Lung cancer is currently the most common malignancy and the leading cause of cancer death in the world (1). Clinically, non–small cell lung carcinoma (NSCLC) represents >80% of lung cancers and can be classified into adenocarcinoma (ADC), squamous cell carcinoma (SQC), and large cell carcinoma. ADC and SQC constitute two major subtypes of NSCLC, and there is a trend that incidence of ADC is increasing worldwide, particularly in women (2, 3). In addition, ADC is the most common histologic type of lung cancers arising in never and former smokers (4, 5). The 5-year survival rate for all stages of NSCLC patients is only ~15%, majorly due to diagnosis at late stage when tumor has progressed and become inoperable. Given the life-threatening nature of lung cancers, it is important to identify biomarkers for their early detection and prognostic, and to obtain a better understanding of the underlying mechanisms with respect to the functional roles of the molecules involved in the development and advances of the cancer. Although many markers from gene expression profiling analysis of lung cancers have

**Purpose:** SOX9 is an important transcription factor required for development and has been implicated in several types of cancer. However, SOX9 has never been linked to lung cancer to date. Here, we show that SOX9 expression is upregulated in lung adenocarcinoma and show how it is associated with cancer cell growth.

**Experimental Design:** Data mining with five microarray data sets containing 490 clinical samples, quantitative reverse transcription-PCR validation assay in 57 independent samples, and immunohistochemistry assay with tissue microarrays containing 170 lung tissue cores were used to profile SOX9 mRNA and protein expression. Short interference RNA suppression of SOX9 in cell lines was used to scrutinize functional role(s) of SOX9 and associated molecular mechanisms.

**Results:** SOX9 mRNA and protein were consistently overexpressed in the majority of lung adenocarcinoma. Knockdown of SOX9 in lung adenocarcinoma cell lines resulted in marked decrease of adhesive and anchorage-independent growth in concordance with the upregulation of p21 (CDKN1A) and downregulation of CDK4. In agreement with higher SOX9 expression level in lung adenocarcinoma, the p21 mRNA level was significantly lower in tumors than that in normal tissues, whereas the opposite was true for CDK4, supporting the notion that SOX9 negatively and positively regulated p21 and CDK4, respectively. Finally, whereas SOX9-knockdown cells showed significantly attenuated tumorigenicity in mice, SOX9 transfectants consistently showed markedly stronger tumorigenicity.

**Conclusions:** Our data suggest that SOX9 is a new hallmark of lung adenocarcinoma, in which SOX9 might contribute to gain of tumor growth potential, possibly acting through affecting the expression of cell cycle regulators p21 and CDK4. Clin Cancer Res; 16(17); 4363–73. ©2010 AACR.
Translational Relevance

The identification of overexpression SOX9 in the majority of lung adenocarcinoma is clinically relevant, as this was based on studies of in silico data mining, quantitative reverse transcription-PCR validation, and immunohistochemistry analyses in >700 clinical samples, which were derived from patients from diverse backgrounds (such as ethnic group, gender, and history of smoking). This further means that SOX9 upregulation is a common and important event for the development and/or progression of a great portion of lung adenocarcinoma. Because SOX9 was shown to be required for cell growth in vitro and to be able to promote tumorigenicity in animal model, SOX9 and its associated genes/pathways might have potential to be developed as targets for anticancer therapy in the future.

been documented (6–9), our knowledge about how lung cancer develop and evolve remains vague, and critical genes and molecular pathways involved in lung cancer development and progression need to be further explored.

