Original Article

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

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Abstract

Background: Epithelial cells affected by somatic mutations undergo transition from a tumour-suppressive to a carcinogenic Smad pathway during sporadic colorectal carcinogenesis, and the specific linker threonine phosphorylation of Smad2/3 in colon epithelial cells indicates stem-like cells. This study extends previous observations to a model of colitis-associated colorectal cancer.

Methods: After Crl:CD-1 mice received an administration of azoxymethane [AOM], the mice were given dextran sodium sulfate [DSS] for 7 days. AOM/DSS-treated mice [AOM/DSS mice] were killed at 10 or 20 weeks. After macroscopic observations, a histopathological analysis was conducted. Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase method [pSmad3C-Ser, pSmad3L-Ser, c-Myc] and immunofluorescent methods [Ki67, β-catenin, cyclin D1, cyclin D1, Sox9, pSmad2/3L-Thr].

Results: The colons from AOM/DSS mice were shorter than those from control mice. The number of colon tumours at Week 20 was higher than at Week 10. The inflammation scores for AOM/DSS mice were greater than for control mice. Immunostaining-positive cells (staining by Ki67, β-catenin [nuclear and cytoplasmic], cyclin D1, and Sox9) were diffusely distributed in colon tumours. The percentage of pSmad3L-Ser-positive cells in colon tumours was higher than in sites of pre-neoplastic colitis, and that in sites of pre-neoplastic colitis was higher than in control mice. pSmad2/3L-Thr-positive cells were sparsely detected around crypt bases in non-neoplastic colon epithelia and at the tops of tumours, and immunohistochemical co-localisation of pSmad2/3L-Thr with Ki67 was not observed. Immunohistochemical co-localisation of pSmad2/3L-Thr with β-catenin and CDK4 was observed.

Conclusions: pSmad3L-Ser signalling is an early event in colitis-associated colorectal cancer, and pSmad2/3L-Thr immunostaining-positive cells might be cancer stem cells.

Keywords: Mouse model, colitis-associated colorectal cancer, carcinogenesis, cancer stem cell, Smad, phosphorylation
1. Introduction

Patients with ulcerative colitis [UC] are at increased risk for developing colorectal cancer [CRC]. The prevalence of UC-associated CRC has steadily increased worldwide. To date, the pathogenesis of UC-associated CRC remains unclear. It is suspected that chronic lesions [aberrant crypt foci] for CRC in rats.

ment with DSS enhances the development of putative precursor lesions [aberrant crypt foci] for CRC in rats. Additionally, certain drugs used to reduce inflammation may prevent the development of CRC.

The major carcinogenic pathways that lead to sporadic CRC, namely chromosomal instability, microsatellite instability, and hypermethylation, also occur in UC-associated CRC.

There have been various attempts to investigate the pathogenesis of UC-associated CRC using animal models. Dextran sodium sulfate [DSS] has been the most widely used chemical to induce colitis.

The DSS animal model is also available for examining UC-associated CRC. Intestinal tumours in rats fed 5% DSS developed between 134 and 215 days. Cooper et al. reported a relationship between the severity of DSS-induced inflammation and colorectal carcinogenesis similar to human UC-associated CRC histopathology. However, these studies require a long experimental period or repeated administration of DSS.

One previous study demonstrated that treatment with DSS enhances the development of putative precursor lesions [aberrant crypt foci] for CRC in rats. Tanaka et al. proposed a novel mouse model of colitis-associated CRC, using azoxymethane [AOM] and DSS [AOM/DSS]. In the AOM/DSS-treated mouse model, exposure to a single dose of AOM followed by 1-week treatment with 2% DSS could induce a number of CRCs within 20 weeks. Moreover, the first CRC was found as early as 6 weeks after administration of AOM.

