Analysis of SOX9 Expression in Colorectal Cancer

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Abstract

*Our purpose was to investigate the role of SOX9, a novel downstream molecule of β-catenin, in colorectal cancer. Expression of SOX9 and β-catenin was detected by immunostaining, quantitative real-time reverse transcription–polymerase chain reaction (Q-PCR), and Western blot in colorectal cancer. The correlation between SOX9 or β-catenin expression and clinicopathologic parameters was also analyzed. Immunostaining, Q-PCR, and Western blot consistently confirmed SOX9 up-regulation in colorectal cancer compared with normal mucosa (*P < *.05). Immunostaining showed more SOX9+ cells in the lower zone of colonic crypts than in the upper zone (*P < *.05). Cancers with strong SOX9 immunostaining were significantly associated with a lower 5-year overall survival (40% [17/43] vs low expression, 69% [66/95];* P <*.01). The Cox proportional hazards model showed that strong SOX9 expression was an independent adverse prognosticator in colorectal cancer (*P < *.05). The detection of SOX9 expression might contribute to predicting clinical outcomes for patients with colorectal cancer.*

Colorectal cancer is the second most common malignancy worldwide. Its prognosis has not been improved significantly during the past 2 or 3 decades. The elucidation of the molecular events underlying the process of colorectal carcinogenesis remains a pressing need. A growing body of evidence suggests that signaling pathways from crypt differentiation appeared to be closely associated with the initiation and progression of colorectal cancer.[1] The Wnt pathway has been identified as one of the key pathways in the initiation and development of colorectal cancer and in gastrulation and morphogenesis and maintenance of crypt stem cell self-renewal.[2,3] β-Catenin is an important molecule in the canonical Wnt pathway. Under normal conditions, β-catenin is under the control of upstream Wnt regulators. Glycogen synthase kinase-3β can phosphorylate the NH2 terminus of β-catenin when Wnt signals are deprived. The phosphorylated β-catenin will bind with APC (adenomatous polyposis coli) protein to form the ubiquitin-mediated proteolytic complex. In most colorectal cancers, APC inactivation or β-catenin mutation inhibits the formation of β-catenin/APC proteolytic complex. Presumably, β-catenin accumulates in the cytoplasm, translocates to the nucleus, binds with T-cell factor 4/lymphocyte enhancer factor 1, and fosters tumor growth and progression.

SOX9, a member of the *SOX* (SRY [sex determining region Y] box) gene superfamily, has become accepted as an important downstream gene of β-catenin.[4-6] SOX9 is required for committed differentiation, such as in chondrogenesis, male sex gonad or respiratory epithelium development, melanocyte differentiation, and the differentiation of Paneth cells in the gut.[7-13] Recent studies have shown the emerging role of SOX9 in cancer.[4,14-17] However, the clinical significance of SOX9 expression in cancer remains unclear.

In this study, we showed the distinct clinicopathologic patterns of SOX9 and β-catenin expression in colorectal cancer by quantitative real-time reverse transcription–polymerase chain reaction (Q-PCR), Western blot analysis, and immunostaining. We confirmed SOX9 overexpression in colorectal cancer as an independent indicator of an unfavorable outcome for colorectal cancer in a Chinese population.

Materials and Methods

Cases

We obtained 188 primary colorectal cancer specimens from 3 separate hospitals in the Zhejiang Province in China between January 1991 and December 2002. We excluded data for patients with hereditary (familial) cancer syndrome and for patients who died within 1 month of the initial surgery. The mean patient age was 65.5 years. Of the patients, 103 were male and 85 were female, 61 with colonic and 127 with rectal cancer. All patients underwent curative resection with tumor-free margins. Adjuvant 5-fluorouracil-based chemotherapy was given to 96 patients, and 92 did not receive chemotherapy. Surviving patients were observed for 2.1 to 12.6 years (mean, 6 years). Of the study patients, 59 died of cancer, with a mean survival of 1.7 years (range, 0.02~5.97 years), and 55 patients survived for fewer than 5 years. The overall 5-year survival was 60.1% (83/138). In addition, 94 sporadic adenomas (including 80 tubular, 7 tubulovillous, and 7 villous) were available for immunostaining.

