Loss of Special AT-Rich Binding Protein 1 Expression is a Marker of Poor Survival in Lung Cancer

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Introduction: Lung cancer is the leading cause of cancer-related mortality and requires more effective molecular markers of prognosis and therapeutic responsiveness. Special AT-rich binding protein 1 (SATB1) is a global genome organizer that recruits chromatin remodeling proteins to epigenetically regulate hundreds of genes in a tissue-specific manner. Initial studies suggest that SATB1 overexpression is a predictor of poor prognosis in breast cancer, but the prognostic significance of SATB1 expression has not been evaluated in lung cancer.

Methods: A cohort of 257 lung cancers was evaluated by immunohistochemistry. Epigenetic silencing of *SATB1* was examined in cell lines by 5-Aza 2-deoxycytidine and trichostatin A treatment, and chromatin immunoprecipitation.

Results: Significant loss of SATB1 expression was found in squamous preinvasive lesions ($p < 0.04$) and in non-small cell lung cancers ($p < 0.001$) compared with matched normal bronchial epithelium. Loss of SATB1 independently predicted poor cancer-specific survival in squamous cell carcinomas (SCCs; hazard ratio: 2.06, 95% confidence interval: 1.2–3.7, $p = 0.016$). Treatment of lung cancer cell lines with the histone deacetylase inhibitor trichostatin A resulted in up-regulation of *SATB1*. *SATB1* was associated with a decrease in the active chromatin mark acetylated histone H3K9 and an increase in the repressive polycomb mark trimethylated H3K27 in a SCC cell line relative to a normal bronchial epithelial cell line.

Conclusions: This is the first study showing that SATB1 expression is lost in early preinvasive squamous lesions and that loss of SATB1 is associated with poor prognosis in lung SCC. We hypothesize that the *SATB1* gene is epigenetically silenced through histone modifications.

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Lung cancer is the most frequent cancer worldwide with the highest cancer-related mortality.[1] This represents a major public health problem, with lung cancer accounting for 1.2 million deaths globally each year.[2] Therapeutic interventions have had little impact on the prognosis of non-small cell lung cancer (NSCLC) and small cell lung cancers (SCLCs), which have 5-year survival rates of 15% and 5%, respectively.[1,3] Lung cancer is histologically and phenotypically heterogeneous, and genetic markers that can further stratify tumors into prognostically relevant or effective treatment subgroups are greatly needed. The poor prognosis may improve with a greater understanding of the molecular basis of the disease, with a focus on molecular markers of risk that may lead to improved detection or treatment strategies.

Lung cancer develops through the accumulation of genetic and epigenetic abnormalities, in preinvasive lesions to invasive lung cancer.[4] One abnormality detected in early squamous preinvasive lesions is widespread histone modifications, such as loss of H4K20 trimethylation.[5] Abnormal modifications to the histone code play an important role in lung cancer tumorigenesis and are predictive of prognosis.[5,6] Special AT-rich binding protein 1 (SATB1) is a tissue-specific chromatin remodeling protein that regulates the epigenetic organization of multiple genes by region-specific histone modifications, which are dictated by the binding of SATB1 to specific ATC-rich DNA sequences.[7–9] SATB1 regulates hundreds of genes simultaneously by tethering specific loop regions of DNA to the nuclear matrix[8] and recruiting other chromatin remodeling proteins to regulate chromatin structure and gene expression over long distances.[10,11]

Initial studies suggest that SATB1 overexpression is associated with tumor progression and metastasis in breast cancer,[10,12] where it is an independent predictor of poor prognosis.[10] Nevertheless, independent validation of the prognostic role of SATB1 expression does not support this finding.[12–14] The first breast cancer study[10] showed that SATB1 expression directs epigenetic modifications resulting in up-regulation of metastasis-associated genes, while down-regulating tumor suppressor genes. By binding to base unpairing regions of DNA, SATB1 is able to act as a docking station for specific chromatin remodel-

ing proteins and transcription factors to coordinate chromatin architecture and nucleosome positioning[11,15–17] and regulate the expression of more than 10% of all genes.[17,18] Therefore, it can be hypothesized that disruption of SATB1 may also result in major changes in gene expression patterns that could contribute to cancer.

SATB1 has also been implicated in other cancers. In two studies of gastric cancer, increased SATB1 expression was found to be an independent adverse prognostic factor.[19,20] In addition, SATB1 was significantly overexpressed in a cohort of 80 laryngeal squamous cell carcinomas (SCC) compared with normal mucosa.[21]

A microarray study comparing gene expression in the lungs of *Myf5-/-*:*Myod-/-* mouse embryos revealed that *SATB1* was down-regulated and was assumed to play a role in pulmonary development and possibly in the differentiation of the alveolar epithelium.[22] Another study investigating messenger RNA (mRNA) expression of *SATB1* in NSCLC found a 13-fold increase in *SATB1* expression relative to normal lung[23]; however, peripheral lung alveoli and bronchial epithelium were pooled in the normal lung sample. In addition, using the Oncomine Research Database, three microarray studies[24–26] were identified which showed significant down-regulation of *SATB1* in lung cancer compared with normal lung, where no distinction was made between peripheral alveoli and bronchial epithelium. Because of the distinct morphological and functional nature of both types of lung tissue, careful examination of SATB1 expression in both normal peripheral lung alveoli and bronchial epithelium is warranted. Clarification of SATB1 protein expression in SCLC and in the histological subtypes of NSCLC is also needed.

SATB1 is located at chromosome 3p, a region which is frequently associated with loss of heterozygosity and gene promoter hypermethylation in NSCLCs.[27] Chromosome 3p alterations are some of the most frequent and earliest molecular abnormalities in lung cancer.[28–30] We have previously described concordant methylation of several chromosome 3p genes in NSCLC,[31] which may be the consequence of a long-range epigenetic effect.[29] Therefore, we hypothesized that SATB1 alteration may also be significant in lung cancer and analyzed SATB1 expression in a cohort of 257 patients. We show that, in contrast to breast cancer and gastric cancer, SATB1 is significantly down-regulated or lost in squamous preinvasive lesions, NSCLC tumors, and metastases. Loss of SATB1 expression is also a marker of poor survival, independent of American Joint Committee on Cancer (AJCC) stage and lymph node status in SCCs. Furthermore, we show that *SATB1* is enriched with repressive histone modifications in a SCC cell line suggesting that *SATB1* may be epigenetically silenced in cancer.

