Epigenetic inactivation of calcium-sensing receptor in colorectal carcinogenesis

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Ca²⁺ is a chemopreventive agent for colon cancer. Ion transport systems are often altered in human cancer. The aim of this study was to clarify the alterations of calcium-sensing receptor (CASR), a member of the G protein-coupled receptor family, in colorectal carcinogenesis. We analyzed the expression of CASR in colorectal cancer cell lines and in cancer and adenoma tissues by RT-PCR and immunostaining. In addition, we analyzed methylation of the CASR promoter by using bisulfite sequence analysis and methylation-specific PCR. CASR mRNA and protein expression was significantly downregulated in most of the cancer cell lines. CpG islands were densely methylated in cancer cell lines with reduced CASR mRNA expression. Treatment with a demethylating agent, 5-aza-2'-deoxycytidine, and/or a histone deacetylase inhibitor, trichostatin A, restored CASR expression in the cancer cell lines. Disruption of CASR expression in CASR-unmethylated HCT-8 cells blocked the enhancing effect of Ca²⁺ on the cytotoxic response to 5-fluorouracil. CASR expression was observed in normal colonic epithelial cells and was retained in most adenoma tissues. CASR mRNA and protein expression was significantly downregulated in cancer tissues. There was an inverse relationship between CASR expression and degree of differentiation. Immunohistochemical CASR staining was reduced more predominantly in less-differentiated cancer tissues and/or in cancer cells at the invasive front, where nuclear/cytoplasmic β -catenin was often localized. CASR methylation was detected in 69% of colorectal cancer tissues and 90% of lymph node metastatic tissues and was significantly correlated with reduced CASR expression. CASR methylation was also detected in 32% of advanced adenoma tissues but was detected in only 9% of adenoma tissues and was not detected in hyperplastic polyp tissues. CASR methylation seems to occur at an early stage and progress in colorectal carcinogenesis. The results suggest that epigenetic inactivation of CASR has an important role in colorectal carcinogenesis.

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Colorectal cancer is one of the most common cancers in the world. Various genetic and epigenetic abnormalities have been identified in colorectal cancer, suggesting a multistep nature of colorectal carcinogenesis.^{1–3} A genetic predisposition has a role in some individuals with colorectal cancer. Diet is also thought to be a major etiological factor for

colorectal cancer. Low intake of calcium and/or vitamin D is suggested to be one of the important risk factors for colorectal cancer.

Ca²⁺ has chemopreventive activity for colorectal cancer.^{4,5} Calcium-sensing receptor (CASR) is a member of the G protein-coupled receptor family. Extracellular Ca²⁺ and CASR serve as a first messenger system in controlling biological function. In addition to the systemic control of Ca²⁺ homeostasis, CASR has been suggested to have diverse functions, such as regulation of various cellular processes in different cell types.^{6–9} Expression of CASR in colonic epithelial cells has been reported. CASR reportedly controls proliferation and differentiation in the human colonic epithelium and colon carcinoma cells.^{10–18}

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Increased thymidylate synthase expression is one the most important mechanisms underlying the resistance to 5-fluorouracil (FU) in colorectal cancer.^{19,20} Interestingly, activation of *CASR* reportedly downregulates the expression of thymidylate synthase and survivin, and upregulates cellular sensitivity to 5-FU in human colorectal cancer cell lines.¹⁵ Moreover, it has been reported that vitamin D mediates its action in human colorectal cancer cells in a *CASR*-dependent manner and downregulates malignant cell behavior.²¹

Ion transport systems are often altered in human cancer. Downregulation of *CASR* protein expression has been reported in colorectal cancer tissues. There are only a few reports showing downregulation of immunohistochemical staining of *CASR* in colorectal cancer tissues.^{11,12,22} However, the numbers of analyzed cases were too small to characterize the alterations of *CASR* expression in colorectal carcinogenesis. In addition, alterations of *CASR* expression in colorectal adenoma have not been reported. Moreover, the mechanism by which *CASR* expression is downregulated in colorectal cancer is not known. It has been reported that Na^+ /monocarboxylate transport, a product of the *SLC5A8* gene, is silenced by promoter hypermethylation in colorectal cancer.²³ If *CASR* is inactivated by DNA methylation, reversal of *CASR* silencing by demethylating agents could be a potential target for effective treatment and/or prevention of colorectal cancer.