Immunohistochemical Analysis

Archival H&E-stained slides were reviewed by 2 pathologists (M.L. and F.X.) in accordance with the 2000 World Health Organization classification. A 2-tiered histologic grading system was applied. We counted tumor budding (a single cancer cell or a small group of cancer cells, <5, at the invasive front) as previously reported.[18] The TNM stage was also reevaluated according to the 2002 International Union Against Cancer classification.

A monoclonal mouse antihuman β-catenin antibody (dilution 1:100) and a polyclonal rabbit antihuman SOX9 antibody (dilution 1:50) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Both antibodies were also used for Western blot analysis. For the study, 4-μm sections from archival paraffin blocks were deparaffinized and heated twice for 10 minutes each in a microwave oven (500 W) before exposure to the first antibodies. Immunoperoxidase staining was carried out using the 2-step EnVision method (DAKO, Glostrup, Denmark) according to the manufacturer's instructions and visualized with 3,3′-diaminobenzidine tetrachloride (Sigma, St Louis, MO). Human ovarian Sertoli cell tumor and pseudopapillary neoplasm of pancreas were used as positive control samples for SOX9 and β-catenin, respectively. The omission of primary bodies was used to prepare negative control samples.

Only nuclear staining was measured for these 2 antibodies. Positive cells were counted by 2 pathologists (B.L. and Y.F.) who were blinded to the clinical outcome. To evaluate the staining distribution pattern in the normal mucosa and adenoma samples, the glands were divided into 2 equal crypt zones (upper and lower), and the positive cells were determined as the number of positive cells per 500 cells in each zone in randomly chosen fields.[19] For clinicopathologic correlation, we used a 4-tiered scoring system (negative to 3+), which took into account the percentage of positive cells and staining intensity, as in our previous study.[20] However, SOX9 was expressed in normal mucosa, so we separately interpreted SOX9– and 1+ as "low expression" and SOX9 2+ and 3+ as "strong expression."

Quantitative PCR

We obtained 50 colorectal cancers with individually matched normal mucosa adjacent to the distant excision margin from the Department of Surgical Pathology, the Second Affiliated Hospital, Zhejiang University, Hangzhou, China, following an institutional review board–approved protocol. Tissue samples were collected by a pathologist who immediately evaluated the tissue quality under a microscope to ensure the presence of at least 70% epithelial cells in the tissue samples. Samples were snap-frozen in liquid nitrogen and stored in a –80°C freezer. Total RNA was isolated from frozen tissues using the Qiagen Rneasy Mini Kit, according to the manufacturer's protocol (Qiagen, Hilden, Germany). Total RNA was quantified by UV spectroscopy, and its quality was examined by formaldehyde denatured agarose gel electrophoresis. Next, 4 μg of total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

The Q-PCR assay was used to detect the differential expression of β-catenin (NM_001904) and SOX9 (NM_000346) between colorectal cancers and individually matched normal mucosa. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (NM_002046) was used as an internal control to normalize samples. Gene-specific primers were designed as follows: SOX9, 5'-AGGTGCTCAAAGGCTACGACT-3' and 5'-AGATGTGCGTCTGCTCCGTG-3'; β-catenin, 5'-TCTTGGCTATTACGACAG-3' and 5'-CCTCTATACCACCCACTT-3'; and GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. PCR was performed by using a SYBR Premix Ex Taq (Perfect Real Time) Kit (TaKaRa Bio, Otsu, Japan) in the Applied Biosystems 7500HT Fast Real-Time PCR System. The specificity of PCR products was checked by the melting temperature and curve. Each sample was measured at least

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3 × 3 times. The relative amount of messenger RNA (mRNA) in each target gene to GAPDH was calculated as the average $2^{-\Delta ct}$, where $\Delta Ct = Ct - Ct_{GAPDH}$.

Western Blot Analysis

Specimens were crushed in liquid nitrogen and lysed in protein lysis buffer containing 7 mol/L of urea, 2 mol/L of thiourea, 4% CHAPS, 65 mmol/L of DTT, 0.2% (pH 3-10) Bio-Lyte (Bio-Rad, Hercules, CA), and complete protease cocktail inhibitors (Roche, Laval, Canada). Lysates were ultrasonicated and cleared by centrifugation at 13,000 rpm at 4°C for 1 hour. Proteins were quantified by using the Bradford method.

