

SOX9 Is Highly Expressed in Nonampullary Duodenal Adenoma and Adenocarcinoma in Humans

Hirotsugu Sakamoto, Hiroyuki Mutoh, Yoshimasa Miura, Miho Sashikawa, Hironori Yamamoto, and Kentaro Sugano

Division of Gastroenterology, Department of Medicine, Jichi Medical University, Shimotsuke, Japan

Background/Aims: SOX9 is a marker for stem cells in the intestine, and overexpression of SOX9 is found in gastric and colon cancer; however, the expression of SOX9 in non-ampullary duodenal adenoma and adenocarcinoma has not yet been evaluated. This study aimed to investigate SOX9 expression in nonampullary duodenal adenoma and adenocarcinoma by immunohistochemistry. **Methods:** We evaluated SOX9 expression in 43 clinical samples (nonampullary duodenal adenoma in 22 lesions and nonampullary duodenal adenocarcinoma in 21 lesions) resected under endoscopic mucosal resection or endoscopic submucosal dissection. **Results:** SOX9 was expressed in part of the base of the normal duodenal mucosa surrounding adenomas and adenocarcinomas. In contrast, SOX9-positive cells were found in more than half of the crypts from the bottom part of the crypt in all of the 43 samples. Moreover, in 15 adenoma samples (68.2%) and 19 carcinoma samples (90.5%), SOX9 was expressed in more than three-quarters of the crypts from the bottom part of the crypt. **Conclusions:** SOX9 is overexpressed in nonampullary duodenal adenoma and adenocarcinoma in humans. (*Gut Liver* 2013;7:513-518)

Key Words: SOX9; Duodenal adenoma; Duodenal adenocarcinoma

INTRODUCTION

Nonampullary duodenal adenoma and adenocarcinoma are uncommon lesions.¹⁻³ With the widespread use of endoscopy, the number of reports of endoscopic therapy for these lesions has recently increased.⁴⁻⁸ We previously reported the efficacy and safety of endoscopic resection for nonampullary duodenal adenoma and adenocarcinoma.⁶ However, the prognosis of advanced nonampullary duodenal adenocarcinoma is still poor.²

Sry (sex determining region Y)-box 9 (SOX9), a member of the Sry-related high-mobility group box transcription factors, is expressed in the nuclei of proliferating stem/progenitor cells found at the bottom third of intestinal crypts from the duodenum to the distal colon.^{9,10} SOX9 is linked to progenitor status in adult liver, exocrine pancreas and intestine and is considered to maintain a precursor cell population during physiological cell replacement and/or regeneration after injury.¹¹ SOX9 is one of the transcriptional target genes of the Wnt signaling pathway.¹² An essential event in the Wnt signaling cascade is the accumulation and nuclear translocation of β -catenin, which then interacts with members of the Lef/Tcf family of transcription factors to activate target genes.¹³

Inactivation of the *SOX9* gene in the intestinal epithelium affects differentiation throughout the intestinal epithelium, with disappearance of Paneth cells and a decrease in goblet cell lineage. Additionally, the morphology of the colon is altered with the appearance of hyperplasia and local crypt dysplasia, suggesting a regulating function for SOX9 in cell proliferation.¹² Moreover, SOX9 is expressed in colon cancer^{9,10,14,15} and gastric cancer.¹⁶ A relationship between these cancers and SOX9 suggests that nonampullary duodenal adenocarcinoma may also be related to SOX9 expression. Therefore, we investigated SOX9 expression in nonampullary duodenal adenoma and adenocarcinoma using clinical specimens (duodenal lesions and adjacent tissues for controls) resected endoscopically.

