissues and increased cell motility, growth and tumorigenicity (3,14). Kaifi *et al* (15) demonstrated that L1CAM was detected in a subset of CRC patients (13% 48/375) and their survival revealed a significantly worse outcome in comparison with L1CAM-negative patients. Aberrant L1CAM protein

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expression is a late event in colorectal carcinogensis and a good biomarker for the management of CRC patients. The activation of Wnt signaling (ß-catenin-TCF), which can transcriptionally regulate the L1CAM expression, is introduced by the APC and/or ß-catenin mutation in initiation step of CRC development. It is unclear why the early event (activation of Wnt signaling) introduces the aberrant L1CAM expression as a late event relating to invasion and metastasis. The molecular mechanism of aberrant L1CAM expression has not been fully elucidated in CRCs.

Colorectal carcinogenesis, for many years a prototypic model for the genetic basis of cancer, is now increasingly cited as an exemple of the role of epigenetic changes in tumorigenesis (16). Site-specific DNA hypermethylation is described in several tumor-suppressor, mismatch-repair and cell-cycle-regulatory genes (16). In concert with site-specific hypermethylation at the CpG island, a progressive and global hypomethylation are also found in lesions across the neoplastic spectrum, from adenomatous polyps to CRCs (17,18). Moreover, DNA hypomethylation has been linked to several mechanisms that could drive neoplastic progression. This occurs at the repetitive sequences residing in satellite or pericentromeric regions directly resulting in the genomic instability (19,20). Site-specific DNA hypomethylation can also lead to the activation of oncogenes, an event that has been documented with the S100A4 metastasis-associated gene in CRC (21).

Thus, genetic and epigenetic alterations cooperate for the gene regulation related to cancer initiation and progression. In the present study, we demonstrated that DNA hypomethylation at the L1CAM gene promoter including putative TCF binding sites were closely associated with aberrant protein expression in CRC.

Materials and methods

Cell lines and tissue samples. Four CRC cell lines (Caco-2, DLD-1, SW620 and SW480) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained under their recommended conditions. A total of 71 CRC patients, who underwent surgical resection between May 2001 and February 2008 at the Iwate Medical University School of Medicine (Morioka, Japan), were involved in our L1CAM-immunohistochemistry and/or DNA methylation analysis. Five and 8 patients, respectively, were subjected to crypt isolation followed by bisulfite-modified genome sequencing, and methylation-specific PCR (MSP) analyses. All tissue samples and clinical data were obtained after the patients provided informed consent.

Immunohistochemistry. Four-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded samples and stained with hematoxylin and eosin. Serial sections were stained using the avidin-biotin system and antigen retrieval methods on a Ventana automated immunostainer with the Ventana immunohistochemistry detection system (Ventana Medical Systems, Tucson, AZ, USA), in accordance with the manufacturer's manual. The primary antibody used for L1CAM was anti-human L1CAM (dilution 1:100, a mouse monoclonal antibody UJ127, NeoMarkers, Fremont, CA, USA). Quantitative comparative analysis of immunohistochemical staining was carried out in each case by two pathologists. The extent of immunohistochemical reactivity for L1CAM was estimated by light microscopy and graded according to the number of immunoreactive cells (proportion score) and staining intensity (intensity score). In brief, proportion scores were graded as follows: 0, no immunoreactive cells were seen; 1, the number of immunoreactive cells was <10%; 2, >10% <50%; 3, >50%. Intensity scores were graded as follows: 0, there was no immunoreactivity; 1, the representative staining intensity was weak; 2, medium; and 3, strong. The final estimation of immunopositivity for L1CAM, when the sum total of proportion score and intensity score was >2.

5-aza-2'-deoxycytidine treatment. We treated 4 CRC cell lines with a demethylating agent (5-aza-2'-deoxycytidine, 5-Aza-dC), as previously described (22). Cells were seeded at a density of $5x10^5$ cells/10-cm plate. After a day, 5-Aza-dC (Sigma, St. Louis, MO, USA) was added to a final concentration of 2 μM. Three days after 5-Aza-dC treatment, the cells were harvested for real-time quantitative PCR (RQ-PCR) and Western blot analysis.

Western blot analysis. The cell suspension was centrifuged at 7,500 rpm for 1 min. After removal of the supernatant, the cell pellet was dissolved in 1.0% NP-40 lysis buffer [50 mM HEPES (pH 7.5)/1 mM EDTA/150 mM NaCl/2.5 mM EGTA/1.0% NP-40]. Cell samples containing equal amounts of protein were mixed with 6 x concentrated loading dye, heated for 4 min at 95˚C and subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on a 7.5% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were then transferred to a PVDF (polyvinylidene difluoride) membrane (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. Primary antibodies for anti-human L1CAM (mouse monoclonal antibody UJ127.11, Abcam, Cambridge, UK) and anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH: Upstate Biotech, Lake Placid, NY, USA) were both diluted 1:1000 in 0.05% Tween-20 phosphate-buffered saline. The membrane was incubated for 60 min at room temperature and washed. For the second antibody, anti-mouse IgG (Amersham Biosciences) was diluted 1:5,000 in blocking buffer. The membrane was then incubated for 45 min at room temperature and washed. Signals were detected with ECL Plus (Amersham Biosciences) and ChemiDoc XRS (Bio-Rad). The intensity of the detected signals was measured by 1-D analysis software (Quantity One, Bio-Rad). For normalization of the target, GAPDH was used as an internal control.

