

Characteristics of carbonic anhydrase 9 expressing cells in human intestinal crypt base

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Abstract. Though recent studies have revealed that stem cells of many tissues are harbored in hypoxic microenvironment, little is known about the relationship between hypoxia and intestinal crypt base, where intestinal stem cells are supposed to exist. In this study, we focused on carbonic anhydrase IX (CA9), a hypoxia-inducible membrane-tethered protein, in normal intestinal crypt base, adenoma and early colorectal cancer. Using surgically resected human colorectal cancer specimen, we searched for the expression pattern and functional association of CA9 in human adult normal intestinal epithelia, adenoma and early colorectal cancer by immunofluorescent and immunohistochemical staining, flow cytometry, and quantitative real-time-polymerase chain reaction. We demonstrated that almost all crypt base slender cells in ileum and crypt base cells with eosinophilic structure in their basal cytoplasm in right and left colon were CA9⁺ with the ratio of 25 to 40%, and that adenoma and T1 colorectal cancer showed broad expression of CA9. Flow cytometrically sorted CA9⁺ population showed increased mRNA level of a Wnt signaling factor *AXIN2*. In conclusion, these observations indicate that CA9 expression in normal crypt base cells has association with intestinal epithelial stemness and CA9 may be involved in the carcinogenesis of colorectal cancer.

Introduction

Vigorous research has shown that the intestinal epithelial stem cells are located at the bottom of the crypt base (1) and consist of proliferative and quiescent types. It is widely accepted that the proliferative stem cells are crypt base columnar (CBC) cells and are positive for the leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) (2) and that quiescent stem cells are located at +4 position from the bottom of the crypt base (3) and positive for *Bmi-1* (4), *Hopx* (5), *mTert* (6) and *Lrig1* (7). The intestinal stem cells are thought to be supported by their adjacent Paneth cells in small intestine (8) through *Wnt* (9), *Notch* (10) and epidermal growth factor (*EGF*) (11) signaling and adversely influenced by villus cells through bone morphogenetic protein (*BMP*) signaling (12).

Carbonic anhydrase 9 (CA9) is a membrane-bound isozyme of 12 enzymatically active CAs in human, and catalyzes the reversible reaction between carbon dioxide (CO₂) and water to the bicarbonate ion and protons at its extracellular catalytic site. High expression of CA9 has been reported in limited cell types of normal tissues (13,14) and the relationship between CA9 and advanced status of cancer has been intensely studied (15-19). However, CA9 expression in normal human intestinal epithelial cells and early stage colorectal cancer (CRC) remains incompletely understood.

In this study, we assessed the characteristics and distribution pattern of CA9 positive cells in human intestinal epithelial cells using clinical samples and in T1 CRC.

Materials and methods

Tissue samples. Surgically resected human adult intestinal normal tissues and T1 CRC tissues were obtained from 20 patients with CRC (2 cecum, 5 ascending, 2 transverse, 3 descending, 5 sigmoid colon, 3 rectum; 54 to 84 years old,

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Table I. Primer sequences and TaqMan probes used for quantitative real-time RT-PCR.

Gene	Primer	UPL probe
<i>GAPDH</i> (NM_002046.3)	5'-AGCCACATCGCTCAGACAC-3' 5'-GCCCAATACGACCAAATCC-3'	60
<i>ENO2</i> (NM_001975.2)	5'-ACTTTGTCAGGGACTATCCTGTG-3' 5'-TCCCTACATTGGCTGTGAACT-3'	27
<i>AXIN2</i> (NM_004655.3)	5'-AGAGCAGCTCAGCAAAAAGG-3' 5'-CCTTCATACATCGGGAGCAC-3'	88

6 female and 14 male, 14 normal mucosa, 3 adenoma and 3 T1 CRC tissues) after informed consent from Osaka University Medical Hospital with approval of the Research Ethics Board. Normal intestinal epithelia were collected from patients without evidence of symptomatic or microscopic inflammation, and distance of >3 cm to the tumors.