Members of SOX gene family share homology with the high-mobility group box of the sex-determining region Y (Sry), which encodes transcription factors that bind to high-mobility group domains of DNA (10). SOX transcription factors play vital roles in the regulation of embryonic development, cell fate determination, differentiation, and proliferation (11, 12). Several SOX genes, including SOX2, SOX4, SOX7, SOX9, SOX11, and SOX17, have been known to be expressed in the developing lung, and many of them have been suggested to be involved in the abnormalities of lung morphogenesis and function (see ref. 13 for review). It is well recognized that genes and pathways critical for development may also play important roles in cancer development and progression. We set forth to screen among the developmentally related SOX family genes for possible candidates as lung cancer biomarkers. We began with a meta-analysis in public lung cancer gene expression databases inquiring if there is any unique expressing pattern among the SOX genes. In this regard, SOX4 was reported as a target of gene amplification in lung cancer (14); more intriguingly, the well-known embryonic regulator SOX2 was recently shown as an amplified lineage-specific survival oncogene for lung and esophagus SQC (15). Aside from these two genes, there has been no evidence linking other SOX family members with lung cancers. In this study, we conducted a comprehensive expression profiling analysis of SOX9 in lung cancer, especially lung ADC. We provided several lines of evidence showing that SOX9 expression was frequently upregulated in lung ADC. We also showed that lung cancer cell proliferation and anchorage-independent growth regulated by differential expression of cell cycle regulators p21 and CDK4 could be a pathway through which SOX9 affected cancer cell growth.

Materials and Methods

Public domain data

Information about public microarray gene expression data sets used in this study is listed in Supplementary Table S1. For the Boston data set (7), raw data in CEL format were downloaded and subjected to GC-RMA probe-level normalization using GeneSpring 10.02 (Agilent). For E-MEXP-23 (16), GSE7670 (17), GSE10072 (18), and GSE7880, probe-level normalized data available at sources indicated at Supplementary Table S1 were used. Before further statistical analysis, the probe-level normalized data were further normalized to the median of intensity of all probes in a sample-wise manner and subject to log2 transformation.

Cell cultures and DNA construct

Human lung ADC cell line NCI-HT1299 was obtained from the American Type Culture Collection. CL1-series human lung ADC cell lines (CL1-0, CL1-1, CL1-2, CL1-3, CL1-5, and CL1-5F4) were obtained from Dr. Cheng-Wen Wu (National Health Research Institutes, Miaoli, Taiwan). All the above cell lines used had been examined to exclude Mycoplasma contamination. Cells were cultured in RPMI 1640 (Invitrogen) with 10% fetal bovine serum (Invitrogen) at 37°C in 5% of CO2. For construction of the full-length human SOX9 plasmid, the coding sequences of SOX9 in EST clone (IMAGE:4299305) were PCR amplified with forward primer 5′-GGATCCCAT-GAATCTCTGGACCCCT-3′ and reverse primer 5′-GAATTCTCAAGGTCGAGTGAGCTGTGTGT-3′, and then subcloned into the pCMV-Tag2V expression vector (Stratagene). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen), and cells were subcultured in selection media containing 1 mg/mL G418 (Invitrogen) if stable transfectants were to be maintained. In this way, a stable clone (mixed population) that stably expresses SOX9 was selected and established from the SOX9-null CL1-0 cell line.

Clinical sample

Fifty-seven clinical lung tissue samples, which is comprised of 29 ADC and 28 normal lung samples, were obtained from surgical resection collected at Chang Gung Memorial Hospital, Taoyuan with approval from the Internal Review Board and informed consents from all participants. Human lung cancer tissue microarrays LC810 and LC1006 were obtained from US Biomax; CC4, CCN4, and CCA3 were from Super Biochips. In total, those 5 tissue microarrays contained 47 lung ADC and 123 normal lung tissues. Corresponding clinical information of each specimen on those tissue arrays was provided by the manufacturers.

Short interference RNA

For short interference RNA (siRNA) experiment, pooled siRNA (SMART pool) containing the following sequences (Dharmacon) were used to target SOX9 gene expression...
according to the manufacturer's instruction: 5′-GGAA-CAGCGGCAAGGACGAAGCC-3′, 5′-GACAATCCCCCGAGACAGAAGG-3′, 5′-GAACCCUGUAGCGCGCAAG-3′, 5′-GACCCUGUAGCGCGCAAG-3′, and 5′-GGACGCUGGUGAAGGACAGAAGG-3′.

RNA extraction
Total RNA from clinical tissue samples and those of cultured cell lines were extracted using Trizol (Invitrogen) reagent and RNase kit (Qiagen), respectively, according to the manufacturer's instruction. The quantity of total RNA was determined by NanoDrop ND-1000 (Wilmington). The quality of total RNA samples were examined by Bioanalyzer 2100 (Agilent) to avoid seriously degraded RNA. RNA samples with RNA integrity numbers (RIN) of <7 were excluded from this study.