These findings made *CASR* relevant to further investigations in colorectal carcinogenesis. Thus, we systematically analyzed the expression and epigenetic regulation of *CASR* in colorectal cancer cell lines and tissues and in adenoma tissues.

Materials and methods

Cell Lines

Colorectal cancer cell lines, Caco-2, COLO 320DM, DLD-1, LoVo, HT-29, SW480, SW48, SW620, HCT-8, HCT 116, and hepatoma cell line JHH7, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), Riken Cell Bank (Tokyo), or the American Type Culture Collection (Rockville, MD, USA), and cultured as recommended. Cells were maintained at 37 °C in an atmosphere of humidified air with 5% CO_2 .

Tissue Samples

Tissue samples were purchased from Genomics Collaborative (Laurel, MD, USA) or obtained from Japanese patients who had undergone surgical treatment or endoscopy. Informed consent was obtained from each patient. Tissue microarray of colorectal adenocarcinoma tissues was purchased from SuperBioChips Laboratories (Seoul, Korea). Advanced adenoma was defined as an adenoma of

10 mm or more in diameter and/or an adenoma with high-grade dysplasia.^{24,25} In all, 90 colorectal adenocarcinoma tissues (mean age: 67 years old; 54 male and 36 female; 39 right sided and 51 left sided (distal to the splenic flexure)), 10 lymph node metastatic tissues (mean age: 71 years old; 6 male and 4 female), 28 advanced adenoma tissues (mean age: 56 years old; 15 male and 13 female; 12 right sided and 16 left sided), 22 adenoma tissues (mean age: 54 years old; 12 male and 10 female; 9 right sided and 13 left sided), and 14 hyperplastic polyp tissues (mean age: 53 years old; 8 male and 6 female; 6 right sided and 8 left sided) were analyzed. The sample was processed for pathological examination using hematoxylin and eosin staining for the evaluation of the tumor cell content. Only specimens containing >80% tumor cells were used for analysis. The seventh edition of the tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer and the International Union against Cancer was used for the pathological diagnosis and classification of variables.

Semiquantitative RT-PCR and Real-Time RT-PCR

Total RNA from cell lines and tissue samples were extracted using an extraction kit (RNeasy Mini kit; Qiagen, Valencia, CA, USA). cDNA was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA) with random hexamers. PCR was carried out using primers specific for the *CASR* gene and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes. The primer sequences used were 5'-CGGGG TACCTTAAGCACCTACGGCATCTAA-3' and 5'-GCT CTAGAGTTAACGCGATCCCAAAGGGCTC-3' for *CASR*, and 5'-TGGACATCAATGAGTGCCTC-3' and 5'-CACATTCTGGTGAGCATTCG-3' for *GAPDH*. *GAPDH* served as an internal control of the reaction. To perform semiquantitative RT-PCR, the ranges of linear amplification for the *CASR* gene and for the *GAPDH* gene were studied by using standard curves.²⁶ Thus, the optimal number of PCR cycles was determined. After PCR, the PCR products were electrophoresed in 2% agarose gels. Real-time RT-PCR was carried out by using SYBR green real-time PCR system. The primer sequences used were 5'-AAGGGGACATTATCCTTGG-3' and 5'-GCTGGGC TGCTGTTTATCTC-3'. A comparative threshold cycle (C_T) was used to determine gene expression relative to the control (calibrator). All reactions were carried out at least in duplicate and controlled without reverse transcriptase.

