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SOX17 Methylation Inhibits Its Antagonism of Wnt Signaling Pathway in Lung Cancer

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Abstract

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Disclosure
J.G.H. is a consultant to MDxHealth. The other authors report no conflicts of interest.

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The purpose of this study was to explore epigenetic changes and functions of *SOX17* in human lung cancer. Five lung cancer cell lines and 88 primary lung cancer samples were examined in this study. Methylation-specific polymerase chain reaction (MSP), semi-quantitative reverse-transcription PCR, immunohistochemistry, luciferase reporter assays, colony-formation assays, and western blotting were used to analyze methylation changes and functions of SOX17 in lung cancer. *SOX17* methylation was found in 60.2% of primary human lung cancer samples, and promoter region methylation of *SOX17* silenced its expression. *SOX17* methylation was associated with female patients and lung cancer differentiation. Colony-formation assays revealed that SOX17 suppressed lung cancer cell proliferation. Re-expression of *SOX17* inhibited Wnt signaling in H23 lung cancer cell line. SOX17 acts as a Wnt signaling inhibitor.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide among both men and women (Sullivan *et al*., 2005; Suemitsu *et al*., 2008). Lung cancer comprises two major histological groups: non-small cell lung cancer (NSCLC) and small cell lung cancer, which account for 80.4% and 16.8% of cases, respectively (Travis *et al*., 1995). More than 60% of lung cancer patients are diagnosed at an advanced stage (Henschke *et al*., 2008; Spira *et al*., 2004), and an effective early-detection marker for lung cancer is thus desirable. Aberrant epigenetic changes are regarded as important mechanisms in lung carcinogenesis (Yang *et al*., 2011).

Unlike in colorectal cancer, mutations of the components in Wnt signaling pathway were relatively rare in lung cancer. Sunaga *et al*. (2001) found that β-catenin gene (*CTNNB1*) was mutated in certain lung cancer cell lines and primary lung cancers. Shigemitsu *et al*. (2001) reported that a small portion of lung cancers had a mutation in *CTNNB1*, and Ohgaki *et al*. (2004) demonstrated that *APC* (adenomatous polyposis coli) mutation occurred in a small portion of squamous cell lung cancer and small cell lung cancer. But disruption of the Wnt signaling pathway by hypermethylation of the promoter region of genes antagonizing the β-catenin/T cell factor (TCF)-signaling pathway was frequently reported in lung cancer, including *RUNX3*, *SFRP1*, *WIF1*, and *APC* (Mazieres *et al*., 2004; Fukui *et al*., 2005; Toyooka *et al*., 2006; Licchesi *et al*., 2008). Our recent study indicated that Dishevelled-associated antagonist of β-catenin 2 (DACT2) was frequently methylated in human cancer (Jia *et al*., 2012).

SRY-box containing gene 17 (*SOX17*) belongs to the high-mobility group (HMG)-box transcription factor superfamily, which is homologous to the sex-determining gene SRY (Gubbay *et al*., 1990). SOX17 has been reported to promote the degradation of β-catenin/TCF via a GSK3β-independent mechanism in the Wnt signaling pathway, and has been recognized as an important antagonist and inhibitor of the canonical Wnt signaling pathway (Sinner *et al*., 2007; Jia *et al*., 2010; Sinner *et al*., 2004). Frequent SOX17 gene methylation has been detected in colon, liver, and breast cancers (Zhang *et al*., 2008; Jia *et al*., 2010; Fu *et al*., 2009). However, the methylation status and effects of *SOX17* on lung carcinogenesis remain unclear. We therefore investigated the epigenetic changes and

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functions of SOX17 in human lung cancer, using lung cancer cell lines and primary human lung cancer tissues.

Materials and Methods

Human tissue samples and cell lines

A total of 88 cases of NSCLC were collected immediately after surgical resection at the Chinese PLA General Hospital. Among these, matched cancer and adjacent normal tissue paraffin blocks were available for 29 cases. All definitive pathological diagnoses were classified as tumor stage I (n=36), stage II (n=25), stage III (n=25), or stage IV (n=2), according to UICC (International Union Against Cancer) staging criteria. All tissues were collected according to the guidelines of institutional review board of the Chinese PLA General Hospital.