Real-time quantitative PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using SuperScript™ III first-strand synthesis SuperMix (Invitrogen). Primers and a fluorogenic probe were designed with the Primer Express software (Applied Biosystems; ABI, Foster City, CA, USA) (Table I). The RQ-RT-PCR assay was done using an ABI PRISM 7500 Sequence Detector (ABI), as previously described (23). For normalization of each target in the samples, the copy number of GAPDH was used as an internal control. Plasmids for standard curve method

Table I. Primer or fluorogenic probe sequences for bisulfate genome sequence, methylation-specific PCR and real-time quantitative PCR.

Name	Sequence
BGS-1-F	5'-GGA TAT GGG AGG GGA TAA ATA AGG G-3'
BGS-1-R	5'-CCT AAC CTC ACC CCC ACA TTA TAT C-3'
BGS-2-F	5'-GTA ATG AGT TAG TGG AGA GAA AGA G-3'
BGS-2-R	5'-CAA ACC CCA ACT TCT ACA AAT CCT T-3'
BGS-3-F	5'-GGT AGT AAT TGG AAA GGA GAG AGG T-3'
BGS-3-R	5'-CCC ATA ATA CCA ACT TCA ATT CCC C-3'
BGS-4-F	5'-GGG GGT GGG GTA TTT AAG TTA GGA TG-3'
BGS-4-R	5'-CCC CAA TAC CCC CTC CTA AAC TCA AA-3'
BGS-5-F	5'-TTT GAG TTT AGG AGG GGG TAT TGG GG-3'
BGS-5-R	5'-CCC CCC CAA CAC ACA CAC ACA AAA C-3'
BGS-6-F	5'-TTT TAA TAG GAT TTT GAT TGG GTT TTT AGG-3'
BGS-6-R	5'-CAC CCT AAC CCC TAA TAC CAA C-3'
BGS-7-F	5'-GGG GAG GGG AGG GTT GGA GAA G-3'
BGS-7-R	5'-ACT CCC TTC AAC TAC TCA CAT TCC T-3'
MSP-M-F	5'-ATC GTT CGG TTT TAG GTT TTT GGG AGT A-3'
MSP-M-R	5'-AAC CGA AAA TCT CCG ACC GAA AAA TAT C-3'
MSP-U-F	5'-ATT GTT TGG TTT TAG GTT TTT GGG AGT A-3'
MSP-U-R	5'-ACC AAA AAT CTC CAA CCA AAA AAT ATC C-3'
RTQPCR-F	5'-ACG GAA CAG TCT CCA CGG C-3'
RTQPCR-R	5'-GGT ACA CGG TCA CAC CCA GC-3'
probe	FAM-TTC CCC ACA GAT GAC ATC AGC CTC AA-TAMRA

BGS, bisulfite genome sequence; M, methylated; U, unmethylated and RTQPCR, real-time quantitative PCR.

were diluted in a precise series, ranging from 5 pg to 0.005 fg ($2x10^6$ to 2 copies). The normalized values of L1CAM mRNA were expressed as the ratio of L1CAM copy number per 10^4 copy number of GAPDH.

DNA extraction. Genomic DNA was isolated from CRC cell lines using a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA). Non-cancerous and cancerous glands were isolated (>1000 crypts in each sample) by the method of Nakamura *et al* (24) and subsequently their genomic DNAs were purified using the same kit. For the extraction of DNA from microdissected materials, we prepared 10-μm thick sections and serial 4-μm thick sections (for hematoxylin and eosin staining) were cut from formalin-fixed and paraffin-embedded samples, as previously described (25). DNAs were extracted by MagneSil Genomic, Fixed Tissue System (Promega Co., Madison, WI, USA) and MagneSphere Magnetic Separation Products (Promega), according to the supplier's instructions.

Bisulfite-modified genome sequencing. Bisulfite-modified genome sequencing was performed, as previously described (23,25,26). DNA was treated with bisulfite modification and examined for the methylation status of 97 CpG sites in the L1CAM promoter containing 4 putative TCF4-binding sites. The consensus TCF4-binding sequences were furnished by Dr Nancy Gavert and Dr Avri Ben-Ze'ev (Department of Molecular Cell Biology, Weizmann Institute of Science, and Rehovot, Israel). Bisulfite modification was done using a Methylamp™ DNA modification kit (Epigenetek Inc., Broadway, NY, USA). Modified DNA was PCR-amplified using 7 primer sets are listed in Table I. PCR amplification was performed under the following conditions; only the set of BGS-5-F/-R (95˚C for 1 min followed by 5 cycles of 95˚C for 30 sec and 64˚C for 15 sec and 72˚C for 90 sec, 5 cycles of 95˚C for 30 sec and 62˚C for 15 sec and 72˚C for 90 sec, and then 40 cycles of 95˚C for 30 sec and 60˚C for 15 sec and 72˚C for 90 sec) and the others were (95˚C for 1 min followed by 5 cycles of 95˚C for 30 sec and 64˚C for 1 min, 5 cycles of 95˚C for 30 sec and 62˚C for 1 min, 5 cycles of 95˚C for 30 sec and 60˚C for 1 min, and then 35 cycles of 95˚C for 30 sec and 58˚C for 1 min). The final reaction volume for all PCRs was 50 μl and the mixture contained 0.2 μM each primer, 1 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of Platinum Taq DNA polymerase (Invitrogen) and 30 μl of distilled water. Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. PCR products were purified with a QIAquick gel extraction kit (Qiagen, Hilden, Germany). The PCR fragments were ligated to pGEM-T Easy Vectors (Promega) and transformed into DH5α competent cells (Toyobo Co., Ltd., Osaka, Japan). Ten subcloned colonies were chosen at random. Plasmid DNA was purified by a PI-200 DNA automatic isolation system (Kurabo, Osaka, Japan). Cycle sequencing used a primer of the SP6 promoter and a BigDye Terminator v3.1 cycle sequencing kit (ABI) and an ABI Prism 3100 DNA Sequencer (ABI).