Histopathology, immunohistochemical and immunofluorescent analyses of intestinal tissue. Human colorectal tissue was fixed in 10% buffered formalin and embedded in paraffin. Sequential 5- μ m sections were stained with hematoxylin and eosin (H&E) for histopathological analyses, and for immunohistological analyses with antibodies to CA9 (2D3, 1:500; Abcam, Cambridge, MA, USA and EPR4151, 1:200; Epitomics, Burlingame, CA, USA), neuron specific enolase (NSE) (1:100; Assaybiotech, Sunnyvale, CA, USA), CD68 (PG-M1; Dako, Carpinteria, CA, USA) and polypyrimidine tract-binding protein 1 (PTBPI) (M01, 3 μ g/ml; Abnova, Taipei, Taiwan). Antigen retrieval (10 mmol/l citrate buffer, pH 6 at 100°C for 40 min) was performed on paraffin-embedded tissues. Visualization was performed using either fluorescent-conjugated species-specific secondary antibodies or the avidin-biotin-peroxidase method (Vectastain Elite ABC reagent kit; Vector Laboratories, Burlingame, CA, USA). Nuclear counterstaining was performed with hematoxylin or ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). All-in-one type fluorescence microscopy (BZ-8000; Keyence, Osaka, Japan) with digital photographic capability was used to visualize cells at several magnifications.

Calculation of the frequency of CA9⁺ cells of the intestinal crypts. The frequency of CA9⁺ cells at specific positions relative to the crypt bottom were evaluated using crypts which intact overall longitudinal sections were available in sections of immunohistochemical staining with anti-CA9 antibody. The counting of intestinal epithelial CA9⁺ and CA9⁻ cells was performed three times independently using 20, 70 and 150 crypts for ileum, right colon and left colon, respectively. The frequency of CA9⁺ cells was calculated by the ratio of the total number of CA9⁺ cells to the total number of CA9⁻ and CA9⁺ cells at each cell position relative to crypt bottom.

Crypt isolation and cell dissociation. The intestinal tissues were washed with cold phosphate-buffered saline (PBS) until the supernatant was clear. Next, they were incubated in 8 mmol/l ethylenediaminetetraacetic acid (EDTA) cold chelation buffer (distilled water with 5.6 mmol/l Na₂HPO₄, 8.0 mmol/l KH₂PO₄,

96.2 mmol/l NaCl, 1.6 mmol/l KCl, 43.4 mmol/l sucrose, 54.9 mmol/l D-sorbitol, 0.5 mmol/l DL-dithiothreitol) (20) for 10 min on ice and the intestinal crypts were stripped and collected with angled Debaquey forceps under a stereomicroscope (SZX10; Olympus, Tokyo, Japan). After washing with cold chelation buffer, the isolated crypts were incubated with TrypLE Express (Invitrogen) including 2,000 U/ml DNase (Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37°C, passed through 40 μ m cell strainers, treated with BD Pharm Lyse (BD Biosciences, San Jose, CA, USA) for the lysis of red blood cells and then washed with cold chelation buffer.

Flow cytometry. Dissociated intestinal crypt cells were blocked with FcR blocking reagent (BD Biosciences) and incubated with antibodies as follows; anti-human CA9 (APC-conjugated; R&D Systems, Minneapolis, MN, USA), anti-human CD31 (FITC-conjugated; eBiosciences, San Diego, CA, USA), anti-human CD44 (PE-conjugated) and lineage cocktail 1 (Lin1) (FITC-conjugated) (both from BD Biosciences). 7-AAD (BD Biosciences) was used to eliminate dead cells. Cells were analyzed and isolated by using FACSAria II equipped with FACSDiva software (BD Biosciences). The live single epithelial crypt base cells (21,22) (7-AAD⁻/CD31⁺/Lin1⁻/CD44⁺) were evaluated for the CA9 expression and sorted accordingly.

RNA preparation and quantitative real-time-polymerase chain reaction (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen). In all cases, 400 ng of total RNA was reverse-transcribed with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. For quantitative assessments, quantitative real-time reverse transcriptase analysis was performed with the LightCycler TaqMan master kit (Roche Diagnostics, Tokyo, Japan) and the LightCycler 480 system (Roche Applied Science, Indianapolis, IN, USA). Primers are listed in Table I.