Five lung cancer cell lines (H23, A549, H157, H446, and 95D) were included in this study. All lung cancer cell lines were previously established from NSCLC primary tumors, and maintained in 90% RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were passaged 1:3 after reaching 80% confluence (approximately 106 cells) in 75-cm^2 culture flasks (NEST Biotechnology, Jiangsu, China).

5-Aza-2′-deoxycytidine (DAC) treatment

H23, A549, H157, H446, and 95D cells were split to low densities (30% confluence) 12 hours before treatment. Cells were treated with DAC (Sigma, St. Louis, MO) at a concentration of 2 mM. Growth medium conditioned with DAC at 2 μM was exchanged every 24 hours for a total of 96 hours of treatment. At the end of the treatment course, RNA was extracted from the cells as described below.

RNA isolation and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD). Agarose gel (1%) electrophoresis and spectrophotometric analysis (A260:280 nm ratio) were used to evaluate RNA quality and quantity. RNA was stored at -80°C prior to use. First-strand cDNA was synthesized according to Trizol manufacturer's instructions. A total of 5 mg total RNA was used to synthesize first-strand cDNA using random 6-mer primers and a Superscript II-reverse transcriptase kit (Invitrogen, Carlsbad, CA). Following first-strand synthesis, the reaction mixture was diluted to 100 μl with water. Subsequently, 2.5 μl of diluted cDNA mixture was used for PCR amplification in a final reaction volume of 25 μl. PCR amplification of SOX17 was carried out using the following primers: 5′-GGCTGGCGCAGCAGAATC-3′ (forward) and 5′- AGCCCTGCTCGGGGAACT-3′ (reverse). The primer set for the *SOX17* gene was designed to span intronic sequences between exons to control for genomic DNA contamination. A total of 32 cycles of amplification were performed for each RT-PCR experiment. Glyceraldehyde 3-phosphate dehydrogenase was amplified as a control using 25 cycles to ensure cDNA quality and quantity for each RT-PCR. Amplified products were analyzed on 1.5% agarose gels.

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Methylation-specific PCR (MSP)

Genomic DNA from lung cancer tissue from 88 cases and five cell lines was prepared using the proteinase-K method. After chloroform/phenol extraction, DNA was precipitated in ethanol, dissolved in low-TE buffer and stored at −20°C. Genomic DNA from lung cancer tissues and cell lines was then modified with bisulfite, as previously described (Herman *et al.*, 1996). MSP primers were designed according to genomic sequences flanking the presumed translation start sites. Primer sequences were oligo-synthesized (Invitrogen, Beijing, China) to allow bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles to be detected by MSP. MSP of *SOX17* was carried out using the following primers: 5′-GGGGCGTTCGTAGTGTTA TTAGGTC-3′ (M-sense); 5′-AAACACTAAAATACC CCGAAAACTACG-3′ (M-antisense); 5′-TTAGGGGT GTTTGTAGTGTTATTAGGTT-3′ (U-sense) and 5′-TAAAACACTAAAATACCCCAAAAACTACA-3′ (U-antisense). Each MSP reaction included approximately 200 ng of bisulfite-treated DNA, 25 pmoles of each primer, 100 pmoles of dNTPs, 2.5 μl 10xPCR buffer, and 1 unit of Taq polymerase (Invitrogen, Beijing, China) in a final reaction volume of 25 μl. Cycle conditions were: 95°C for 5 minutes, 1 cycle; 35 cycles of 95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 5 minutes, 1 cycle. MSP products were analyzed using 2% agarose gel electrophoresis.

Immunohistochemistry (IHC)

IHC was performed on 4-μm-thick serial sections derived from formaldehyde-fixed paraffin blocks of lung cancer tissue and paired distant adjacent normal tissue. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 minutes in methanol containing 0.3% hydrogen peroxide. Antigen retrieval was performed for 45 minutes at 96°C in target retrieval solution, followed by a cooling-off period of 20 minutes. Incubation with the primary mouse antibodies [anti-β-catenin at 1:200 dilution (ZSGB Biotechnology, Beijing, China); anti-SOX17 at 1:50 dilution (OriGene Technologies, Rockville, MD), respectively] was carried out overnight at 4°C. The catalyzed signal amplification system (GBI, Mukilteo, WA) was then used to detect β-catenin and SOX17 staining, according to the manufacturer's instructions. The correlations between *SOX17* methylation and the locations of β-catenin and SOX17 were examined using χ^2 tests. Values of $P<0.05$ were considered to be statistically significant.

cDNA construction

Full-length *SOX17* cDNA (GenBank accession number NM_022454) derived from normal colon mucosa mRNA by RT-PCR was cloned into pcDNA3.1/V5-His B vector (Invitrogen, Beijing, China) via KpnI and XhoI sites, as described previously. Deletion mutants of *SOX17* were generated by PCR.

