〈Original Article〉

Pyrroline-5-carboxylate synthetase in fecal is specifically increased by colorectal cancer

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Summary Colorectal cancer (CRC) is one of the most popular cancers in developed countries, but there are no good screening biomarkers for diagnosing early-stage CRC. Therefore, novel biomarkers for the early detection of CRC are urgently needed. Previous studies have shown that cancer cells produce large quantities of delta-1-pyrroline-5-carboxylate synthase (P5CS).

In this study, a 1,2-dimethylhydrazine (DMH)-induced CRC mouse model was used to test the hypothesis that the fecal P5CS concentration could be used as a biomarker of CRC. As a result, we found that the CRC model mice displayed markedly increased fecal P5CS concentrations compared with the controls. In addition, an immunohistochemical investigation revealed that strong P5CS expression was only observed in adenocarcinoma cells. Our preliminary data show that P5CS could be used as a specific screening biomarker of colorectal cancer.

Key words: P5CS, Colorectal cancer, Fecal, CRC mouse model

1. Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer death in developed countries[1, 2]. Fecal occult blood testing (FOBT) is the generally accepted screening method for diagnosing CRC[3-5]. Moreover, there are other effective diagnostic methods for CRC. The CRC diagnostic procedure other than FBOT, such as colonoscopy, CT colonography, and the combined use of multiple CT modalities, are more precise and dependable for diagnosing CRC. For example, CT colonography displays about 90% sensitivity for detecting CRC or adenomas of 10 mm or more in diameter[6]. Examinations involving a combination of conventional screening methods have also been used for the diagnosis of CRC and were found to be effective. However, these approaches are need for bowel preparation and higher cost. Therefore, it is necessary to establish a new screening method for the early diagnosis of CRC that is practical, highly

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sensitive and specific, and non-invasive[7].

The pentose phosphate pathway is activated by delta-1-pyrroline-5-carboxylate (P5C) which is supplied by P5CS. Activation of the pentose phosphate pathway is leading to enhanced nucleic acid synthesis. The P5C, on the other hand, is converted by ornithine aminotransferase (OAT) to ornithine which stimulates folic acid synthesis after changing to polyamine; therefore both together resulting in stimulated nucleic acids DNA and RNA biosynthesis. In consequence, P5CS is a key enzyme of this pathway (Fig. 1)[8-11]. Previously, Matsuzawa, T. et al. found in 1998 and described that the mRNA levels of P5CS increased specifically in human colorectal cancer tissues[12]. Feces contain many desquamated colon epithelium cells. Thus, it is expected that P5CS of colorectal cancer origin is present in the feces of colorectal cancer patients.

We hypothesized that the detection of P5CS in stools is a promising biomarker for diagnosing CRC. In this study, we generated a DMH-induced CRC mouse model[13, 14] and attempted to detect P5CS in its feces. We also tried to detect P5CS histochemically in the middle and distal colon tissues of CRC-induced and non-induced mice.

2. Materials and methods

2.1. Reagents

Dextran sodium sulfate (DSS) with a molecular weight of 36,000-50,000 was purchased from Meito Sangyo Co., Ltd. (Nagoya, Japan). Anti-P5CS antibody used was recognizable N-terminal sequence of P5CS was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Histofine Simple Stain Max-PO MULTI was purchased from NICHIREI BIOSCIENCES INC (Tokyo, Japan). The ECL Plus Western Blotting Detection System was purchased from GE Healthcare UK Ltd (Chiltern, UK). The DAB (3,3'-diaminobenzidine) chromogen tablets, which were used to detect peroxidase reactions during the immunohistochemical examinations, and all other chemical reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All reagents were of analytical reagent grade.