Recently, cancer stem cell [CSC] theory has emerged as an attractive hypothesis for cancer development and progression. The theory suggests that cancers consist of subsets of cells with functional heterogeneity. In the CSC model, one small subset of cancer cells has the characteristics of stem cells. These CSCs have the capabilities of both self-renewal and differentiation into diverse cancer cells, and play a decisive role in maintaining capacity for malignant proliferation, invasion, metastasis, and cancer recurrence. Assuming CSCs are relatively resistant to the therapies developed to eradicate the non-CSC component of cancers, the CSC model provides a theoretical basis for developing therapies that target the minority CSC population and presents a new perspective for the treatment of cancer.

Smads, central mediators that convert signals from receptors for the transforming growth factor-β [TGF-β] superfamily members to the nucleus, are modular proteins with conserved Mad-homology 1, intermediate linker, and Mad-homology 2 domains. A catalytically active TGF-β type I receptor phosphorylates COOH-terminal serine [Ser] residues of receptor-activated Smads, which include Smad2 and Smad3, a highly similar protein.

The specific linker Ser or threonine [Thr] residues undergo regulatory phosphorylation by proline-directed [Ras-associated] kinases, including extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and cyclin-dependent kinase [CDK4].

TGFB type I receptor and proline-directed kinases differentially phosphorylate Smad2 and Smad3 to create some phosphosignals.

Additionally, certain drugs used to reduce inflammation may prevent the development of CRC.

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The present study extends our previous observations to a mouse model of colitis-associated CRC. This investigation of Smad2/3 phosphorylation profiles clarifies the proposition that additional carcinogenic pSmad3L-Ser signalling induced by chronic inflammation is an important early event in colitis-associated CRC. Furthermore, this study also explores whether pSmad2/3L-Thr can serve as a biomarker for CSCs of a mouse model of colitis-associated CRC.

2. Materials and Methods

2.1. Mice

Five-week-old male Crl:CD-1 [ICR] mice were purchased from Charles River Laboratories [Charles River Laboratories Japan, Yokohama, Japan]. All mice were bred at the Kansai Medical University animal facility under specific pathogen-free conditions. Mice were offered commercially available food pellets [F2; Funabashi Farm, Chiba, Japan] and tapwater. The Ethics Committee for the Use of Experimental Animals of Kansai Medical University approved all experimental protocols.

2.2. Chemicals

AOM, a colon carcinogen, was purchased from Sigma-Aldrich [Sigma-Aldrich Japan K.K., Tokyo, Japan]. DSS with a molecular weight of 36,000–50,000 was purchased from MP Biomedicals [MP Biomedicals, Solon, OH, USA]. DSS for induction of colitis was dissolved in water at a concentration of 2%.

2.3. Experimental design

ICR mice received a single intraperitoneal injection of AOM [10 mg/kg body weight]. One week after the AOM injection, the mice were given 2% DSS in their drinking water for 7 days. Mice treated with AOM/DSS [AOM/DSS mice] were killed 10 [Week 10; n = 11] or 20 [Week 20; n = 17] weeks after the AOM administration [Figure 1]. Control mice were untreated.

Figure 1. Experimental protocol for the establishment of a mouse model of colitis-associated colorectal cancer. 1, azoxymethane [AOM], 10 mg/kg body weight, intraperitoneal injection; 2, 2% dextran sodium sulfate [DSS] in drinking water—basic diet and tapwater.
Colons were flushed with saline, excised, cut open longitudinally along the main axis, and washed with saline again. The length of the colon [from the ileocecal junction to the anal verge] was measured. After macroscopic observations, they were cut and fixed in 10%-buffered formalin. Paraffin-embedded sections were made using routine procedures.