Colony-formation assay

H23 cells were grown in 6-well culture plates for 24 hours before transfection. Cells were transfected with empty control vector or *SOX17* expression construct, according to the manufacturer's instructions (Roche Applied Science, San Diego, CA). Cells were diluted

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and reseeded at 800 cells per well in 6-well culture plates in triplicates 24 hours later. Growth medium conditioned with G418 (Invitrogen, Beijing, China) at 500 μg/ml, was exchanged every 24 hours. After 14 days, cells were fixed with 75% ethanol for 30 minutes and stained with 0.2% crystal violet for visualization and counting.

Luciferase reporter assay

H23 cells were seeded at 5×10^4 cells/well in 24-well culture plates 24 hours before transfection. To examine transcriptional activity driven by β-catenin/TCF, H23 cells were transfected with 200 ng/well pGL3-OT (*TCF/LEF*-responsive reporter containing three consensus TCF-binding sites), 30 ng/well pRL-TK control vector (Promega) as an internal control reporter, and 600 ng/well β-catenin construct. Basal transcriptional activity in H23 cells was tested by transfection with empty vectors. H23 cells were then transfected with 200 ng/well pGL3-OT, 30 ng/well pRL-TK, 150 ng/well β-catenin constructs, and 200 ng/ well *SOX17* constructs (wild-type and *SOX17* constructs 50-414, 135-414 and 1–353) with FuGENE 6 (Roche Applied Science).

At 36 h after transfection, relative luciferase activities were measured using a GLOMAX luminometer (Promega, Madison, WI) and normalized for background *Renilla* luciferase activities via the Dual Luciferase Reporter Assay system (Promega), according to the manufacturer's instructions. The luciferase assay was performed twice for each experiment.

Protein preparation and western blotting

Transfected cells were lysed in ice-cold Tris buffer (20 mmol/l Tris; pH 7.5) containing 137 mmol/l NaCl, 2 mmol/l EDTA, 1% Triton X, 10% glycerol, 50 mmol/l NaF, 1 mmol/l dithiothreitol, and a protease inhibitor cocktail (Roche Applied Science). The protein lysates were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes (Hybond-P, Amersham, Pittsburgh, PA). After blocking with 5% nonfat milk and 0.1% Tween-20 in TBS, the membranes were incubated with mouse anti-SOX17 (OriGene Technologies), rabbit cyclin D1 (Bioworld Technology, Minneapolis, MN), or rabbit anti-β-actin (Beyotime Biotechnology, Shanghai, China) antibodies. The blots were visualized using enhanced chemiluminescence (Pierce Bioscience, Rockford, IL).

Statistical analysis

Statistical analysis was carried out using χ^2 tests. Values of $P<0.05$ were considered statistically significant.

Results

Expression of SOX17 was regulated by DNA methylation in lung cancer cell lines

Semi-quantitative RT-PCR was employed to detect the expression levels of *SOX17* in lung cancer cell lines (H23, A549, H157, H446, and 95D). Among these five lung cancer cell lines, *SOX17* was weakly expressed in A549 and H157 cells, normally expressed in H446 and 95D cells, and silenced in H23 cells (Figure 1A).

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Partial promoter region methylation of *SOX17* was detected in four cell lines (A549, H157, H446, and95D), and complete methylation was found in H23 cells. Methylation was correlated with loss or reduction of the *SOX17* gene expression (Figure 1B).

To further explore the regulation of *SOX17* expression by promoter region hypermethylation, the five lung cancer cell lines were treated with DAC, a DNA methylation transferase inhibitor. Re-expression of *SOX17* occurred in H23 cells, and increased expression of *SOX17* was detected in A549 and H157 cells. No changes of expression were found in H446 or 95D cells (Figure 1A). The correlation between promoter methylation and reduced or absent *SOX17* expression was further confirmed by western blot analysis of protein levels in H23 and A549 cells (Figure 1C). These results demonstrate that *SOX17* expression was regulated by promoter region hypermethylation in lung cancer cell lines.

