ANTICANCER RESEARCH *41*: 4789-4799 (2021)
doi:10.21873/anticanres.15294

Linker Threonine-phosphorylated Smad2/3 Is a Biomarker of Colorectal Neoplastic Stem-like Cells that Correlates With Carcinogenesis

SACHI MIYAMOTO, TOSHIRO FUKUI, SHUNSUKE HORITANI, YUJI TANIMURA, YASUSHI MATSUMOTO, RYO SUZUKI, YU TAKAHASHI, MASANOBU KISHIMOTO, TAKASHI TOMIYAMA, AKIYOSHI NISHIO, KAZUICHI OKAZAKI and MAKOTO NAGANUMA

Third Department of Internal Medicine, Division of Gastroenterology and Hepatology, Kansai Medical University, Hirakata, Japan

Abstract. *Background/Aim: This study analysed threonine-phosphorylated Smad2/3 (pSmad2/3L-Thr) expression and investigated whether pSmad2/3L-Thr is related to the transition from human colorectal adenoma (CRA) to carcinoma (CRC). Materials and Methods: Immunofluorescent staining was performed forβ-catenin, p53, CDK4, Ki67, Sox9, aldehyde dehydrogenase (ALDH) 1, and pSmad2/3L-Thr. Results: We analysed specimens of diffuse p53-positive CRCs arising from p53-negative CRAs. Percentage of p53, nuclear β-catenin, Ki67, CDK4, and pSmad2/3L-Thr-positive cells at the site of CRCs was significantly higher than that at the site of CRAs. At the site of normal colorectal mucosae, few epithelial cells were stained positively for pSmad2/3L-Thr. At the site of CRCs, pSmad2/3L-Thr-positive cells showed co-localization with p53, nuclear β-catenin, and ALDH1. At any site, pSmad2/3L-Thr-positive cells showed co-localization with CDK4. Conclusion: pSmad2/3L-Thr correlates with human CRC carcinogenesis, and pSmad2/3L-Thr-positive cells show human colorectal stem cell-like and cancer stem cell characteristics.*

Colorectal adenoma (CRA) is composed of highly proliferating dysplastic cells with molecular and genetic alterations and has a specific tendency to progress to colorectal carcinoma (CRC) (1). The colorectal adenoma-carcinoma sequence embodies the well-known facts about the continuous development of cancer resulting from the accumulation of genomic mutations. Decades ago, Vogelstein *et al*. proposed a multistep molecular mechanism for colorectal tumorigenesis. This led to low grade dysplastic adenomas progressing to high grade dysplastic adenomas, following adenomatous polyposis coli (APC) (5q21) inactivation, nuclear β-catenin accumulation, hypomethylation, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-ras*) mutations (2). Allelic loss in the 17p region of the chromosome, which contains the *TP53* gene associated with a mutation in the second allele, could cause inactivation of the two alleles. This loss of p53 function has been shown to be caused by a mutation that occurs later in the transition from CRA to CRC (3).

The p53 transcriptional factor is an important tumor suppressor that controls several signaling pathways associated with carcinogenesis (4). The wild-type *TP53* gene co-ordinates the anti-proliferative function essential for cell cycle arrest during G1 phase of the cell cycle. It aims to repair the DNA of highly damaged cells and prevent the growth of potential cancer clones. When cells fail to repair damaged DNA, they undergo apoptosis. However, this function is disrupted when wild-type p53 is inactivated by mutant p53 (5).

Recently, the cancer stem cell (CSC) hypothesis has been accepted as a leading concept in the development and progression of cancer. This hypothesis suggests that malignant tumors are composed of several cell populations and that one small cell population retains the characteristics of stem cells. CSCs have both the ability to self-renew and differentiate into a variety of cancer cells and play an important role in maintaining the ability of cancer to grow, invade, metastasize, and recur (6). Given that CSCs are comparatively resistant to therapies developed to eradicate populations of non-CSCs, the CSC hypothesis provides a theoretical basis for developing new therapies targeting a small population of CSCs and presents a new perspective of cancer treatment (7).

Correspondence to: Toshiro Fukui (ORCID: 0000-0003-1131-4067), Third Department of Internal Medicine, Division of Gastroenterology and Hepatology, Kansai Medical University, 2-5-1 Shinmachi, Hirakata, Osaka 573-1010, Japan. Tel: +81 728040101, Fax: +81 728042524, e-mail: fukuitos@hirakata.kmu.ac.jp

Key Words: Phosphorylation, carcinogenesis, colorectal neoplasm, Smad protein, cancer stem cell.

Smads, central mediators that signal the nucleus from receptors for the transforming growth factor-β (TGF-β) superfamily proteins, are regulatory proteins with well-conserved Mad-homology 1, intermediate linker, and Mad-homology 2 domains (8). A catalytically active TGF-β type I receptor phosphorylates the C-terminal serine (Ser) residues of receptor-activated Smads. These Smads contain very similar proteins, Smad2 and Smad3 (9). The particular Ser or threonine (Thr) residues in the linker domain are phosphorylated by proline-directed kinases such as extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and cyclin-dependent kinase (CDK) (10-12). TGF-β type I receptors and proline-directed kinases specifically phosphorylate Smad2 and Smad3 to produce several phosphoisoforms: C-terminally phosphorylated Smad2/3 (pSmad2/3C), linker phosphorylated Smad2/3 (pSmad2/3L), and both linker and C-terminally phosphorylated Smad2/3 (pSmad2/3L/C) (13). Phosphorylated Smad2 and Smad3 promptly oligomerize with Smad4, translocate to the nucleus, and regulate the transcription of target genes.

Our previous study confirmed significant expression of Smad2/3 phosphorylated at the specific linker Thr residues (pSmad2/3L-Thr) in mouse colon epithelial cells. This suggests that these cells are colon epithelial stem-like cells (14). In the subsequent study, our observations have been extended to a mouse model of colitis-associated CRC (15). The study has revealed that pSmad2/3L-Thr works as a biomarker for CSCs in the model of colitis-associated CRC. The anti-pSmad2/3L-Thr sera utilized in these two studies, recognized the same Smad3 linker phosphorylation as the specific phosphorylation of the Thr residue by CDK4 shown by Matsuura *et al*. (11, 13). On the other hand, during the development of sporadic CRC in humans, epithelial cells affected by somatic mutations, such as K-ras gene mutations, change from the tumor-suppressing pSmad3C-Ser pathway to the carcinogenic pSmad2/3L-Ser pathway (16).

In this study, we observed the expression profile of pSmad2/3L-Thr in human sporadic colorectal neoplasms and investigated whether pSmad2/3L-Thr is related to the transition from CRA to CRC and can be a potential biomarker for CSCs.

Materials and Methods

Specimens. Pathologists with interest in gastrointestinal neoplasia evaluated the histological characteristics according to the Japanese classification criteria of CRC (17). The principal histological distinction was between benign neoplasm including adenoma (low grade dysplasia in the West) and malignant neoplasm including adenocarcinoma (high grade dysplasia or carcinoma in the West) (18, 19).