PATIENTS AND METHODS

Cell Lines, Quantitative Polymerase Chain Reaction, and Western Blot Analysis

The following cell lines were obtained from ATCC (Manassas, VA) and grown according to recommendations: H1299 (metastatic NSCLC), H1975 (adenocarcinoma [ADC]), H292 (mucoepidermoid carcinoma), H358 (bronchioalveolar carcinoma), H427 (ADC), H460 (large cell carcinoma), H520 (SCC), A549 (ADC), H187 (SCLC), NL20 (normal bronchial epithelial cell line), and MDA-MB-231 (breast cancer cell line). Total RNA was harvested from cell cultures using the RNeasy Mini kit (Qiagen, Valencia, CA), incorporating DNase I treatment (Qiagen). Complementary DNA synthesis was performed using the TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA) and the ABI 7900 Real-Time PCR system (Applied Biosystems). *SATB1* (Accession No. NM 002971.3) primers used for quantitative polymerase chain reaction (qPCR) were forward 5′ CAT GTT CCA GCA GAG CAG ATT CAG 3′ (3491–3514), reverse 5′ CAC CGT GGG TTG CCG TGG 3′ (3628–3611), and probe 5′ d FAM-CCA CAG CAG CAG CCA CAG ACA GGC CC-BHQ-1 3′. TaqMan assays were used for the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene (Applied Biosystems). qPCRs were performed in triplicate for at least three separate cultures per cell line.

For Western blot analysis, lysates from three separate cultures for each cell line were prepared using RIPA buffer supplemented with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM sodium orthovanadate (Sigma-Aldrich, St Louis, MO). Equivalent amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA) to electro blot. Mouse anti-SATB1 monoclonal antibody (611182, BD Biosciences, Franklin Lakes, NJ) 1:250 was used for Western blotting with mouse antiglyceraldehyde 3-phosphate dehydrogenase monoclonal antibody (AM4300, Ambion, Austin, TX) 1:10,000, used as a reference gene. Western blots were scanned and densitometry performed using Image J.[32]

Patient Cohort

Lung tissue samples were obtained from patients treated at the Royal Prince Alfred Hospital, Sydney, Australia, between 1996 and 2002. Surgery was performed by the one cardiothoracic surgeon (B.C.M.). Ethical approval for the study was obtained from the Royal Prince Alfred Ethics Review Committee (X02-0216 and X06-0167), granted with a specific waiver of consent, with all tissue specimens analyzed anonymously. Only lung cancer-specific deaths were included in the survival analysis. The cohort totaled 257 patients and included 14 patients with SCLC (5%) and 243 patients with stages I to IV NSCLCs (95%). Female patients (95) made up 37% of the cohort and males (162) 63%. Median age at diagnosis was 67 years (range: 37–87 years), and median survival time was 37 months. Smoking history was available for 196 patients of which 36 (18%) were current smokers, 149 (76%) were exsmokers, and 11 (6%) had no history of smoking. None of the patients received adjuvant chemotherapy. Histological tumor subtypes were assessed using the World Health Organization classification,[33] and within the NSCLCs, there were 107 (44%) ADCs (including 16 [7%] bronchioloalveolar carcinomas [BACs]), 42 (17%) large cell carcinomas (LCCs), 7 (3%) large cell neuroendocrine carcinomas (LCNECs), and 87 (36%) SCCs. NSCLC tumors were staged using the AJCC tumor, node and metastasis (TNM) classification classification.[34] The cohort

consisted of 151 (59%) stage I, 81 (32%) stage II, 19 (7%) stage III, and 6 (2%) stage IV tumors. Forty-one patients with regional lymph node metastases were included in the cohort. Precursor lesions were also assessed when available, and there were 27 cases of bronchial squamous epithelial metaplasia, 3 with low-grade dysplasia, and 11 cases of bronchial SCC in situ. Follow-up information of at least 5 years was available for this study.

Tissue Microarray Samples and Immunohistochemistry Staining

Tissue microarrays were constructed using 2 to 4 tissue cores of normal bronchial epithelium obtained from bronchi or terminal bronchioles, 1 to 2 tissue cores of normal peripheral lung alveoli, and 3 to 6 tumor cores, per patient. Each core was 1 mm in diameter and was selected from areas previously marked by a specialist lung pathologist (W.A.C.) as the most representative. Monoclonal mouse anti-SATB1 (611182, BD Biosciences) was used for immunohistochemistry. Antigen retrieval was performed with Dako buffer pH 9.0 (s2367, Dako, Carpentaria, CA) at 125°C for 5 minutes in a Pascal pressure chamber (Dako), and the primary antibody was incubated for 60 minutes at 1:20 dilution. The Envision FLEX system (Dako) was used as a secondary antibody. Mouse IgG1 was included as a negative control and normal peripheral lung alveoli as a negative tissue control. Spleen and normal bronchial epithelium were used as positive controls. SATB1 staining was also confirmed in breast cancer tissue with normal breast staining negatively for SATB1 expression (data not shown). SATB1 expression was evaluated by estimating the average percentage of cells stained positively. Staining was evaluated independently by C.I.S. and a specialist lung pathologist W.A.C., without knowledge of the patient's clinical details, and the average of the two percentages was used for analysis. Significant discrepancies were reviewed before reaching a consensus. SATB1 expression data were dichotomized to positive or negatively stained, with positively stained tissue set at a threshold of $\geq$5% of cells stained positively. This threshold was arrived at, as percentages of $\leq$5% were not accurately indicative of positive staining. This cutoff resulted in 119 patients with less than 5% and 139 patients with $\geq$5% SATB1 expression. The patient cohort contained insufficient examples of ADC preinvasive lesions, therefore only squamous preinvasive lesions were analyzed.

Treatment of Cell Lines with 5-Aza 2-Deoxycytidine and Trichostatin A

A549 and NCI-H520 cell lines were seeded at 1×10^5 cells/ml in 100 mm culture dishes and prepared for each treatment in triplicate. After 24 hours, the cells were treated with either 5 μM 5-Aza 2-deoxycytidine (5-AzaC, Sigma-Aldrich, St Louis, MO) (for 24 hours, then fresh medium for 48 hours) or trichostatin A (TSA, Sigma) at 50, 100, or 200 nM concentrations for 48 hours. The equivalent volume of ethanol was added to mock control cultures. For cotreatment of cells with 5-AzaC and TSA, 5-AzaC was added initially for 24 hours, after which it was removed and TSA added for an additional 48 hours. After treatment, the cells were harvested for RNA analysis.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out with normal NL20 and NCI-H520 SCC cells using the EZ-Magna ChIP kit (Millipore) according to the manufacturers instructions. Formaldehyde-crosslinked chromatin was sonicated and immunoprecipitated with antibodies to histone H3K9Ac (Millipore no. 06-599), histone H3K27Me3 (Millipore no. 07-449) and Rabbit IgG (Jackson ImmunoResearch, West Grove, PA, no. 011-000-120) as a control. ChIP experiments were performed in duplicate. DNA enrichment was quantified using SYBR green (Applied Biosystems) real-time qPCR in triplicate. ChIP primers include *SATB1* (Accession No. NT 022517.18) F: 5′ GTT TTA ATC CCC ATC CCT TTT CCG CC 3′ (18406963-18406938) and *SATB1* R: 5′ GTC CGG GGA ACG GGA GCG AGC GA 3′ (18406803-18406825). Internal control primers: *MYOD* (Accession No. NT 009237.18) F: 5′ CCG CCT GAG CAA AGT AAA TGA 3′ (17681689–17681709), *MYOD* R: 5′ GGC AAC CGC TGG TTT GG 3′ (17681763–17681747), *GAPDH* control primers (Acession No. NT009759.16) F: 5′TCG ACA GTC AGC CGC ATC R 3′(6583701–6583719), and R: 5′CTA GCC TCC CGG GTT TCT CT 3′ (6583769–6583750). For each chromatin sample, the average Ct value was obtained for the immunoprecipitated material and for the input chromatin. The difference in Ct values (delta Ct) reflects the difference in the amount of material that was immunoprecipitated relative to the amount of input chromatin or the internal control genes *GAPDH* and *MYOD* as described in ABI PRISM 7700 Sequence Detection System User Bulletin no 2, P/N 4303859.