Gene expression microarray profiling
Complementary RNA targets were synthesized, amplified, labeled, and purified using the TargetAmp Nano-G Bioti-rRNA Labeling kit (Epigenet) according to the manufacturer's instruction. Briefly, 500 ng of total RNA were reversed transcribed and subject to second strand cDNA synthesis. The double-stranded cDNA was then subject to in vitro transcription and complementary RNA labeling with biotin-dUTP. Hybridization of labeled probe to Illumina BeadChips Human HT-12v3 was conducted according to protocol recommended by Illumina. Each HT-12 chip has totally 48,804 unique 50-mer oligonucleotides probes with 15-fold feature redundancy in average. These probes target 27,901 redundancy in average. These probes target 27,901

Quantitative reverse transcription-PCR
Total RNA was reversed transcribed using SuperScript III (Invitrogen) and oligo(dT) primers according to the manufacturer's instruction. Taqman-PCR amplification was done in the LightCycler instrument (Roche Applied Science) with LightCycler Taqman Master kit (Roche Applied Science) using gene-specific primers and probes designed with the Universal Probe Library system (Roche Applied Science). TATA box binding protein (TBP) was used as the internal control to normalize technical variations. The following primers (all in 5′ to 3′ direction) and the Universal Probe Library probes used were as follows: SOX9-fw, GTACCACACCTTTCAACGC, SOX9-rev, TCGCTTCTCTCCAGAAGCTCTC, SOX9 probe, #61; TBP-fw, GTATCCCTCCGCTCCATGCCTC, TBP-rev, ATCCGAGCCTCCAGAAGGC, TBP probe, #51; p21-fw: TGGGTTGTCACCTCTGGA, p21-rev: TGAATTCAGCACCCGATCGAGCTC, p21 probe: #51; CDK4-fw: GTGCACTGCGTTAGTCATGTC, CDK4-rev: TGTGTGCGTAAAAGCTCGA, CDK4 probe: #25. If not particularly indicated, triplicate measurements were done for each sample. Method used for quantification of relative mRNA level has been described elsewhere (20).

Western blotting
Methods for proteins separation by SDS-PAGE and Western blotting were as previously described (21). The primary antibodies used were rabbit anti-SOX9 antibody (Chemicon AB5535, 1:4,000; ref. 22), rabbit anti-CDK4 (Santa Cruz SC-601, 1:1,000), rabbit anti-p21 (Santa Cruz SC-397, 1:1,000), mouse anti–p21 protein (Santa Cruz SC-534907, 1:4,000), and mouse anti–α-tubulin (Santa Cruz SC-58666, 1:2,000) antibodies.

Immunohistochemistry
The immunohistochemistry (IHC) staining was done using a rabbit anti-SOX9 antibody (Chemicon AB5535, 1:50) following protocol previously described (21). The immunoreactivity pattern and histologic appearance of all tissue microarray slides were examined and scored by two independent pathologists. The intensity of nuclear expression and/or cytoplasmic immunostaining was scored on a scale from 0 (no staining) to 3 (strongest intensity). In brief, a score from 0 to 3 (0, 1+, 2+, 3+) was defined as: score 0 indicates negative staining or <10% of positive tumor cells were stained; score 1 indicates 11% to 50% were stained; score 2 indicates 51% to 80% were stained; and score 3 indicates >80% of the tumor cells were stained.

Cell proliferation assay
Cells treated with various conditions were seeded in 24-well plates for time intervals indicated. MTT (Calbiochem) assays were used according to the manufacturer's instruction.

Anchorage-independent growth
Cells (∼1×10⁶) were seeded in top layer of medium containing 0.3% of soft agar (Cambrex Bioscience) and then plated over hardened bottom layer of medium containing 0.5% of agar in 3-cm culture dish. Triplicate experiments were done for each condition. Colonies formed were stained with crystal violet and counted at 14 to 20 days dependent on growth rate. The ImageJ (Web site for Image software: http://rsb.info.nih.gov/ij/) software was used to determine the number of colony.