2.4. Histopathological analysis
Histopathological alterations were identified in haematoxylin and eosin–stained sections. Colitis was scored according to the following morphological criteria described by Cooper et al. \(^{21} \) 0, normal colonic mucosa; 1, loss of one-third of the basal crypts, with mild inflammation and oedema in the mucosa; 2, loss of two-thirds of the basal crypts, with moderate inflammation in the mucosa; 3, loss of all crypts, with severe inflammation in the mucosa, but with retention of the surface epithelium; and 4, loss of all crypts and the surface epithelium, with severe inflammation in the mucosa, muscularis propria, and submucosa. Colon neoplasms were diagnosed according to the description by Ward. \(^{33} \)

2.5. Domain-specific antibodies against the phosphorylated Smad2 and Smad3
Polyclonal anti-pSmad3C-Ser [Ser 423/425], anti-pSmad3L-Ser [Ser 208/213], and anti-pSmad2/3-L-Thr [Smad2: Thr 220, Smad3: Thr 179] sera were raised against the phosphorylated COOH-terminal and linker regions of Smad2 and Smad3 by immunising rabbits with synthetic peptides. \(^{26,30,34} \) Relevant antisera were affinity-purified using phosphorylated peptides as previously described. \(^{36} \)

2.6. Immunohistochemistry
Immunohistochemical staining was performed on formalin-fixed paraffin-embedded sections using the avidin-biotin immunoperoxidase method [pSmad3C-Ser, pSmad3L-Ser, c-Myc] and immunofluorescent method [Ki67, β-catenin, CDK4, cyclin D1, Sox9, pSmad2/3-L-Thr], as described previously. \(^{35} \) Briefly, paraffin-embedded sections were deparaffinised and rehydrated through xylene and ethanol washes. Non-enzymatic antigen retrieval was performed by heating sections to 121°C in a 0.01 \( \text{M} \) sodium citrate buffer [pH 6.0] for 36, 37 min. After incubation, sections were rinsed in Tris-buffered saline [TBS] and blocked with 3% bovine serum albumin in TBS for 5 min.

Primary antibodies [Abs] were diluted in TBS containing 0.1% Tween 20 and were incubated at 4°C in a humid chamber. The primary Abs used in this study included rabbit anti-pSmad3C-Ser, rabbit anti-pSmad3L-Ser, rabbit anti-c-Myc [Abcam, Cambridge, UK], rat anti-Ki67 [Dako, Glostrup, Denmark], mouse anti-β-catenin [BD Biosciences, San Jose, CA, USA], mouse anti-CDK4 [Santa Cruz Biotechnology, Santa Cruz, CA, USA], rabbit anti-cyclin D1 [Abcam], rabbit anti-Sox9 [Santa Cruz Biotechnology], and rabbit anti-pSmad2/3-L-Thr.

After incubation with the primary and biotin-conjugated secondary Abs using the avidin-biotin immunoperoxidase method [pSmad3C-Ser, pSmad3L-Ser, c-Myc], the sections were incubated with avidin-biotin peroxidase complex [ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA]. Then, sections were reacted with a fresh mixture of 0.05% 3,3′-diaminobenzidine and 0.005% \( \text{H}_2\text{O}_2 \) in TBS. Nuclei were counterstained with haematoxylin.

In the immunofluorescent method [Ki67, β-catenin, CDK4, cyclin D1, Sox9, pSmad2/3-L-Thr], appropriate species-specific AlexaFluor 488 or 568–conjugated Abs [Invitrogen, Carlsbad, CA, USA] were used as the secondary Abs. Slides were mounted with VECTASHIELD Mounting Medium with DAPI [4′,6-diamidino-2-phenylindole] [Vector Laboratories] to counterstain nuclei and preserve fluorescence. Images were obtained with a fluorescence microscope [Olympus, Tokyo, Japan].

Well-oriented portions from the base to the surface of the mucosa and tumour were selected for counting immunostaining–positive cells in 10 successive crypts for each colon, and results were averaged.

2.7. Statistical analysis
All values were expressed as mean (± standard error of the mean [SEM]). Data were analysed by one-way analysis of variance followed by Fisher’s protected least significant difference test. A \( p \)-value of less than 0.05 was accepted as statistically significant.

