Promoter Methylation of Genes in and around the Candidate Lung Cancer Susceptibility Locus *6q23-25*

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Abstract

Chromosomal aberrations associated with lung cancer are frequently observed in the long arm of chromosome 6. A candidate susceptibility locus at *6q23-25* for lung cancer was recently identified; however, no tumor suppressor genes inactivated by mutation have been identified in this locus. Genetic, epigenetic, gene expression, and *in silico* screening approaches were used to select 43 genes located in *6q12-27* for characterization of methylation status. Twelve (28%) genes were methylated in at least one lung cancer cell line, and methylation of 8 genes was specific to lung cancer cell lines. Five of the 8 genes with the highest prevalence for methylation in cell lines (*TCF21, SYNE1, AKAP12, IL20RA*, and *ACAT2*) were examined in primary lung adenocarcinoma samples from smokers (n = 100) and never smokers (n = 75). The prevalence for methylation of these genes was 81%, 50%, 39%, 26%, and 14%, respectively, and did not differ by smoking status or age at diagnosis. Transcription of *SYNE1, AKAP12*, and *IL20RA* was completely silenced by hypermethylation and could be restored after treatment with 5-aza-2-deoxycytidine. Significant associations were found between methylation of *SYNE1* and *TCF21, SYNE1* and *AKAP12*, and *AKAP12* and *IL20RA*, indicating a coordinated inactivation of these genes in tumors. A higher prevalence for methylation of these genes was not associated with early-onset lung cancer cases, most likely precluding their involvement in familial susceptibility to this disease. Together, our results indicate that frequent inactivation of multiple candidate tumor suppressor genes within chromosome *6q* likely contributes to development of sporadic lung cancer. [Cancer Res 2008;68(6):1707–14]

Introduction

Lung cancer remains the leading cause of cancer death among both men and women, with an estimated 160,440 deaths and 173,000 new cases annually in the United States alone (1). It kills more people than breast, colon, and prostate cancer combined. Lung tumors develop through mutation or epigenetic silencing via promoter hypermethylation of numerous genes involved in cell cycle control, growth regulation, signaling, apoptosis, and adhesion (2). Some of the earliest changes detected in the bronchial epithelium of smokers include loss of heterozygosity (LOH) at chromosomes *3p21, 9p21,* and *17p13* (3). Within these areas of allelic loss, inactivation of the remaining allele by promoter hypermethylation of *RASSF1A* and *p16* genes and by mutation of the *p53* gene is commonly seen in non–small cell lung cancers (NSCLC) (4). Methylation of the *p16* gene is one of the earliest changes in lung cancer development, occurring in the field of epithelial damage induced by carcinogens within tobacco and increasing in prevalence during histologic progression of adenocarcinoma and squamous cell carcinoma (5–7).

Another chromosome region where LOH is frequently observed in adenocarcinomas from both smokers and never smokers is *6q*. LOH within *6q22-27* occurs in 30% to 55% of tumors (8), whereas deletions between *6q14* and *6q24* have been detected in 60% of primary lung tumors (9). Moreover, this locus has been associated with lung cancer susceptibility. A recently conducted genome-wide linkage analysis of 52 extended pedigrees with a minimum of three family members with aerodigestive cancer identified a lung cancer susceptibility locus at chromosome *6q23-25* (10). Smoking at any level also was found to increase risk in carriers of the inherited susceptibility locus on chromosome *6q*. The high frequency for LOH and the potential existence of a susceptibility locus within *6q23-25* supports the existence of tumor suppressor genes inactivated through the classic Knudson's two-hit model in which complete loss of gene function arises through loss of one allele and mutation of the second allele (11). However, only one candidate tumor suppressor gene, *p34*, localized to *6q25* has been identified, but this gene was not found to be associated with familial lung cancer susceptibility (12). This scenario is reminiscent of the chromosome *3p14-25* in which LOH is commonly seen in lung tumors, although no major tumor suppressor gene inactivated by mutation has been identified in this locus. Rather, genes inactivated by promoter hypermethylation at prevalences ranging from 30% to 58% have been identified within this locus. These include *RASSF1A, BLU, SEMA38*, and *retinoic acid receptor β* (4).