MSP. Microdissected DNA material (10 ng/reaction) subjected to MSP analysis. Two sets of primers were designed to specifically amplify either the methylated (M) or

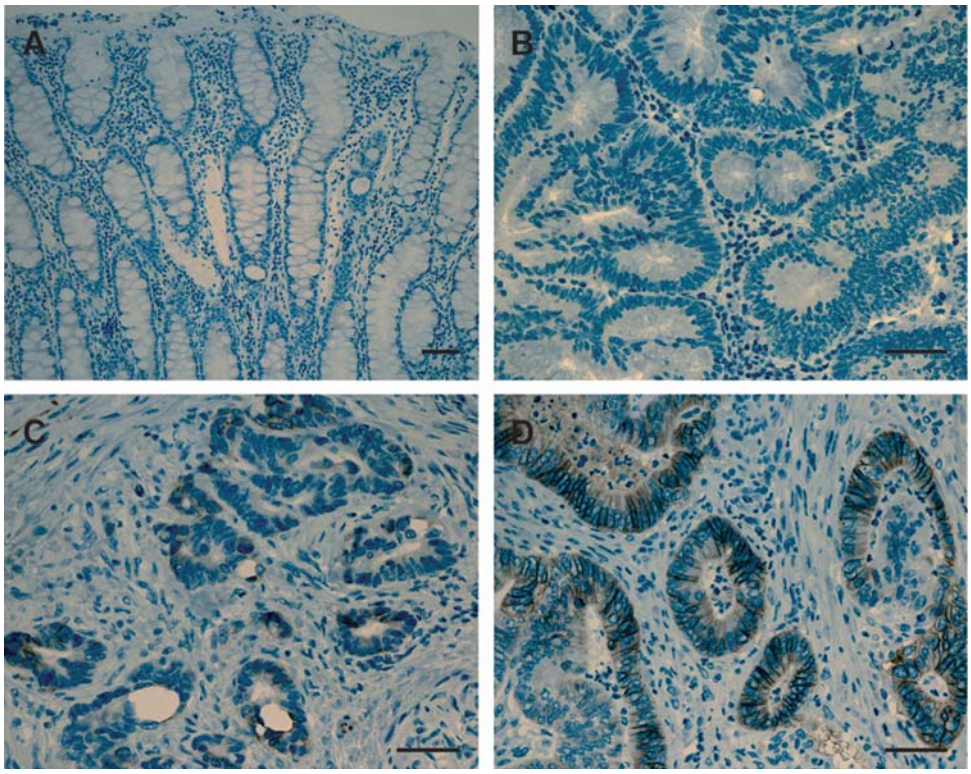

Figure 1. Immunohistochemistry for L1CAM in normal colonic epithelium and primary colorectal cancers. (A) Normal colonic epithelium is negative for the L1CAM protein. (B-D) L1CAM immunoreaction for primary colorectal cancers. Quantitative comparative analysis (proportion and intensity scores) of immunohistochemical staining for TUBBB3 protein was performed (see Materials and methods). Assessment of 3 cases is represented (c, negative; d, medium; e, strong). Bar, 100 μm.

unmethylated (U) bisulfite-modified sequence (Table I). The final reaction volume for all PCRs was 50 μl containing 0.2 μM each primer, 25 μl of AmpliTaq Gold PCR Master Mix (ABI) and 21 μl of distilled water. Bisulfite-modified DNA (10 ng) was subjected to specific-PCR amplification: M (95˚C for 5 min followed by 38 cycles of 95˚C for 30 sec and 55˚C for 15 sec and 72˚C for 30 sec), U (95˚C for 5 min followed by 38 cycles of 95˚C for 30 sec and 52˚C for 15 sec and 72˚C for 30 sec).

Statistical analysis. The correlation between the expression of L1CAM protein and clinicopathological variables were analyzed by Fisher's exact or Kruskal-Wallis tests. Survival data were analyzed according to the method of Kaplan-Meier survival curves and log-rank test. P-value of <0.05 was considered significant.

Results

Immunohistochemistry for L1CAM protein in primary CRC and survival analysis. We examined immunohistochemically L1CAM protein expression in 71 patients with primary CRCs. No immunoreaction was observed in any normal colonic epithelia (Fig. 1A). Sublocalization of L1CMA protein was positive in the cell surface membrane (Fig. 1C and D). Immunoreactivity for L1CAM protein varied among tumors (Fig. 1B, C and D) and intratumoral heterogeneity in each case was also observed. We performed quantitative comparative analysis of immunohistochemical staining by two pathologists. Positive immunoreactivity was observed in 31 (43.7%) of 71 cases. Table II summarizes the relationship between L1CAM immunoreactivity and clinicopathological variables of cases in the total series. L1CAM expression was correlated with advanced stage, and presence of lymph node and distant metastases (P<0.05) (Table II).