Colorectal tissue specimens of CRCs arising from CRAs (CRAs containing CRCs, adenocarcinomas arising from adenomas of the colon or rectum) were obtained from the Department of Pathology, Kansai Medical University Medical Center. All specimens were from patients undergoing endoscopic polypectomy or mucosal resection from January 2012 to January 2018 at Kansai Medical University Medical Center. The patients' age ranged from 52 to 78 years (68.0±1.89 years, n=15). All CRAs included tubular adenomas (n=15). CRCs included tubular adenocarcinomas classified histologically as well differentiated (n=12) and moderately differentiated (n=3). CRCs were confined to the mucosa (Tis, n=8) or submucosa (T1, n=7). Distal normal tissues paired with the above lesions were also obtained. Subject to the document uploaded to the Kansai Medical University web page declaring an opt-out policy that any possible patient and/or relatives could refuse to participate in this study, it was approved by the Ethics Committee of Kansai Medical University (approval number: 2012101). Therefore, informed consent was obtained in the form of an opt-out policy undertaken by the participants.

Domain-specific antibody against Smad2 and Smad3 phosphorylated at the specific linker Thr residues. Polyclonal rabbit anti-pSmad2/3L-Thr (Smad2: Thr 220, Smad3: Thr 179) sera was produced against the phosphorylated linker Thr regions of Smad2 and Smad3 by immunizing rabbits with synthetic peptides (13-15). These antisera were affinity-purified using phosphorylated peptides as previously reported (20).

Immunostaining. Immunofluorescent staining was performed on formalin-fixed paraffin-embedded sections (14, 15, 21). Nonenzymatic antigen retrieval was conducted by heating sections to 121˚C for 10 min in 0.01 M sodium citrate buffer (pH 6.0). After cooling, sections were blocked with 3% bovine serum albumin in tris-buffered saline (TBS) for 5 min.

Primary antibodies (Abs) were diluted with TBS containing 0.1% Tween 20 and incubated in a humidified chamber at 4˚C. The primary Abs used in this study included monoclonal mouse anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-p53 (Dako, Glostrup, Denmark), monoclonal mouse anti-CDK4 (Santa Cruz Biotechnology), monoclonal mouse anti-Ki67 (Dako), monoclonal rabbit anti-Sox9 (Abcam, Cambridge, UK), monoclonal mouse anti-aldehyde dehydrogenase (ALDH) 1 (BD Biosciences, San Jose, CA, USA), and polyclonal rabbit anti-pSmad2/3L-Thr. Suitable species-specific AlexaFluor (488 or 568)-conjugated Abs (Invitrogen, Carlsbad, CA, USA) were used as secondary Abs. Slides were mounted with VECTASHIELD mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) to stain the nuclei and maintain fluorescence. Images were captured with a fluorescent microscope (Olympus, Tokyo, Japan).

After immunofluorescent staining, the same sections were stained with hematoxylin and eosin (H&E) and observed under a light microscope.

Evaluation of immunofluorescent staining in CRC arising from CRA. To confirm the accuracy of the differential diagnosis between CRAs and CRCs, which is sometimes difficult depending only on the histological examination by H&E staining, all the specimens were screened for p53 expression using the fluorescent immunostaining methodology described above. We chose and subsequently analysed the specimens that had diffuse p53-positive CRCs arising from p53-negative CRAs.

After fluorescent immunostaining, three representative sites were captured at a magnification of ×200 using a fluorescence

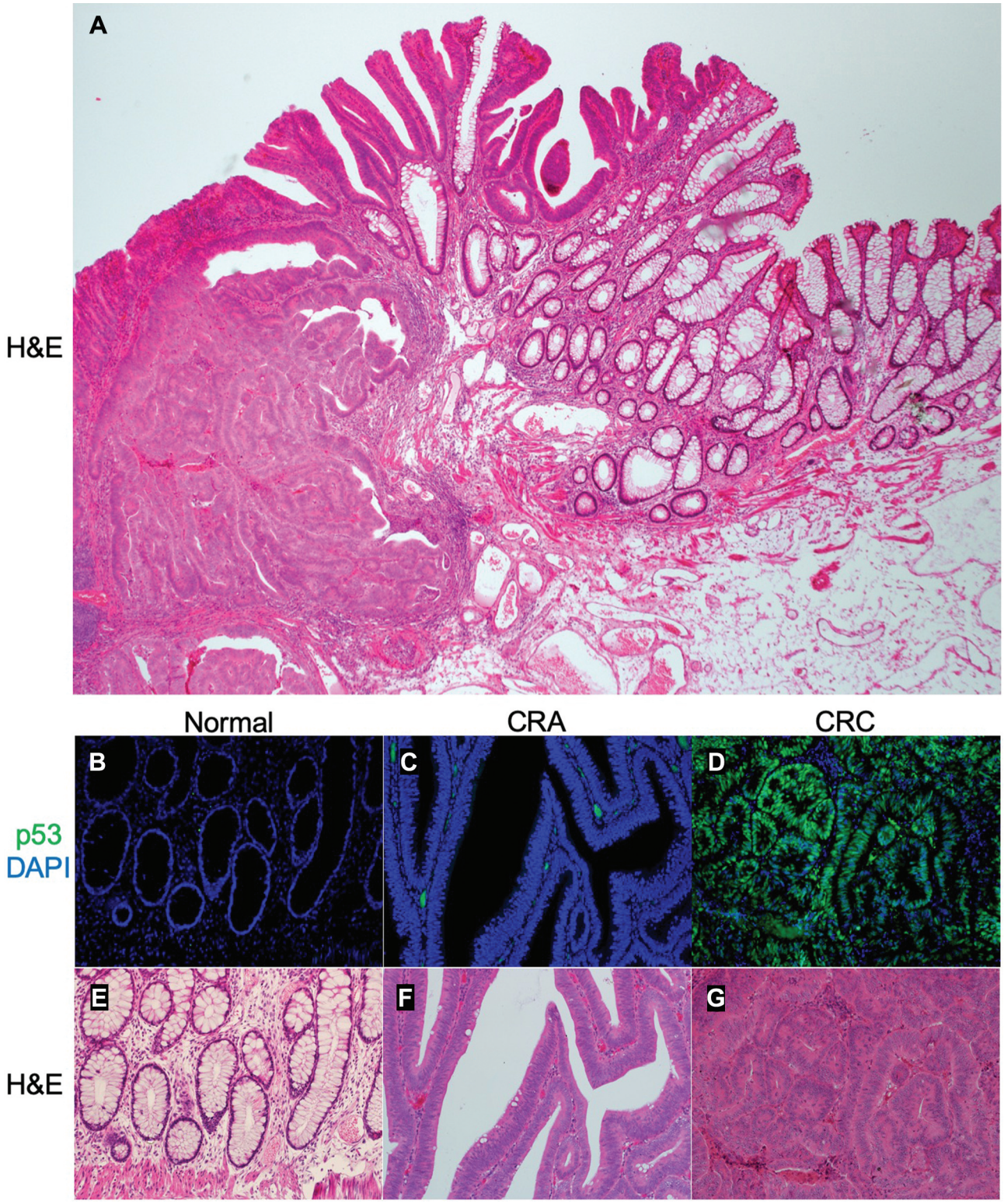

Figure 1. *Immunofluorescent staining for p53 in colorectal carcinoma (CRC) arising from colorectal adenoma (CRA). (A) A representative image of hematoxylin and eosin (H&E)-stained CRC arising from CRA specimen is shown. Carcinoma, adenoma, and normal mucosa can be seen from the left side of the image. (B-D) Immunofluorescent staining for p53 (green) in (B) normal mucosa, (C) CRA, and (D) CRC in CRC arising from CRA is shown. DAPI (blue) is used for nuclear staining. (E-G) H&E-stained images of (E) normal mucosa, (F) CRA, and (G) CRC using the specimens after immunofluorescent staining are shown. Original magnification: ×40 (A) and ×200 (B-G).*