Chromosome 6 is one of the gene-rich and CpG island–rich chromosomes that contains ~1,557 genes and 1,070 CpG islands (13, 14). The fact that chromosome *6q* accumulates genetic aberration in the form of LOH could also make this region a hotspot for silencing of genes by promoter hypermethylation. Support for this supposition is growing as genes silenced by methylation in lung tumors are now being identified within *6q*. Estrogen receptor α, which maps to *6q25*, has been shown by our laboratory to be silenced by promoter hypermethylation in 20% and 36% of lung tumors from smokers and never smokers, respectively (15). Recently, Smith et al. (16) identified a tumor suppressor gene, *TCF21*, within the *6q23-24* locus that is normally expressed in lung airway epithelial cells but silenced in aerodigestive tumors. The purpose

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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of this investigation was to identify novel genes in and around the candidate lung cancer susceptibility locus *6q23-25* that are inactivated by promoter hypermethylation and to compare their prevalence in adenocarcinomas from smokers and never smokers. A multifaceted strategy was undertaken that used comparative genomic hybridization (CGH), a transcriptome microarray, *in silico* screening, and a candidate approach to select and evaluate the methylation patterns of 43 genes.

Materials and Methods

Cell lines, tumors, and control samples. Twelve lung cancer cell lines (H23, H358, H1568, H1993, H2023, H2085, H2228, H2009, Calu-3, Calu-6, SKLU-1, and SW-900) and 4 breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and MDA-MB-435) obtained from the American Type Culture Collection (ATCC) and maintained in ATCC-recommended medium were used in this study. A total of 175 frozen lung adenocarcinomas (100 from smokers and 75 from never smokers) were obtained from frozen tumor banks at Johns Hopkins and the Mayo Clinic (Supplementary Table S1). Each tumor was histologically classified as adenocarcinoma according to the current WHO criteria and considered suitable for study based on the presence of >80% tumor cells (17). Peripheral blood mononuclear cells (PBMC) isolated from never smokers using Histopaque-1077 (n = 20) and human bronchial epithelial cells (HBEC) isolated from cancer-free smokers who underwent bronchoscopy (n = 20) were used as controls.

CGH analysis. Regional copy number alterations were examined using array CGH analysis as described (18). Briefly, DNA from four lung cancer cell lines (H1435, H1568, H2009, and Calu-6) was isolated using the Puregene DNA isolation kit (Gentra Systems). Genomic DNA was fragmented, random prime labeled, and hybridized to an oligonucleotide microarray that contains 22,500 elements designed for expression profiling (Human 1A V2, Agilent Technologies), for which 16,097 unique map positions were defined (Build 35). The median interval between mapped elements is 54.8 kb; 96.7% of intervals are <1 Mb and 99.5% are <3 Mb. Fluorescence ratios of scanned images of the arrays were calculated as the average of two paired arrays (dye swap), and the raw array CGH profiles were processed to identify statistically significant transitions in copy number using a segmentation algorithm as described (18). Significant copy number changes are determined based on segmented profiles only.