We further examined the relationship between L1CAM immunoreactivity and disease outcome in 45 patients whose follow-up data were available. They consisted of 30 negative (67%) and 15 positive (33%) patients (median follow-up of 34 months: range, 1 to 67 months). Eleven (24%) of 45 patients died of disease relapse. The Kaplan-Meier curve for patients who were LICAM-positive exhibited a trend of worse outcome than individuals who had L1CAM-negative immunoreactivity, however, no statistical significance was obtained (P=0.33, log-rank test, Fig. 2).

Expression of L1CAM mRNA/protein in CRC cell lines and 5-aza-dC treatment. Two CRC cell lines (SW480 and SW620) strongly expressed L1CAM protein and immunoreactivity of Caco-2 and DLD-1 was undetectable (Fig. 3A). L1CAM mRNA expression levels were at the endogenous level and compatible with their protein expression level (Fig. 3B). Treatment with the demethylating agent 5-Aza-dC, induced L1CAM mRNA/protein expression in L1CAM-negative cell lines, whereas that in L1CAM-positive cell lines did not alter (Fig. 4). These results indicate that L1CAM expression is regulated by epigenetic modification in CRC cell lines. We further examined the methylation status at 5' regions of CpG islands including the core promoter and 4 putative TCF-binding sites of the L1CAM gene in CRC cell lines.

Table II. Correlation between L1CAM immunoreactivity and clinicopathological variables in 71 patients with CRC.

Variable	L1CAM immunoreactivity		
	Positive (n=31)	Negative (n=40)	P-value
Depth			
T1, T2	2	7	0.152
T3, T4	29	33	
Lymph node metastasis			
No	6	17	0.034[a]
Yes	25	23	
Differentiation			
Well	5	3	0.876
Moderate	21	33	
Poor	4	3	
Mucinous	1	1	
Distant metastasis			
No	18	34	0.012[a]
Yes	13	6	
Peritoneal metastasis			
No	22	33	0.193
Yes	9	7	
Stage			
0, I, II	3	16	0.004[a]
III, IV	28	24	
Location of tumor			
Colon	24	29	0.843
Rectum	7	11	

[a]Indicates statistically significant.

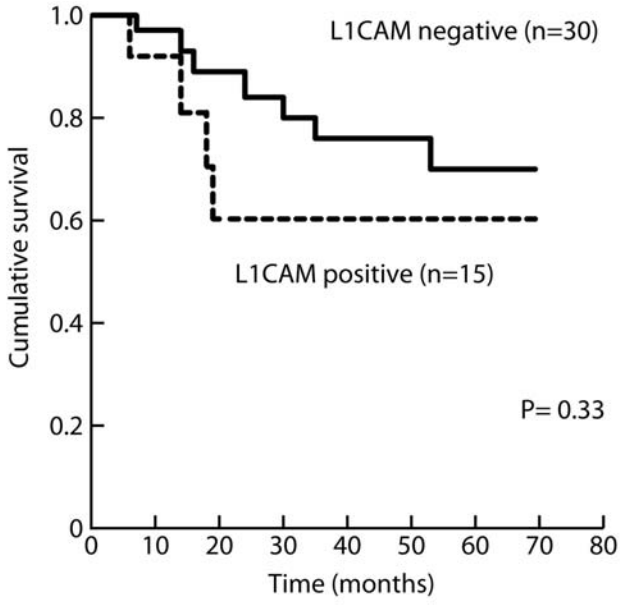

Figure 2. Survival curves for 45 colorectal cancer patients according to L1CAM immunoreactivity. A trend of worse outcome in L1CAM-positive patients was observed, however, there was no statistical significance (P=0.33, long-rank test).

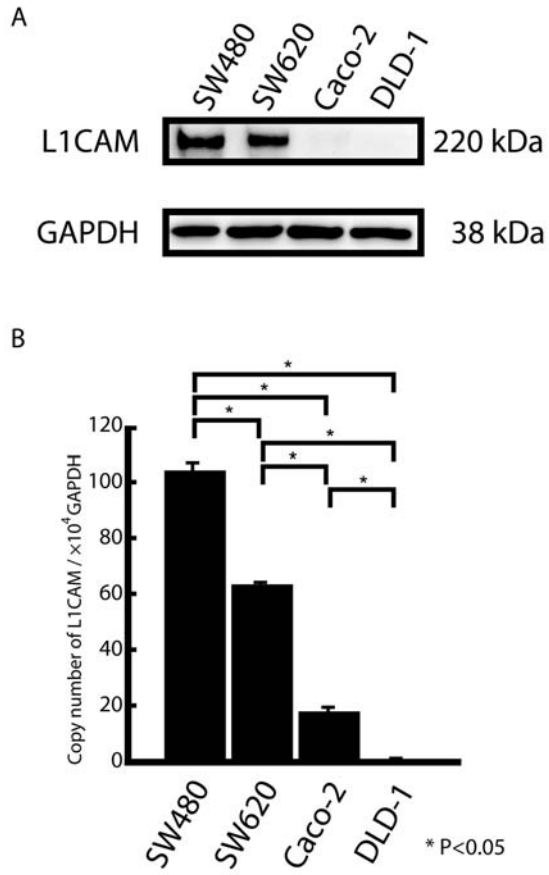

Figure 3. Results of Western blot analysis and real-time quantitative PCR for L1CAM mRNA/protein expression in 4 colorectal cancer cell lines. (A) SW480 and SW620 exhibited overexpression of L1CAM protein, whereas Caco-2 and DLD-1 were negative, respectively. Twenty micrograms of whole-cell lysate was applied to each lane. Equal loading was confirmed by blotting of GAPDH. (B) Quantitative results for L1CAM mRNA are compatible with the results of protein expression.