Statistical analysis. The relationships among gene expressions, cell counts, and tumor volume were analyzed using ANOVA. All tests were analyzed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). A value of P<0.05 was taken as statistically significant.

Results

CA9 protein expression in human intestinal crypt base. To assess anatomical pattern of CA9 expression in human normal

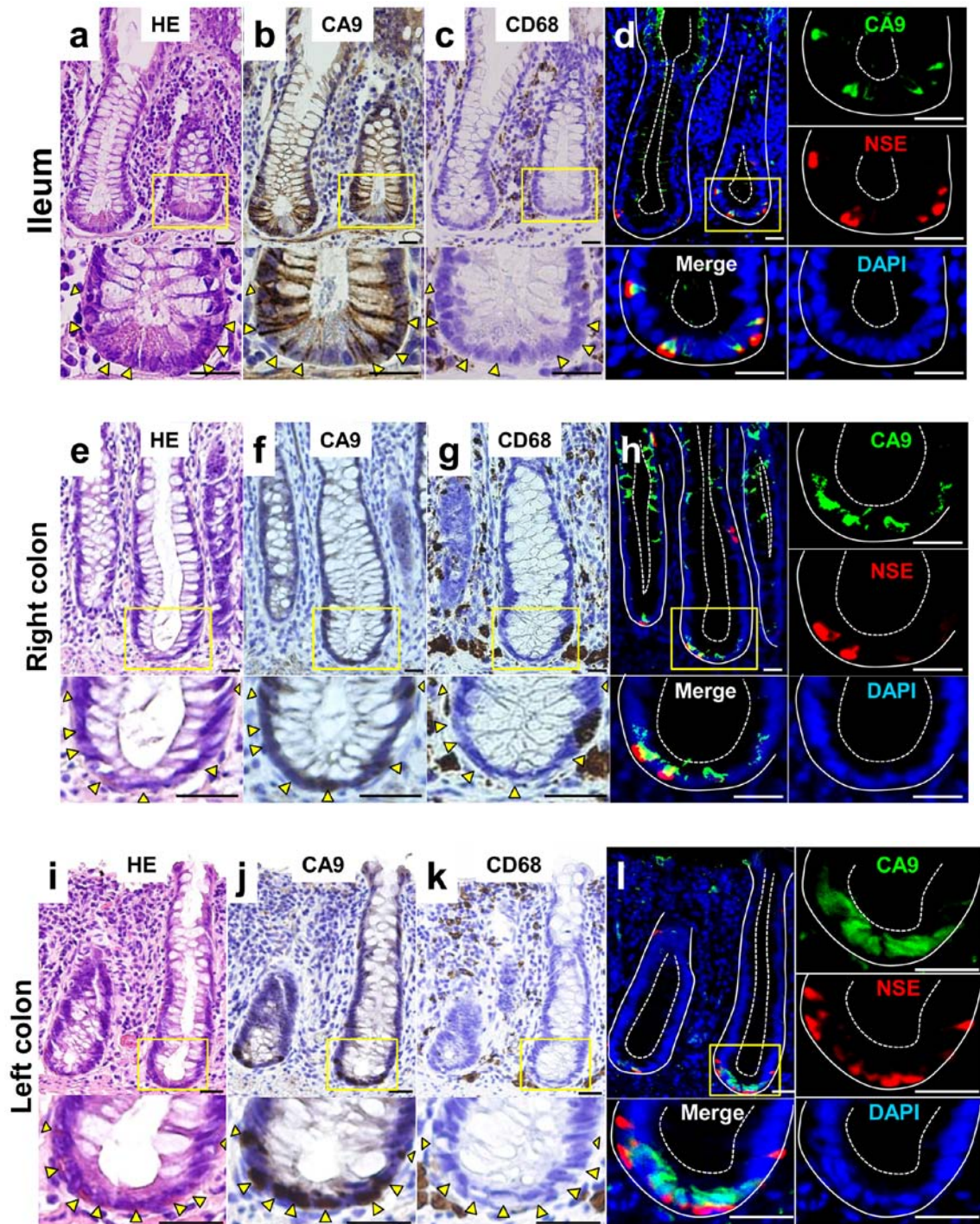


Figure 1. CA9 expression pattern in the human ileum and colon. Representative staining patterns of human ileum (a-d), right colon (e-h) and left colon (i-l). (a, e and i) Hematoxylin and eosin (H&E) staining. (b, f and j) Immunohistochemical (IHC) staining with anti-CA9 antibody. (c, g and k) IHC staining with anti-CD68, a macrophage marker. (d, h and l) Immunofluorescent staining with antibodies to CA9 (green), neuron specific enolase (NSE) (red) and counterstained with DAPI (blue). Larger magnification views of the crypt base boxed in yellow are shown at the bottom on the right. IHC staining sections were visualized with diaminobenzidine (brown) and counterstained with hematoxylin. Arrowheads point to cells which express CA9. White solid lines demarcate epithelial-mesenchymal boundary, and dashed lines mark the apical epithelial surface in (d, h and l). Scale bar, 25 μ m. Original magnification, top, x20; bottom, x40.

intestinal epithelia, formalin-fixed paraffin-embedded serial sections of ileum, right colon and left colon were stained with H&E, CA9, CD68 and NSE (Fig. 1). The CA9 expression was confined to cell membrane and was mainly observed in slender cells at the bottom of small intestinal and in colon crypts with mosaic pattern. CA9⁺ cells commonly contained eosinophilic structure in basal cytoplasm. Among those evaluated, none of the CA9⁺ cells located in the intestinal epithelia were positive

for CD68, a marker for macrophage, which means that although intestinal lamina propria contains abundant macrophages (23), macrophages were not the source of CA9⁺ cells. Almost all of the CA9⁺ cells in the crypt base were also stained with NSE.

Frequency of CA9⁺ intestinal epithelial cells in association with their position. To quantify the frequency of the CA9⁺ intestinal epithelial cells in association with their position