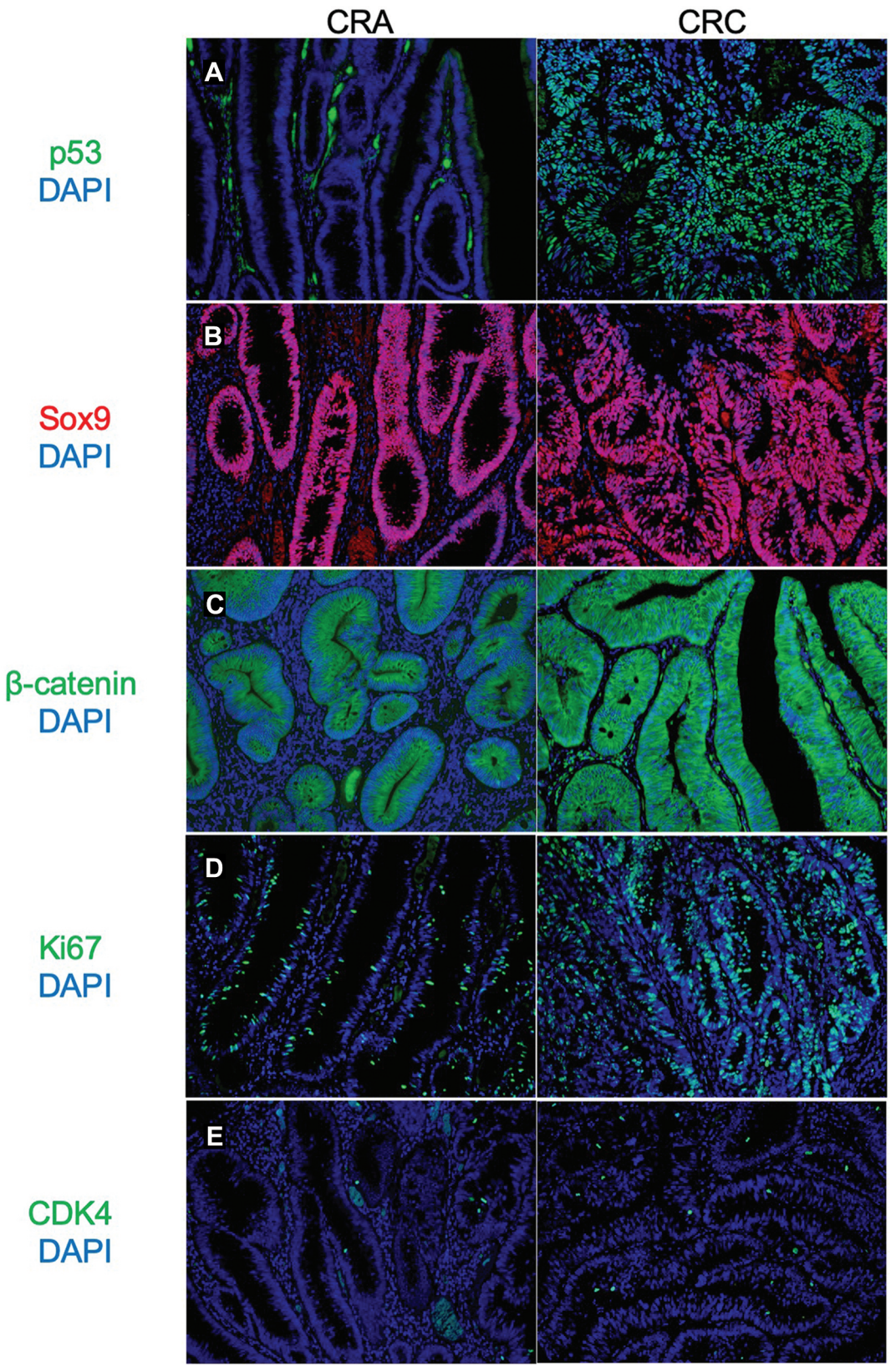

Figure 2. *Continued*

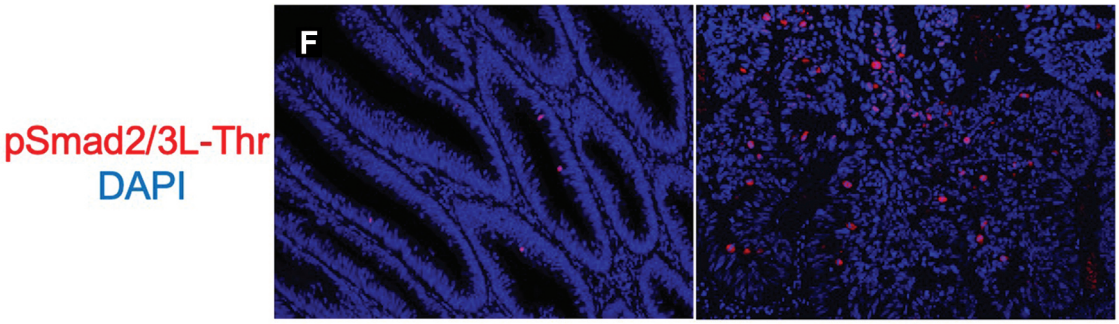

Figure 2. *Immunofluorescent staining for p53, Sox9, β-catenin, Ki67, CDK4, and pSmad2/3L-Thr in colorectal carcinoma (CRC) arising from colorectal adenoma (CRA). (A-F) Immunofluorescent staining for (A) p53, (B) Sox9, (C) β-catenin, (D) Ki67, (E) CDK4, and (F) pSmad2/3L-Thr (green: p53, β-catenin, Ki67, CDK4; red: Sox9, pSmad2/3L-Thr) of CRAs (left panels) and CRCs (right panels) in CRCs arising in CRAs are shown using DAPI (blue) nuclear staining. Original magnification: ×200.*

microscope and analysed using inForm Image Analysis software (PerkinElmer, Waltham, MA, USA), and then the results were averaged. This software analysis allows us to objectively count the percentage of immunostaining-positive cell populations for each biomarker, improving accuracy of the statistical analysis.

Statistical analysis. Values were expressed as the mean±standard error of the mean (SEM). We analysed data using paired *t*-test. A *p*-value less than 0.05 was considered statistically significant. Data were collected and statistically evaluated with the StatView software (version 5.0).