MATERIALS AND METHODS

1. Patients and samples

By using our endoscopy database, we identified all patients in whom a nonampullary duodenal adenoma or adenocarcinoma was resected using endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) between August 2005

Correspondence to: Hiroyuki Mutoh

Division of Gastroenterology, Department of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke 329-0498, Japan
Tel: +81-285-58-7348, Fax: +81-285-44-8297, E-mail: muto@jichi.ac.jp

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Table 1. Patient Characteristics

Characteristic	Adenoma	Carcinoma	Total
Total no. of patients	18 (47.4)	20 (52.6)	38 (100)
Age, yr	54.3±11.6 (24-69)	65.2±10.1 (45-83)	60.0±12.0 (24-83)
Sex			
Female	7 (38.9)	14 (70.0)	21 (55.3)
Male	11 (61.1)	6 (30.0)	17 (44.7)
Total no. of lesions	22 (51.2)	21 (48.8)	43 (100)
Method			
EMR	7 (31.8)	4 (19.0)	11 (25.6)
ESD	15 (68.2)	17 (81.0)	32 (74.4)

Data are presented as number (%) or mean±SD (range).

EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection.

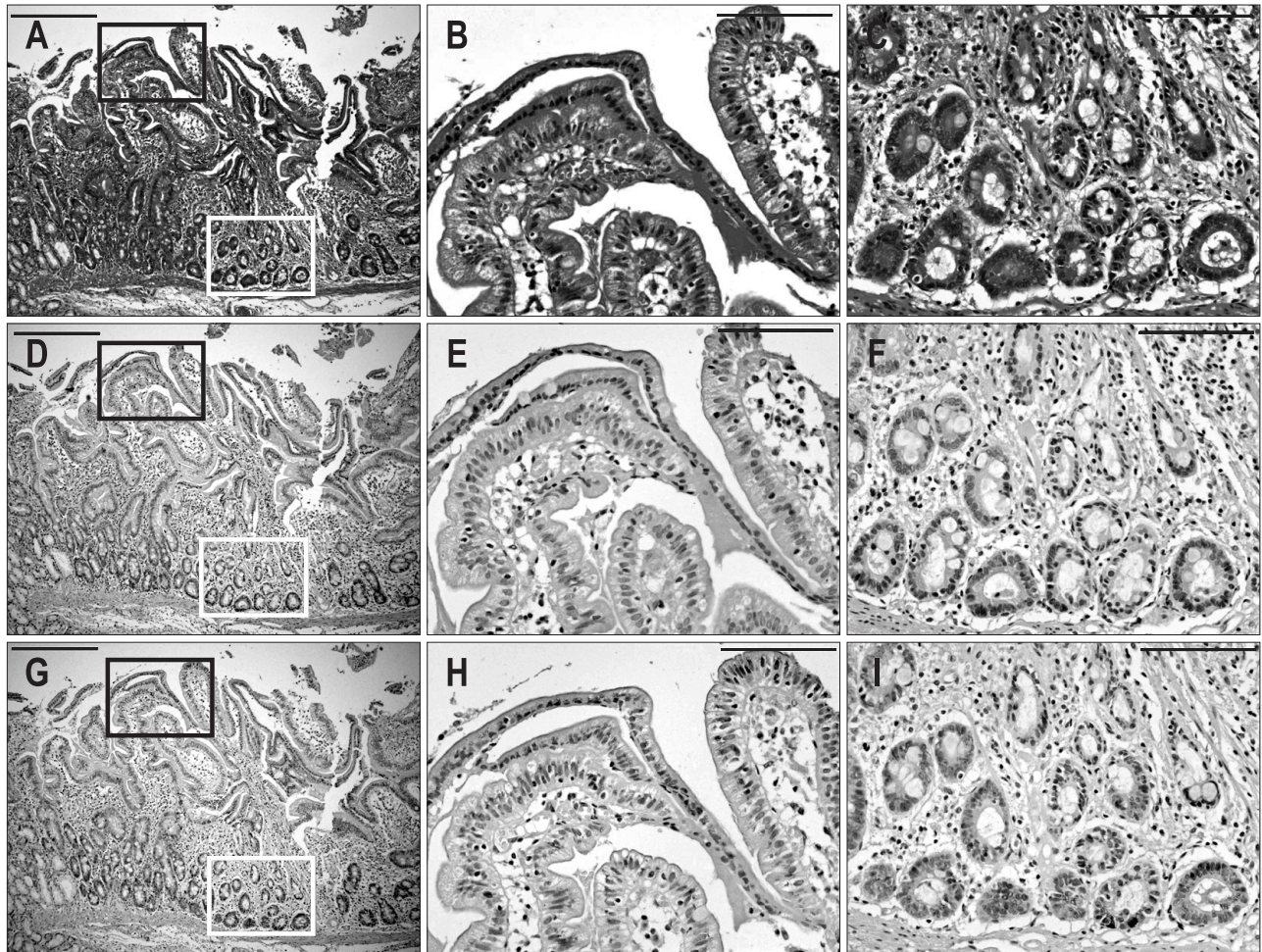


Fig. 1. (A, B, C) Hematoxylin and eosin staining, (D, E, F) immunohistochemical staining for SOX9, and (G, H, I) proliferating cell nuclear antigen (PCNA) in normal duodenal mucosa. Panels A, D, and G are serial sections for normal duodenal mucosa. The areas surrounded by a yellow square in panels A, D, and G are magnified in panels B, E, and H, respectively. The areas surrounded by a green square in panels A, D, and G are magnified in panels C, F, and I, respectively. SOX9 and PCNA are expressed in the lower third of the normal duodenal mucosa. Scale bar: A, D, and G, 300 μ m; B, C, E, F, H, and I, 100 μ m.

and February 2011, at Jichi Medical University Hospital. The diagnoses were confirmed by at least two pathologists. All of the samples were fixed by paraffin. Approval from the Institute