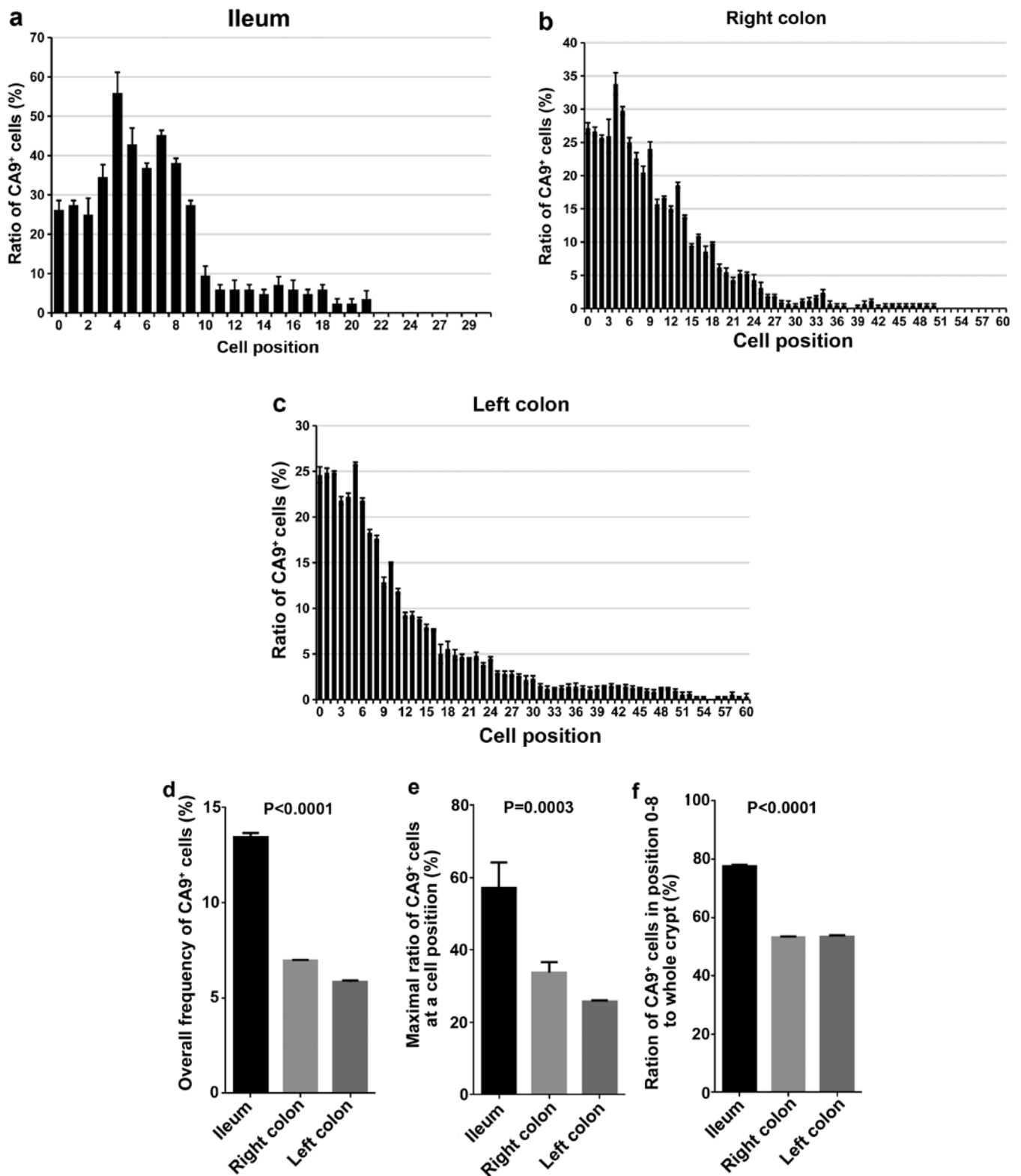


Figure 2. The frequency of CA9⁺ cells in the human ileum and colon. The frequency of CA9⁺ cells appeared at specific positions relative to the crypt bottom in human ileum (a), right colon (b) and left colon (c). The overall frequency of CA9⁺ (d), maximal frequency of CA9⁺ cells at a position (e) and the ratio of CA9⁺ cells located in crypt base region (f) in ileum, right colon and left colon. Most of the CA9⁺ cells were found to be almost alternately positioned at the base of crypts, and rarely found in distal positions.

relative to crypt bottom, the counting of CA9⁻ and CA9⁺ cells of intestinal epithelia in ileum, right colon and left colon was performed (Fig. 2a-c). Total frequency of CA9⁺ cells was 13.5 ± 0.2, 7.0 ± 0.1 and 5.9 ± 0.1%, the maximal frequency of CA9⁺ cells in relation to cell position was 56.0 ± 9.0% (cell

position 3), 33.8 ± 2.9% (cell position 4), 25.8 ± 0.3% (cell position 5) and the ratio of CA9⁺ cells in crypt base (cell position 0-8) was 77.7 ± 0.5, 53.1 ± 0.4 and 53.5 ± 0.4 in ileum, right colon and left colon, respectively (Fig. 2d-f). These data indicate that CA9⁺ cells are mainly located in the crypt base,