Bisulfite Sequencing and MSP

Bisulfite modification of genomic DNA was performed using a Methylamp DNA modification kit (Epigentek, Brooklyn, NY, USA). In the chemical modification of cytosine to uracil by bisulfite

treatment, all cytosines are converted to uracil, but those that are methylated are resistant to this modification and remain as cytosine. Bisulfite sequencing was carried out²⁷ using primers, 5'-TG TTAGGGGTTAGGGATAAGG-3' and 5'-ACTCATTC TACAAAACCTCAAATCAAAA-3', designed to amplify the *CASR* promoter region. MSP analysis was carried out using primers corresponding to the *CASR* promoter region sequences. One can design PCR primers to distinguish methylated from unmethylated DNA in bisulfite modified DNA, taking advantage of the sequence differences resulting from bisulfite modification. Sequences of methylation-specific primers were 5'-GGTTCGGGTTTT TAAGTAGC-3' and 5'-TCAAACGTTACCTATACC GC-3'. Sequences of unmethylation-specific primers were 5'-TTAGGTTTGGGTTTTTAAGTAGT-3' and 5'-TC AAACATTACCTATACCACAAA-3'. After PCR, the PCR products were electrophoresed in 2% agarose gels.

5-Aza-2'-Deoxycytidine (5-aza-dC) and/or Trichostatin A Treatment

To examine the role of CpG methylation and histone deacetylation in silencing of *CASR*, cancer cells were treated with 1 μ M of 5-aza-dC (Sigma, St Louis, MO, USA) for 72 h or with 100 nM of trichostatin A for 24 h. Cells were also treated with 1 μ M of 5-aza-dC for 72 h followed by 100 nM of trichostatin A for an additional 24 h. The timing and sequencing of 5-aza-dC and/or trichostatin A was based on similar preliminary studies as well as published studies.²⁸ After the treatment, expression of *CASR* was analyzed by RT-PCR and immunoblotting.

Immunoblotting

Immunoblotting for *CASR* was carried out as described previously.^{29,30} Cell lines were homogenized in lysis buffer (50 mM Tris-HCl (pH 7.4) 1% Triton X-100, 1 mM diethyldithiocarbamic acid, 1 mM EDTA, 1% Tween 20, 10 μ M leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice. After sonication and centrifugation (10 000 g at 4 °C), protein content of the supernatant was measured by the method of Bradford. Solubilized proteins (100 μ g) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride transfer membrane. After being blocked with 5% blocking agent, the membrane was incubated with an anti-human *CASR* rabbit polyclonal antibody (Millipore, Billerica, MA, USA). The protein was visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, UK), and the membranes were stripped and reprobed with mouse anti-actin monoclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA). Results were analyzed using a multiimage analyzer (Bio-Rad, Richmond, CA, USA).

Development of *CASR* Short Hairpin RNA (shRNA) Stable Transfectants

CASR shRNA (*CASR*-shRNA) and control shRNA (Ctrl-shRNA) plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA). Plasmids were transfected into *CASR*-unmethylated HCT-8 cells using shRNA Plasmid Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's protocol. Stable *CASR*-shRNA or Ctrl-shRNA transfectants were established by selection of cells with puromycin. Clones of stable transfectants were obtained by the use of cloning cylinders.

Cytotoxicity Assays

HCT8 cells and transfectants were maintained in Ca^{2+} -free Spinner-modified minimal essential medium (Sigma) supplemented with sodium bicarbonate and 5% fetal bovine serum as described previously.^{11,12,15} To assess the effect of Ca^{2+} on the cytotoxic response to drugs, the medium of cells was replenished with a medium containing physiological concentrations of Ca^{2+} (1.4 mM) and 5-FU (15 μ M). The medium of a parallel set of cultures was replenished with a medium only containing 5-FU without the addition of exogenous Ca^{2+} . After the treatment for 24 or 48 h, WST-8 assay was used to determine the cytotoxic response to 5-FU. WST-8 assay was carried out using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Immunohistochemistry

Sections (5 μ m thick) of formalin-fixed and paraffin-embedded tissue and tissue microarray were dewaxed in xylene and rehydrated in alcohol. The sections were then heated to 105 °C in target retrieval solution (DakoCytomation, Carpinteria, CA, USA) in an autoclave for 10 min for antigen retrieval. Endogenous peroxidase activity was suppressed using a solution of 3% hydrogen peroxide in methanol for 5 min. After being rinsed twice in phosphate-buffered saline (PBS), the sections were incubated for 18 h at 4 °C with an anti-human *CASR* rabbit polyclonal antibody (1/100, abcam, Cambridge, MA, USA) or an anti-human β -catenin monoclonal antibody (Santa Cruz Biotechnology).³¹ The antibodies were diluted in antibody diluent with background reducing components (0.05 mol/l Tris-HCl buffer containing 0.1% Tween and 0.015 mol/l sodium azide, DakoCytomation). Normal rabbit or mouse immunoglobulins were substituted for each primary antibody as negative controls. After washing three times in PBS, the sections were treated with biotinylated goat anti-rabbit or anti-mouse immunoglobulin (DakoCytomation) for 10 min and then with horseradish peroxidase-avidin complex, diluted as recommended by the