3. Results
3.1. Macroscopic observations
As compared with colons from control mice [Figure 2a], flat, nodular, polyoid, or polyp-like colon tumours were found in the middle and distal colons from AOM/DSS mice at Weeks 10 [Figure 2b] and 20 [Figure 2c]. The mean colon lengths for AOM/DSS mice at Weeks 10 [10.96 ± 0.28 cm, \( n = 11 \)] and 20 [11.17 ± 0.30 cm, \( n = 17 \)] were significantly lower than for control mice [12.35 ± 0.20 cm, \( n = 10 \)]. [Figure 2d, \( p < 0.01 \)]. The mean number of colon tumours per mouse at Week 20 [10.18 ± 1.43, \( n = 17 \)] was significantly larger than that at Week 10 [4.55 ± 0.56, \( n = 11 \)]. [Figure 2e, \( p < 0.01 \)].

3.2. Microscopic observations
In contrast to control mice [Figure 3a], pre-neoplastic colitis was identified in the middle and distal colons in AOM/DSS mice at Weeks 10 [Figure 3b] and 20 [Figure 3d]. There was shortening of crypts with infiltration of inflammatory cells and oedematous change. The inflammation scores at Weeks 10 [1.55 ± 0.25, \( n = 11 \)] and 20 [1.24 ± 0.11, \( n = 17 \)] were thus greater than those of control mice [0.00 ± 0.00, \( n = 10 \)]. [Figure 3g, \( p < 0.001 \)]. Although there was no significant difference between AOM/DSS mice at Weeks 10 and 20 [\( p = 0.14 \)], the mean score at Week 20 was somewhat smaller than that at Week 10.

Most colon tumours at Weeks 10 [Figure 3c] and 20 showed the characteristics of intramucosal adenocarcinoma. The nuclei were enlarged, round, or ovoid, with prominent nucleoli; nuclear polarity was almost completely lost; numerous mitoses were present; and goblet cells were completely absent. A few of them were observed to invade beyond the muscularis mucosae [Figure 3e, f].

3.3. Immunofluorescent staining against markers involved with proliferation, neoplastic transformation, and stem cells
Ki67-positive cells were detected around crypt bases in both normal mucosae of control mice [Figure 4a] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 4b]. In colon tumours at Weeks 10 [Figure 4c] and 20 [Figure 4d], Ki67-positive cells were diffusely distributed throughout the tumours.

Immunofluorescent staining of β-catenin showed weak positivity in the cell membrane of both normal mucosae of control mice [Figure 4e] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 4f]. In colon tumours from AOM/DSS mice at Weeks 10 [Figure 4g] and 20 [Figure 4h], strongly β-catenin-positive cells were distributed all around the tumours, and their expression was observed predominantly in the nucleus and cytoplasm of tumour cells.
Strongly CDK4-positive cells were sparsely detected around crypt bases in both normal mucosae of control mice [Figure 4i] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 4j]. In colon tumours at Weeks 10 [Figure 4k] and 20 [Figure 4l], strongly CDK4-positive cells were scattered throughout the tumours.

Immunofluorescent staining of cyclin D1 showed no positive cells in either normal mucosae of control mice [Figure 4m] or sites of pre-neoplastic colitis from AOM/DSS mice at Weeks 10 and 20 [Figure 4n]. In colon tumours at Weeks 10 [Figure 4o] and 20 [Figure 4p], strongly cyclin D1-positive cells were distributed all around the tumours, and their expression was observed predominantly in the nuclei of tumour cells.

Sox9-positive cells were detected around crypt bases in both normal mucosae from control mice [Figure 4q] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 4r]. In colon tumours at Weeks 10 [Figure 4s] and 20 [Figure 4t], Sox9-positive cells were diffusely distributed throughout the tumours.