SOX17 was frequently methylated in human primary lung cancer and loss of SOX17 expression was related to methylation

SOX17 methylation status in primary lung cancer tissues was investigated using MSP. *SOX17* was methylated in 60.2% of cases (53/88) and unmethylated in 39.8% (35/88). In comparison, in 29 available adjacent normal tissue samples, *SOX17* was methylated in only 17.2% (5/29) and unmethylated in 82.8% (24/29) (Figure 2A). The ratio of methylated/ unmethylated *SOX17* was significantly higher in lung cancer compared with adjacent normal tissue ($P<0.05$). IHC was performed on 29 available paraffin-embedded paired lung cancer and adjacent normal tissue samples. Reduced or absent *SOX17* expression was detected in 25 cancer cases, with normal expression in only four cases. In contrast, reduced or absent *SOX17* expression was found in only 6 adjacent normal tissues, while normal expression was found in 23 cases. *SOX17* expression levels thus differed significantly between lung cancer and adjacent normal tissues (Figure 2B). Among the 25 cases of lung cancer with absent or reduced *SOX17* expression, 22 cases were methylated (88.0%). Among the 6 cases of adjacent normal lung tissue sample with absent or reduced *SOX17* expression, 4 cases were methylated (66.7%). These results suggest that promoter region hypermethylation is significantly related to absent or reduced *SOX17* expression ($P<0.05$).

SOX17 methylation was significantly associated to female sex in lung cancer patients ($P<0.05$), and to poor differentiation of lung cancer ($P<0.05$, Table 1). These results suggest that *SOX17* was silenced by promoter region methylation in lung cancer, and that *SOX17* methylation may induce lung carcinogenesis. *SOX17* methylation may be a susceptibility factor for lung cancer in women.

Colony formation was inhibited by restoration of SOX17 expression in H23 cells

The effects of *SOX17* on lung carcinogenesis were evaluated by colony-formation assays. The efficiency of colony formation was significantly inhibited by restoration of *SOX17* expression in H23 cells (Figure 3), suggesting that SOX17 suppressed lung cancer cell proliferation.

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Wnt signaling was suppressed by re-expression of SOX17 in H23 cells

The effect of *SOX17* on Wnt/β-catenin signaling was assessed using the TCF-LEF luciferase reporter system. Luciferase activity was significantly higher in H23 cells with forced β-catenin expression compared with the control empty vector group (Figure 4A). The structure of *SOX17*, deleted into different length fragments, is shown in Figure 4B. There was no suppressive effect on β-catenin/TCF-LEF transcription following transfection with pGL3-OT, pRL-TK, β-catenin, or *SOX17* fragment 135–414 (with HMG domain deleted). Transfection with *SOX17* expression vector fragments 50–414 and 1–353 produced similar inhibitory effects (Figure 4C).

Western blotting in H23 cells was employed to further confirm the inhibitory effect of *SOX17* on the Wnt signaling pathway. The expression of cyclin D1, the downstream gene of β-catenin, was reduced in *SOX17* re-expressed H23 cells (Figure 4D).

Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide; however, less than 20% of patients are diagnosed at an early stage (Tanner *et al*., 2010). Researchers have recently made progress in identifying prospective lung cancer biomarkers (Greenberg *et al*., 2007). Epigenetic change is now well established as an important molecular mechanism for gene silencing, and is involved in a series of human primary carcinomas, including lung cancer (Brock *et al*., 2008). Our previous study demonstrated that promoter region hypermethylation of *SOX17* plays an important role in colorectal cancer and hepatocellular carcinoma (Zhang *et al*., 2008; Jia *et al*., 2010). SOX17 has also been reported to be involved in the development of lung cancer (Park *et al*., 2006; Lange *et al*., 2009). However, the regulation of *SOX17* expression and its function in human lung carcinogenesis remains unclear. The results of the current study showed that *SOX17* was frequently methylated in human lung cancer, and that *SOX17* expression was in turn regulated by promoter region hypermethylation. *SOX17* methylation was associated with female sex. These results suggest that *SOX17* methylation may serve as a potential marker for lung cancer detection, and also suggest that females are more susceptible than males to *SOX17*-related lung carcinogenesis. The mechanism of females being more susceptible to *SOX17*-related lung cancer remains unclear. In the sea bass, *SOX17* expression was reported significantly higher in females (Navarro-Martin *et al*., 2009). *SOX17* methylation was also related to lung cancer differentiation. Colony-formation assays indicated that SOX17 suppressed lung cancer cell proliferation, indicating a suppressive role for SOX17 in lung carcinogenesis. The results of further experiments suggest that SOX17 inhibits the Wnt signaling pathway in lung cancer cells. All the above results demonstrate that SOX17 acts as a tumor suppressor in lung cancer by inhibiting Wnt signaling.