Clonogenic assay
Limited diluted cells (∼1×10⁵) were seeded with regular culture medium in triplicate in six-well plates. After 2 to 3 weeks, stained foci composed of at least 50 cells were counted using similar methods as in the anchorage-independent growth assay.
Flow cytometry
At 24, 48, and 72 hours after siRNA treatment, cells were harvested and washed with PBS, then fixed with 70% ethanol at −20°C. After washing with PBS again, cells were resuspended in solution containing TritonX-100 (0.1%), RNase 0.2 mg/mL, and propidium iodide (20 μg/mL) for 30 minutes in the dark. Samples were analyzed for DNA content using flow cytometry. The cell cycle phases were analyzed using the CELLQuest software (Becton Dickinson).

CDK2 kinase assay
CDK2 kinase activity was measured as the extent of phosphorylation of H1 protein in the presence of radioactive ATP as previously described elsewhere (23).

Animal experiments
For CL-15 F4 cells, 1.5 × 10^6 cells transfected with siRNA were injected s.c. into five severely combined immunodeficient (SCID) mice (CB-17) each of test (siSOX9 RNA) and control (scramble RNA) groups at 48 hours posttransfection. For SOX9 transfectant clones, 1.0 × 10^6 cells were prepared for injection into six mice (CB-17) each of test (SOX9 transfectant) and control (empty vector) groups. The volume of tumor formed was measured over a 2-month period postinjection. Tumor volume was calculated according to the formula of length × width × width/2. For each time point, tumor volume was the average of volume of all tumors in tumor-bearing mice of the same group. All procedures follow an animal use protocol approved by the Institutional Animal Care and Use Committee of NHRI.

Statistical analysis
Parametric Student’s t test or nonparametric Wilcoxon test was used to assess the significance of difference between conditions of interest, dependent on whether data of interest was apparently normally distributed. The association between SOX9 protein level (score of the IHC staining) and the quantified clinicopathologic features of tumors were examined by χ² test. Cox proportional hazard regression was used for survival analysis. In all analysis, a P value of <0.05 was considered as statistically significant.

Results

In silico mRNA profiles of SOX9 in lung cancer tissues and quantitative reverse transcription-PCR validation
In a study using previously established lung ADC cell lines (24), we found that the expression of SOX9 was abundant in the highly tumorigenic lung ADC cell lines CL1-5 and CL-15F4 whereas absent in the low tumorigenic cell lines CL1-0 and CL1-1 (Supplementary Fig. S1), implicating a potential role of SOX9 in the development and/or progression of certain lung cancer. To scrutinize this possibility, we first asked whether the SOX9 gene is differentially expressed between cancer and normal cells or among cells from subtypes of NSCLC. We conducted an in silico gene expression analysis using several public data sets (Supplementary Table S1). From the Boston data set (7), the transcript levels of SOX9 in ADC, SQC, and normal lung tissues from NSCLC patients were compared. Interestingly, with normal lung tissues, both ADC and SQC tissues showed significant upregulation of SOX9 (Fig. 1A), with an obvious higher significance of upregulation in ADC (P = 0.0014, Wilcoxon test) than in SQC (P = 0.028), whereas no apparent difference in SOX9 expression level between ADC and SQC was observed. For simplicity, we primarily focused on analysis of SOX9 expression in lung ADC alone. To validate the SOX9 overexpression in lung ADC, more panels of lung cancer gene expression data set having SOX9 expression data in ADC samples were further investigated. Consistently, despite diverse sample populations of those data sets, and regardless whether only matched pairs of normal/tumor tissues from identical patients were used, the significant upregulation of SOX9 in lung ADC relative to normal lung tissue was repeatedly observed across data sets (Supplementary Fig. S2A, Student’s t test; Fig. 1B and C). In addition to the difference observed in tumor/normal comparison, recurrent lung ADC tends to have a significantly higher SOX9 expression level than that of primary ADC lesions (Supplementary Fig. S2B; P = 0.028, Student’s t test), which was not the case in SQC (P = 0.16) from the GSE7880 data set. These in silico data offered a rough overview of the SOX9 expression pattern in lung ADC, which strongly suggested that SOX9 overexpression may play a role during tumorigenesis and/or progression of lung ADC.