3.4. Immunohistochemical staining against markers involved with Smad3 phosphoisoform-mediated signalling

Immunohistochemical staining for pSmad3C-Ser uniformly showed many positive cells in each crypt in both normal mucosae from control mice [Figure 5a] and sites of pre-neoplastic colitis in AOM/DSS
mice at Weeks 10 and 20 [Figure 5b]. In colon tumours at Weeks 10 [Figure 5c] and 20 [Figure 5d], pSmad3C-Ser-positive cells were distributed all around the tumours, and their expression was observed in tumour cell nuclei. The percentages of pSmad3C-Ser-positive cells in control mice [83.39 ± 2.07%, n = 10], sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 [81.72 ± 1.04%, n = 28], and colon tumours from AOM/DSS mice at Weeks 10 and 20 [83.51 ± 1.13%, n = 28] showed no significant differences [Figure 5m].

Immunohistochemical staining of pSmad3L-Ser showed some positive cells predominantly in each crypt base in normal mucosae from control mice [Figure 5e], and more positive cells broadly in sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 5f]. In colon tumours at Weeks 10 [Figure 5g] and 20 [Figure 5h], pSmad3L-Ser-positive cells were distributed all around the tumours, and their expression was observed in the tumour cell nuclei. The percentage of pSmad3L-Ser-positive cells in sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [72.26 ± 1.25%, n = 28] was significantly greater than that of control mice [63.62 ± 4.21%, n = 10] [Figure 5n, p < 0.01]. Furthermore, the percentage of pSmad3L-Ser-positive cells in colon tumours from AOM/DSS mice at Weeks 10 and 20 [90.30 ± 0.64%, n = 28] was

Figure 3. Histopathology of colons from [a] control mice, [b, c] AOM/DSS mice at Week 10, and [d, e, f] AOM/DSS mice at Week 20; and [g] colon inflammation score [0–4]. Pre-neoplastic colitis was found in the middle and distal colons in AOM/DSS mice at Weeks 10 [b] and 20 [d]. Most of the colon tumours showed the characteristics of intramucosal adenocarcinoma [c]. A few were observed to invade beyond the muscularis mucosae [e, f]. Histopathology was conducted using haematoxylin and eosin stain; original magnification, ×100 [a–e] and ×40 [f]. Scale bars: 50 μm [a–e] and 200 μm [f]. White, grey, and black bars represent control mice, AOM/DSS mice at Week 10, and AOM/DSS mice at Week 20, respectively. Data are expressed as the means (± standard error of the mean [SEM]) of 10 control mice, 11 AOM/DSS mice at Week 10, and 17 AOM/DSS mice at Week 20. Results were compared using a one-way analysis of variance followed by Fisher’s protected least significant difference test [***p < 0.001; N.S., not significant].
significantly greater than that in sites of pre-neoplastic colitis at Weeks 10 and 20 \( p < 0.001 \). Immunohistochemical staining of c-Myc showed several positive cells predominantly in each crypt base in normal mucosae of control mice [Figure 5i], and more positive cells on sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 5j]. In colon tumours at Weeks 10 [Figure 5k] and 20 [Figure 5l], c-Myc-positive cells were distributed all around the tumours. The expression patterns of c-Myc were similar to those of pSmad3L-Ser.

3.5. Double immunofluorescent staining for pSmad2/3L-Thr with markers involved in proliferation and neoplastic transformation

pSmad2/3L-Thr-positive cells were sparsely detected around crypt bases in both normal mucosae of control mice [Figure 6a] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 6b]. Although pSmad2/3L-Thr-positive cells were observed beside and between Ki67-positive cells, immunohistochemical co-localisation of pSmad2/3L-Thr with Ki67 was not observed. Also, in colon tumours from AOM/DSS mice at Weeks 10 [Figure 6c] and 20 [Figure 6d], pSmad2/3L-Thr-positive cells were sparsely detected beside and between Ki67-positive cells within tumour cells, and immunohistochemical co-localisation of pSmad2/3L-Thr with Ki67 was not observed.