Conclusion

Promoter region hypermethylation of *SOX17* occurs frequently in lung cancer, and represents an important mechanism for downregulating gene expression. SOX17 inhibits proliferation of lung cancer cells, and plays an important role in antagonizing Wnt/β-catenin

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signaling in lung cancer. Epigenetic changes in *SOX17* may serve as useful diagnostic and prognostic biomarkers in lung cancer.

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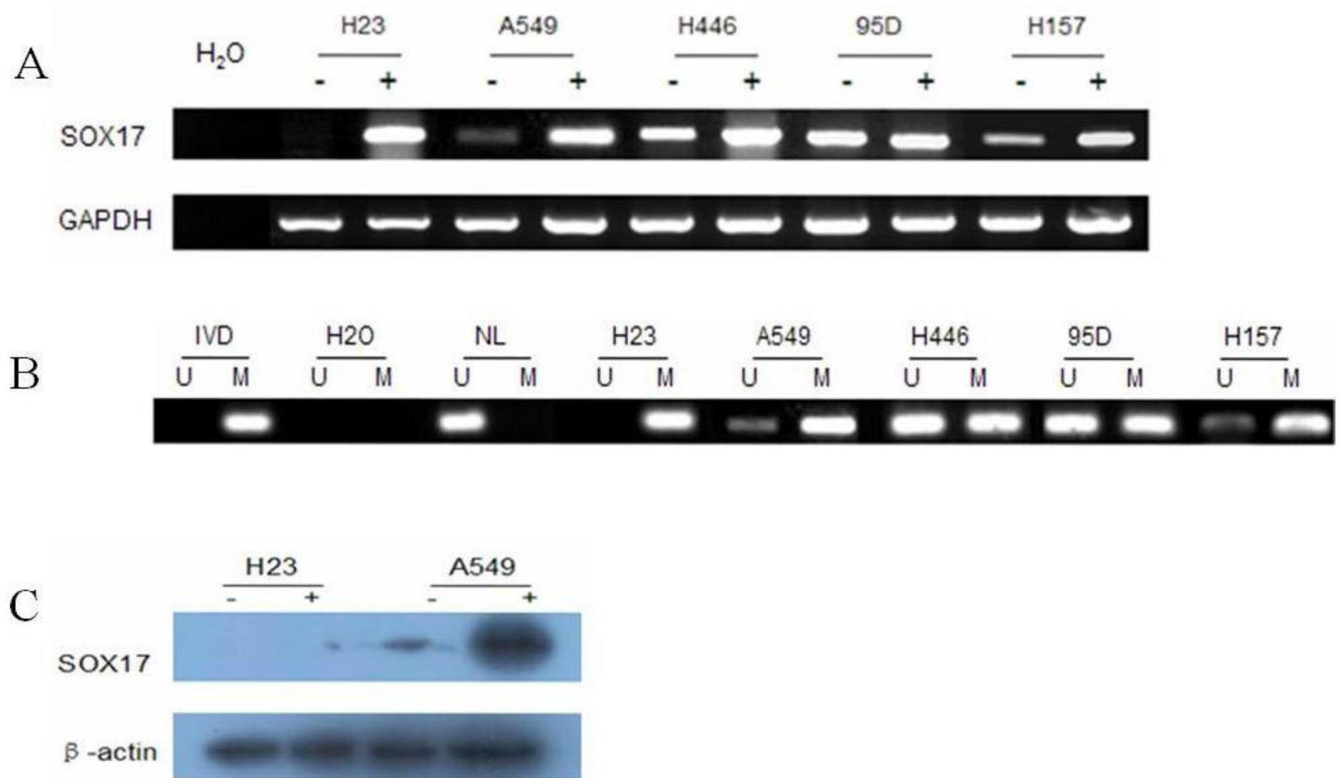

Figure 1.