Statistical Analysis

SATB1 expression between cell lines, and between primary tumors and normal tissue were compared with Student's *t* test (two sided). Associations between SATB1 expression and clinicopathological parameters were compared with Fisher's exact test (two sided). The Kaplan-Meier log-rank and Cox proportional regression models were used for survival analysis, determining hazard ratios (HRs) with 95% confidence intervals (CIs). Statistical analyses were performed with Stata/SE 9.2 (Stata Corp., College Station, TX) and SPSS software (IBM, Chicago, IL). *p* values of less than 0.05 were considered statistically significant.

RESULTS

Differential SATB1 Expression in Normal Lung and Lung Cancer Cell Lines

SATB1 expression was analyzed in eight NSCLC cell lines (H1299, H1975, H292, H358, H427, H460, H520, and A549), a SCLC cell line (H187), and a normal bronchial epithelial cell line (NL20) at the mRNA transcript level by real-time qPCR analysis (Figure 1*A*). The MDA-MB-231 breast cancer cell line was included as a positive control, which confirmed relative mRNA and protein expression of SATB1. The majority of NSCLC cell lines (6/8) had significantly reduced expression of *SATB1* relative to the normal

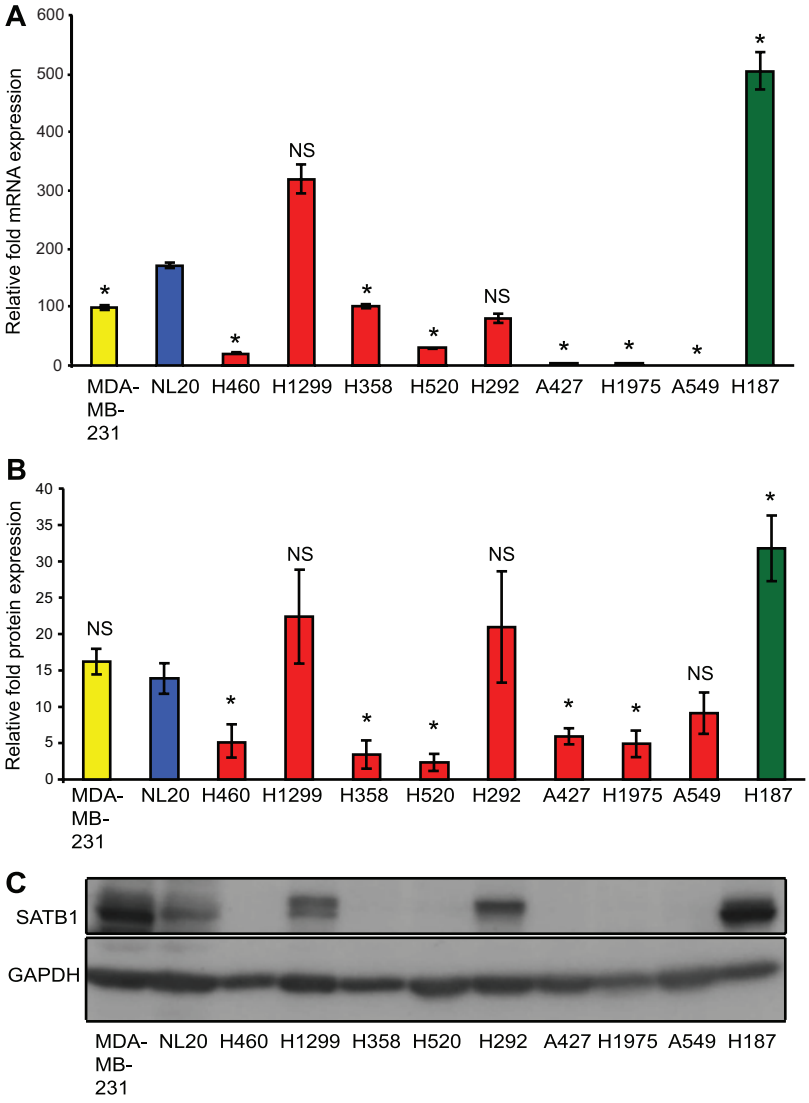

FIGURE 1. Differential special AT-rich binding protein 1 (SATB1) expression in normal lung and lung cancer cell lines. The normal bronchial epithelial cell line NL20 (blue) was compared with non-small cell lung cancer (NSCLC) cell lines (red); H520, $p = 0.010$ (squamous cell carcinoma); H358, $p = 0.032$ (bronchioloalveolar); H1975, $p < 0.001$ (adenocarcinoma [ADC]); H460, $p = 0.042$ (large cell carcinoma); A427, $p = 0.004$ (ADC); A549, $p < 0.001$ (ADC); H292, $p = 0.152$ (mucoepidermoid carcinoma); H1299, $p = 0.144$ (metastatic NSCLC); and a small cell lung cancer cell line (SCLC; green; $p = 0.022$) at the messenger RNA (mRNA) level by quantitative polymerase chain reaction (qPCR, normalized to Glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) (*A*). Relative protein levels (*B*) were compared between the NL20 (blue); NSCLC cell lines (red; H520, $p = 0.014$; H358, $p = 0.006$; H1975, $p = 0.028$; H460, $p = 0.009$; A427, $p = 0.044$; A549, $p = 0.374$; H292, $p = 0.403$; H1299, $p = 0.239$); and SCLC cell line (green; $p < 0.001$). The breast cancer cell line MDA-MB-231 (yellow) was included as a positive control (*A*, $p = 0.032$; *B*, $p = 0.066$). *Defines significance <0.05 relative to normal NL20, NS, not significant. Error bars represent ±standard error of the mean. Representative Western blot (*C*) showing SATB1 (lower band) and GAPDH.

bronchial epithelial cell line NL20 ($p < 0.042$). The SCLC cell line showed significantly increased expression of *SATB1* relative to NL20 cells ($p = 0.022$). The same trend of expression was observed at the protein level, with the majority of NSCLC cell lines (5/8) showing significantly lower abundance of SATB1 expression relative to NL20 cells (Figures 1*B, C*, $p < 0.044$). The SCLC cell line showed significantly more abundant expression of SATB1 protein relative to NL20 cells ($p < 0.001$).