manufacturer, for 10 min. The slides were then washed in PBS and developed in 0.05 mol/l Tris-HCl (pH 7.5) containing 0.6 mg/ml 3-3' diaminobenzidine at room temperature. The sections were counterstained in Mayer's hematoxylin and mounted. Cancer cases were categorized into the following three groups corresponding to immunostaining patterns of β -catenin: membranous, membranous staining pattern similar to that in normal colon mucosa; weak, no staining or weaker staining than normal colon mucosa; and nuclear/cytoplasmic, diffuse staining in the nucleus and/or cytoplasm. When a cancer contained multiple staining patterns, the case was categorized into the dominant staining pattern.

Statistical Analysis

The results were assessed for associations with clinicopathological parameters using the following statistical tests: Student's *t*-test for age, the Mann-Whitney test for lymph node metastasis and pTNM stage, and the χ^2 -test or Fisher's exact test for the remaining parameter. A *P*-value of <0.05 was considered significant. A *P*-value between 0.05 and 0.10 was considered as trend toward an association.

Results

Reduced Expression of *CASR* mRNA in Colorectal Cancer Cell Lines

We examined *CASR* mRNA expression in cancer cell lines by using semiquantitative RT-PCR (Figure 1). The *CASR* mRNA expression was frequently lost or considerably reduced in cancer cell lines (Figure 1). There was no correlation between *CASR* expression and histopathology. Concordant results were obtained by real-time RT-PCR (data not shown).

Bisulfite Sequencing and MSP of the *CASR* Promoter in Colorectal Cancer Cell Lines

Methylation status of the CpG islands of *CASR* was analyzed in colorectal cancer cell lines. The bisulfite

Figure 1 Expression of *CASR* mRNA in colorectal cancer cell lines analyzed by semiquantitative RT-PCR analysis. RT-PCR was carried out using cDNA from 11 colorectal cancer cell lines. *GAPDH* served as a control. The *CASR* mRNA expression was frequently lost or considerably reduced in cancer cell lines.

sequencing method was used to analyze the methylation status of 11 CpG sites in the 218-bp fragment of the *CASR* promoter including the transcriptional start site (Figure 2a). These CpG islands were densely methylated in cancer cell lines with no or reduced *CASR* mRNA expression but not in the HCT-8 cell line expressing *CASR*. Methylation status of *CASR* was further analyzed in cancer cell lines by using MSP (Figure 2b). A methylated PCR product was detected in cell lines with no or reduced *CASR* mRNA expression. Only an unmethylated PCR product was detected in the HCT-8 cell line expressing *CASR*. Thus, downregulation of *CASR* mRNA expression was correlated with hypermethylation of the promoter.

Reactivation of *CASR* Expression by 5-aza-dC/trichostatin A Treatment in Colorectal Cancer Cell Lines

To further examine the role of CpG methylation and histone deacetylation in silencing of *CASR*, cancer cells were treated with 5-aza-dC and/or trichostatin A. Treatment with 5-aza-dC restored *CASR* expression, and combined treatment with 5-aza-dC and trichostatin A restored *CASR* expression synergistically at mRNA and protein levels in colorectal cancer cell lines (Figure 3).

Figure 2 Bisulfite sequencing (a) and MSP (b) analyses of *CASR* promoter in colorectal cancer cell lines. Methylation status of the CpG islands of *CASR* was analyzed in colorectal cancer cell lines. (a) The bisulfite sequencing method was used to analyze methylation status of 11 CpG sites in the 218-bp fragment of the *CASR* promoter including the transcriptional start site. Unmethylated CpG sites are indicated by open boxes and methylated CpG sites are indicated by filled boxes. These CpG islands were densely methylated in cancer cell lines with no or reduced *CASR* mRNA expression but not in the HCT-8 cell line expressing *CASR*. The arrows represent primers for MSP. (b) Methylation status of *CASR* was further analyzed in cancer cell lines by using MSP. NC, normal colorectal mucosa; IVD, *in vitro* methylated DNA. A methylated PCR product was detected in cell lines with no or reduced *CASR* mRNA expression. Only an unmethylated PCR product was detected in the HCT-8 cell line expressing *CASR*.