Results

Immunofluorescent staining for p53 in CRC arising from CRA. CRCs arising from CRAs were diagnosed by the pathologists following examination of H&E-stained sections (Figure 1A). CRC was defined based on a combination of severe cytologic dysplasia, structural atypia, and nuclei atypia, regardless of the invasion status. After confirming the pathological diagnosis, immunofluorescent staining for p53 (green) was performed on continuous sections using DAPI (blue) nuclear staining.

No p53-positive cells were found at the site of both normal colorectal mucosae (Figure 1B) and CRAs (Figure 1C). At the site of CRCs, most neoplastic cells were p53-positive in the nuclei (Figure 1D). The differences in the results of immunofluorescent staining for p53 between CRAs and CRCs were obvious. After immunofluorescent staining, sections were stained with H&E, and normal colorectal mucosae (Figure 1E), CRAs (Figure 1F), and CRCs (Figure 1G) were re-confirmed using a light microscope.

Immunofluorescent staining for p53, Sox9, β-catenin, Ki67, CDK4, and pSmad2/3L-Thr in CRC arising from CRA. Immunofluorescent staining for p53 (green; Figure 2A), Sox9 (red; Figure 2B), β-catenin (green; Figure 2C), Ki67 (green; Figure 2D), CDK4 (green; Figure 2E), and pSmad2/3L-Thr (red; Figure 2F) was performed in CRCs (right panels of each figure) arising from CRAs (left panels of each figure) using DAPI (blue) nuclear staining.

Most neoplastic cells were positively stained in the nuclei by immunofluorescent staining for p53 at the site of CRCs, but no neoplastic cells were positively stained at the site of CRAs (Figure 2A). Almost all of the neoplastic cells were stained positively in the nuclei by immunofluorescent staining for Sox9, but there was no difference in the frequency of Sox9-positive cells at the site of CRAs and CRCs (Figure 2B). All of the neoplastic cells were positively stained in the cytoplasm by immunofluorescent staining for β-catenin at the site of CRAs and CRCs, but also occasionally positive in the nuclei at the site of CRCs (Figure 2C). Some of the neoplastic cells were stained positively in the nuclei by immunofluorescent staining for Ki67 at the site of CRCs, but the neoplastic cells were occasionally stained positively at the site of CRAs (Figure 2D). Several neoplastic cells were stained positively in the nuclei by immunofluorescent staining for CDK4 at the site of CRCs, while a few of the neoplastic cells were stained positively at the site of CRAs (Figure 2E). Several neoplastic cells were stained positively in the nuclei and cytoplasm by immunofluorescent staining for pSmad2/3L-Thr at the site of CRCs, while a few of the neoplastic cells were stained positively at the site of CRAs (Figure 2F).

Percentage of the cells with positive immunofluorescent staining for p53, Sox9, nuclear β-catenin, Ki67, CDK4, and pSmad2/3L-Thr in the neoplastic cells of CRC arising from CRA. Percentage of p53 (Figure 3A), Sox9 (Figure 3B), nuclear β-catenin (Figure 3C), Ki67 (Figure 3D), CDK4 (Figure 3E), and pSmad2/3L-Thr-positive cells (Figure 3F)

in the neoplastic cells of CRCs arising from CRAs was analysed (n=15). The software was used to separately calculate immunostaining-positive neoplastic cell count/total neoplastic cell count for CRA and CRC.

Percentage of p53-positive cells at the site of CRCs (71.753±3.432%) was significantly higher than that at the site of CRAs (0.000±0.000%) (Figure 3A; $p<0.0001$). Percentage of Sox9-positive cells at the site of CRCs (97.000±0.331%) was somewhat higher than that at the site of CRAs (91.047±3.028%), but no significant difference was observed between the two (Figure 3B; $p=0.0769$). Although there was some dispersion in the values between the samples, percentage of nuclear β-catenin-positive cells at the site of CRCs (16.157±4.635%) was significantly higher than that at the site of CRAs (0.000±0.000%) (Figure 3C; $p=0.0040$). Percentage of Ki67-positive cells at the site of CRCs (36.047±3.202%) was significantly higher than that at the site of CRAs (19.100±2.478%) (Figure 3D; $p<0.0001$). Although each value was small, the percentage of CDK4-positive cells at the site of CRCs (11.733±1.354‰) was significantly higher than that at the site of CRAs (4.133±0.661‰) (Figure 3E; $p<0.0001$). Although each value was small, the percentage of pSmad2/3L-Thr-positive cells at the site of CRCs (11.400±1.253‰) was significantly higher than that at the site of CRAs (4.067±0.665‰) (Figure 3F; $p<0.0001$).

Immunofluorescent staining for pSmad2/3L-Thr with p53, β-catenin, Ki67, CDK4, or ALDH1 in the normal colon epithelial and neoplastic cells of the specimen of CRC arising from CRA. Double immunofluorescent staining for pSmad2/3L-Thr (red; white arrowheads in Figure 4) with p53 (green; Figure 4A), β-catenin (green; Figure 4B), Ki67 (green; Figure 4C), CDK4 (green; Figure 4D), or ALDH1 (green; Figure 4E) was performed in the normal colon epithelial and neoplastic cells of the specimens of CRCs arising from CRAs using DAPI nuclear staining (blue).

At the site of normal colorectal mucosae, a very small number of epithelial cells near the crypt bases were stained positively in the nuclei and cytoplasm by immunofluorescent staining for pSmad2/3L-Thr (upper left panels of each figure).

As no epithelial cells were positively stained by immunofluorescent staining for p53 at the site of normal colorectal mucosae (middle left panel of Figure 4A) and CRAs (middle panel of Figure 4A), pSmad2/3L-Thr-positive cells did not show immunohistochemical co-localization with p53. pSmad2/3L-Thr-positive cells were sparsely distributed and showed immunohistochemical co-localization with p53 at the site of CRCs (right panels of Figure 4A). Cells were positively stained for β-catenin only in the cell membranes at the site of normal colorectal mucosae (middle left panel of Figure 4B), but also in the cytoplasm at the site of CRAs and CRCs (middle and middle right panels of Figure 4B), and also in the nuclei at the site of CRCs (middle right panel of Figure 4B); pSmad2/3L-Thr-positive cells showed immunohistochemical co-localization with β-catenin, respectively (Figure 4B). At the site of normal colorectal mucosae, a few epithelial cells in the proliferating zones just above the crypt bases were positively stained for Ki67 (middle left panel of Figure 4C), while several and some of the neoplastic cells were stained positively at the site of CRAs and CRCs, respectively (middle and middle right panels of Figure 4C). At any site, most of the pSmad2/3L-Thr-positive cells demonstrated immunohistochemical co-localization with Ki67 (filled arrowheads; Figure 4C), but several pSmad2/3L-Thr-positive cells did not co-localize with Ki67 (open arrowheads; Figure 4C). At any site, pSmad2/3L-Thr-positive cells demonstrated immunohistochemical co-localization with CDK4 (Figure 4D). Fluorescence expression of CDK4 in pSmad2/3L-Thr-positive cells was easily detectable, and a few other CDK4-positive cells were also detected in the cells other than pSmad2/3L-Thr-positive cells. Because no epithelial cells were positively stained for ALDH1 at the site of normal colorectal mucosae (middle left panel of Figure 4E) and CRAs (middle panel of Figure 4E), pSmad2/3L-Thr-positive cells did not show immunohisto-chemical co-localization with ALDH1. pSmad2/3L-Thr-positive cells were sparsely distributed and demonstrated immunohistochemical co-localization with ALDH1 at the site of CRCs (right panels of Figure 4E).