SATB1 Nuclear Expression is Decreased in Primary NSCLC Tumors

SATB1 is a matrix attachment binding protein and is functional in the nucleus. Immunohistochemical analysis of nuclear SATB1 expression was performed in 257 lung cancers and matching normal bronchial and peripheral lung alveoli tissues. SATB1 expression was absent in peripheral lung alveoli (Figure 2*D*). The majority of patients (96%) showed nuclear SATB1 expression in the pseudostratified columnar epithelium of normal bronchi or terminal bronchioles (Figures 2*A* and 3*B*) but not homogeneously in all cells. On average, 28.7% of normal bronchial epithelial cells stained positive for SATB1 in the nucleus (Figure 2*M*). The level of SATB1 nuclear expression was significantly decreased in cancer tissue in all NSCLC subtypes and lymph node metastases (Figure 2*M*), where the average number of cells showing SATB1 nuclear expression was 16.9% in LCC ($p < 0.001$), 13.7% in SCC ($p < 0.001$), and 7.1% in ADC ($p < 0.001$). There was no significant difference between SCLC ($p = 0.574$), SCLC metastases ($p = 0.380$), and normal bronchial epithelium (Figure 2*M*). When SATB1 expression was dichotimized to ≥ or less than 5% nuclear expression, loss of SATB1 expression (<5%) occurred in 52.3% of NSCLC tumors compared with 6% of normal tissue specimens. In SCLCs, there was no statistical difference in the level or frequency of SATB1 nuclear expression between tumors and the normal bronchial tissue specimens (Figure 2*M*), and no significant difference in clinicopathological characteristics (Table 2). Loss of nuclear SATB1 expression was more

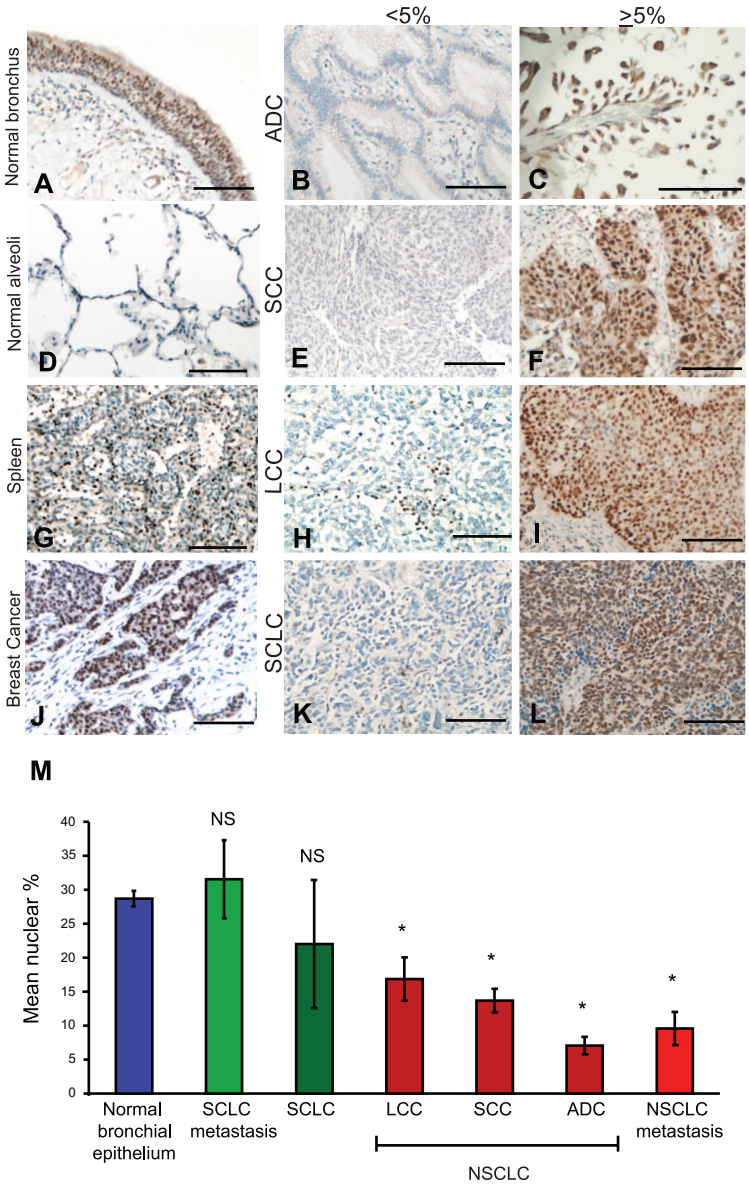

FIGURE 2. Immunohistochemistry analysis of special AT-rich binding protein 1 (SATB1) expression in tumor samples. SATB1 was expressed in normal bronchial epithelial cells (*A*) but not in peripheral lung alveoli (*D*). SATB1 stained positively in T lymphocytes in spleen tissue (*G*) and stained positively in breast tumors (*J*). Representative staining is also shown for SATB1 expression in adenocarcinoma (ADC) <5% (*B*) and ≥5% (*C*), in squamous cell carcinoma (SCC) <5% (*E*) and ≥5% (*F*), in large cell carcinoma (LCC) <5% (*H*) and ≥5% (*I*) and in small cell lung cancer (SCLC) <5% (*K*) and ≥5% (*L*). Scale = 500 μm. SATB1 expression (*M*) was compared with normal bronchial epithelium in SCLCs ($p = 0.574$), SCLC metastases ($p = 0.380$), LCC ($p < 0.001$), SCC ($p < 0.001$), ADC ($p < 0.001$), and NSCLC metastases ($p < 0.001$). *Defines significance <0.001 relative to normal. NS, not significant. Error bars represent ±standard error of the mean.

frequent in ADC tumors than in SCC ($p < 0.001$) or LCC ($p = 0.005$) (Table 1). In NSCLCs, SATB1 expression (≥5%) was associated with poor differentiation ($p = 0.019$) (Table 1). In early stages of NSCLC, such as stage 1A, the majority of cases were SATB1 positive ($p = 0.041$) (Table 1). In contrast, the majority of stage 3 and stage 4 tumors were SATB1 negative, but this was not statistically significant ($p > 0.106$).

We observed no significant difference in the expression of nuclear SATB1 in normal bronchial epithelial cells from patients with NSCLC with or without a history of smoking ($p = 0.371$). Of the 10 never smokers, 50% displayed loss of SATB1 expression in tumor tissue, which was the same frequency as patients who were current smokers (Table 1, $p = 0.854$). There was also no significant difference in SATB1 expression between tumor tissue from patients who had never smoked or had ever smoked ($p = 1.000$). The majority of never smokers had ADC (27%) or BAC (55%).

Cytoplasmic staining of SATB1 (data not shown) was also observed, but only 0.8% of cells stained positive in the normal bronchial epithelium. In contrast to the decrease in nuclear expression in NSCLCs, the level of cytoplasmic expression of SATB1 was increased in NSCLCs ($p < 0.001$). The percentage of cells staining positive for SATB1 was 7.4% for LCC, 29.4% for SCC, 9.3% for ADC, and 14.0% for NSCLC metastatic lymph node tumors. Nevertheless, cytoplasmic SATB1 expression was not associated with survival or any other clinicopathological parameters. Only 1.7% of cells in SCLCs showed cytoplasmic SATB1 expression, and this was not significantly different from normal bronchial epithelium ($p = 0.510$).