2.2. Animal treatment

The 6-week-old male ICR mice used in this study were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were maintained at the Education and Research Center of Animal Models for Human Disease, Fujita Health University, according to the

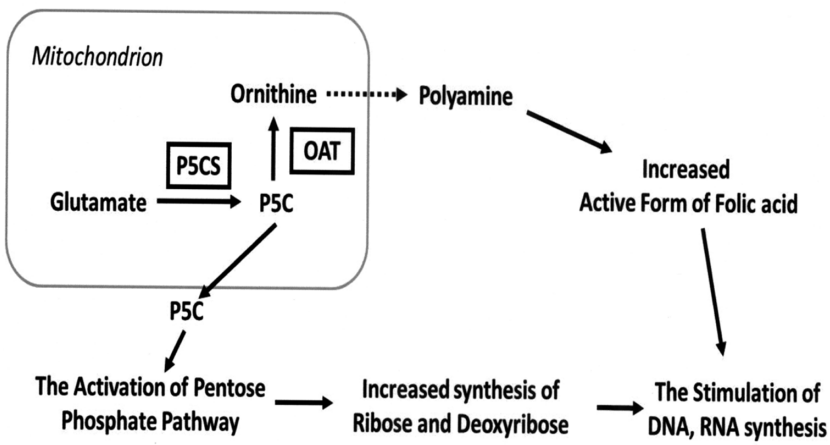

Fig. 1 The possible role of the P5CS metabolic pathway in colorectal cancer.
Mitochondrial P5CS participates in P5C and ornithine syntheses and the activation of the pentose phosphate pathway, which are in turn involved in the stimulation of DNA and RNA syntheses.

institutional animal care guidelines. All animals were housed in plastic cages (one mouse/cage) under controlled humidity (55 ± 10%), light (12h:12h light:dark cycle), and temperature (23 ± 2℃) conditions and allowed free access to drinking water and a pelleted basal diet (MF-Food, Oriental Yeast Co. Ltd., Tokyo, Japan). After being quarantined for 7 days, the mice were randomly divided into three groups.

The colitis-associated colon cancer (CACC) mouse model was induced by treating the mice with 1,2-dimethyl-hydrazine (DMH) and dextran sulfate sodium (DSS). The mice were intraperitoneally injected with DMH (20 mg/kg body weight) and then were given 2% DSS (w/v) in their drinking water for a week after the administration of DMH. The mice in group 1 (G1), the control group, were only given 2% DSS in their drinking water for a week and then were raised for 9 weeks. The mice in groups 2 (G2) and 3 (G3), the colon cancer groups, were treated with DMH and DSS, as described above, and then were raised for 5 weeks and 9 weeks, respectively.

After the mice had been dissected, their colons and recta were excised, opened longitudinally, stretched flat with needles on paper, and fixed in 10% neutral buffered formalin for histological analysis.

2.3. Immunohistochemistry

After formalin fixation, paraffin-embedded blocks of tumor and normal tissue were cut into 4 ㎛ sections. Each section was mounted on a silane-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 0.3% H_2O_2/methanol to block endogenous peroxidase activity. Thereafter, the slides were blocked in 0.1M citrate buffer (pH 6.0) supplemented with 1% skimmed milk for 10 min. Then, the anti-P5CS antibodies were added to the blocking solution at a dilution of 1:100 (21.4 g/ml protein), and the slides were incubated for 1 h at 37℃. The antigen-antibody complex was visualized using Histofine Simple Stain Max-PO DAB tablet. Subsequently, the slides were counterstained with hematoxylin.

2.4. Detection of P5CS in feces

After 50 mg mouse fecal samples had been homogenized in 1.0 ml of lysis buffer containing 1 mM Tris-HCl (pH 7.5), 1% Triton X-100, 5 mM ethylenediamine tetraacetic acid (EDTA), 20 mM NaF, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM dithiothreitol (DTT), the lysate was sonicated on ice for 30 seconds and centrifuged at 15,000×g for 10 min at 4℃ to remove fecal debris. The supernatant was then diluted to a protein concentration of 2 mg/mL with lysis buffer, and equal amounts of protein (4 μg) were loaded into each lane. Each sample was separated by 10% SDS-polyacrylamide gel electrophoresis (CompactPAGE AE-7500, ATTO, Tokyo, Japan) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Clear blot P, ATTO, Japan) via 30 min electrotransfer at 100 mA (CompactBLOT, ATTO, Tokyo, Japan).