SOX17 expression was regulated by DNA methylation in lung cancer cell lines. A. Expression of *SOX17* was detected by semi-quantitative RT-PCR in five lung cancer cell lines (H23, A549, H157, H446, and 95D) before and after treatment with 2 μmol/l DAC (+) for 96 hours. B. MSP results for *SOX17* in five lung cancer cell lines. U, unmethylated; M, methylated. Amplification efficiency was verified using methylated (*in vitro* methylated DNA, IVD) and unmethylated controls (normal blood lymphocyte DNA, NL). C. Expression of SOX17 in H23 and A549 cells before and after treatment with 2 μmol/l DAC (+) for 96 hours was further analyzed using western blots.

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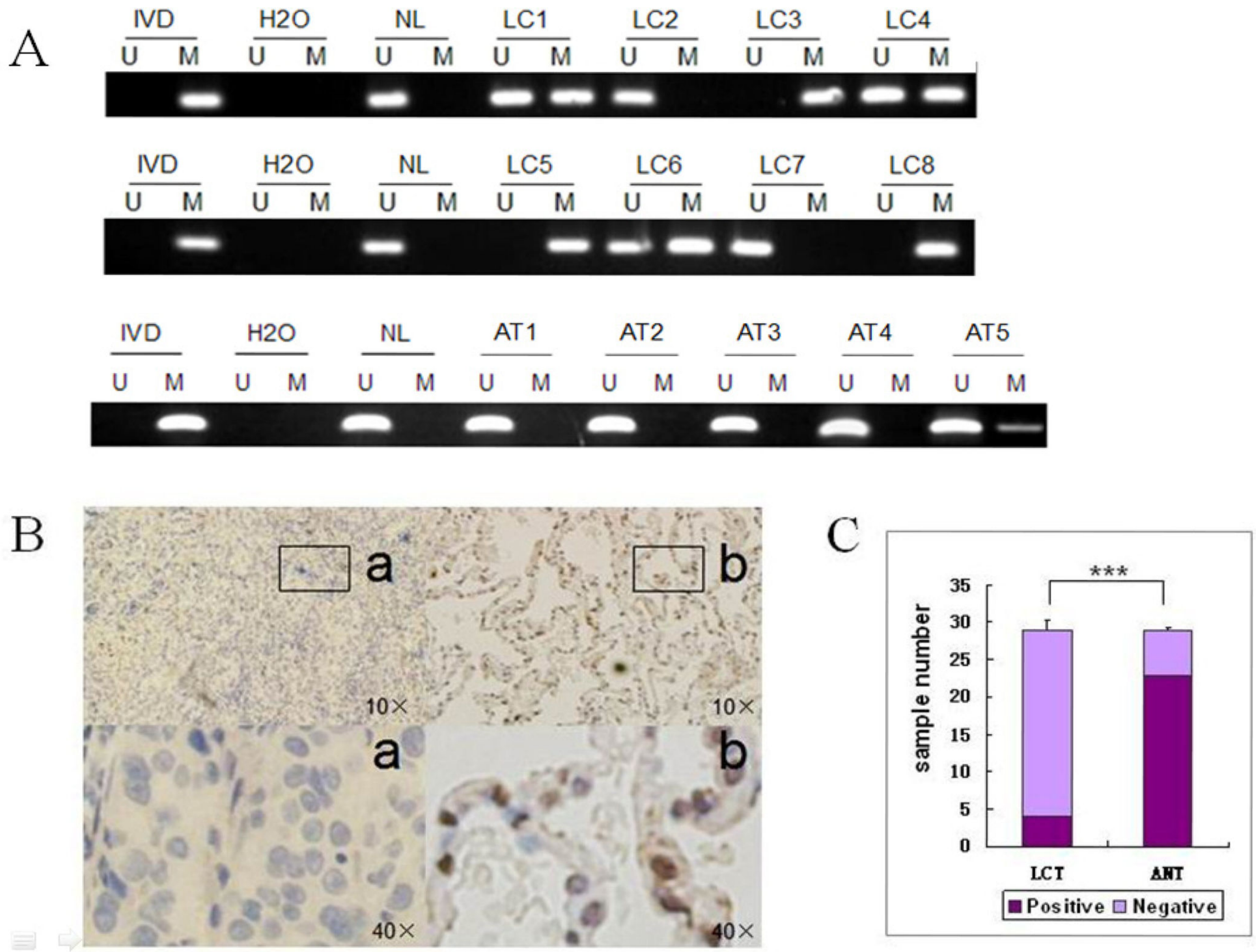

Figure 2.