To verify the upregulation of SOX9 in lung ADC tissues, an independent set of clinical tissue–derived RNA samples (n = 57) containing 29 ADC and 28 normal tissues adjacent to tumors were collected and their SOX9 mRNA expression level measured by quantitative reverse transcription-PCR (qRT-PCR). Consistent with the previous in silico data, when all normal and tumor samples were compared, SOX9 mRNA was significantly upregulated in lung ADC compared with normal tissues (P = 1.5 × 10^-6, Fig. 1D); when only matched pairs of ADC tissues and their corresponding surrounding normal lung tissues (n = 15) were compared, the upregulation of SOX9 mRNA remained highly significant (P = 1.5 × 10^-7). These data convinced us that SOX9 overexpression was frequent in lung ADCs.

IHC analysis of SOX9 in lung ADC
We then asked whether there is a concordant change between the protein and mRNA expression of SOX9 in lung ADC. To do this, five slides of commercially available tissue microarrays of two independent sources were obtained and analyzed by IHC. Among those arrays, there are 47 ADC and 123 normal lung tissue samples available for the purpose of this study. As shown in a representative case (Fig. 2A), all normal pneumocytes invariably showed positive but relatively weaker (1+) SOX9 staining. However, the
majority of lung ADC (27 of 47, 57%) cases showed much stronger (2+ or 3+) staining (Fig. 2B and C) than normal tissues ($P < 0.0001$), whereas the remaining ADC (~40%) has comparable level of SOX9 intensity with normal tissues (data not shown). A statistical analysis of SOX9 IHC staining intensity with available clinical information from those samples indicated a trend of association with stage ($P = 0.06$, $\chi^2$ test), whereas there was no significant association...
with grade of differentiation or lymph node status (Supplementary Table S2). This analysis indicates that SOX9 protein expression is upregulated in the majority of lung ADC.

**Knockdown of SOX9 caused inhibition of cell growth**

Having established the association between SOX9 overexpression and lung ADC, we next asked whether SOX9 is functionally linked to cancer phenotypes. Using RNA interference technique, several lung ADC cell lines that express high level of SOX9, including NCI-H1299, CL1-5, and CL1-5F4, were treated with siRNA of SOX9 and their phenotypic change examined. As revealed by cell proliferation assay, the monolayer cell growth of CL1-5 and CL1-5F4 was significantly decreased to various extents when SOX9 expression was knocked down (Fig. 3A). The anchorage-independent colony formation in soft agar of NCI-H1299 and CL1-5F4 cells was also significantly attenuated by the silencing of SOX9 expression (Fig. 3B). In clonogenic assay, which evaluates the ability of a limited number of cancer cells seeded in culture medium to grow into colony, CL1-5 and CL1-5F4 cells also showed similar suppressive effect upon knockdown of SOX9, with CL1-5F4 showing most significant inhibition (Fig. 3C). In those three cell lines, silencing of SOX9 consistently resulted in a decrease of growth capability, suggesting that SOX9 is important for cell proliferation of lung ADC cells under different growth conditions.

**Upregulation of p21 and downregulation of CDK4 were involved in SOX9 knockdown-mediated growth inhibition**

To further interrogate the mechanisms underlying the reduced growth upon SOX9 knockdown, we performed microarray gene expression analysis for three cell lines used above to obtain genes whose expression were concordantly affected. Among the genes that were consistently differentially expressed in SOX9 knockdown, NCI-H1299, CL1-5, and CL1-5F4 cells (data not shown), we further searched for genes that are involved in the regulation of cell growth. Surprisingly, CDK4 was found as the most consistently downregulated gene, whereas p21 was the most consistently upregulated. These microarray data were further confirmed by qRT-PCR (Fig. 4A) and Western blot analysis (Fig. 4B). Under SOX9 knockdown condition, the transcriptional downregulation of CDK4 and upregulation of p21, which encodes a cyclin dependent kinase inhibitor protein, strongly suggests that there might be a corresponding change in the distribution of cell cycle phases. Consistently, a significant decrease in G1 (P = 0.03) and increase in G2-M (P = 0.009) phases under SOX9 knockdown condition was observed in CL1-5 cells (Fig. 4C). These results indicate that SOX9-related alteration in cancer cell growth could be mediated by modulation of the expression of cell cycle regulators CDK4 and p21. Notably, such possibility was further supported by...
evidence from a CDK2 kinase activity assay, which showed that CDK2 kinase activity was significantly attenuated when SOX9 was silenced in CL1-5 and CL1-5F4 cells (Supplementary Fig. S3), possibly due to the above-mentioned elevation in the expression of p21, a natural inhibitor of CDK2 activity.