Discussion

There are some differences in the diagnostic criteria for gastrointestinal epithelial tumors between Western and Japanese pathologists (18, 19). In the West, CRC is defined by the invasion of atypical epithelial cells from the muscularis mucosa into the submucosa, especially with the desmoplastic reaction. In Japan, CRC is defined based on the combination of cellular and structural atypia of neoplastic tissue, regardless of the status of cell invasion. Consequently, intramucosal carcinoma in Japan is often diagnosed as high grade dysplasia in the West. The present study did not aim to distinguish between the two, but to analyse the expression of biomarkers including pSmad2/3L-Thr in the development of CRC based on the adenoma-carcinoma sequence. Therefore, in order to not get confused by the differential diagnosis between CRA and CRC, we screened all the sections for the expression of p53, whose functional loss is a late event in the CRA to CRC transition (3). We subsequently analysed various biomarkers that may be involved in the development and progression of cancer in the specimens that had diffuse p53-positive CRCs arising from p53-negative CRAs.

Sox9 is a transcriptional factor expressed in the progenitor and stem cell zone of normal colon epithelial cells (22). In the present study, most of the neoplastic cells were diffusely positive for Sox9; there was no difference in the frequency of Sox9-positive cells at the site of CRAs and CRCs. Sox9

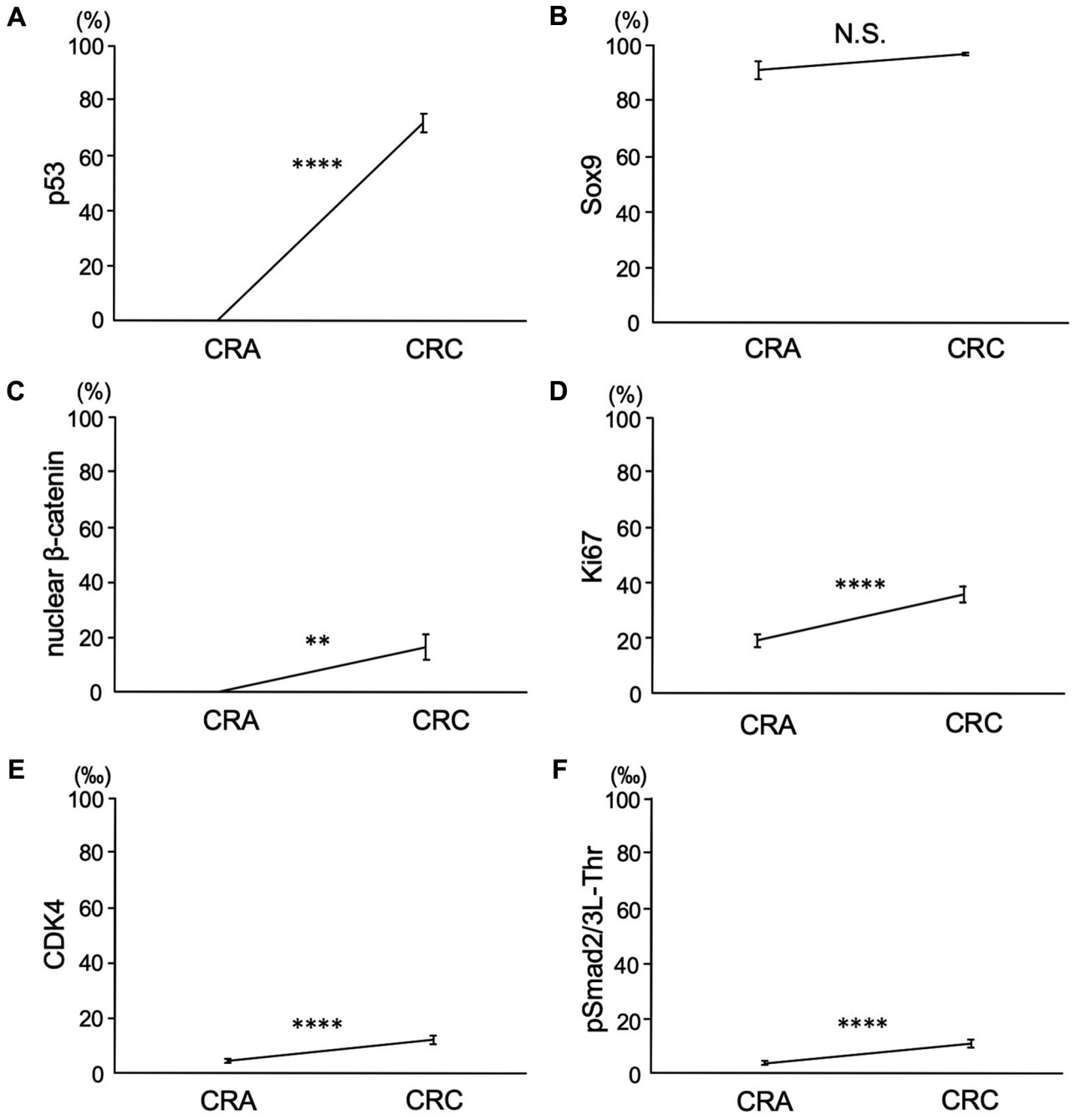

Figure 3. *Percentage of the cells with positive immunofluorescent staining for p53, Sox9, nuclear β-catenin, Ki67, CDK4, and pSmad2/3L-Thr in the neoplastic cells of colorectal carcinoma (CRC) arising from colorectal adenoma (CRA). (A-F) Percentage of (A) p53, (B) Sox9, (C) nuclear β-catenin, (D) Ki67, (E) CDK4, and (F) pSmad2/3L-Thr-positive cells at the site of CRAs and CRCs were compared in CRCs arising from CRAs. Immunostaining-positive neoplastic cell count/total neoplastic cell count was calculated separately for CRA and CRC using the software. Data are expressed as the mean±standard error of the mean and were analysed using a paired t-test (**p<0.01; ****p<0.0001; N.S.: not significant).*

plays important roles in tumorigenesis and is over-expressed in many types of human cancers, including CRC, whose expression correlates with malignant features and progression (23).