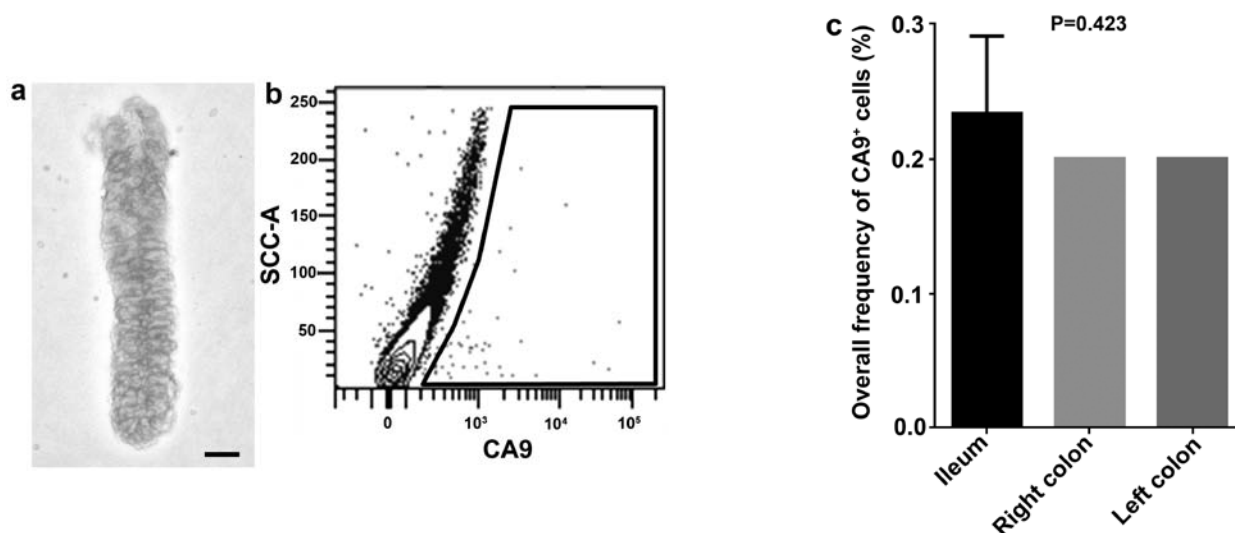


Figure 3. Flow cytometry analysis of human intestinal epithelia with CA9. (a) Representative image of an isolated intestinal epithelial crypt. (b) Representative dot plots of human CRC cell lines incubated with an anti-CA9 antibody. (c) Frequency of CA9⁺ cells detected by flow cytometry in ileum, right colon and left colon. Scale bar, 25 μ m. The frequency of CA9⁺ cells was smaller than that of IHC.

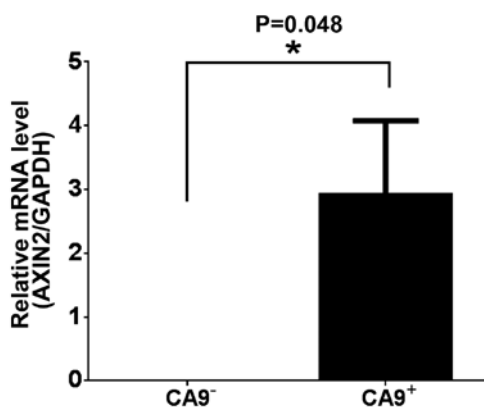


Figure 4. Comparison of the expression of *AXIN2* in CA9⁻ and CA9⁺ human intestinal epithelial cells. The expression of *AXIN2*, a Wnt pathway-associated gene, was evaluated by the ratio normalized to GAPDH expression in human intestinal epithelial CA9⁻ and CA9⁺ cells as determined by qRT-PCR. CA9⁺ cells showed significantly higher level of *AXIN2* than CA9⁻ cells.

and ileum contains more CA9⁺ cells in the crypt base than right or left colon.

CA9⁺ intestinal epithelial cells in fresh human clinical samples. To investigate the biological function of CA9⁺ cells in intestinal epithelial crypt base, flow cytometric analysis was performed on freshly isolated human intestinal epithelial cells. The percentage of CA9⁺ cells was 0.23 ± 0.06 , 0.20 ± 0.00 and $0.20 \pm 0.00\%$, in ileum, right colon and left colon, respectively, without significant difference among the locations ($P=0.4226$) (Fig. 3).

Correlation of CA9 and the Wnt pathway gene AXIN2. To elucidate the characteristic of the CA9⁺ cells in intestinal epithelial cells, expression of *AXIN2*, a direct target gene of Wnt pathway (24), was analyzed on freshly isolated CA9⁻ and CA9⁺ dissociated human intestinal epithelial cells by qRT-PCR. The expression of *AXIN2* in CA9⁺ cell population was significantly higher than that of CA9⁻ ($P=0.048$) (Fig. 4).