Concordance of expression patterns of SOX9, CDK4, and p21 in lung ADC

We further asked whether the above association among SOX9, CDK4, and p21 found in lung cancer cell lines is also clinically relevant. To address this, we again evaluated two previously used public data sets, the Boston data set and GSE7670, to retrieve the mRNA expression of p21 and CDK4 therein. Consistently, both data sets showed significantly higher CDK4 transcript level and lower p21 level in tumor tissues (Fig. 5A and B). Thus, the expression levels of SOX9, CDK4, and p21 agreed with those observed in lung cancer cell lines described above. To verify the correlated expression patterns in an independent sample set and analysis, we examined our own clinical sample cohorts for qRT-PCR quantification of CDK4 and p21. Again, the aforementioned relationship of SOX9, CDK4, and p21 was observed in our independent sample set (Fig. 5C). In conclusion, these in vitro and in vivo data suggest a negative correlation between expression of SOX9 and p21, and a positive correlation between SOX9 and CDK4 in lung ADC.
Expression of SOX9 affects tumor growth in animal model

To further assess the role of SOX9 in tumorigenicity, we performed two animal experiments of xenograft induction in severe combined immunodeficient mice. In one experiment, the SOX9 siRNA–pretreated CL1-5F4 cells, which showed significant reduction in cell growth in vitro as previously mentioned, were s.c. injected into mice (n = 5), and their tumor formation/growth were compared with mice (n = 5) of the control group injected with the cells pretreated with nontargeting (scramble) siRNA. The results showed that the test and control groups had similarly high positive rate (80%) of tumor formation (four of five mice), which is consistent to the previous study showing the high tumorigenic potential of the CL1-5F4 cells (24). However, in terms of tumor growth kinetics, the mice injected with SOX9-knockdown cells showed significantly slower rate of tumor growth than those of control group (Fig. 6A). Alternatively, in another experiment, we selected a SOX9 transfectant (stable) clone from SOX9-null CL1-0 cell line for similar studies. When SOX9 transfectants were injected into the mice (n = 6), they showed a higher positive rate of xenograft formation than those injected with vector-transfected control: four of six in SOX9 transfectant versus two of six in control. Furthermore, the tumors from SOX9-transfected line also grew faster and larger than those derived from control CL1-0 cells (Fig. 6B). Together, these two animal experiments indicate that expression of SOX9 promotes tumor growth in vivo.

Discussion

SOX9 was originally known as a chondrogenic transcription factor involved in bone formation and testis development, whereas its mutation has been linked to campomelic dysplasia and autosomal sex reversal (25, 26). Subsequently, SOX9 was shown to be a multifaceted transcription factor indispensable for the development of many other organs/tissues, such as pancreas (27, 28), prostate (29, 30), intestine (31), and pigment cell (32). Given the scenario that developmentally required genes are also likely oncogenes upon their deregulation, it is not surprising that SOX9 is associated with cancers: To date, abnormal expression of SOX9 has been linked to mesenchymal chondrosarcoma (33), pancreatic cancer (34), prostate cancer (30, 35), colorectal cancer (36, 37), cutaneous basal cell carcinoma (38), and melanoma (39, 40). In the fetal lung, SOX9 is widely expressed in respiratory epithelial cells, but conditional inactivation of SOX9 specifically in respiratory epithelium did not affect lung morphogenesis or function (41). Even so, in a model portraying the genes/network regulating branching during lung morphogenesis, unique expression pattern of SOX9 in the distal epithelia buds containing progenitor/stem cells marks itself a plausible factor that affects lung growth and thus proliferation of progenitor cells (42). In the present study, we have related SOX9 expression to human lung cancer.