Figure 3 Reactivation of *CASR* by 5-aza-dC and/or trichostatin A treatment in colorectal cancer cell lines. To examine the role of CpG methylation and histone deacetylation in silencing of *CASR*, cancer cells were treated with 5-aza-dC and/or trichostatin A. As indicated, cells were incubated for 72 h with 1 μ M 5-aza-dC and/or for 24 h with 100 nM trichostatin A. After the treatment, expression of *CASR* was analyzed by RT-PCR and immunoblotting. Treatment with 5-aza-dC restored *CASR* expression, and combined treatment with 5-aza-dC and trichostatin A restored *CASR* expression synergistically at mRNA and protein levels in colorectal cancer cell lines.

Block of the Enhancing Effect of Ca^{2+} on the Cytotoxic Response to 5-FU by Disruption of *CASR* Expression

shRNA approach was used to target the *CASR* to determine the role of *CASR* in mediating the action of extracellular Ca^{2+} . Expression of *CASR* was significantly downregulated in *CASR* shRNA-transfected HCT-8 cells, but not in control shRNA-transfected cells (data not shown). After treatment with 5-FU for 24 h, extracellular Ca^{2+} enhanced the sensitivity to 5-FU in control shRNA-transfected HCT-8 cells, but not in *CASR* shRNA-transfected HCT-8 cells (Figure 4). After treatment with 5-FU for 48 h, similar results were obtained (data not shown).

Reduced Expression of *CASR* mRNA in Colorectal Tumor Tissues

Downregulation of *CASR* mRNA expression was observed in 24 (60%) of 40 cancer tissues compared with expression in their matched normal tissues (Figure 5 and data not shown). Reduced mRNA expression of *CASR* was observed in 21 (70%) of 30 pT2–pT4 cancer tissues and in 3 (30%) of 10 pT1 cancer tissues. Reduced mRNA expression of *CASR* was also observed in 4 (21%) of 14 advanced adenoma tissues but in only one (8%) of 12 adenoma tissues and in none of 10 hyperplastic polyp tissues (data not shown).

Expression of *CASR* and β -Catenin Protein in Colorectal Tumor Tissues

Figure 6 shows representative results of immunohistochemistry for *CASR* and β -catenin in colorectal

Figure 4 Block of the enhancing effect of Ca^{2+} on the cytotoxic response to 5-FU by disruption of *CASR* expression. shRNA approach was used to target the *CASR* to determine the role of *CASR* in mediating the action of extracellular Ca^{2+} . Parental cells and transfectants were exposed to 5-FU for 24 h and their cytotoxic response to 5-FU was evaluated by using WST-8 assay. *, **, and *** indicate $P < 0.01$. After treatment with 5-FU for 24 h, extracellular Ca^{2+} enhanced the sensitivity to 5-FU in control shRNA-transfected HCT-8 cells, but not in *CASR* shRNA-transfected HCT-8 cells.

Figure 5 Expression of *CASR* mRNA in colorectal cancer tissues analyzed by real-time RT-PCR analysis. Real-time RT-PCR was carried out by using SYBR Green real-time PCR system. N and T, matched samples from non-tumor and tumor tissue, respectively. *CASR* mRNA expression was reduced in seven of eight cancer tissues compared with expression in their matched normal tissues.

cancer tissues. There was no detectable immunoreactivity with the control normal rabbit or mouse immunoglobulins (data not shown). *CASR* was stained in columnar epithelial cells of normal colonic crypts and used as positive controls. In 51 (57%) of 90 cancer tissues, *CASR* expression in cancer cells was weak or lost when compared with that in adjacent normal colonic epithelial cells in the same section. Reduced *CASR* expression was observed in 45 (64%) of 70 pT2–pT4 cancer tissues and in 6 (30%) of 20 pT1 cancer tissues.