Aliquots of protein extracts (~60 µg) were first separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Pierce Biotechnology, Rockford, IL). After blocking with tris(hydroxymethyl)aminomethane-buffered saline–polysorbate 20 containing 5% skim milk, the PVDF membrane was incubated with the primary antibody, SOX9 (dilution 1:1,000) or β-catenin (dilution 1:1,000), overnight at 4°C, followed by horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:10,000 for 1 hour. Bound antibodies were detected by enhanced chemiluminescent reagent (Pierce Biotechnology). As a control for equal protein loading, blots were restained using a rabbit antihuman actin antibody (Santa Cruz Biotechnology).

Statistical Analysis

We used the SPSS, version 13.0, software package (SPSS, Chicago, IL) for statistical analysis. The association among categorical data was analyzed by using the χ^2 test, F test (Student-Newman-Keuls), Wilcoxon test, Mann-Whitney *U* test, or Friedman test, as appropriate. The correlation between SOX9 expression and β-catenin expression was analyzed by using the Spearman correlation. Survival curves were generated by the Kaplan-Meier method, and univariate survival distributions were compared with the use of the log-rank test. The multivariate Cox proportional hazards model was used for detection of independent prognosticator. The 2-tailed *P* value for significance was established at .05.

Results

Immunostaining Pattern of SOX9 in Colorectal Adenoma and Cancer

SOX9 expression in normal mucosa was predominantly located within the lower part of the crypts, the proliferating compartment **Image 1A** and **Image 1B**; it was significantly higher in the lower zone of the crypt than in the upper zone (mean ± SD, 15.3% ± 13.9% vs 9.6% ± 13.1%; $P < .001$). In colorectal adenomas, SOX9+ cells were distributed along the whole crypt. SOX9 expression was also more common in the lower part of adenoma samples than in the upper part (mean ± SD, 50.7% ± 25.1% vs 32.9% ± 30.0%; $P < .001$) **Image 1D**. SOX9 expression was up-regulated in colorectal adenomas compared with paired mucosa (mean ± SD, 39.8% ± 30.9% vs 24.9% ± 18.2%; $P < .001$) **Image 1C**. In most colorectal cancers, SOX9 expression was randomly distributed but was much higher than in the normal mucosa (mean ± SD, 36.7% ± 30.3% vs 7.9% ± 9.7%; $P < .001$) **Image 1E** and **Image 1G**. SOX9 expression showed no significant difference between colorectal adenoma and cancer ($P > .05$). Meanwhile, nuclear β-catenin was more common in colorectal adenoma and cancer **Image 1I** than in matched normal mucosa, which invariably showed a membranous or cytoplasmic staining pattern ($P < .05$). We did not find a significant correlation between SOX9 expression and nuclear β-catenin in colorectal cancer ($P > .05$).

Relationship of Strong SOX9 Expression to Clinicopathologic Parameters and Prognosis in Colorectal Cancer

We found that strong SOX9 expression was present in 50 (53%) of 54 adenomas and 64 (34.0%) of 188 cancers (Images 1C, 1D, and 1E) **Image 1F** but was not strongly expressed in normal mucosa (Images 1A and 1B). Strong SOX9 expression was invariably associated with colorectal adenoma and cancer ($P < .001$). As shown in **Table 1**, strong SOX9 expression and nuclear β-catenin (data not shown) were less common in mucinous and signet-ring cell carcinoma (mucin-producing carcinoma) than in non–mucin-producing colorectal cancer ($P < .05$; χ^2 test) (Images 1D and 1E) **Image 1H**. However, strong SOX9 expression was not significantly associated with high-grade histologic features ($P > .05$). Strong SOX9 expression showed a lower overall 5-year survival in colorectal cancer (strong expression, 40% [17/43] vs low expression, 69% [66/95]; $P < .01$), whereas nuclear β-catenin was not strongly expressed ($P > .05$; χ^2 test). Strong SOX9 expression did not significantly correlate with the histologic type and grading in adenomas ($P > .05$).