Immunohistochemical co-localisation of pSmad2/3L-Thr with β-catenin was observed around crypt bases in both normal mucosae from control mice [Figure 6e] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 6f]. However, immunofluorescent staining was positive for pSmad2/3L-Thr and β-catenin in the nuclei and cell membranes, respectively. Immunohistochemical co-localisation of pSmad2/3L-Thr with β-catenin was observed in colon tumours from AOM/DSS mice at Weeks 10 [Figure 6g] and 20 [Figure 6h]. Nuclei and nuclei or cytoplasm of tumour cells showed positive immunofluorescent staining of pSmad2/3L-Thr and β-catenin, respectively.

Immunohistochemical co-localisation of pSmad2/3L-Thr with CDK4 was observed around crypt bases in both normal mucosae from control mice [Figure 6i] and sites of pre-neoplastic colitis in AOM/DSS mice at Weeks 10 and 20 [Figure 6j]. In colon tumours from AOM/DSS mice at Weeks 10 [Figure 6k] and 20 [Figure 6l], immunohistochemical co-localisation of pSmad2/3L-Thr with CDK4 was observed in tumour cell nuclei. After dividing colon tumours \( n = 28 \) longitudinally into three sections of equal length, more pSmad2/3L-Thr-positive cells were detected at the tumour top \([79.70 ± 3.69\%]\) [Figure 6m, \( p < 0.001 \)] than at the tumour base \([8.54 ± 1.79\%]\) and at the middle \([11.76 ± 2.72\%]\).

4. Discussion

UC-associated CRC is closely related to long-standing tissue injury and chronic inflammation. The relationship between chronic inflammation and carcinoma dates back to Virchow, who hypothesised that the origin of carcinoma was at sites of chronic inflammation. Although the relationship has been well established in general, the molecular mechanisms involved in the process remain unclear. Chronic inflammation leads to increased oxidative stress. Leukocytes and other phagocytic cells generate reactive oxygen and nitrogen species that can damage epithelial cells. Chronic inflammation also promotes the apoptosis of normal epithelial cells, which can lead to a compensatory proliferative response by the remaining tissue. In AOM/DSS mice, pre-neoplastic colitis was found persistently even 20 weeks after the AOM injection [18 weeks after the DSS administration]. Colon tumours were predominantly located...
in the distal colon of AOM/DSS mice, where DSS colitis was most severe. With a longer duration of the inflammation, the number of colon tumours increased significantly. These results are consistent with characteristics of UC-associated CRC.

Ki67 antigen is a nuclear matrix protein that is expressed in proliferating cells, but not in quiescent cells. In non-neoplastic colon epithelia from control and AOM/DSS mice, Ki67-positive cells were observed around crypt bases. The zone of the crypt in which Ki67-positive cells are located is referred to as the proliferating zone. However, in neoplastic colon tumours, Ki67-positive cells were observed throughout the tumours.

β-catenin is a key component of the cadherin-mediated cell-cell adhesion system and an important molecule in the Wnt-APC signal transduction system. It regulates transcription of genes related to growth, development, and differentiation of cells. Although β-catenin is detected in the cell membrane in non-neoplastic colon epithelial cells, accumulation of nuclear and cytoplasmic β-catenin is associated with colon carcinogenesis. D-type cyclins have been discovered to be factors whose expression is increased by growth signals. They are considered to be mediators of signalling that link extracellular stimuli to cell-cycle machinery. In AOM-induced mouse colon tumour, nuclear cyclin D1 overexpression is observed. In accordance with these earlier studies, we detected nuclear and cytoplasmic β-catenin accumulation and nuclear cyclin D1 overexpression in neoplastic colon tumours from AOM/DSS mice.