CA9 protein expression in adenoma and T1 CRC. To investigate the relationship between CA9 expression and tumorigenesis, formalin-fixed paraffin-embedded sections were stained with CA9 in adenoma (3 samples) and T1 CRC (3 samples). Three (100%) and 3 (100%) were positive for CA9 in adenoma and T1 CRC, respectively. In adenomas, the CA9 expression was confined to cell membrane and was observed with mosaic-like pattern at the bottom of crypt-like structures where PTBP1, a hypoxia-related protein (25), is abundantly observed (Fig. 5a and c). In T1 cancer, CA9 expression was also confined to cell membrane but almost all cells were positive for CA9, and there was no apparent difference of staining positivity in the same tumor tissue (Fig. 5b and d).

Discussion

In this study, we precisely revealed that CA9⁺ cells exist in human adult crypt base of ileum, right colon and left colon epithelia, and we also showed that the CA9⁺ cells in the crypt base are suspected to be associated with intestinal stem cells morphologically and functionally. We also showed possible association of CA9 expression with carcinogenesis.

In human adult normal intestinal epithelial crypt, CA9⁺ cells were slender and mainly distributed with mosaic pattern, which is consistent with morphological characteristic of previously reported intestinal epithelial stem cells (2-8). It is noteworthy that the CA9 expressed in normal colorectal epithelia is reported to be a splicing variant lacking C-terminal part of the catalytic domain and it is different from the full-length form expressed in CRC and increased by hypoxia (26). This may explain the reason CA9⁺ cells are arranged in intestinal crypt bases with mosaic pattern regardless of the distance from blood vessels. The antibodies, which we used in this study, can also be used in flow cytometry under non-denatured condition. Since the reported splicing variant of CA9 is lacking trans-membranous domain, the C-terminal of the full-length protein, the CA9 protein levels which we analyzed in this study contains both forms of CA9. Although NSE is commonly considered