Univariate analysis by Kaplan-Meier plots revealed that TNM stage III or IV increased tumor budding (≥15), high histologic grade, and strong SOX9 expression were significantly associated with an unfavorable overall survival ($P < .01$). Kaplan-Meier curves did not show any survival differences in colorectal cancer indicated by other parameters, including expression of β-catenin, sex, age, tumor location, and chemotherapy ($P < .05$). A characteristic plot of strong SOX9 expression is shown in **Figure 1**. Multivariate Cox analysis indicated that TNM stage, tumor budding, and SOX9 expression were independent variables ($P = .022$). Histologic grading was excluded in this model ($P = .140$). TNM stage III or IV,

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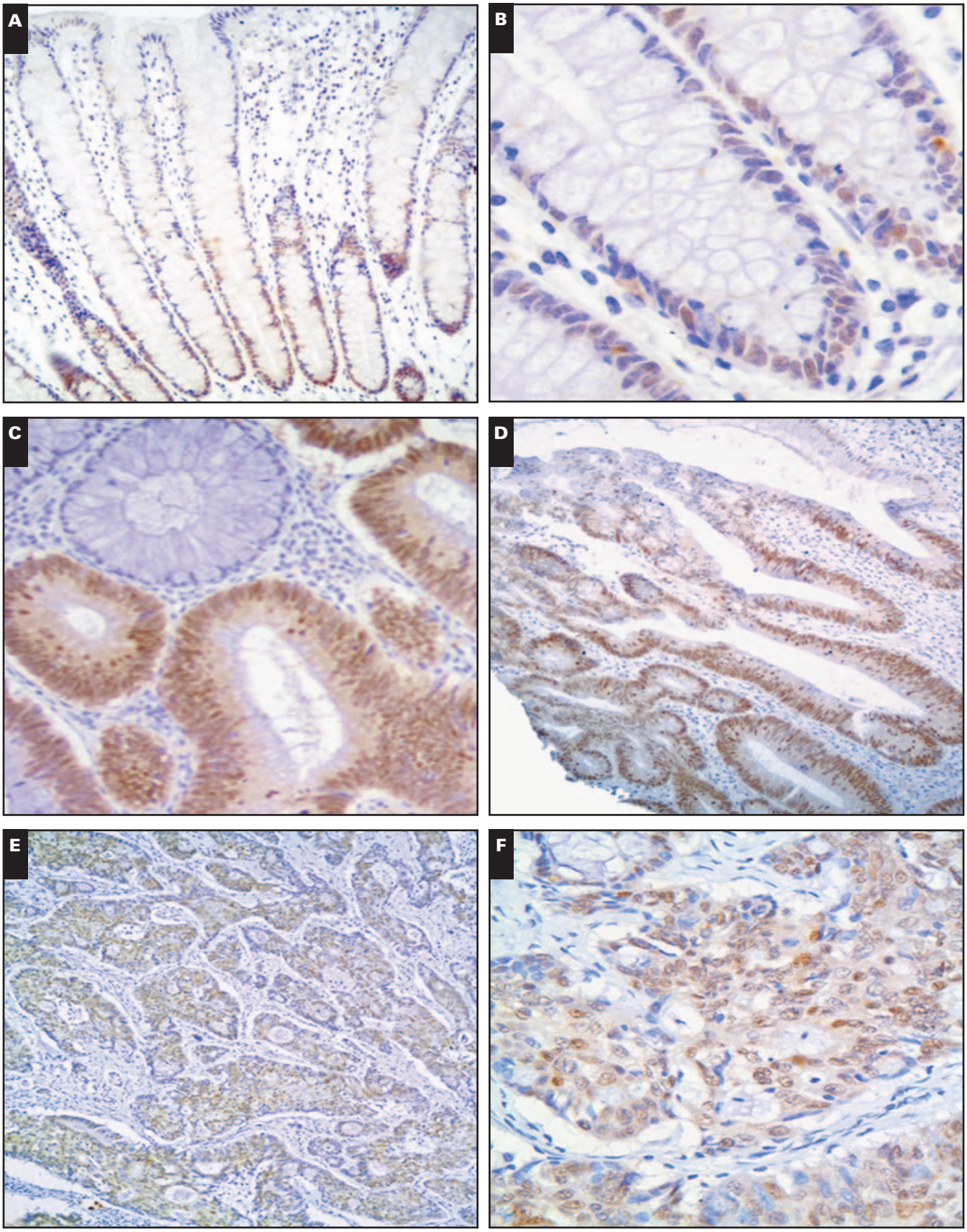