The membranes were subsequently washed 3 times with PBST buffer (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 27 mM KCl, 137 mM NaCl, and 0.1% Tween-20; pH 7.4) and then incubated for 1 h with a secondary antibody (horseradish peroxidase-labeled anti-mouse IgG; Bio-Rad Laboratories, Inc., Hercules, CA) diluted 1: 20000 in blocking solution. The P5CS bands were visualized using the ECL Plus Western Blotting Detection System, and their chemiluminescence intensities were quantified using the LAS-1000 system (Fujifilm, Tokyo, Japan). Comparisons between the groups were interpreted with reference to the results of one-tailed paired t-tests.

3. Results

3.1. Macroscopic and histological observations

Macroscopically, no polyps were found in G1. In contrast, polypoid tumors were observed in the middle and distal colons of all mice in G2 and G3 (Fig. 2). The tumors of the CRC-induced mice were histologically analyzed by hematoxylin-eosin staining. As a result, a large number of the polypoid tumors were diagnosed as well- or moderately-differentiated adenocarcinoma. A representative tumor from a DMH-induced CRC model mouse is shown in figure 3.

3.2. Detection of P5CS in feces

Next, we investigated the fecal P5CS concentrations of each group using Western blotting. In this experiment, the fecal P5CS concentration was determined from the relative luminescence intensity of the band around 75kDa. As a result, P5CS was detected in the feces of all groups (Fig. 4a). In addition, the P5CS levels of G2 and G3 were approximately 40-fold and 1450-fold higher than those of G1, respectively (Fig. 4b).

3.3. Immunohistochemical observations

Western blotting showed that G2 and G3 exhibited significantly higher concentrations of P5CS in their feces than G1. Therefore, we confirmed that P5CS is present in both normal and malignant colorectal cells.

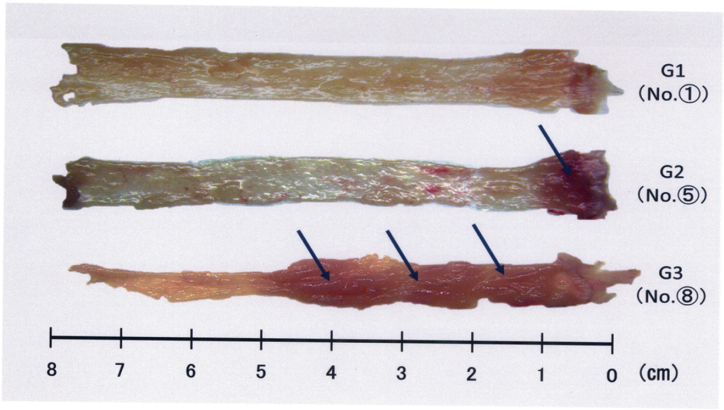

Fig. 2 Representative macroscopic view of the colons of the mice in G1, G2, and G3. The black arrows indicate tumors.