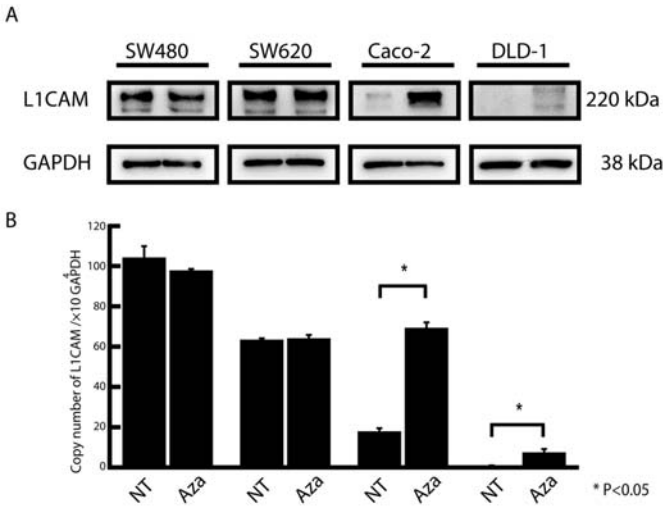

Figure 4. L1CAM mRNA/protein expression in 4 colorectal cancer cell lines treated with a demethylation agent (Aza, 2 μM 5-Aza-CdR). NT, untreated control. (A) Western blot analyses for L1CAM protein indicate that the expression level of L1CAM is not altered in the strongly L1CAM-overexpressing cell lines SW480 and SW620. In contrast, 2 cell lines lacking L1CAM expression, Caco-2 and DLD-1, exhibit an increase of L1CAM protein expression. Twenty micrograms of whole-cell lysate was applied to each lane. Equal loading was confirmed by blotting of GAPDH. (B) Quantitative results for L1CAM mRNA are compatible with those of protein expression. *P<0.05.