β-catenin regulates the transcription of genes involved in cell growth, development, and differentiation. Although expressed on the cell membrane of normal colon epithelial cells, accumulations of cytoplasmic and nuclear β-catenin correlate

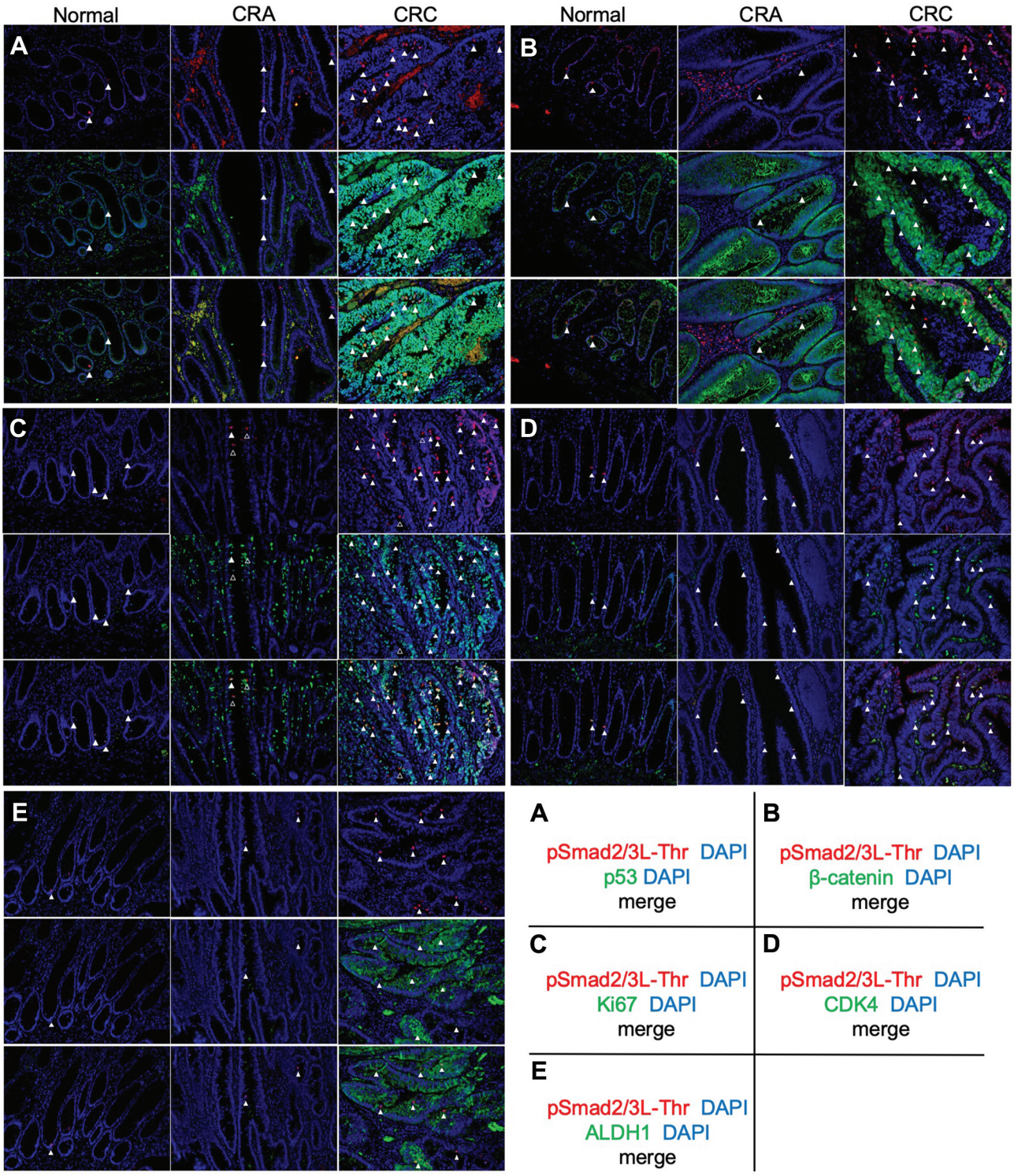

Figure 4. *Immunofluorescent staining for pSmad2/3L-Thr with p53, β-catenin, Ki67, CDK4, or aldehyde dehydrogenase (ALDH) 1 in the normal colon epithelial and neoplastic cells of the specimen of colorectal carcinoma (CRC) arising from colorectal adenoma (CRA). (A-E) Double immunofluorescent staining for pSmad2/3L-Thr (red; white arrowheads) with (A) p53, (B) β-catenin, (C) Ki67, (D) CDK4, or (E) ALDH1 (green) of normal mucosae (left panels), CRAs (middle panels), and CRCs (right panels) in CRCs arising from CRAs are shown using DAPI nuclear staining (blue). Lower panels are merged images. (C) Open arrowheads indicate Ki67-negative pSmad2/3L-Thr-positive cells. Original magnification: ×200.*

with the sequential stages in colorectal carcinogenesis (24). Similar to previous studies, we observed β-catenin accumulation only in the cell membranes at the site of normal colorectal mucosae, in the cytoplasm at the site of CRAs and CRCs, and also in the nuclei at the site of CRCs. The infrequent expression of β-catenin in the nuclei of CRCs in the present study might have been due to the peculiar states of CRCs arising from CRAs in their early carcinogenesis (24).

Expression of D-type cyclins is increased by stimulation with growth factors. D-type cyclins form a complex with CDK4 or CDK6 and phosphorylate and inactivate Rb family proteins. This is essential for the progression of the cell cycle from G0/G1 to S phase (25). Matsuura *et al*. reported that the active complex of CDK4 and D-type cyclin also phosphorylates particular sites in Smad3 (and, almost certainly, homologous Smad2), promoting cell cycle progression from G1 (or G0) to S phase (11, 25). It is very interesting that anti-pSmad2/3L-Thr Ab used in the present study recognized the same linker-phosphorylated Smad3 as the specifically phosphorylated Thr residue reported by Matsuura *et al*. (11, 13). Additionally, CD34-positive hemopoietic stem cells present in G0 phase suppress the expression of most CDKs and cyclins except CDK4 (26). The findings in this study re-confirmed that pSmad2/3L-Thr co-localizes with CDK4 at the site of normal colorectal mucosae, CRAs, and CRCs, as demonstrated in earlier studies in mice (14, 15, 21, 27).

Ki67 is expressed in proliferating cells, excluding quiescent G0 cells, throughout the cell cycle (28). In mice, pSmad2/3L-Thr-positive normal intestinal cells have been shown to be Ki67-negative and slow-cycling 5-bromo-2-deoxyuridine label-retaining cells (14). In the present study, pSmad2/3L-Thr-positive cells were both Ki67-positive and -negative, but they were consistently located around the crypt base of the normal human colorectal epithelium and were positive for CDK4 and cell membrane β-catenin, similar to the findings of previous research (15). Based on these findings, we regarded them as normal intestinal stem-like cells with properties similar to those of pSmad2/3L-Thr-positive cells previously observed in mice (14, 15, 21, 27). We inferred that pSmad2/3L-Thr might be observed in human specimens for a longer period of time, which could attribute to the double positive population of pSmad2/3L-Thr and Ki67 and account for the differences between human and mouse samples. Presumably, this expression of pSmad2/3L-Thr is preserved during the quiescent and early proliferative phases of the cell cycle. Wrighton *et al*. reported that the on-off response of pSmad2/3L-Thr as a transcriptional factor is controlled by a variety of mechanisms that use dephosphorylation of the linker domains by some specific phosphatases and degradation by ubiquitination (29). It is suggested that the expression of pSmad2/3L-Thr changes depending on the animal species and the situations and circumstances in which the cells are placed.