Research Ethics Committee was obtained for the use of clinical materials described in the present study. Informed consent was obtained from all patients capable of completing the full diag-

nostic workup, and/or their parents, before the procedure, and the risk was explained to them in accordance with the Institutional Review Board guidelines.

2. Histopathology and immunohistochemistry

Duodenal tissue specimens were fixed in neutral-buffered 10% formalin for 12 to 24 hours, washed in 70% ethanol, processed by standard methods, embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin (for histopathological assessment).

Sections were used for immunohistochemistry as described previously.^{17,18} Primary antisera were diluted in phosphate buffered saline (PBS) and incubated overnight at 4°C. The next day, slides were washed in PBS and incubated with horseradish peroxidase-labeled secondary antibody at 37°C for 60 minutes. Color development was carried out by incubating the

sections with 3,3-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) as the chromogenic substrate. Finally, the sections were lightly counterstained with hematoxylin, mounted and viewed under a light microscope.

Our panel of primary antisera included the anti-SOX9 antibody (dilution of 1:1,000, rabbit polyclonal; Millipore, Temecula, CA, USA) and the antiproliferating cell nuclear antigen (anti-PCNA) antibody (dilution of 1:2,000, mouse monoclonal; Sigma-Aldrich, St. Louis, MO, USA).

3. Statistical analysis

Univariate analysis was performed with Fisher's exact test for the comparison of expression frequencies among the groups. The 2-sided p-value less than 0.05 was considered statistically significant. All statistical analyses were carried out using Excel Tokei 2010 for Microsoft Excel 2010 (SSRI, Tokyo, Japan).