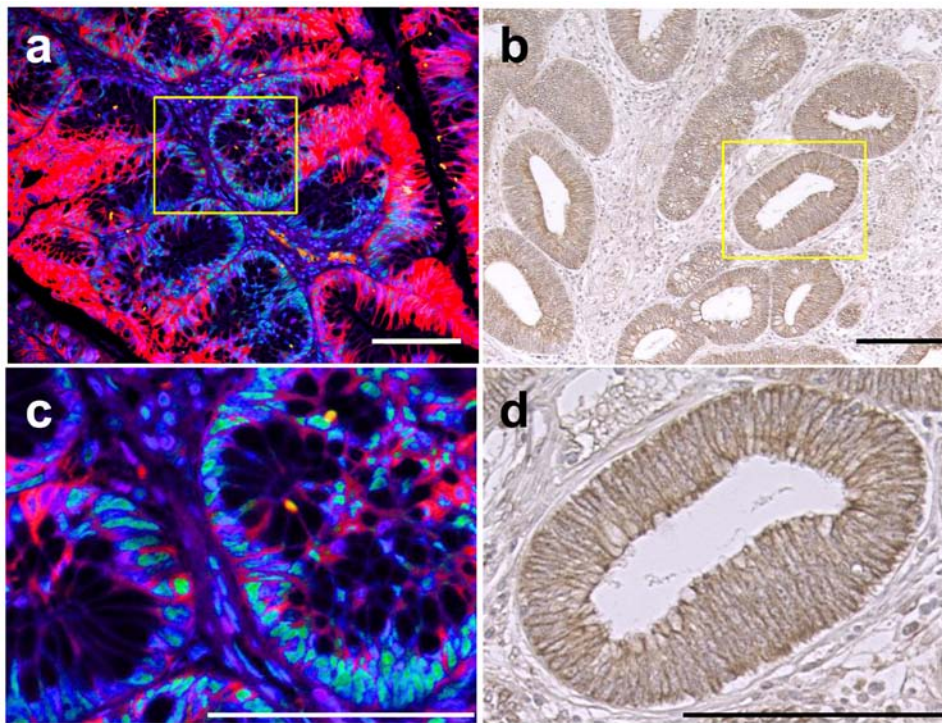


Figure 5. CA9 expression pattern in adenoma and T1 colorectal cancer tissue. (a and c) Immunofluorescent-staining merged images of tubular adenoma with antibodies to CA9 (red), polypyrimidine tract-binding protein (PTBP1) (green) and counterstained with DAPI (blue). (b and d) IHC staining images of T1 adenocarcinoma with anti-CA9 antibody, visualized with diaminobenzidine (brown) and counterstained with hematoxylin. Larger magnification views of the typically stained pattern boxed in yellow (a and b) are shown (c and d). Scale bar, 100 μm . Original magnification (a and b) x20; (c and d), x40.

to be a marker for enteroendocrine cells (27,28), intestinal quiescent stem cells were recently shown to be the precursors which were committed to mature into differentiated secretory cells of the Paneth and enteroendocrine lineage (29). In addition, NSE functions as a glycolytic enzyme by converting 2-phospho-D-glycerate into phosphoenolpyruvate and exhibits proliferative and protective effects on cultured neuron cells (30). These facts imply that it is reasonable for the morphologically stem-like cells in the intestinal crypt base to express NSE in this study. It was notable that CA9⁺ cells in crypt base expressed *AXIN2*, a direct target gene of Wnt pathway (24), which indicates that they have increased activity of Wnt pathway, a critical pathway for intestinal epithelial stem cells (2). Based on these findings, it can be supposed that the CA9⁺ cells in human adult normal intestinal epithelia are associated with stemness, although CA9 has been associated with hypoxia in embryonic and fetal intestinal epithelia (14), and CA9 per se is not imperative in the development or maintenance of intestinal epithelia under normal condition (31).

In the process of carcinogenesis and progression, cancer cells are required to overcome hypoxia and acidosis caused by over-population and increased distance from blood vessels (32). CA9 is induced by hypoxia (33-35) and regulates pH of microenvironment (36). In addition, CA9 has been shown to support carcinogenesis itself, promote cell migration, invasion angiogenesis and metastasis (37-41) and to be associated with cancer stemness (42) and resistance to the therapies (41-44). All these aspects are consistent with our results yielded from clinical samples and data, where the expression of CA9 is mostly associated with poor prognosis or progressed stage (16-19,44-49). In this study, we showed that