Loss of Nuclear SATB1 Expression is an Early Event in SCC

Lung cancers have been reported to arise from a series of progressive pathological lesions.[35–37] Preinvasive changes

TABLE 1. Clinicopathological Characteristics and SATB1 Nuclear Expression in NSCLCs

	SATB1 Negative (<5% Expression)	SATB1 Positive (≥5% Expression)	*p*
Pathology			
Well differentiated	16 (62%)	10 (38%)	0.100
Moderately differentiated	56 (52%)	51 (48%)	0.244
Poorly differentiated	44 (40%)	66 (60%)	**0.019**
Histology type			
SCC (vs. total tumors)	29 (33%)	58 (67%)	**0.001**
ADC (vs. total tumors)	68 (64%)	39 (36%)	**<0.001**
LCC (vs. total tumors)	19 (39%)	30 (61%)	0.200
ADC vs. SCC	—	—	**<0.001**
ADC vs. LCC	—	—	**0.005**
SCC vs. LCC	—	—	0.577
AJCC stage			
1A	18 (35%)	34 (65%)	**0.041**
1B	46 (49%)	48 (51%)	0.793
2A	4 (33%)	8 (67%)	0.382
2B	37 (54%)	31 (46%)	0.202
3A	3 (75%)	1 (25%)	0.351
3B	3 (43%)	4 (57%)	1.000
4	5 (83%)	1 (17%)	0.106
Smoking history			
Current smokers	17 (50%)	17 (50%)	0.854
Exsmoker	70 (47%)	79 (53%)	0.793
Never smoked	5 (50%)	5 (50%)	1.000
Unknown	24 (48%)	26 (52%)	1.000
Lymphatic invasion			
Absent	97 (48%)	105 (52%)	0.866
Present	19 (46%)	22 (54%)	0.866
Blood vessel invasion			
Absent	94 (48%)	100 (52%)	0.749
Present	22 (45%)	27 (55%)	0.749
Perineural invasion			
Absent	104 (47%)	119 (53%)	0.350
Present	12 (60%)	8 (40%)	0.350
Pleural invasion			
Absent	86 (48%)	93 (52%)	0.885
Present	30 (47%)	34 (53%)	0.885
Bronchial resection margin			
Not involved	106 (47%)	120 (53%)	0.452
Involved	10 (59%)	7 (41%)	0.452

Clinicopathological characteristics were compared between patients with SATB1-negative tumors (<5%) and SATB1-positive tumors (≥5%) in non-small cell lung cancers (NSCLC) Bolded values denote statistically significant *p* value.

SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; SATB1, special AT-rich binding protein 1; AJCC, American Joint Committee on Cancer.

TABLE 2. Clinicopathological Characteristics and SATB1 Nuclear Expression in SCLCs

	SATB1 Negative (<5% Expression)	SATB1 Positive (≥5% Expression)	*p*
Pathology			
Poorly differentiated	2 (14%)	12 (86%)	N/A
AJCC stage			
1A	0 (0%)	3 (100%)	1.000
1B	0 (0%)	2 (100%)	1.000
2A	0 (0%)	0 (0%)	N/A
2B	0 (0%)	1 (100%)	1.000
3A	1 (25%)	4 (75%)	1.000
3B	1 (33%)	2 (67%)	0.396
4	0 (0%)	0 (0%)	N/A
Smoking history			
Current smokers	0 (0%)	2 (100%)	0.533
Exsmokers	0 (0%)	0 (0%)	N/A
Never smoked	0 (0%)	1 (100%)	0.672
Unknown	2 (18%)	9 (82%)	0.425
Lymphatic invasion			
Absent	2 (14%)	12 (86%)	N/A
Present	0 (0%)	0 (0%)	N/A
Blood vessel invasion			
Absent	1 (9%)	10 (91%)	0.396
Present	1 (33%)	2 (67%)	0.396
Perineural invasion			
Absent	2 (14%)	12 (86%)	N/A
Present	0 (0%)	0 (0%)	N/A
Pleural invasion			
Absent	2 (15%)	11 (85%)	1.000
Present	0 (0%)	1 (100%)	1.000
Bronchial resection margin			
Not involved	2 (14%)	12 (86%)	N/A
Involved	0 (0%)	0 (0%)	N/A

Clinicopathological characteristics were compared between patients with SATB1-negative tumors (<5%) and SATB1-positive tumors (≥5%) in small cell lung cancers (SCLC).

N/A, not applicable; AJCC, American Joint Committee on Cancer; SATB1, special AT-rich binding protein 1.

have been well defined for SCC, whereas preinvasive changes leading to ADC or LCC are less well understood.[4,38,39] We wanted to determine whether loss of SATB1 expression occurred before invasive SCC tumor formation, and if so, at which preinvasive stage this could be detected. We observed a significant reduction in the average level of SATB1 nuclear expression in all categories of preinvasive squamous lesions analyzed (Figure 3*A*), including squamous metaplasia ($p < 0.001$) (Figure 3*C*), low-grade dysplasia ($p < 0.001$) (Figure 3*D*), and carcinoma in situ ($p = 0.04$) (Figure 3*E*), along with SCC ($p < 0.001$) (Figure 3*F*), carcinoma in situ ($p = 0.042$), and lymph node metastasis ($p < 0.001$) (Figure 3*G*), compared with normal bronchial expression (Figure 3*B*). This suggests that loss of SATB1 expression is an early event in squamous cell carcinogenesis and is detectable in the most initial stage of preinvasive changes.

Loss of SATB1 Expression is Associated with Poorer Survival in SCC and in Patients with NSCLC with History of Smoking

Significance of SATB1 expression for disease-specific survival was assessed (Figure 4). Noncancer-related deaths and patients who were still alive at the time of analysis were