Figure 6 Immunohistochemistry for CASR and β -catenin in colorectal cancer tissues. Immunohistochemistry with an anti-human CASR rabbit polyclonal antibody and an anti-human β -catenin monoclonal antibody was carried out in 90 colorectal cancer tissues. (a) Normal epithelial tissues positive for CASR (left) and cancer cells negative for CASR (right), (b) cancer tissue positive for CASR, (c) cancer tissue with membranous β -catenin staining. (d) Cancer tissue negative for CASR. In 51 (57%) of 90 cancer tissues, CASR expression in cancer cells was weak or lost when compared with that in adjacent normal colonic epithelial cells. (e) Cancer tissue with nuclear/cytoplasmic β -catenin staining. Original magnification: a, $\times 100$; b–e, $\times 400$. CASR expression was reduced more predominantly in less-differentiated cancer tissues or in cancer cells at the invasive front, where nuclear/ cytoplasmic β -catenin was often localized. Nuclear/ cytoplasmic β -catenin expression was more frequently observed in cases with reduced CASR expression than in cases with normal CASR expression ($P < 0.01$).

Downregulation of CASR expression was inversely correlated with degree of differentiation ($P < 0.01$). Membranous staining of β -catenin was observed in normal epithelium and used as positive controls. CASR expression was reduced more predominantly in less-differentiated cancer tissues or in cancer cells at the invasive front, where nuclear/cytoplasmic β -catenin was often localized. Nuclear/cytoplasmic β -catenin expression was more frequently observed in cases with reduced CASR expression than in cases with normal CASR expression ($P < 0.01$). Reduced expression of CASR was observed in 6 (21%) of 28 advanced adenoma tissues but only in 2 (9%) of 22 adenoma tissues and in none of 14 hyperplastic polyp tissues (data not shown).

CASR Methylation in Colorectal Tumor Tissues

CASR methylation status was analyzed in colorectal tumor tissues (Figure 7). CASR methylation was detected in 62 (69%) of 90 cancer tissues (Figure 7a) and it was inversely correlated with degree of differentiation ($P < 0.01$). CASR methylation was significantly correlated with reduced mRNA and protein expression of CASR ($P < 0.01$). CASR methylation was detected in 53 (76%) of 70 pT2–pT4

cancer tissues, 9 (45%) of 20 pT1 cancer tissues, and 9 (90%) of 10 lymph node metastatic tissues. CASR methylation was detected in 9 (32%) of 28 advanced adenoma tissues but only in 2 (9%) of 22 adenoma tissues and in none of 14 hyperplastic polyp tissues (Figure 7b and c).

Discussion

In this study, we demonstrated frequent downregulation of CASR mRNA expression in colorectal cancer cell lines including moderately differentiated ones. As a mechanism of CASR downregulation in cancer, we speculated that CASR may be silenced by promoter hypermethylation. By using bisulfite sequence analysis and MSP, we demonstrated that silencing of CASR was correlated with hypermethylation of the promoter in colorectal cancer cell lines. To confirm the role of epigenetic alterations in transcriptional repression of the CASR gene, we treated colorectal cancer cell lines, in which CASR was methylated, with 5-aza-dC alone or in combination with trichostatin A. Treatment with 5-aza-dC restored the CASR expression in cancer cell lines. Moreover, combined treatment with 5-aza-dC and trichostatin A restored CASR expression synergistically, indicating that CpG methylation and histone

Figure 7 MSP analysis of *CASR* in colorectal tumor tissues. *CASR* methylation status was analyzed in colorectal tumor tissues by MSP. (a) *CASR* methylation status in normal (N), cancer (T), and lymph node metastatic (LM) tissues. U and M, unmethylated and methylated PCR products, respectively. NL, normal lymphocytes; IVD, *in vitro* methylated DNA. In total, *CASR* methylation was detected in 62 (69%) of 90 cancer tissues and it was inversely correlated with degree of differentiation ($P < 0.01$). (b) *CASR* methylation status in advanced adenoma (AA), adenoma (AD), and hyperplastic polyp (HP) tissues. U and M, unmethylated and methylated PCR products, respectively. NC, normal colorectal mucosa. In total, *CASR* methylation was detected in 9 (32%) of 28 advanced adenoma tissues but only in 2 (9%) of 22 adenoma tissues and in none of 14 hyperplastic polyp tissues. (c) Frequency of *CASR* methylation in colorectal tumor tissues. Progressive epigenetic silencing of *CASR* was observed in colorectal carcinogenesis.

deacetylation has an important role in silencing the *CASR* gene.