the colorectal adenomas had mosaic pattern of CA9 expression in basal region, similar to but more aberrant than normal epithelia, and that T1 CRC had CA9 expression in entire area of the tumor, contrary to the normal intestinal epithelia which CA9⁺ ratio is as low as 10%. Thus, it could be reasonable to suspect that the CA9⁺ cells are associated with carcinogenesis.

This study have some limitations as follows. First, we analyzed the CA9⁺ cells only in immunohistochemistry and flow cytometry. In addition, analyzed sample number is small for reading strong evidence. Second, although the morphology of CA9⁺ cells were same as the CBC cells which were reported as the stem cells of mouse small intestine, there has not been any evidence that the same event was also justified in human samples. Third, although there was a gap of the ratio of CA9⁺ cells among the anatomical location, we were unable to explain its meaning. The gaps of the ratio of CA9⁺ cells between IHC and flow cytometry may be explained by the fact that the sensitivity of the antibodies used for flow cytometry and those for IHC was different (50) and that the methodology of calculation of positivity was two-dimensional in IHC and three-dimensional in flow cytometry. Forth, although in flow cytometry, we could detect CA9 high cells in mucosal epithelial cells, in immunohistochemistry, we could not classify the epithelial cells according to CA9 staining intensity. However, according to Fig. 1b, epithelial cells on villi were weakly positive and CBC cells were highly positive for CA9 suggesting that CA9⁺ cells would be morphologically identical for CBC cells. Further studies would be needed for understanding the CA9 functions. This study propose the possibility that CA9 could be a new marker of human adult intestinal epithelial stem cells and that it is associated with carcinogenesis in CRC.

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