pSmad2/3L-Thr-positive neoplastic cells were always Ki67-negative in the mouse CRC model (15). However, in this study, Ki67-positive and -negative cells were detected in human CRA and CRC samples. They were always CDK4-positive, as was the case with pSmad2/3L-Thr-positive neoplastic cells detected in the mouse CRC model. As shown in the present study, pSmad2/3L-Thr-positive neoplastic cells were positive for p53 in human CRC samples and positive for cytoplasmic (or nuclear) β-catenin in human CRA and CRC samples. Moreover, a small population of Ki67-negative pSmad2/3L-Thr-positive neoplastic cells was identified, thus they were considered slow-cycling CSCs with similar properties to pSmad2/3L-Thr-positive cells observed in the mouse CRC model. Expression of pSmad2/3L-Thr is thought to be observed in quiescent and early proliferative cells of human specimens, similar to the normal colorectal epithelium.

Numerous studies have shown that the expression of several biomarkers increases during the progression of CRA to CRC (30). We examined the alterations in the expression of p53, Sox9, nuclear β-catenin, Ki67, CDK4, and pSmad2/3L-Thr in the human colorectal adenoma-carcinoma sequence by fluorescent immunostaining using specimens of CRC arising from CRA. The examination of expression within the same specimen reflects more important events in the early stages of carcinogenesis when compared to that within separate CRA and CRC specimens. It is probably because of this early state in the carcinogenesis process of CRC that the frequency of nuclear β-catenin expression in CRCs was low, while the expression of p53 and Ki67 was relatively high (24). On the other hand, Sox9 showed no significant difference in expression between CRA and CRC samples; its expression was already high in CRA, which is considered to be an early driver in the tumorigenesis process of CRC (31). Expression of pSmad2/3L-Thr was also significantly different between CRA and CRC, which increased with malignant transformation. This corresponded to the findings of previous reports indicating that pSmad2/3L-Thr-positive cells in neoplasms are CSCs capable of neoplastic formation and progression and that neoplasms that express more CSC biomarkers suggest more progressed stages of the human colorectal adenoma-carcinoma sequence (15, 32). When colorectal mucosa becomes tumorigenic, CSCs are activated and the number of CSC biomarker-positive cells increases. Such dynamic change of biomarkers for CSCs reflects ongoing colorectal carcinogenesis and could be a hallmark of the adenoma-carcinoma transition.

ALDH1 activity has been reported to be able to identify stem-like subsets of human hemopoietic and solid malignancies, including CRC. Increased expression of ALDH1 correlates with rapid tumor progression and bad prognosis (32). pSmad2/3L-Thr-positive cells were mainly observed in the ALDH1-positive regions of CRCs.

Therefore, we verified that pSmad2/3L-Thr-positive cells function as CSCs for human CRCs, which is consistent with the *in vivo* findings using the mouse CRC model.

In conclusion, pSmad2/3L-Thr correlates with the carcinogenesis of human CRC. This study supports the following hypothesis: pSmad2/3L-Thr-positive cells indicate human colorectal stem-like cells and CSCs in normal mucosae and neoplasms, respectively.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

All Authors have contributed to the study conception and design and agreed on the content of the final manuscript. S.M. carried out the material preparation and experiments, conducted data collection and analyses, and helped draft the manuscript. T.F. conceived the study, carried out data analyses, performed statistical analyses, and drafted the manuscript. S.H., Y.Tan., Y.M., R.S., Y.Tak., M.K., T.T., A.N., K.O., and M.N. carried out data analyses and provided significant advice and consultation. All Authors read and approved the final manuscript.

Acknowledgements

The Authors would like to thank Editage (www.editage.jp) for the English language review. This study was supported by a Grant-in-Aid for Scientific Research (C) (16K09330, 25460938) from the Japan Society for the Promotion of Science and a research grant from Kyoto Health Management Research Foundation in 2020.

References

1 Jackman RJ and Mayo CW: The adenoma-carcinoma sequence in cancer of the colon. Surg Gynecol Obstet *93(3)*: 327-330, 1951. PMID: 14866716.

2 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL: Genetic alterations during colorectal-tumor development. N Engl J Med *319(9)*: 525-532, 1988. PMID: 2841597. DOI: 10.1056/NEJM198809013190901

3 Mulder JW, Wielenga VJ, Pals ST and Offerhaus GJ: p53 and CD44 as clinical markers of tumour progression in colorectal carcinogenesis. Histochem J *29(6)*: 439-452, 1997. PMID: 9248852. DOI: 10.1023/a:1026403404652

4 Rivlin N, Brosh R, Oren M and Rotter V: Mutations in the p53 tumor suppressor gene: Important milestones at the various steps of tumorigenesis. Genes Cancer *2(4)*: 466-474, 2011. PMID: 21779514. DOI: 10.1177/1947601911408889

5 Suzuki H, Igarashi S, Nojima M, Maruyama R, Yamamoto E, Kai M, Akashi H, Watanabe Y, Yamamoto H, Sasaki Y, Itoh F, Imai K, Sugai T, Shen L, Issa JP, Shinomura Y, Tokino T and Toyota M: IGFBP7 is a p53-responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype. Carcinogenesis *31(3)*: 342-349, 2010. PMID: 19638426. DOI: 10.1093/carcin/bgp179

6 Campbell LL and Polyak K: Breast tumor heterogeneity: cancer stem cells or clonal evolution? Cell Cycle *6(19)*: 2332-2338, 2007. PMID: 17786053. DOI: 10.4161/cc.6.19.4914

7 Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL and Wahl GM: Cancer stem cells – perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res *66(19)*: 9339-9344, 2006. PMID: 16990346. DOI: 10.1158/0008-5472.CAN-06-3126

8 Massagué J: TGF-beta signal transduction. Annu Rev Biochem *67*: 753-791, 1998. PMID: 9759503. DOI: 10.1146/annurev.biochem.67.1.753

9 Wrana JL: Crossing Smads. Sci STKE *2000(23)*: re1, 2000. PMID: 11752591. DOI: 10.1126/stke.2000.23.re1

10 Kretzschmar M, Doody J, Timokhina I and Massagué J: A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. Genes Dev *13(7)*: 804-816, 1999. PMID: 10197981. DOI: 10.1101/gad.13.7.804

11 Matsuura I, Denissova NG, Wang G, He D, Long J and Liu F: Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature *430(6996)*: 226-231, 2004. PMID: 15241418. DOI: 10.1038/nature02650

12 Mori S, Matsuzaki K, Yoshida K, Furukawa F, Tahashi Y, Yamagata H, Sekimoto G, Seki T, Matsui H, Nishizawa M, Fujisawa J and Okazaki K: TGF-beta and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. Oncogene *23(44)*: 7416-7429, 2004. PMID: 15326485. DOI: 10.1038/sj.onc.1207981