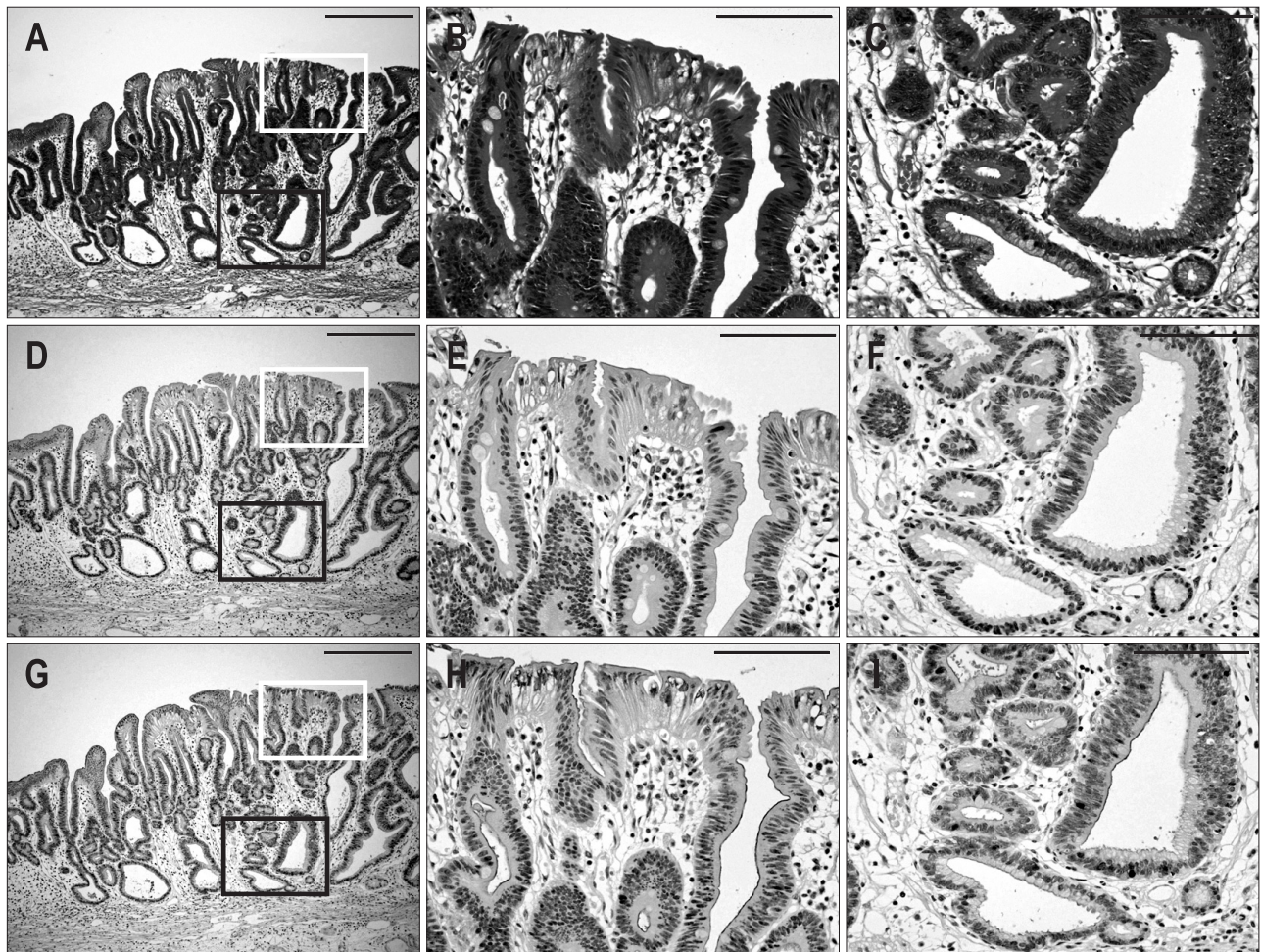


Fig. 2. (A, B, C) Hematoxylin and eosin staining, (D, E, F) immunohistochemical staining for SOX9, and (G, H, I) proliferating cell nuclear antigen (PCNA) in nonampullary duodenal adenoma. Panels A, D, and G are serial sections for nonampullary duodenal adenoma. The areas surrounded by a yellow square in panels A, D, and G are magnified in panels B, E, and H, respectively. The areas surrounded by a green square in panels A, D, and G are magnified in panels C, F, and I, respectively. SOX9 and PCNA are expressed in most of the nonampullary duodenal adenoma cells. Scale bar: A, D, and G, 300 μm ; B, C, E, F, H, and I, 100 μm .