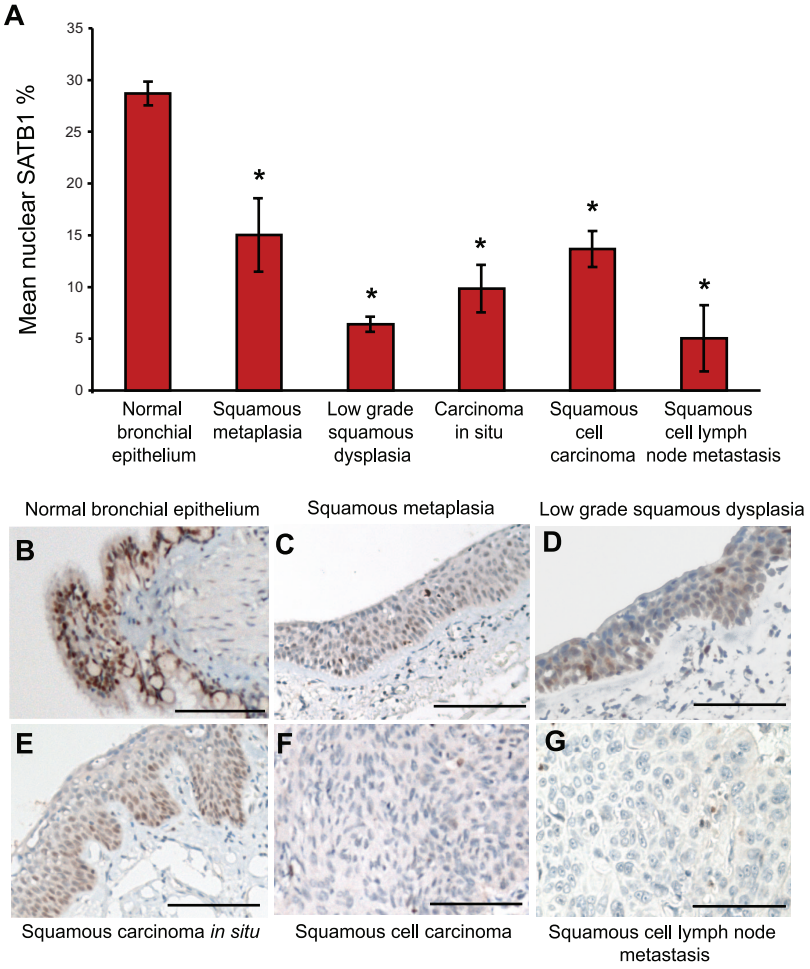

FIGURE 3. Immunohistochemistry analysis of special AT-rich binding protein 1 (SATB1) expression in normal lung and preneoplastic lesions. *A,* SATB1 expression was analyzed by immunohistochemistry and found to be significantly lost in squamous metaplasia ($p < 0.001$), low-grade squamous dysplasia ($p < 0.001$), carcinoma in situ ($p = 0.04$), squamous cell carcinoma ($p < 0.001$), carcinoma in situ ($P = 0.042$) and squamous lymph node metastasis compared with normal bronchial epithelial cells compared. *Denotes $p < 0.05$ significance relative to normal. Error bars represent ±standard error of the mean. Representative staining is shown for normal bronchial epithelium (*B*), squamous metaplasia (*C*), squamous low grade dysplasia (*D*), squamous cell carcinoma in situ (*E*), squamous cell carcinoma (*F*), and squamous cell lymph node metastasis (*G*). Scale = 500 μm.

censored resulting in 152 patients with NSCLC and 9 patients with SCLC. Loss of SATB1 expression was associated with poorer survival in the patients with NSCLC who had ever smoked ($p = 0.049$) but not in the whole NSCLC cohort ($p = 0.09$). In the NSCLC subgroups, loss of SATB1 expression was significantly associated with decreased disease-specific survival in SCC ($p < 0.01$) but not in LCC ($p = 0.40$) or ADC ($p = 0.23$). In patients with SCLC, loss of SATB1 expression also correlated with poorer disease-specific survival ($p = 0.03$). Nevertheless, after censoring, the SATB1-negative group contained only two patients of a total nine patients; therefore, this survival difference requires validation with a larger sample group.

In a multivariate analysis, loss of SATB1 expression was found to be an adverse prognostic factor in SCCs, independent of AJCC stage, lymphatic, and blood vessel invasion (HR: 2.06, 95% CI: 1.2–3.7, $p = 0.016$) (Table 3). In patients with NSCLC who had ever smoked, loss of SATB1 expression was not an independent prognostic factor (HR: 1.41, 95% CI: 0.9–2.15, $p = 0.114$) (Table 3).

SATB1 Expression is Epigenetically Regulated in Lung Cancer Cell Lines

In a study of 11 lung carcinoma samples in the Cosmic cancer database,[40] *SATB1* mutations were found to be very rare, with only one single point mutation in one of the 11 samples investigated. Similarly, mutations in *SATB1* appeared to be rare in all other cancer tissue types. Therefore, we began by examining whether loss of SATB1 expression was due to epigenetic silencing. Two lung cancer cell lines (H520 and A549) were treated with the demethylating agent 5-AzaC, an inhibitor of histone deacetylase—TSA, or a combination of both. We observed an increase in expression of *SATB1* in both H520 and A549 cell lines, after 200 nM TSA treatment alone ($p < 0.01$, $p = 0.03$) and a further increase in expression in cells treated with both 5 μM 5-AzaC and 200 nM TSA ($p < 0.01$ and $p < 0.01$), suggesting that *SATB1* expression is epigenetically repressed in these lung cancer cell lines (Figures 5*A*, *B*).

To determine whether the down-regulation of SATB1 was associated with chromatin remodeling, we performed ChIP on the SCC cell line NCI-H520 and the normal bronchial epithelial cell line NL20. NCI-H520 cells express SATB1 at low levels, compared with the NL20 cells where SATB1 expression is at moderately high levels (Figure 1*A*). In the NCI-H520 cells, the *SATB1* promoter was found to be deacetylated at histone H3K9 (Figure 5*C*) relative to the NL20 cell line. Furthermore, in the NCI-H520 cells, *SATB1* was significantly enriched for the repressive chromatin mark