Image 1 Immunostaining of SOX9 in colorectal cancer. **A** and **B**, Overall staining pattern of SOX9 in normal colonic mucosa and a predominant staining pattern in the lower zone of normal mucosa. More SOX9+ cells were also found in the lower zone of adenoma (**D**, upper left) than in the upper zone (**D**, lower right). Colorectal adenomas (**C** and **D**) and carcinomas (**E** and **F**) had strong nuclear SOX9 expression, but low expression was common in mucin-producing carcinomas (**H**).

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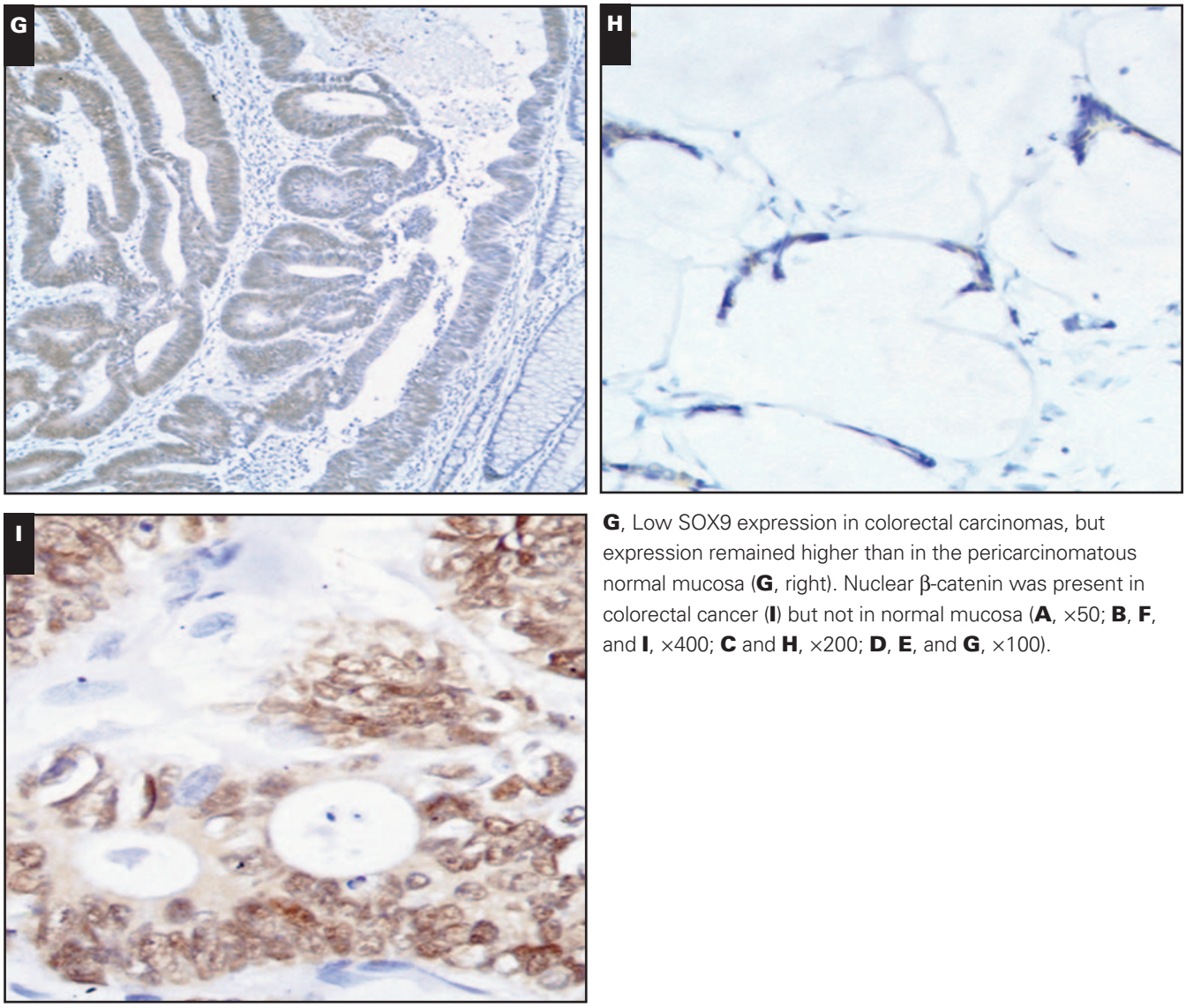