RESULTS

1. Patient characteristics

Thirty-eight patients were included in this study. A total of 22 nonampullary duodenal adenomas (18 patients) and 21 nonampullary duodenal adenocarcinomas (20 patients) were resected during the study period. We did not find any patients who had both nonampullary duodenal adenoma and adenocarcinoma. The mean age of the 38 patients (21 males and 17 females) was 60.0 ± 12.0 years (range, 24 to 83 years). EMR was performed in 11 lesions (25.6%) and ESD was performed in 32 lesions (74.4%) (Table 1).

2. Restricted expression of SOX9 in normal human duodenum

SOX9 is a reported biomarker of crypt stem or progenitor cells and a target of β -catenin/TCF-activated transcription in the intestine. We investigated the expression of SOX9 in normal

duodenal mucosa (tissue adjacent to all duodenal tumors) (Fig. 1A–C). SOX9-positive cells were found in the bottom one-third of the crypt in all of the 43 samples (Fig. 1D–F). To investigate whether SOX9-positive cells at the base of the duodenal mucosa were proliferative, PCNA immunostaining was performed to visualize actively cycling cells using adjacent sections. SOX9-positive epithelial cells in the duodenal mucosa were actively cycling because SOX9-positive cells were positive for PCNA in adjacent sections of duodenal mucosa (Fig. 1G–I).

3. Immunohistochemical staining for SOX9 in human nonampullary duodenal adenoma and adenocarcinoma

We then studied the expression of SOX9 and PCNA in duodenal adenoma (Fig. 2A–C) and adenocarcinoma (Fig. 3A–C), using clinical specimens resected endoscopically. Immunohistochemical analysis for nonampullary duodenal adenoma and adenocarcinoma samples showed that nuclear SOX9-positive cells were found in more than half of the crypts from the bottom