SOX17 methylation and expression in primary lung cancer tissues. A. Representative *SOX17* methylation results in primary lung cancer and normal lung tissues. LC1-8, primary lung cancer; AT1-AT5, adjacent normal tissues. B. SOX17 expression in primary lung cancer and adjacent normal tissues analyzed by immunohistochemistry. a. Nuclear-negative SOX17 in lung cancer tissue (LCT). b. Nuclear-positive SOX17 in adjacent normal tissue (ANT). C. Expression of SOX17 in primary lung cancer and adjacent tissues shown by histograms (χ^2 tests, *** $P<0.001$).

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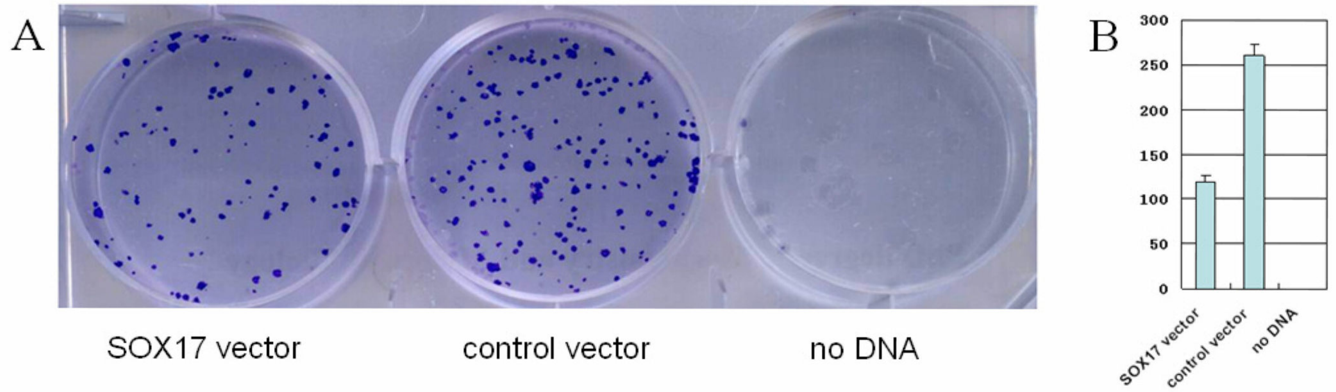

Figure 3.

Restoration of *SOX17* expression inhibited clonogenicity in H23 cells. A. H23 cells transfected with empty control vector or *SOX17* expression vector were reseeded at 800 cells/well and selected with 500 μg/ml G418 for 14 days. Cells were observed and counted after fixing with 75% ethanol and staining with 0.2% crystal violet. B. Experiments were repeated in triplicates and the average colony number for each transfected group is expressed by a bar graph.