G, Low SOX9 expression in colorectal carcinomas, but expression remained higher than in the pericarcinomatous normal mucosa (**G**, right). Nuclear β-catenin was present in colorectal cancer (**I**) but not in normal mucosa (**A**, ×50; **B**, **F**, and **I**, ×400; **C** and **H**, ×200; **D**, **E**, and **G**, ×100).

more tumor budding (≥15), and strong SOX9 expression were associated with adverse overall survival **Table 2**.

Further Validation of SOX9 Overexpression in Colorectal Cancer by Q-PCR and Western Blot

Q-PCR and Western blot were done to further confirm SOX9 expression in colorectal cancer. Q-PCR showed that SOX9 mRNA expression was elevated in 35 of 50 colorectal cancers compared with expression in individually matched normal mucosa. Despite interindividual variations in the content of SOX9 expression, this gene was significantly up-regulated in colorectal cancer (mean ± SD, normal samples, 0.1065 ± 0.1615; cancer samples, 0.233 ± 0.478; $P < .05$). However, β-catenin mRNA expression was not significantly changed in cancer (mean ± SD, normal samples, 0.1047 ± 0.0225; cancer samples, 0.0795 ± 0.0282; $P > .05$).

To determine whether there is any difference in the relative protein levels between normal and colon cancer tissues, Western blot was performed in 21 colorectal cancers and individually matched to normal mucosa samples. Western blot showed that expression of SOX9 and β-catenin protein was up-regulated in 16 and 15 cancer tissues compared with normal mucosa samples, respectively **Figure 2**. Expression of SOX9 and β-catenin protein was significantly higher in colorectal cancer (mean ± SD, SOX9 normal samples, 0.3293 ± 0.3863 vs cancer samples, 0.907 ± 0.6413; $P < .01$; β-catenin normal samples, 0.3397 ± 0.2921 vs cancer samples, 0.6024 ± 0.498; $P < .05$). SOX9 showed a consistent expression pattern between its relative expression level of mRNA and protein ($P < .05$) in colorectal cancer, whereas β-catenin did not ($P > .05$).

Discussion

Colorectal cancer is one of the most aggressive cancers worldwide. Curing this disease will require the identification of molecular biomarkers for prognosis or novel targets for

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Table 1
Relationships Between Clinicopathologic Features and SOX9 Expression in Colorectal Cancer

	SOX9 Expression		
	Strong (n = 64)	**Low (n = 124)**	***P***
Sex			.877
Male	36	67	
Female	28	57	
Age (y)			.759
≥65	30	62	
<65	34	62	
Location			.413
Rectum	46	81	
Colon	18	43	
Overall 5-y survival*†			.001
Yes	17	66	
No	26	29	
Histologic type*‡			.016
Non–mucin-producing cancer	62	103	
Mucin-producing cancer	2	21	
Histologic grading			.328
Low-grade	18	45	
High-grade	46	79	
Tumor budding			.116
<15	42	96	
≥15	22	28	
Serosa/adventitia involvement			.14
Yes	48	79	
No	16	45	
Lymph node involvement			.761
Yes	32	65	
No	32	59	
TNM stage†			1
I/II	33	64	
III/IV	30	56	
Nuclear β-catenin†			.118
Positive	33	46	
Negative	31	73	

* Statistically significant difference; χ^2 test.
† Data in some cases are not available or excluded due to the statistical requirements (5-y survival).
‡ Mucin-producing cancer includes mucinous cancer and signet-ring cell cancer.