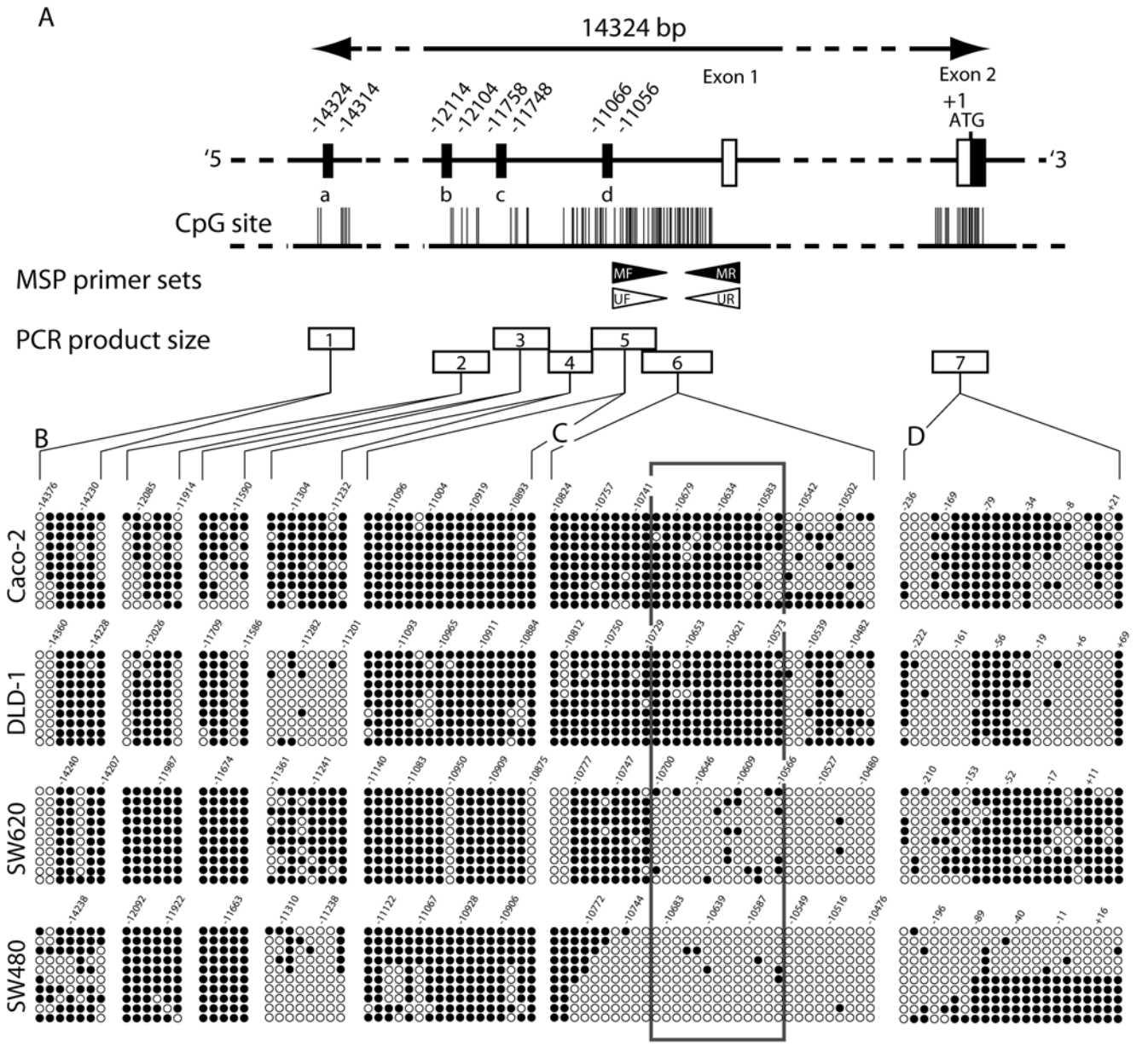

Figure 5. Diagram of the L1CAM gene promoter region and methylation status in 4 CRC cell lines. (A) Diagram of the upstream region of the L1CAM gene. Four TCF4-binding sites are indicated by the black boxes labeled a-d. The vertical lines mark the location of CpG dinucleotide sites. The size and location of the amplified PCR products are indicated by open boxes labeled 1-7. MSP primer sets were indicated by open and filled arrows (MF/MR and UF/UR). (B) Methylation status of CpG sites upstream of the L1CAM gene at TCF4-binding sites (PCR products 1-5 in Fig. 5A). Each circle represents a CpG dinucleotide site. The filled circles are methylation-positive and the open circles are methylation-negative. The number at the top indicates the position of each CpG site from the adenine of the start codon. (C) Methylation status at the core promoter (PCR product 6 in Fig. 5A). The methylation status of 13 CpG sites surrounded by open square were well correlated with L1CAM mRNA/protein expression. (D) Methylation status at CpG island around the start codon (PCR product 7 in Fig. 5A).

Methylation status in CRC cell lines. The methylation status of L1CAM gene was investigated using the bisulfite-modified genome sequencing method. Fig. 5A represents the diagram of CpG sites within the L1CAM promoter region containing 4 putative TCF4-binding sites. The distinct pattern of cytosine methylation was found between L1CAM-positive and negative cell lines at the 13 CpG sites within the core promoter (-10,566 to -10,700 bp; numbers, upstream of the adenine of the start codon) (Fig. 5C). Inverse correlation of DNA methylation status and mRNA/protein expression was confirmed. Methylation status at CpG sites of 4 TCF-binding sites and around the start codon varied among cell lines and there was no relation to L1CAM expression.

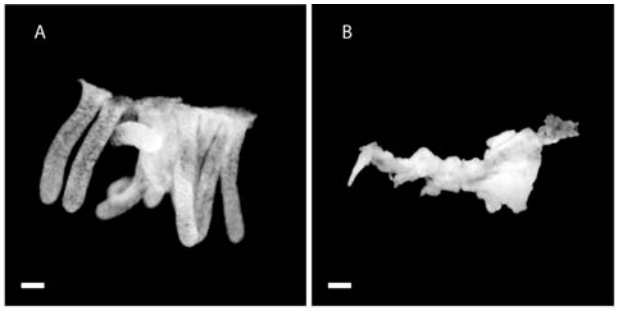

Figure 6. Features of cancerous and non-cancerous glands isolated from a colorectal cancer patient. (A) Non-cancerous and (B) cancerous glands. Bar, 100 μm.