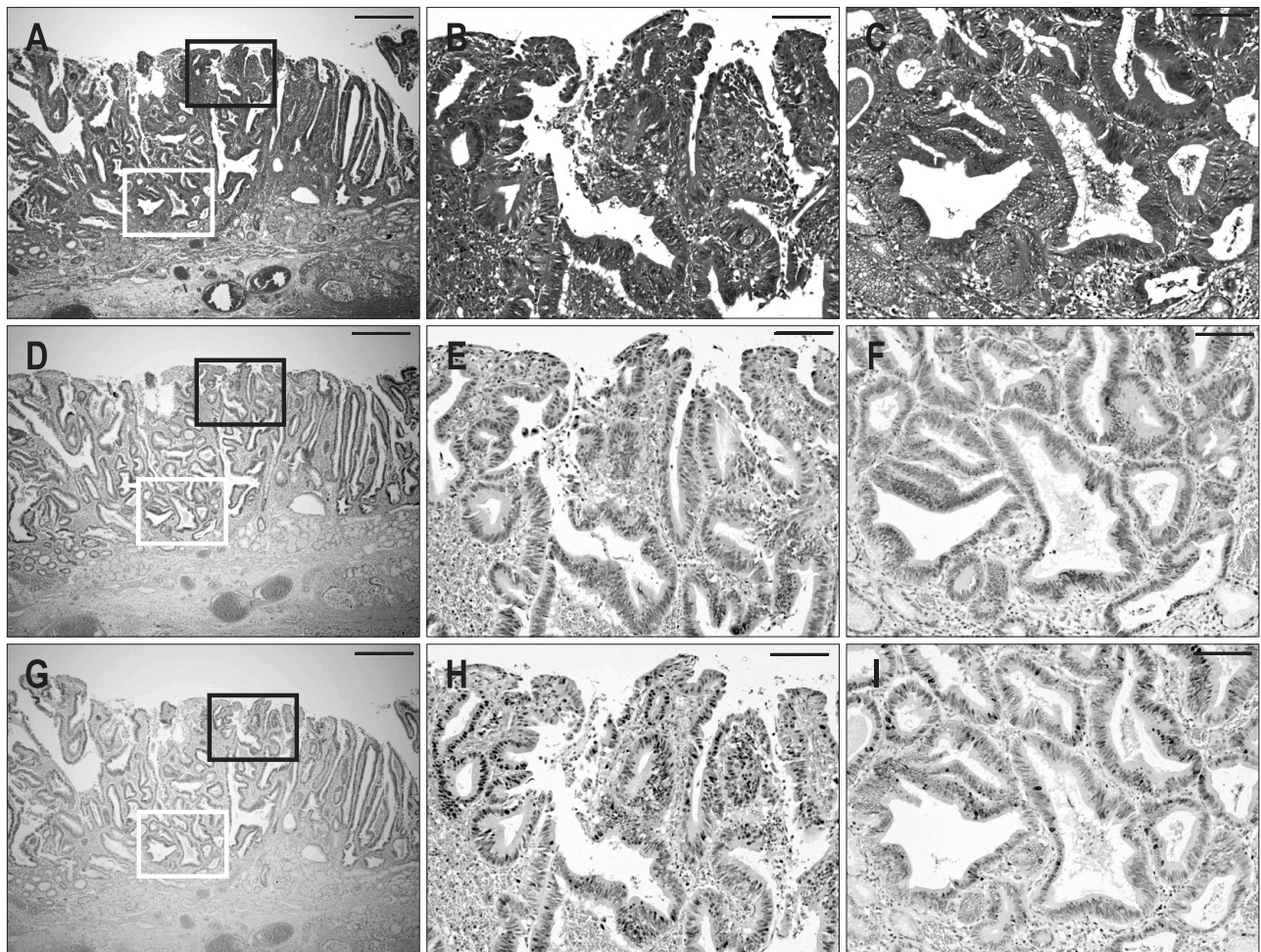


Fig. 3. (A, B, C) Hematoxylin and eosin staining, (D, E, F) immunohistochemical staining for SOX9, and (G, H, I) and proliferating cell nuclear antigen (PCNA) in nonampullary duodenal adenocarcinoma. Panels A, D, and G are serial sections for nonampullary duodenal adenocarcinoma. The areas surrounded by a yellow square in panels A, D, and G are magnified in panels B, E, and H, respectively. The areas surrounded by a green square in panels A, D, and G are magnified in panels C, F, and I, respectively. SOX9 and PCNA are expressed in most of the nonampullary duodenal adenocarcinoma cells. Scale bar: A, D, and G, 500 μ m; B, C, E, F, H, and I, 100 μ m.

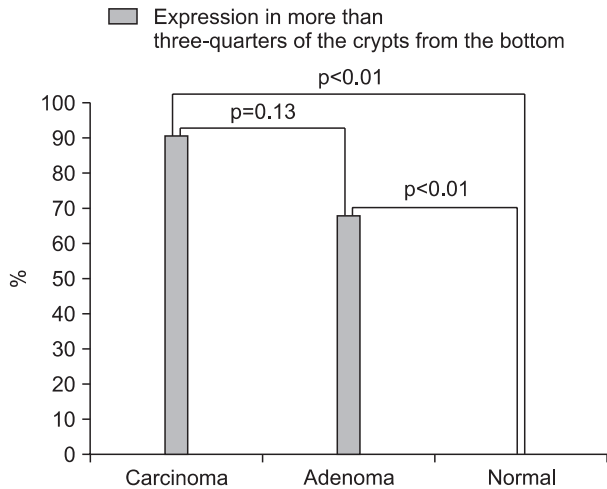


Fig. 4. SOX9 expression. In 15 samples of adenoma (68.2%) and 19 samples of carcinoma (90.5%), SOX9 was expressed in more than three-quarters of the crypts from the bottom part of the crypt. Significant differences were observed between adenoma and normal duodenal mucosa ($p<0.01$, Fisher exact test) and between carcinoma and normal duodenal mucosa ($p<0.01$, Fisher exact test); however, there was no significant difference between adenoma and carcinoma (68.2% vs 90.5%; $p=0.13$, Fisher exact test).