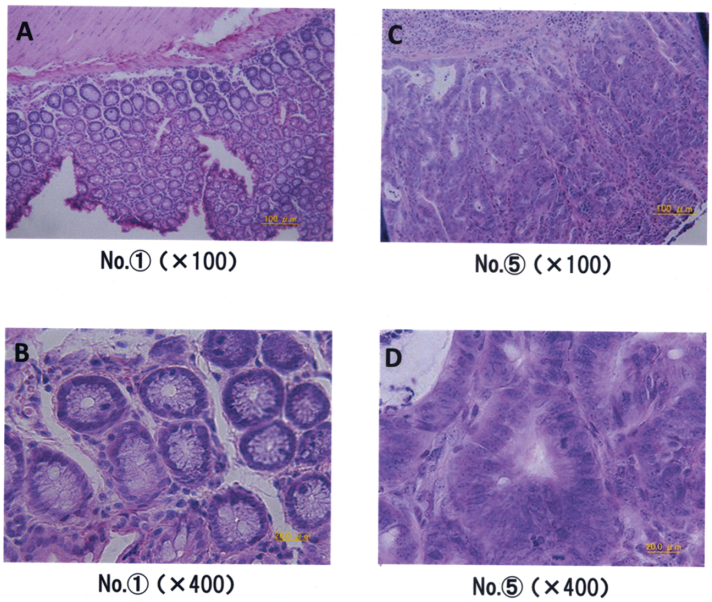

Fig. 3 H&E stained sections of the descending colons of the G1 (No.1) and G2 (No.5) mice. A and B: normal colon tissue in G1. C and D: well- differentiated adenocarcinoma in G2. A and C: ×100. B and D: ×400.

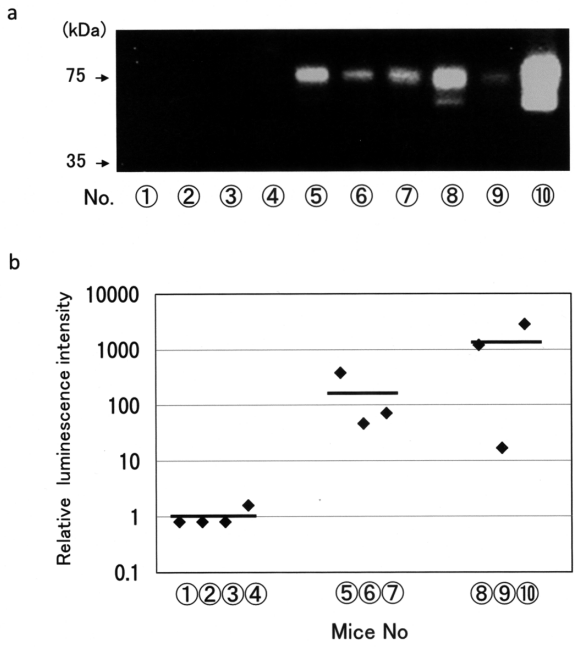

Fig. 4 The fecal concentration of P5CS according to Western blotting. We measured the protein content of fecal samples obtained from each group, and 4ｵg of protein from each sample were separated by SDS-PAGE on 10% polyacry lamide gel before being transferred to PVDF membranes. a: The P5CS-specific band is shown. Mouse numbers 1-4, 5-7, and 8-10 belonged to G1, G2, and G3, respectively. b: The luminescence intensity of P5CS is plotted on the graph. G2 and G3 displayed increased P5CS concentrations compared with G1. The line represents the median value. The t-test was used to determine statistical significance. (*$p < 0.05$)