13 Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, Uemura Y, Seki T, Taketani S, Fujisawa J and Okazaki K: Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-beta signal in later stages of human colorectal cancer. Cancer Res *69(13)*: 5321-5330, 2009. PMID: 19531654. DOI: 10.1158/0008-5472.CAN-08-4203

14 Kishimoto M, Fukui T, Suzuki R, Takahashi Y, Sumimoto K, Okazaki T, Sakao M, Sakaguchi Y, Yoshida K, Uchida K, Nishio A, Matsuzaki K and Okazaki K: Phosphorylation of Smad2/3 at specific linker threonine indicates slow-cycling intestinal stem-like cells before reentry to cell cycle. Dig Dis Sci *60(2)*: 362-374, 2015. PMID: 25185661. DOI: 10.1007/s10620-014-3348-3

15 Suzuki R, Fukui T, Kishimoto M, Miyamoto S, Takahashi Y, Takeo M, Mitsuyama T, Sakaguchi Y, Uchida K, Nishio A and Okazaki K: Smad2/3 linker phosphorylation is a possible marker of cancer stem cells and correlates with carcinogenesis in a mouse model of colitis-associated colorectal cancer. J Crohns Colitis *9(7)*: 565-574, 2015. PMID: 25908723. DOI: 10.1093/ecco-jcc/jjv073

16 Yamagata H, Matsuzaki K, Mori S, Yoshida K, Tahashi Y, Furukawa F, Sekimoto G, Watanabe T, Uemura Y, Sakaida N, Yoshioka K, Kamiyama Y, Seki T and Okazaki K: Acceleration of Smad2 and Smad3 phosphorylation via c-Jun NH(2)-terminal kinase during human colorectal carcinogenesis. Cancer Res *65(1)*: 157-165, 2005. PMID: 15665291.

17 Japanese Society for Cancer of the Colon and Rectum: Japanese classification of colorectal, appendiceal, and anal carcinoma. 3rd English edition. Japanese Society for Cancer of the Colon and Rectum (ed.). Tokyo, Kanehara & Co., 2019.

18 Schlemper RJ, Kato Y and Stolte M: Review of histological classifications of gastrointestinal epithelial neoplasia: differences in diagnosis of early carcinomas between Japanese and Western

pathologists. J Gastroenterol *36(7)*: 445-456, 2001. PMID: 11480788. DOI: 10.1007/s005350170067

19 Yao T and Shiono S: Differences in the pathological diagnosis of colorectal neoplasia between the East and the West: Present status and future perspectives from Japan. Dig Endosc *28(3)*: 306-311, 2016. PMID: 26295687. DOI: 10.1111/den.12535

20 Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, Yamagata H, Matsushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J and Inoue K: p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. Hepatology *38(4)*: 879-889, 2003. PMID: 14512875. DOI: 10.1053/jhep.2003.50384

21 Fukui T, Kishimoto M, Nakajima A, Yamashina M, Nakayama S, Kusuda T, Sakaguchi Y, Yoshida K, Uchida K, Nishio A, Matsuzaki K and Okazaki K: The specific linker phosphorylation of Smad2/3 indicates epithelial stem cells in stomach; particularly increasing in mucosae of Helicobacter-associated gastritis. J Gastroenterol *46(4)*: 456-468, 2011. PMID: 21229365. DOI: 10.1007/s00535-010-0364-8

22 Ramalingam S, Daughtridge GW, Johnston MJ, Gracz AD and Magness ST: Distinct levels of Sox9 expression mark colon epithelial stem cells that form colonoids in culture. Am J Physiol Gastrointest Liver Physiol *302(1)*: G10-G20, 2012. PMID: 21995959. DOI: 10.1152/ajpgi.00277.2011

23 Matheu A, Collado M, Wise C, Manterola L, Cekaite L, Tye AJ, Canamero M, Bujanda L, Schedl A, Cheah KS, Skotheim RI, Lothe RA, López de Munain A, Briscoe J, Serrano M and Lovell-Badge R: Oncogenicity of the developmental transcription factor Sox9. Cancer Res *72(5)*: 1301-1315, 2012. PMID: 22246670. DOI: 10.1158/0008-5472.CAN-11-3660

24 Brabletz T, Jung A and Kirchner T: Beta-catenin and the morphogenesis of colorectal cancer. Virchows Arch *441(1)*: 1-11, 2002. PMID: 12111194. DOI: 10.1007/s00428-002-0642-9

25 Malumbres M and Barbacid M: Mammalian cyclin-dependent kinases. Trends Biochem Sci *30(11)*: 630-641, 2005. PMID: 16236519. DOI: 10.1016/j.tibs.2005.09.005

26 Furukawa Y, Kikuchi J, Nakamura M, Iwase S, Yamada H and Matsuda M: Lineage-specific regulation of cell cycle control gene expression during haematopoietic cell differentiation. Br J Haematol *110(3)*: 663-673, 2000. PMID: 10997979. DOI: 10.1046/j.1365-2141.2000.02253.x

27 Takahashi Y, Fukui T, Kishimoto M, Suzuki R, Mitsuyama T, Sumimoto K, Okazaki T, Sakao M, Sakaguchi Y, Yoshida K, Uchida K, Nishio A, Matsuzaki K and Okazaki K: Phosphorylation of Smad2/3 at the specific linker threonine residue indicates slow-cycling esophageal stem-like cells before re-entry to the cell cycle. Dis Esophagus *29(2)*: 107-115, 2016. PMID: 25168378. DOI: 10.1111/dote.12277

28 Weidner N, Moore DH 2nd and Vartanian R: Correlation of Ki-67 antigen expression with mitotic figure index and tumor grade in breast carcinomas using the novel "paraffin"-reactive MIB1 antibody. Hum Pathol *25(4)*: 337-342, 1994. PMID: 8163266. DOI: 10.1016/0046-8177(94)90140-6

29 Wrighton KH, Willis D, Long J, Liu F, Lin X and Feng XH: Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor-beta signaling. J Biol Chem *281(50)*: 38365-38375, 2006. PMID: 17035229. DOI: 10.1074/jbc.M607246200

30 Juniku-Shkololli A, Manxhuka-Kerliu S, Ahmetaj H, Khare V and Zekaj S: Expression of immunohistochemical markers of progression in pre-cancerous and cancerous human colon: correlation with serum vitamin D levels. Anticancer Res *35(3)*: 1513-1520, 2015. PMID: 25750305.

31 Wolff RK, Hoffman MD, Wolff EC, Herrick JS, Sakoda LC, Samowitz WS and Slattery ML: Mutation analysis of adenomas and carcinomas of the colon: Early and late drivers. Genes Chromosomes Cancer *57(7)*: 366-376, 2018. PMID: 29575536. DOI: 10.1002/gcc.22539

32 Cui G, Xu G, Zhu L, Pang Z, Zheng W, Li Z and Yuan A: Temporal and spatial changes of cells positive for stem-like markers in different compartments and stages of human colorectal adenoma-carcinoma sequence. Oncotarget *8(28)*: 45311-45322, 2017. PMID: 28484082. DOI: 10.18632/oncotarget.17330

Received July 31, 2021
Revised August 21, 2021
Accepted August 23, 2021