We showed that extracellular Ca^{2+} enhanced the cytotoxic response of *CASR*-unmethylated HCT-8 cells to 5-FU. This enhancing effect of extracellular Ca^{2+} required a functional *CASR* because knocking down *CASR* expression abrogated the effect of extracellular Ca^{2+} .

The results of *in vitro* studies were further supported by results for primary tumor tissues. *CASR* mRNA expression in cancer cells was frequently downregulated in colorectal cancer tissues including pT1 cases. Downregulation of *CASR* expression was also observed at the protein level analyzed by immunohistochemistry. A significant inverse association was found between *CASR* downregulation and degree of differentiation. Moreover, *CASR* mRNA and protein expression was downregulated in advanced adenoma tissues at a lower frequency than that in cancer tissues. In contrast,

CASR expression was retained in adenoma tissues and hyperplastic polyp tissues. These results suggest that downregulation of *CASR* expression occurs in the early stage and progresses in colorectal carcinogenesis.

One of the supposed functions of extracellular Ca^{2+} and the *CASR* in colorectal cancer cells is suppression of β -catenin/T-cell factor activation.^{11,14} In a small series of colorectal cancer tissues ($n = 6$), a relationship between *CASR* expression and β -catenin staining pattern was reported.¹⁴ In this study, nuclear/cytoplasmic β -catenin expression was more frequently observed in cases with reduced *CASR* expression than in cases with normal *CASR* expression. As various genetic and epigenetic alterations are involved in β -catenin/T-cell factor activation, the relationship between β -catenin expression and *CASR* expression seems not to be simple. Further analyzes are needed to address these issues.

By using MSP, we detected *CASR* methylation in 69% of cancer tissues and it was inversely correlated with degree of differentiation. *CASR* methylation was significantly correlated with reduced mRNA and protein expression of *CASR*. Interestingly, *CASR* methylation was detected in 90% of lymph node metastatic tissues. These results suggest that *CASR* downregulation by DNA methylation contributes to abnormal differentiation and malignant progression. Moreover, *CASR* methylation was detected in 32% of advanced adenoma tissues but was detected in only in 9% of adenoma tissues and was not detected in hyperplastic polyp tissues. *CASR* methylation in advanced adenoma tissues was less significantly correlated with reduced protein expression, suggesting monoallelic and/or heterogeneous methylation. These results further support the notion that progressive epigenetic silencing of *CASR* has an important role in colorectal carcinogenesis.

Activation of *CASR* reportedly downregulates the expression of thymidylate synthase and survivin, and upregulates cellular sensitivity to 5-FU in human colon carcinoma cells.¹⁵ Liu *et al*¹⁵ reported that functional *CASR* is necessary for tumor suppressive function of vitamin D and that this function is abrogated by knocking down *CASR* expression. Therefore, from the viewpoint of chemotherapy and/or chemoprevention using Ca^{2+} and/or vitamin D, the results obtained in this study are important. If the *CASR* gene is methylated, reversal of *CASR* silencing by demethylating agents might be a potential target for effective treatment and/or prevention of colorectal cancers. Calcimimetics enhance *CASR* agonist activity.¹⁶ The use of calcimimetics might be beneficial in cases characterized by reduced *CASR* levels. However, systemic use of allosteric agonists of the *CASR* might be associated with potentially major adverse effects.¹⁶ Development of organ- or tissue-specific targeting methods will be necessary to avoid these adverse effects.

Hypermethylation of the *CASR* gene may provide a useful marker for detecting preinvasive and early stage cancer amenable to cure and/or for predicting the risk of cancer. Analysis of DNA methylation and/or expression of *CASR* in precancerous or early carcinoma lesions may enable prediction of which lesions will respond to the differentiation-inducing or chemopreventive activity of extracellular Ca^{2+} and/or vitamin D.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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