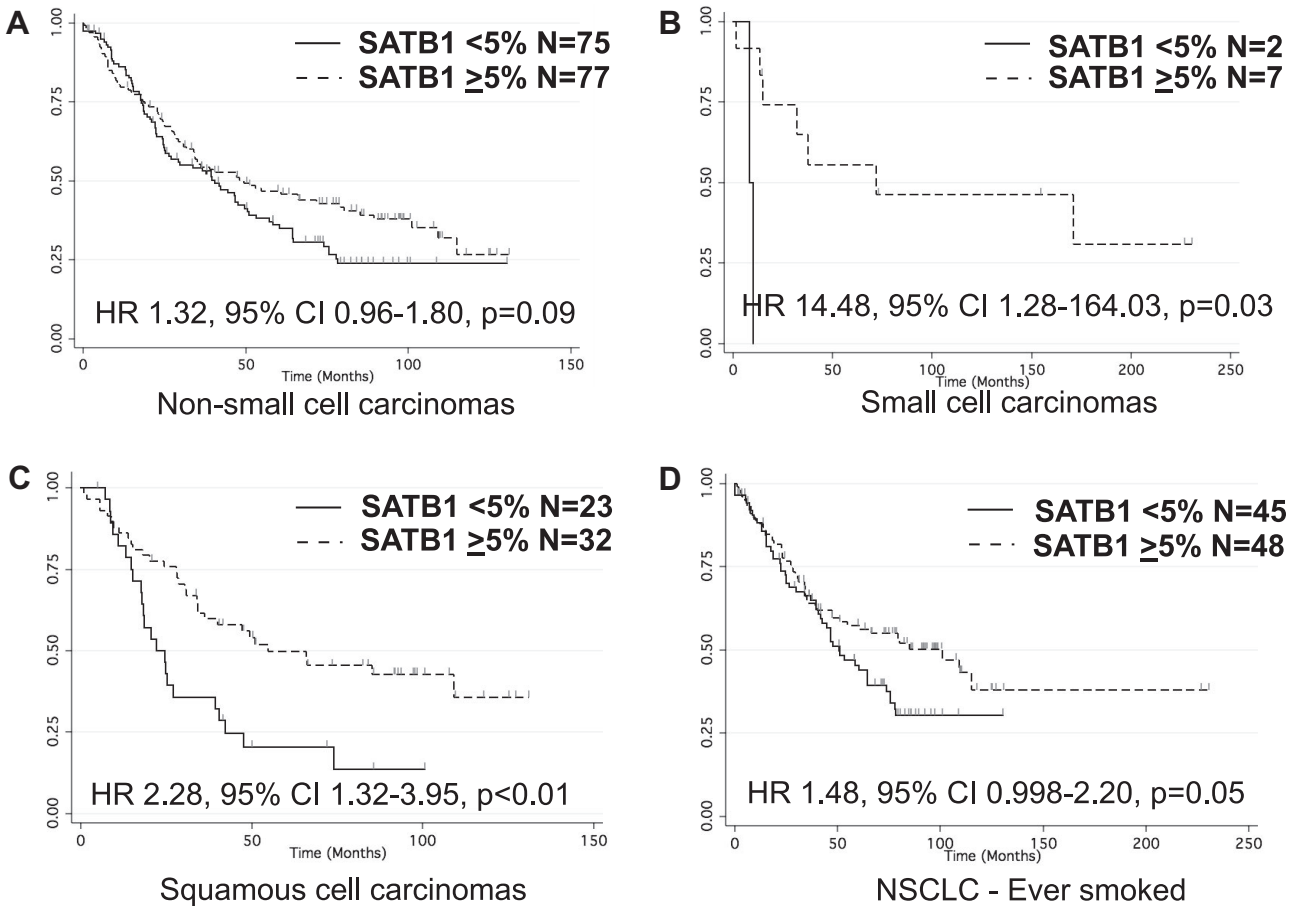

FIGURE 4. Disease-specific survival and special AT-rich binding protein 1 (SATB1) expression in patients with lung cancer. Kaplan-Meier analyses for lung cancer-specific survival stratified by SATB1 expression in non-small cell lung cancer (*A*), small cell lung cancer (*B*), squamous cell lung cancer (*C*), and patients with non-small cell lung cancer who had ever smoked (*D*).

trimethylated histone H3 lysine 27 (H3K27Me3) relative to the NL20 cells (Figure 5*C*). *SATB1* is, therefore, silenced by deacetylation of histone H3K9 with enrichment of H3K27Me3 in NCI-H520 cells, suggesting that chromatin remodeling is associated with the down-regulation of *SATB1*.

DISCUSSION

SATB1 is a cell-type-specific nuclear protein and plays an active role in higher-order chromatin organization that regulates gene expression, and it is essential for normal T-cell development.[41] SATB1 tethers loops of DNA to the nuclear matrix, recruiting chromatin-remodeling factors that play an important role in modifying gene expression.[7] SATB1 can act as a transcriptional activator or a repressor, by recruiting other transcriptional activators or repressors, depending on the specific genomic sites of SATB1 binding. In the breast cancer cell line MDA-MB-231, SATB1 binding to specific regions leads to recruitment of histone acetylases (such as p300) and results in up-regulation of specific SATB1 bound genes.[10] In thymocytes and MDA-MB-231 cells, SATB1 also facilitates histone deacetylase 1 binding and consequent repression of other genes.[10,11] Recently, it was shown in breast cancer that SATB1 directly up-regulates metastasis-associated genes[10] and is involved in multidrug resistance,[42] with higher expression levels also correlating with shorter overall survival times.[10] High expression of SATB1 is also associated with poor overall survival in gastric cancers.[19,20] Nevertheless, these studies have not been independently validated,[12–14] and one study found increased SATB1 expression corresponded with improved disease-free survival in estrogen receptor-positive patients with breast cancer.[13] Regardless of this dichotomy, by its role as a "genome organizer," SATB1 is thought to potentially play a key role in cancer progression, deserving of further investigations.

We have shown for the first time that SATB1 is expressed in the normal bronchial epithelial cells of the lung but is significantly reduced or lost in NSCLCs, based on immunohistochemical analysis of resected tumors and Western blot and qPCR analysis in lung cancer cell lines. SATB1 is significantly reduced in early squamous neoplastic preinvasive lesions and in SCCs and lymph node metastases. Although squamous metaplasia is considered reversible, squamous dysplasias and carcinoma in situ are most frequently associated with progression to SCC.[35,38] Our finding suggests that cells of the bronchial epithelium undergo sig-

TABLE 3. Multivariate Analysis of SATB1 and Clinicopathological Parameters

	Variable	Hazard Ratio	95% Confidence Interval	*p*
Squamous cell carcinoma				
Univariate	SATB1 negative	2.28	1.32–3.95	**0.004**
	AJCC stage	1.81	1.23–2.68	**0.005**
	Lymphatic invasion	4.45	2.40–8.26	**<0.001**
	Blood vessel invasion	5.10	2.73–9.56	**<0.001**
Multivariate	SATB1 negative	2.06	1.15–3.72	**0.016**
	AJCC stage	1.47	0.75–2.88	0.263
	Lymphatic invasion	2.65	1.09–6.43	**0.031**
	Blood vessel invasion	1.96	0.80–4.83	0.142
(NSCLC) Ever smoked				
Univariate	SATB1 negative	1.48	0.998–2.20	**0.050**
	AJCC stage	1.80	1.36–2.40	**<0.001**
	Lymphatic invasion	3.12	1.86–5.24	**<0.001**
	Blood vessel invasion	2.15	1.21–3.79	**0.016**
Multivariate	SATB1 negative	1.41	0.92–2.15	0.114
	AJCC stage	1.93	1.21–3.07	**0.006**
	Lymphatic invasion	2.70	1.43–3.10	**0.002**
	Blood vessel invasion	1.05	0.51–2.16	0.887

Univariate and multivariate analyses were performed using Cox proportional hazard model in squamous cell carcinomas and in patients with non-small cell lung cancer (NSCLC) who had ever smoked. Bolded values denote statistically significant *p* value.

AJCC, American Joint Committee on Cancer; SATB1, special AT-rich binding protein 1.