Sox9 is a high-mobility group box transcription factor that is expressed in the progenitor and stem cell zone of the small intestine and colon. In non-neoplastic colon epithelia from control and AOM/DSS mice, Sox9-positive cells were observed around crypt bases. These cells are expected to include transit-amplifying progenitor and colon epithelial stem cells. In neoplastic colon tumours from AOM/DSS mice, Sox9-positive cells were diffusely observed in most of the tumour cells. Sox9 is thought to have important roles for carcinogenesis, and it is overexpressed in a wide range of human cancers including CRC where its expression is correlated with malignant character and progression.

**Figure 5.** Immunohistochemical staining using [a–d] pSmad3C-Ser, [e–h] pSmad3L-Ser, and [i–l] c-Myc in control mice [a, e, i], sites of pre-neoplastic colitis in AOM/DSS mice [b, f, j], colon tumours from AOM/DSS mice at Week 10 [c, g, k], and colon tumours from AOM/DSS mice at Week 20 [d, h, l]. Haematoxylin was used for nuclear staining. Insets show enlargements of the selected areas. Original magnification, ×200 [a–l]. Scale bars: 50 μm. [m] Percentage of pSmad3C-Ser-positive cells. [n] Percentage of pSmad3L-Ser-positive cells. White, grey, and black bars represent control mice, sites of pre-neoplastic colitis in AOM/DSS mice, and colon tumours from AOM/DSS mice, respectively. Data are expressed as the means (± standard error of the mean [SEM]) of 10 control mice, 28 sites of pre-neoplastic colitis in AOM/DSS mice, and 28 colon tumours from AOM/DSS mice. Results were compared using a one-way analysis of variance followed by Fisher’s protected least significant difference test [∗∗p < 0.01 and ∗∗∗p < 0.001; N.S., not significant].
D-type cyclins form complexes with CDK4 or CDK6. These complexes phosphorylate and inactivate the retinoblastoma protein family. This process is indispensable for progression of the cell cycle from the G0-G1 transition to the S phase. Matsuura et al. reported that active complexes of CDK4 and D-type cyclins also phosphorylate the specific sites of Smad3 [and, presumably, homologous Smad2] and facilitate progression of the cell cycle from the G1 [or G0] to the S phase. Interestingly, the anti-pSmad2/3L-Thr sera used in the present study recognise the same linker phosphorylation of Smad3 as the specific phosphorylation of Thr residue reported by Matsuura et al. Furukawa et al. reported that CD34-positive haematopoietic stem cells in the G0 phase generally suppress expression of CDKs and cyclins, except for CDK4. Also in this study, immunohistochemical co-localisation of pSmad2/3L-Thr with CDK4 was observed in non-neoplastic epithelia at the crypt base. In addition, our previous studies have already demonstrated that pSmad2/3L-Thr-positive cells were BrdU label-retaining, slow-cycling, and Ki67-negative quiescent cells in the G0 phase, and were located adjacent to actively proliferating cells [in the proliferating zone]. Taken together, CDK4 and D-type cyclins may phosphorylate Smad3 [and Smad2] at the specific linker Thr residue at the beginning of the G0-G1 transition, and these cells may transiently express more pSmad2/3L-Thr than any other cells. Therefore, we have consistently proposed that pSmad2/3L-Thr is useful for identifying stem-like cells immediately before they re-enter the cell cycle from the dormant state of the G0 phase in the oesophagus, stomach, small intestine, and colon.

According to the CSC theory, a minority of undifferentiated cancer cells, which exhibit characteristics similar to normal stem cells, drive tumour growth and spread. Although CSCs have the capacity for self-renewal, they are comparatively quiescent. They have proliferative capacity but are often not cycling. They have been shown to have significantly longer cell-cycle times than proliferating non-CSCs. This is presumably due to the arrest of CSCs at the G0 phase. Although CRC has been intensively studied, the cell of origin for cancer formation is still poorly understood. Two possible hypotheses have been suggested: the bottom-up and top-down theories.
The bottom-up theory proposes that a normal stem cell is the first transformed cell, giving rise directly to cancer cells or reprogramming itself and acquiring CSC behaviour before inducing cancer. However, histological evidence suggests the top-down model of CRC development, in which CRC might arise from late progenitors or even an early differentiated cell.