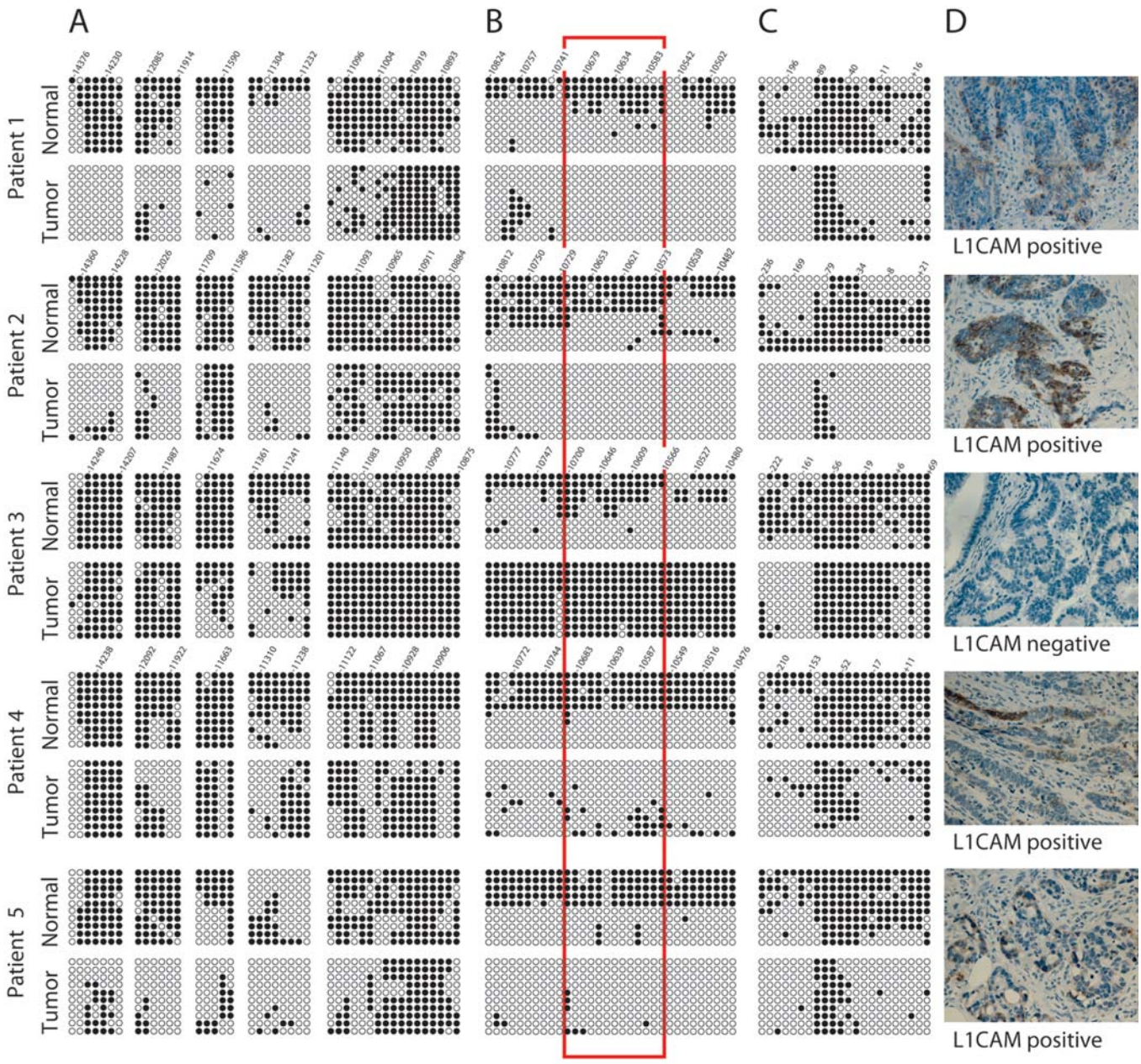

Figure 7. Methylation status at the L1CAM gene promoter and immunohistochemistry for L1CAM protein in 5 patients with colorectal cancers examined by the crypt isolation method. Each circle represents a CpG dinucleotide site. The filled circles are methylation-positive and the open circles are methylation-negative. The number at the top indicates the position of each CpG site from the adenine of the start codon. (A) Methylation status of CpG sites at TCF4-binding sites of the L1CAM gene (PCR products 1-5 in Fig. 5A). Each circle represents a CpG dinucleotide site. The filled circles are methylation-positive and the open circles are methylation-negative. The number at the top indicates the position of each CpG site from the adenine of the start codon. (B) Methylation status at the core promoter (PCR product 6 in Fig. 5A). The methylation status of 13 CpG sites surrounded by open square were well correlated with L1CAM mRNA/protein expression. (C) Methylation status at CpG island around the start codon (PCR product 7 in Fig. 5A). (D) Immunohistochemistry for L1CAM protein in each patient examined by the crypt isolation method.

Crypt isolation and methylation status at the L1CAM promoter in primary CRC and normal colonic epithelium. We also investigated the methylation status of the L1CAM gene promoter in 5 of 71 patients assessed by immunohistochemistry. For the elimination of interstitial tissues, we isolated both cancerous and non-cancerous glands by the crypt isolation technique (Fig. 6) and then analyzed by the bisulfite-modified genome sequencing method. In all patients, the normal colonic epithelium exhibited at least half of DNA clones heavily methylated at the 13 CpG sites within the core promoter (Fig. 7B). Conversely, the methylation status of cancerous glands exhibited marked demethylation in 4 L1CAM-positive tumors (Fig. 7D). In only a L1CAM-negative tumor, these were heavily methylated (Fig. 7B, patient 3). Flanking CpG sites at the 4 putative TCF-binding sites were relatively hypermethylated in the normal glands in comparison with the cancerous glands (Fig. 7A). Similarly to the core promoter region, demethylation at TCF-binding sites was frequently observed in cancerous glands in 4 L1CAM-positive tumors (Fig. 7A). A L1CAM-negative tumor was relatively DNA hypermethylated at TCF-binding sites in comparison to L1CAM-postive tumors. Thus, bisulfite-

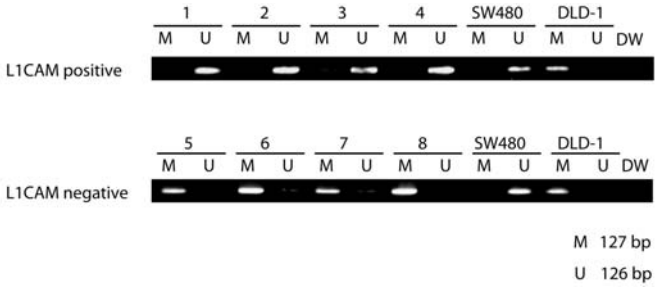

Figure 8. Methylation-specific PCR analyses for L1CAM gene promoter in 8 patients with colorectal cancer. Numbers are patient number. M, methylated primer and U, unmethylated primer. SW480 and DLD-1 were used for controls for unmethylated and methylated-specific primers. Results of L1CAM immunostaining-positive (A) and -negative tumors (B).

modified genome sequencing results of CRC cell lines and primary CRCs suggested that both hypomethylation at CpG methylation within TCF-binding sites and the core promoter region might contribute to L1CAM aberrant expression.