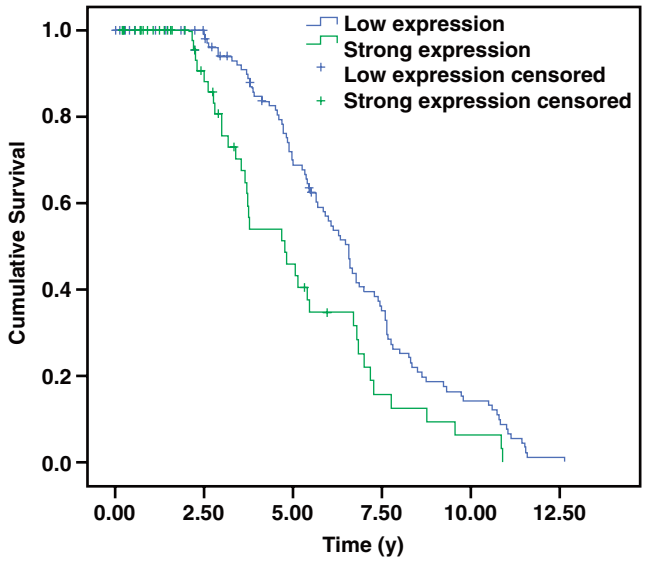

Figure 1 Kaplan-Meier curve showing survival differences by SOX9 expression. Colorectal cancers with strong SOX9 expression show a better overall survival than cancers with low expression. The Cox proportional hazards model indicated that strong SOX9 expression is an independent adverse prognostic indicator in colorectal cancer (relative risk, 1.381; 95% confidence interval, 1.051-1.815; $P < .05$).

Table 2
Multivariate Cox Analysis of Survival in Colorectal Cancer

Variable	RR	95.0 CI for RR	*P*
Strong SOX9 expression	1.381	1.051-1.815	.021
TNM stage	1.846	1.357-2.511	<.001
Tumor budding	1.435	1.089-1.891	.010

CI, confidence interval; RR, relative risk.

therapeutic intervention. In this study, we suggest that SOX9, a recently demonstrated Wnt downstream transcription factor,[4-6] is a potent candidate by showing its overexpression and association with an adverse prognosis in colorectal cancer.

We found that SOX9 expression was predominantly in the proliferation compartment (lower zone) of normal colorectal mucosa, the putative site of stem cells, thereby implying that SOX9 may be a putative stem or progenitor cell biomarker of the colonic epithelium. Previous studies showed the role of SOX9 in committed differentiation, such as chondrocyte differentiation, outer root sheath differentiation, and the formation of the hair stem cell compartment.[5,21] Blache and colleagues[4] first reported that SOX9 can inhibit intestinal crypt differentiation in the colon by repressing 2 intestine-specific differentiation genes, *CDX2* and *MUC2*. Recent studies indicated that SOX9 is required for Paneth cell differentiation in the intestinal epithelium.[12,13] SOX9 inactivation influenced differentiation throughout the intestinal epithelium by showing a disappearance of Paneth cells.

Strong SOX9 expression was more common in colorectal cancer at the mRNA and protein levels than in normal mucosa. A growing body of evidence has shown that SOX9 and its homologues were up-regulated in several cancers.[4,16,21-27] Only one study showed SOX9 up-regulation in colorectal cancer.[4] However, this study included only a small number of human colorectal cancers and, more important, did not analyze specific clinicopathologic relevance. The present study not only confirmed SOX9 overexpression at the mRNA and protein levels in colorectal cancer, but also had several other distinct features. First, we showed that strong SOX9 expression was associated with an adverse prognosis in colorectal cancer. To our knowledge, the present study is the first clinical investigation of the prognostic significance of SOX9 expression in cancer. Second, we found that strong SOX9 expression

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and nuclear β-catenin staining are rare in colorectal mucin-producing cancers (mucinous and signet-ring cell cancers), although they are invariably associated with colorectal cancer. Wong et al[28] also reported that nuclear β-catenin was absent in mucinous and signet-ring cell carcinoma. Finally, we also provided the first details about SOX9 expression in colorectal adenomas. The finding that the nuclear SOX9 staining distribution was more frequent in the lower part of colorectal adenomas is consistent with the classic bottom-up model of colorectal carcinogenesis.[29]