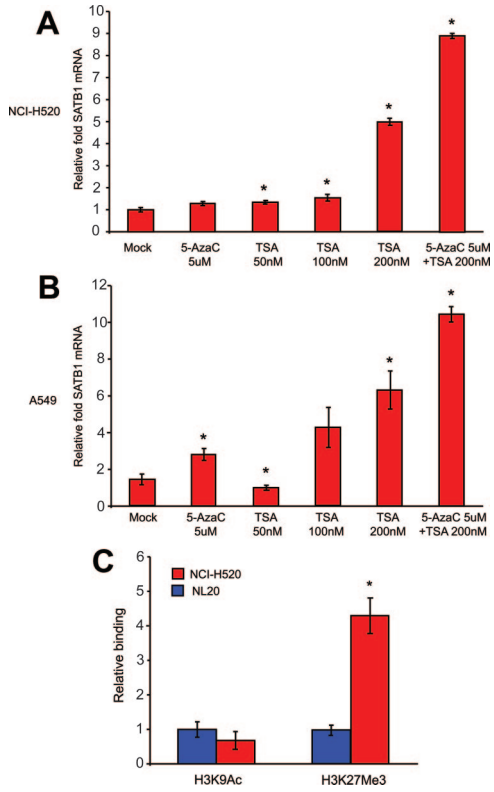

FIGURE 5. Epigenetic regulation of special AT-rich binding protein 1 (SATB1). *A*, In the squamous cell line NCI-H520, SATB1 messenger RNA (mRNA) expression was significantly increased in cells treated with 50 nM trichostatin A (TSA) ($p = 0.024$), 100 nM TSA ($p = 0.021$), 200 nM TSA ($p < 0.001$), and 5 μM 5-Aza-2-deoxycytidine (5-AzaC) + 200 nM TSA ($p < 0.001$). *B*, In the adenocarcinoma cell line A549, SATB1 mRNA expression was significantly increased in cells treated with 5 μM 5-AzaC ($p = 0.010$), 50 nM TSA ($p = 0.025$), 200 nM TSA ($p = 0.031$), and 5 μM 5-AzaC + 200 nM TSA ($p < 0.001$). *Denotes $p < 0.05$ compared with mock control. Expression was quantitated by real-time polymerase chain reaction (PCR) and normalized using GAPDH expression. *C*, Chromatin immunoprecipitation was performed on NL20 and NCI-H520 cells using H3K9Ac (active mark) and H3K27Me3 (repressive mark) antibodies. The amount of immunoprecipitated DNA (relative binding) was quantitated by real-time PCR and was calculated as a ratio of immunoprecipitated DNA relative to internal control genes: GAPDH (H3K9Ac) and MyoD (H3K27Me3). Data are shown relative to normal NL20. *Denotes $p < 0.05$ compared with NL20. Error bars represent ±standard error of the mean.

nificant loss of SATB1 expression early during squamous carcinogenesis, and this can be detected in the most initial preneoplastic lesions.

Our studies reveal that loss of SATB1 expression is a significant adverse prognostic factor in SCCs, independent of tumor stage and lymphatic metastatic invasion. SCCs usually arise centrally in the lung and are thought to derive from cells of the major bronchi.[43] This may explain why loss of SATB1 expression is pertinent to SCCs and not to ADCs, which are thought to derive from cells of the peripheral alveoli,[44–46] where SATB1 expression is absent. If SATB1 expression in ADCs was compared with peripheral alveoli rather than the bronchus, SATB1 could be seen as significantly overexpressed, such as in breast, gastric, and laryngeal cancer. Nevertheless, regardless of which normal tissue SATB1 expression is compared with in ADC, it was not prognostically significant in these patients. Despite primary SCLCs showing on average, no significant difference in SATB1 expression compared with normal bronchial epithelium, those patients with SCLC with loss of SATB1 expression still suffered significantly worse survival outcomes ($p = 0.03$). Further investigation into the prognostic value of SATB1 in a larger sample population of SCLCs is warranted.

The phenotypically "normal" small airway epithelium of smokers has been shown to have approximately 300 genes up- or down-regulated compared with normal airway epithelium of nonsmokers.[47] We compared normal tissue from smokers and nonsmokers and observed no significant difference in the expression of SATB1 in normal bronchial epithelial cells from patients with or without a history of smoking.

Interestingly, although SATB1 is a master epigenetic regulator of gene expression, we show that the *SATB1* gene itself is epigenetically regulated through histone modifications in a SCC cell line. In SCC cells, we show that *SATB1* is enriched for the repressive chromatin mark trimethylated H3K27 with moderate deacetylation of the active chromatin mark histone H3K9 compared with the normal cell line. This

silencing is reversed on inhibition of histone deacetylase using TSA. Therefore, we speculate that loss of SATB1 expression in primary SCC lung tumors may also be controlled by epigenetic silencing mechanisms. Further studies are needed to determine whether this epigenetic silencing phenomenon is widespread in SCCs. Chromatin modifications such as loss of histone H4K16 acetylation and histone H4K20 trimethylation have been detected in lymphomas, liver, breast, and colon cancers.[48] In lung tumorigenesis, global loss of H4K20 trimethylation correlates with poor survival.[5] Clearly aberrant modification of histones plays a significant role in cancer development.

Through its role as an epigenetic regulator, both overactivation and loss of SATB1 function is expected to have widespread genomic consequences. SATB1 orchestrates the epigenetic organization of multiple genes by region-specific histone modifications, by recruiting proteins such as HDAC 1 and 2, histone acetylase p300, and imitation switch ATPase (ISWI).[7,10,11] Apart from the profound effect of SATB1 up-regulation on tumorigenesis, loss of SATB1 function may also be critical because it causes widespread disruption of histone modifications. These may result in the loss of activation or loss of repression of other genes, which contribute to tumorigenesis. In contrast with several studies in other cancers,[10,19–21] where SATB1 is absent in normal tissue, and the aberrant expression of SATB1 correlates with tumor progression and poor survival, in lung cancer the reverse is observed.

There is currently no data indicating the specific role of SATB1 in bronchial epithelial cells of the lung. The bronchial epithelium functions as a barrier to inhaled environmental particulates supported by ciliated and secretory cells. The airway epithelium undergoes constant renewal and responds quickly to injury with ongoing dedifferentiation and proliferation.[49] An explanation for the opposing findings of SATB1 expression in different tissues could be in the role of SATB1 as a differentiation gene in normal bronchial cells, similar to its role in T cells, where it is required for normal cell development. The *SATB1* null mouse has multiple defects in T cells at every stage of development and is not viable beyond 3 weeks of age.[41] SATB1 functions to coordinate gene expression for T-cell development and maturation. We speculate that if SATB1 is acting in a similar way in the lung, to coordinate gene expression for normal bronchial cell maintenance, then it is conceivable that loss of a key differentiation gene may stall precursor cells in a less differentiated and more pluripotent state that renders them more tumorigenic. SATB1 has also been shown to play a role in stem-cell differentiation and deregulation of pluripotency. SATB1 negatively regulates the pluripotency genes Nanog, Klf4, and Tbx3 in mouse embryonic stem cells and is required for proper stem-cell differentiation.[50] In *SATB1* null embryonic stem cells, Nanog expression is elevated, resembling cells where self-renewal is strongly favored over differentiation.[50] In the context of the lung, SATB1 could also be important for the balance between differentiation and pluripotency in a tissue environment undergoing rapid repair. Further investigations are needed to explore the functional role of SATB1 in the bronchial epithelium.

In conclusion, loss of SATB1 expression is a prognostically significant factor in tumors derived from bronchial epithelial cells, such as SCCs. Loss of SATB1 expression may have profound biologic consequences because of the epigenetic deregulation of hundreds of genes at once. The potency of SATB1 to potentially affect the regulation of up to 10% of the genome may help explain why it is a strong prognostic marker, independent of the most established predictive factors in lung cancer, and also may explain why it can be both a positive and negative marker in cancers from different tissues. Investigation into SATB1 target genes in the context of lung cancer could help stratify patients in terms of prognosis, and justifies further research, opening up new avenues for the study of lung cancer treatment and chemoprevention.

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