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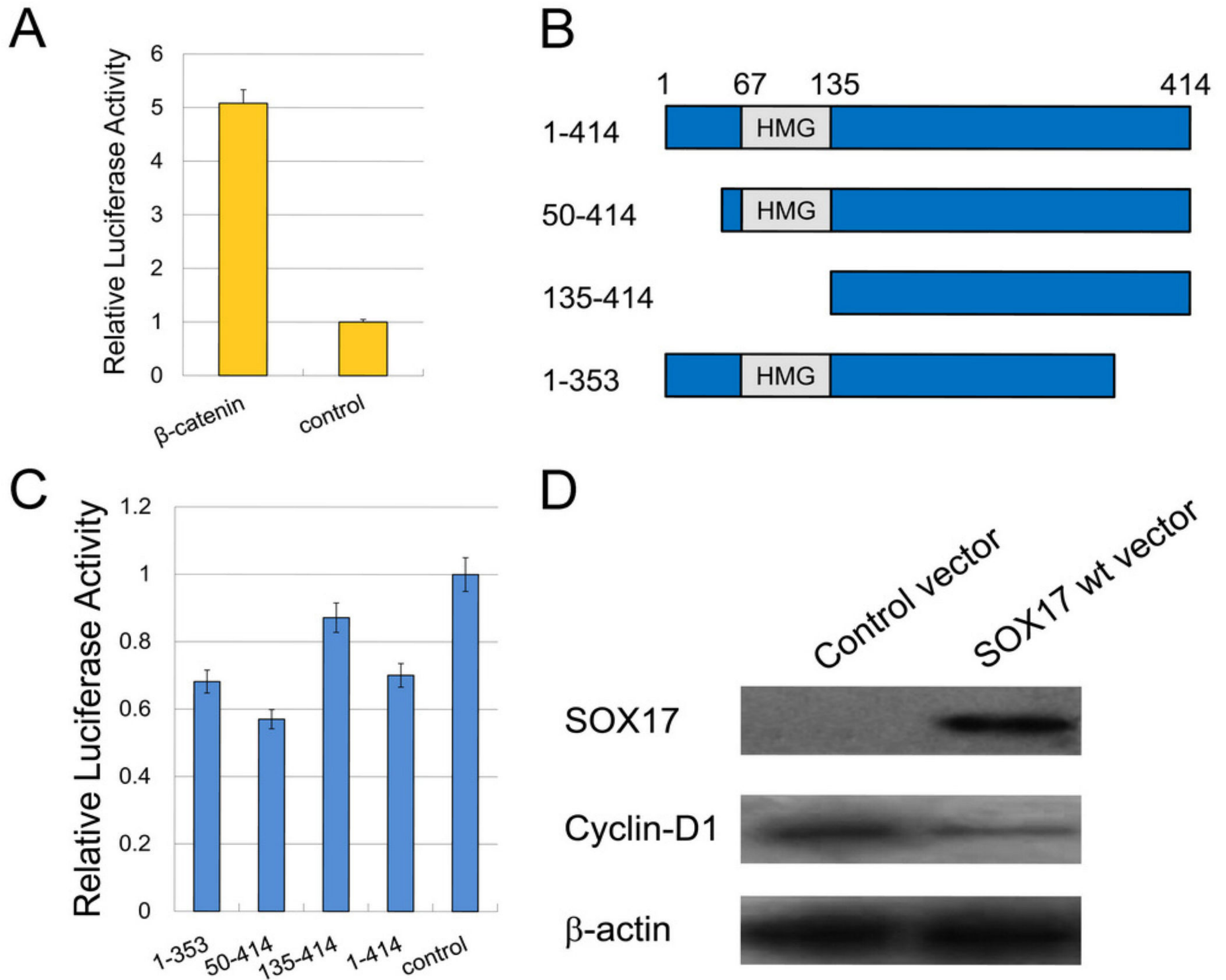

Figure 4.

SOX17 influenced the transcriptional activation of β-catenin and its downstream genes in the Wnt signaling pathway. A. H23 cells were transfected with β-catenin and empty vectors, respectively, to detect relative luciferase activities. B. The architecture of SOX17 with an HMG box is depicted in schematic form. Different deletion mutants (SOX17 constructs 50–414, 135–414, and 1–353) were generated by PCR. C. H23 cells were co-transfected with β-catenin and different SOX17 expression vectors (wild-type or mutant). Transfection with wild-type and mutant SOX17 showed greater or less suppressive effects on β-catenin/TCF-LEF transcriptional activity. D. Transfection with wild-type SOX17 inhibited cyclin D1, a downstream gene of the Wnt signaling pathway.

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Table 1

Clinicopathological Characteristics and SOX17 Methylation Status in Human Lung Cancer

Clinical Parameter	Number of Patients	SOX17 Methylation Status		P Value*
		Methylated	Unmethylated	
		n=53 (60.2%)	n=35 (39.8%)	
Age (years)				
<65	60	37 (61.7%)	23 (38.3%)	0.6863
65	28	16 (57.1%)	12 (42.9%)	
Sex				
Male	60	30 (50.0%)	30 (50.0%)	0.0084
Female	28	23 (82.1%)	5 (17.9%)	
Smoking				
Negative	3	1 (33.3%)	2 (67.6%)	0.9001
Positive	40	22 (55.0%)	18 (45.0%)	
Tumor type				
Squamous cell	34	20 (58.8%)	14 (41.2%)	0.9908
Adenocarcinoma	46	27 (58.7%)	19 (41.3%)	
Tumor differentiation				
Low	38	29 (76.3%)	9 (23.7%)	0.0133
Middle	38	20 (52.6%)	18 (47.4%)	
High	12	4 (33.3%)	8 (66.7%)	
Tumor stage				
I	36	15 (41.7%)	21 (58.3%)	0.9618
II	25	10 (40.0%)	15 (60.0%)	
III	25	9 (36.0%)	16 (64.0%)	
IV	2	1 (50.0%)	1 (50.0%)	

*P values were obtained from χ^2 tests; P<0.05 indicates significant difference.