part of the crypt in all of the 43 samples (Figs 2D-F and 3D-F). Moreover, in 15 samples of adenoma (68.2%) and 19 samples of carcinoma (90.5%), SOX9 was expressed in more than three-quarters of the crypts from the bottom part of the crypt (Fig. 4). There were significant differences between adenoma and normal duodenal mucosa ($p<0.01$, Fisher's exact test), and between carcinoma and normal duodenal mucosa ($p<0.01$, Fisher's exact test). However, there was no significant difference between adenoma and carcinoma (68.2% vs 90.5%; $p=0.13$, Fisher's exact test). There was no cytoplasmic SOX9 expression in any of the samples. PCNA expression was similar to SOX9 expression in adenoma (Fig. 2G-I) and adenocarcinoma (Fig. 3G-I).

DISCUSSION

We clarified SOX9 expression in both duodenal adenoma and adenocarcinoma by examining 43 clinical samples (nonampullary duodenal adenoma in 22 lesions and nonampullary duodenal adenocarcinoma in 21 lesions) resected by EMR or ESD. We found that SOX9 is overexpressed in nonampullary duodenal adenoma and adenocarcinoma in humans.

SOX9 is also highly expressed in cells within human colon adenoma and adenocarcinoma.^{9,10,14,15} The function of SOX9 in adenoma and adenocarcinoma is unknown, but one speculation is that SOX9 may be a marker of cancer-associated stem cells. Indeed, in the current study, SOX9-expressing cells were correlated with PCNA-positive cells. There are two theories for the formation of adenoma. One is the "top-down" theory (colorectal adenomas arise and grow across the mucosal surface and

down into the crypts)¹⁹ and another is the "bottom-up" theory.²⁰ The finding that nuclear SOX9 staining distribution was more frequent in the lower part of duodenal adenomas is similar to the classic bottom-up model of colorectal carcinogenesis.²⁰ We found that PCNA-positive SOX9-expressing adenomatous cells were located in the bottom of crypts where intestinal stem cells reside. Such a distribution could support a bottom-up scenario.

Strong expression of SOX9 has been shown to be linked to poor prognosis of colorectal,¹⁵ lung,²¹ breast,²² and urothelial carcinomas.²³ On the other hand, it has been reported that a lack of SOX9 expression is associated with a higher tumor stage in biliary tract carcinoma.²⁴ In the current study, the association between SOX9 expression and prognosis of duodenal adenocarcinoma was unclear because SOX9 was broadly detected in adenomas as well as adenocarcinomas. Furthermore, in the current study, duodenal adenocarcinomas, which were resected under EMR or ESD, were in the early stage. The current study was not able to clarify whether SOX9 expression is associated with a poor prognosis. However, SOX9 expression of duodenal adenoma and carcinoma was significantly higher than that of normal duodenal mucosa in the current study. Therefore, the presence of SOX9 expression in duodenal adenoma may indicate that SOX9 expression is associated with the early stage of duodenal carcinogenesis.

In this largest clinical survey on SOX9 expression (43 clinical samples), we found that SOX9 overexpression was common in duodenal adenoma and adenocarcinoma. It is not clear in the current study whether SOX9 is related to duodenal carcinogenesis. While SOX9-knockdown cells show significantly attenuated tumorigenicity in mice, SOX9 transfectants consistently show markedly stronger tumorigenicity.²¹ Taken together, the previous report and the current results suggest that SOX9 expression may be closely associated with duodenal carcinogenesis. Further studies need to be conducted on the molecular and biological effects of SOX9 in duodenal adenocarcinoma. Furthermore, determination of whether SOX9 expression correlates with duodenal carcinogenesis and predicts duodenal adenocarcinoma risk in humans may provide new insight into the etiology of duodenal carcinoma. Clarifying the association between SOX9 and carcinogenesis may lead to SOX9 being a potential therapeutic target in duodenal adenocarcinoma.

In conclusion, SOX9 is overexpressed in nonampullary duodenal adenoma and adenocarcinoma in humans. The current study data suggest that SOX9 is a new hallmark of nonampullary duodenal adenoma and adenocarcinoma.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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