The top-down model is supported by the finding that dysplastic cells are routinely found at the luminal surface of the crypts, whereas the cells at the bases of these same crypts appear morphologically normal in the early stages of tumourigenesis. Aberrant crypt foci, which are putative precursor lesions for CRC, in rats treated with AOM and/or DSS are observed by surface examination of the luminal mucosa of colon preparations stained with methylene blue. Furthermore, Schwitalla et al. have suggested that these theories need not exclude each other and that tumour-initiating mutations can occur in both normal stem cells and more differentiated cells, as long as both cells de-differentiate and re-acquire stem cell properties triggered by chronic inflammatory signalling. They have demonstrated that epithelial non-stem cells can re-express stem cell markers and be converted into CSCs, and have provided an additional explanation of why patients with UC have an elevated risk of developing CRC. In neoplastic colon tumours from AOM/DSS mice, pSmad2/3L-Thr-positive cells were present in very small numbers and were significantly detected around the tumour top. As expected in the tumours, immunohistochemical co-localisation of pSmad2/3L-Thr with β-catenin and CDK4 were observed, whereas co-localisation with Ki67 was not observed. In non-neoplastic and neoplastic epithelial cells, pSmad2/3L-Thr-positive cells have consistently indicated stem-like properties. There is a possibility that pSmad2/3L-Thr is a stem-like cell biomarker in other organs and in CSC. Future studies should try further to ascertain that pSmad2/3L-Thr-positive cells may be CSCs in neoplastic lesions.

We previously showed that TGF-β transmitted a signal through TGF-β type I receptor-dependent pSmad3C-Ser signalling, participating in the cytostatic response by repressing transcriptional activity of the c-myc gene. Proline-directed [Ras-associated] kinase-dependent pSmad2/3L-Ser signalling was able to provide carcinogetic potential to epithelial cells via up-regulation of c-myc, plasminogen activator inhibitor-1, and matrix metalloproteinases-1, -2, and -9, resulting in strongly enhanced tumour growth and invasion. Taken together, domain-specific phosphorylation of Smad2 and Smad3 is a key determinant regulating transcriptional activation of several target genes, ultimately selecting either tumour suppression or carcinogenesis. In accordance with our previous investigations, the percentage of pSmad3L-Ser-positive cells in neoplastic colon tumours from AOM/DSS mice was significantly increased compared with that in non-neoplastic epithelia from control and AOM/DSS mice. Furthermore, the percentage of pSmad3L-Ser-positive cells in pre-neoplastic colitis epithelia from AOM/DSS mice was significantly increased compared with normal epithelia from control mice. As for immunohistochemical staining of c-Myc, a small number of c-Myc-positive cells were observed in crypt bases in non-neoplastic colon epithelia in control mice, but many c-Myc-positive cells were observed throughout the tumours of AOM/DSS mice. In comparison with immunoreactivities of Ki67, β-catenin, cyclin D1, and Sox9, pSmad3L-Ser-immunoreactivity is the earliest carcinogenic event, observed even in the pre-neoplastic state of the mouse model of colitis-associated CRC.

In conclusion, pSmad3L-Ser signalling correlates with carcinogenesis of colon tumours from AOM/DSS mice [i.e. in colitis-associated CRC], and this work supports the hypothesis that pSmad2/3L-Thr immunostaining-positive cells are CSCs.

Conflict of interest
The authors have declared that no conflicts of interest exist.

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References


39. Weidner N, Moore DH II, Vartanian R. Correlation of Ki-67 antigen expression with mitotic figure index and tumor grade in breast carci-

40. Miller JR, Moon RT. Signal transduction through beta-catenin and spec-