MSP. We next evaluated the methylation status at the core promoter region in additional 8 patients including 4 L1CAM-positive and -negative tumors, respectively. Positive signals for a U-primer set were observed in 4 patients, all of whom showed positive immunoreactivity for L1CAM, whereas positive signals for an M-primer set were observed in 4 patients, all of whom showed negative immunoreactivity for L1CAM (Fig. 8).

Discussion

In the present study, we evaluated DNA demethylation partly involved in the aberrant L1CAM expression in CRC. The 5'-flanking region sequences and transcriptional regulation of the L1CAM gene were elucidated in mouse genome (27,28). It has been documented that the minimal region needed to induce overexpression of the L1CAM gene contains 4 putative binding sites for the regulation factor with TCF4 (3). For the L1CAM aberrant expression, the requirement of demethylation at the 13 CpG sites within core promoter region was also shown as well as TCF-binding sites. Hence, the combination of 2 molecular mechanisms (activation of the ß-catenin-TCF signaling and demethylation at the CpG islands within the promoter region) might be necessary for the aberrant L1CAM expression in CRC. This is a good explanation why the activation of Wnt signaling assigned as the early genetic event in CRC is involved in the aberrant L1CAM expression associated with late event such as invasion and metastasis. Normal epithelium lacks L1CAM expression, because the ß-catenin-TCF signaling is not activated by the abnormalities of the APC gene and other Wnt related genes in normal colonic glands. If the activation would occur as an early genetic event, the DNA hypermethylation at the CpG islands within the L1CAM promoter region might be a defense for its aberrant expression. DNA hypomethylation at the L1CAM gene promoter associated with progressive global hypomethylation would induce gain of L1CAM expression as a late event. Again, DNA hypomethylation at the L1CAM promoter might cooperate with a genetic event (activation of the ß-catenin-TCF signaling) and be partly involved in the aberrant L1CAM expression as a late event in colorectal carcinogenesis.

The L1CAM gene, as a candidate to be controlled by the activation of the Wnt signaling is reported in cell proliferation, such as cyclin D1 (29,30) and c-myc (31), and in cell adhesion and/or cell migration, such as ADAM10 (14) and Fascin1 (32). *In vitro* and *in vivo* studies of Fascin1 are similar to those of L1CAM. Fascin1 is an actin bundling protein localized along the entire length of filopodia and its depletion leads to a substantially reduced number of filopodia (33). Moreover, several studies showed that Fascin1 significantly increases cell migration in transfilter assays (34,35). Fascin1 is expressed predominantly in neuronal tissue and is absent from normal epithelial cells. However, high levels of Fascin1 expression were reported in many types of cancer cells (36), including CRC (35,37). Fascin1 mRNA and protein expression were increased in primary CRC in a stage-dependent manner (32). Fascin1 was exclusively localized at the invasive front of tumors also displaying nuclear ß-catenin (32). Forced expression of Fascin1 in CRC cell lines increased their migration and invasion and caused cell dissemination and metastasis *in vivo*, whereas suppression of Fascin1 expression by small interfering RNA reduces cell invasion (32). Vignjevic *et al* (32) also found that distant metastases were Fascin1-negative, whereas Fascin1 levels were higher in human primary tumors that developed into distant metastases. They hypothesized that the switchback of Fascin1 mRNA/protein expression might be regulated in the two-phase model for ß-catenin target gene activation suggested by Brabletz *et al* (38). Fascin1 gene might be activated as a phase II gene which is possibly stimulated by the aberrant environmental signals from mesenchymal tissues. The idea is fascinating to understand the switchback phenomenon concerning to the aberrant protein expression, however our results of the L1CAM gene suggested that epigenetic statuses should be considered to resolve the problem.

The Fascin1 mRNA was upregulated by the treatment with a demethylating agent in hepatocellular carcinoma cell lines (39). I was described that the estrogen receptor-ß expression declined as prostate cancer developed in the gland but reappeared in lymph node and bone metastases. It was introduced by the switchback mechanism on the DNA methylation at the promoter region of the estrogen receptor-ß gene. Unlike the genetic mutations that accumulate in a cancer cell, epigenetic mutations (or modifications) can be readily reversed (40). Thus, when a cell adopts a new gene expression pattern as a result of an epigenetic change, the cell and its progeny retain the capability to revert back to a previous gene expression pattern. Domann and Futscher (40) described that neoplastic progression should perhaps not be considered as a continual march toward an increasingly aggressive phenotype, but rather as a stochastic trial and error saunter toward an increasing aggressiveness in a 'two steps forward, one step backward' manner.

Thus, DNA hypomethylation at the L1CAM promoter might cooperate with the activation of Wnt-signaling and contribute to the acquisition of aggressive tumor behavior in CRC.

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