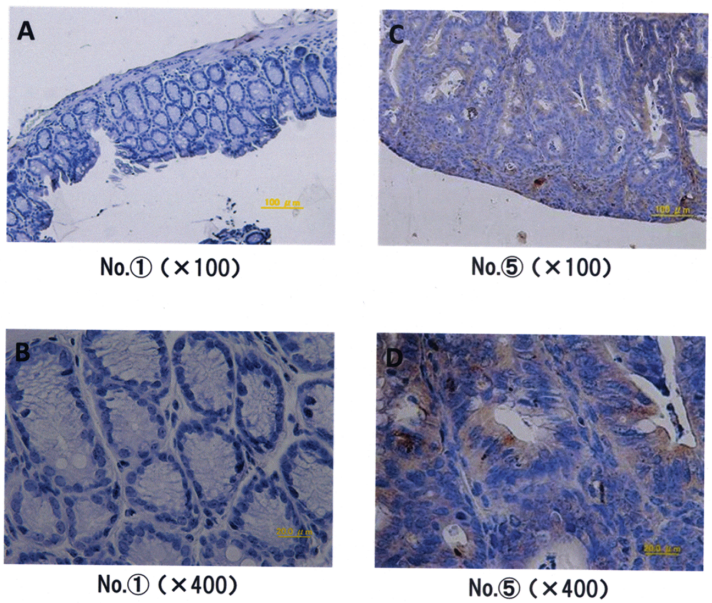

Fig. 5 Immunohistochemically stained sections of the descending colons of mice from G1 (No.1) and G2 (No.5). b: A and B: normal colon tissue in G1. C and D: well-differentiated adenocarcinoma in G2. A and C: ×100. B and D: ×400.

The immunoreaction against P5CS showed dark brown reaction products that varied slightly in their intensity and distribution, but exclusively localized in mitochondria (Fig. 5). In the immunohistochemical study, strong P5CS expression was only observed in adenocarcinoma cells.

4. Discussion

Many forms of CRC can be cured if they are detected in early stage; thus, early diagnosis has been pursued to be important for reducing mortality and improving the treatment compliance in CRC[15]. Therefore, a suitable and minimally invasive screening biomarker for CRC is required. In this study, we successfully detected P5CS in the feces of DMH-induced CRC model mice. In addition, we only observed strong immunohistochemical staining of P5CS in colorectal adenocarcinoma cells.

Previous studies using rat models of CRC reported that early-stage cancer and/or small polypoid tumors were observed in the distal part of the colon and rectal mucosae of almost all rats at 6 weeks after the induction of the model[16, 17]. Similarly, we detected small polypoid tumors and adenocarcinomas in the colons and recta of all animals at 5 weeks after the induction of the model. Although the mice only had small tumors, all of them exhibited P5CS in their feces.

The values of P5CS in the feces obtained from the CRC model mice displayed a large inter-individual variation. We suppose that these differences are due to two reasons. First, feces contain many desquamated colon epithelium cells; however, the amount of desquamated colorectal cancer cells differs between each feces, indicating the amount of desquamated colorectal cancer cells in feces is influenced by tumor size and the location of the tumor in the colon. Second, P5CS is a mitochondrial innermembrane-buried protein, and its polypeptide chain get across the lipid bilayer of mitochondrial membrane seven times. Therefore, it is difficult to solubilize as a whole P5CS protein molecule, and hence, its extraction efficiency varies. Our anti-P5CS antibody recognizes N-teminal twelve amino acids sequence, those are common in mouse and human P5CS and exposing to mitochondrial matrix. Ishikawa, H. et al. found that the anti-P5CS antibody for C-terminal sequences species-specific to human and mouse P5CS, but the one for N-terminal sequence is not. It is necessary to confirm the specificity, reliability, sensitivity and recovery of the fecal P5CS method as a biomarker of CRC before its clinical application.

Standard guaiac-based FOBT only detect 33-75% of CRC[4, 18], and the human hemoglobin-specific fecal immunochemical test detects CRC with a sensitivity of about 60-85%[5]. However, the positive predictive value of FOBT is generally considered to be unsuitable for detecting early-stage CRC. Assessing the fecal concentration P5CS might solve this problem. Previous studies have reported that P5CS participates in the regulation of cell proliferation and that its expression is enhanced in human colon adenocarcinoma cell lines.

Furthermore, Matsuzawa, T. et al. described that the P5CS mRNA level in human colorectal cancer tissue was over one hundred fold dramatically increased compared with normal colon tissue. These findings are matched for our data that the fecal concentration of P5CS was specifically increased hundreds to thousands fold in a CRC mouse model. It is expected that patients with colorectal cancer will exhibit high levels of P5CS in their feces. Thus, P5CS could be applicable as a simple and specific screening biomarker of colorectal cancer.

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