The role of SOX9 in other biologic processes, except committed differentiation, remains to be elucidated. SOX9 transfection in SW480 colonic cancer cells tended to inhibit cell proliferation or trigger apoptosis in vitro.[14] It seems paradoxical that strong SOX9 expression is associated with a worse prognosis in colorectal cancer in this study. However, in that study, transfection may have produced excessive SOX9 expression in colon cancer cells. Thus, the effects are possibly not physiologic. A gene silencing–based approach seems to be more reasonable than transfection. Recent studies in prostate cancer showed that SOX9 down-regulation by RNA interference could inhibit cell proliferation in vitro and the growth of tumor xenografts in vivo.[15,25] We also believe that, in the assessment of prognosis in cancer, the relative weight of potential proliferative or apoptotic markers may vary considerably. The role of SOX9 in committed differentiation may be largely responsible for the poor prognosis in colorectal cancer.

Generally, SOX9 overexpression in colorectal cancer is associated with constituent β-catenin activation.[4] However, the present study did not show a significant correlation between SOX9 and β-catenin expression. Presumably, there should be other underlying mechanisms that are responsible for SOX9 expression. Sonic hedgehog (Shh) signaling can maintain SOX9 overexpression in skin tumors.[21] Insulin receptor substrate-1 can modulate the number of SOX9+ cells in intestinal crypts.[30] We postulate that Shh and insulin/insulin growth factor signaling might be also associated with SOX9 overexpression in colorectal cancer. However, much work needs to be done on the regulatory mechanism of SOX9 expression in colorectal cancer in the future.

In this largest clinical survey on SOX9 expression in vivo, we found that SOX9 up-regulation is common in colorectal adenoma and cancer. More important, strong SOX9 expression is an independent indicator for an adverse prognosis in colorectal cancer. These results imply that SOX9 could be a potential therapeutic target in colorectal cancer. However, further studies should be conducted on the molecular and biologic effects of SOX9 in colorectal cancer.

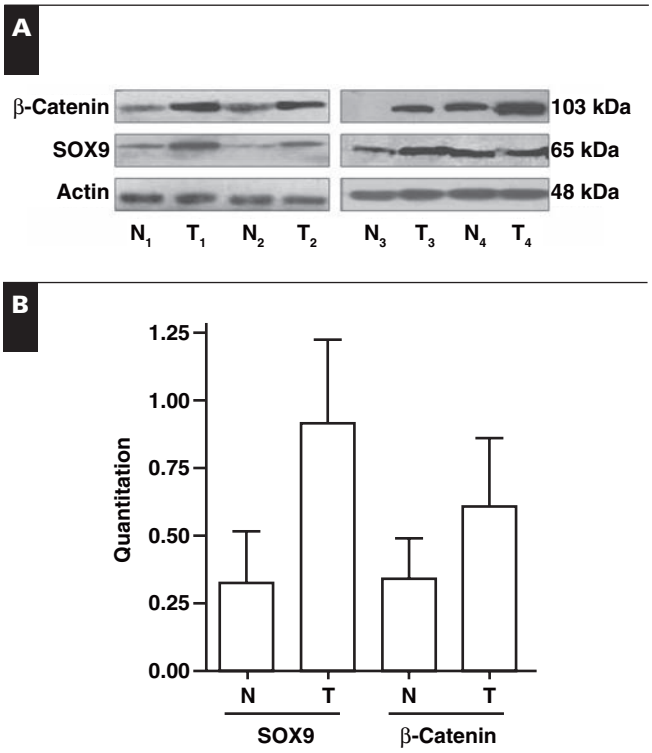

Figure 2 Immunoblots of SOX9 and β-catenin in colorectal cancer. **A**, Western blot analysis revealed up-regulated SOX9 and β-catenin expression in colorectal cancer (T) samples when compared with that in normal (N) colorectal tissues. Depicted are 4 individual matched pairs of colorectal normal–cancer samples. **B**, Immunosignals were quantified by densitometric scanning. The relative quantification of SOX9 or β-catenin expression in the individual tissue samples was calculated as SOX9 or β-catenin expression relative to actin expression (mean ± SD, SOX9 normal sample, 0.3293 ± 0.3863; cancer sample, 0.907 ± 0.6413; and β-catenin normal sample, 0.3397 ± 0.2921; cancer sample, 0.6024 ± 0.498. SOX9, $P < .01$; β-catenin, $P < .05$).

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