Two putative targets of SOX9, namely p21 and CDK4, were identified in this study. As important cell cycle regulators, both targets have been found to be associated with lung cancers (43–46). Furthermore, the association of SOX9 with p21 seems to be in agreement with a recent report of the interaction between SOX9 and p21 promoter in mouse melanoma cells (40). However, although p21 was reported to be positively regulated by SOX9 in mouse melanoma cells, our data suggest a negative regulation of p21 by SOX9 in lung ADC cells, based on the following observations: (a) upregulation of p21 and associated decrease of

Fig. 5. Concordant mRNA expression profiles of p21 and CDK4 in clinical samples. Expression of p21 and CDK4 in the public domain data were retrieved from Boston (A) and GSE7670 (B) data sets as previously described in Fig. 1. Both two data sets showed consistent upregulation of CDK4 and downregulation of p21 in tumors, compared with normal tissues. C, validation assay in our independent sample cohorts using qRT-PCR gives results consistent to those from public microarray data. Methods for data analysis were the same as in Fig. 1.
CDK2 kinase activity upon knockdown of SOX9 and (b) the reverse expression pattern of p21 and SOX9 in tumor and normal tissues consistently found in several public data sets and in validation assay using independent samples. To account for the variation in roles of SOX9 in the regulation of p21 expression in melanoma versus lung cancer cells, we also noted that the SOX9 expression pattern in these two cancer types is entirely opposite: Melanoma cells have underexpressed SOX9 than normal skin cells, whereas lung ADC cells have overexpressed SOX9 than normal lung tissues. Given the distinct background in expression pattern, it is highly plausible that the way SOX9 regulate p21 might be cell type dependent because different SOX partners might be involved in the regulation of target genes (11). However, more investigations are required to further understand whether p21 is a transcriptional target of SOX9 in lung ADC cells. It is also tempting to know if there exists any SOX9 partner, which is also involved in lung cancer development. In addition, intriguingly, Cdk4 might be a target of SOX9 in lung ADC cancer cells. Whether Cdk4 is a transcriptional target of SOX9 also remains to be elucidated. Yet, the SOX9-mediated simultaneous upregulation of p21 and downregulation of Cdk4, whose encoded product is also a target of inhibitory binding of p21, was appealing. Perhaps this implies SOX9 could target important key regulators of the same pathway, which could make control of cell proliferation in lung cancer cells more efficient and flexible, offering the growth advantages over normal cells during cancer formation.

Most of the analyses of SOX9 expression in lung cancer cells in the present study were confined to lung ADC. This does not exclude the role of SOX9 in lung SQC. For example, upregulation of SOX9 was statistically significant in a public lung SQC data set (Fig. 1A), although this data set has smaller sample size in SQC. Moreover, our IHC analysis of lung SQC tissues also revealed a great portion of samples with upregulated SOX9 level (data not shown). Intriguingly, a survival analysis in a public lung SQC data set indicated that SOX9 expression is inversely correlated with recurrence-free survival of patients (data not shown), indicating a possibility that SOX9 could also be linked to lung SQC, although more comprehensive studies are required.

Recently TITF1 (thyroid transcription factor 1, also known as NKX2-1), another gene important for lung morphogenesis, was identified as an amplified lineage-specific oncogene for lung ADC (47, 48). Our finding that SOX9 is overexpressed in lung ADC raises the question whether SOX9 and TITF1 could be in the same gene network/pathway destined to the development of lung ADC. In favor of this possibility, a loop-containing network composed of four genes, including SOX9, FOXA2, HNF3β, TITF1, and CBP (CREB-binding protein) was built using knowledge-based network construction tool (Supplementary Fig. S4). It is tempting to know if this network is functioning in lung ADC cells and relevant to lung ADC tumorigenesis and pathogenesis.

This is the first study reporting the involvement of SOX9 expression in the regulation of cell proliferation and tumorigenicity of lung ADC. Delineating the pathway and mechanism underlying this involvement would help us to understand lung cancer formation and identify potential new targets for cancer therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Yu-Ting Chiu of NHRI Pathology Core for IHC experiments, Chih-Yu Chen and Oscar Tsung-Kai Chang for the assistance in statistical analysis, and Dr. Men-Liang Chen and Dr. Shu-Chen Liu for the critical comments.
Grant Support

References


Clinical Cancer Research

Upregulation of SOX9 in Lung Adenocarcinoma and Its Involvement in the Regulation of Cell Growth and Tumorigenicity


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-0138

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2010/08/31/1078-0432.CCR-10-0138.DC1

Cited articles
This article cites 48 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/16/17/4363.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/16/17/4363.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.