Treatment and genome-wide transcriptome array. Six lung adenocarcinoma cell lines, three (H23, H1568, and H1993) originated from smokers and three (H2023, H2085, and H2228) from never smokers, were used for the transcriptome array. Cells at log phase of growth were grouped in duplicate into control, trichostatin A (TSA) alone, and 5-aza-2-deoxycytidine (DAC) alone treatment groups and treated as follows: control (culture medium), TSA [culture medium containing 300 nmol/L TSA (stock solution 5 μmol/L in ethanol; Sigma) for 18 h], or DAC [culture medium containing 5 μmol/L DAC (stock solution 10 mmol/L in PBS; Sigma) for 96 h with fresh medium containing the drug changed every 24 h]. Cells in the control and TSA groups underwent fresh medium changes in parallel with the DAC group, and TSA treatment was done 18 h before all treatment groups were harvested in TRI reagent (Sigma). Genome-wide transcriptome array analysis was conducted essentially as described (19). Briefly, total RNA was isolated and quantified, and its quality was assessed with the 2100 Bioanalyzer (Agilent Technologies). Sample amplification and labeling procedures were carried out using the Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies), and the labeled cRNA was purified and quantified. RNA spike-in controls (Agilent Technologies) were added to RNA samples before amplification. Samples (0.75 μg) labeled with Cy3 or Cy5 were mixed with control targets (Agilent Technologies), assembled on the oligo microarray, hybridized, and processed according to the Agilent microarray protocol. Scanning was performed with the Agilent G2565BA microarray scanner using settings recommended by Agilent Technologies. All calculations were performed using the R statistical computing platform and packages from the Bioconductor bioinformatics software project (20–22).

DNA extraction and modification. High molecular weight DNA was isolated from cell lines, PBMCs, HBECs, and tumor samples using a standard phenol-chloroform extraction method. Genomic DNA was modified using the EZ DNA Methylation-Gold kit (Zymo Research) as described by the manufacturer, and 40 ng of modified DNA were used per PCR for combined bisulfite modification and restriction analysis (COBRA) or methylation-specific PCR (MSP).

Combined bisulfite modification and restriction analysis. Forty-three CpG islands located in gene promoter regions within *6q* were selected using genetic (array CGH), epigenetic (gene expression array after demethylation), and *in silico* screening (presence of a promoter CpG island and predicted candidate tumor suppressor function). The selection criteria used for each gene are shown in Table 1. The DNA methylation profile of these 43 genes was analyzed in 12 lung cancer cell lines using COBRA. The COBRA primers were designed to amplify gene promoter regions where multiple methylation-sensitive restriction enzyme recognition sites were present. Primer sequences, amplification conditions, and the number of restriction sites within the amplified region of each gene are described in Table 1 and Supplementary Table S2. PCRs were conducted in a total volume of 50 μL, containing 1× PCR buffer with 2.5 mmol/L $MgCl_2$, 62.5 μmol/L of each deoxynucleotide triphosphate, 0.25 pmol each of the forward and reverse primers, and 2.5 units of Taq Gold polymerase (Perkin-Elmer). The reaction mixes were first heat denatured for 10 min at 94°C followed by 40 amplification cycles and 5 min of extension at 72°C. Each PCR cycle consisted of equal time of denaturation at 94°C, annealing (see Supplementary Table S2), and extension at 72°C. The resulting PCR products were digested with the specific enzyme (Supplementary Table S2), mixed with 5 μL of loading buffer, resolved by electrophoresis through 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

MSP and bisulfite sequencing. Promoter regions that showed tumor-specific methylation in the lung cancer cell lines (i.e., no methylation in normal HBECs and PBMCs) from COBRA were further analyzed in primary lung adenocarcinomas from smokers and never smokers using MSP (for primers and amplification conditions, see Supplementary Table S3). For genes with a high prevalence of methylation (>20%), the density of methylation at each CpG within the COBRA-assayed region was further characterized by bisulfite sequencing. Bisulfite-modified DNA from a HBEC line and a primary adenocarcinoma, and lung cancer cell lines was amplified (using the COBRA primers that do not discriminate between methylated and unmethylated DNA) and cloned into pCR II cloning vector (Invitrogen), and five clones were sequenced per sample. Within the promoter regions of *SYNE1, AKAP12*, and *IL20RA*, 41, 33, and 30 CpGs, respectively, were analyzed by bisulfite sequencing, and the density of promoter methylation of each gene was described as percent of methylated CpGs from the total number of CpGs analyzed in five clones.

Gene expression analysis. Sham-, TSA-, and DAC-treated cell lines were harvested in TRI reagent, and RNA was isolated following TRI reagent instructions (Molecular Research Center, Inc.). Total RNA (3 μg) was reverse transcribed using the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen) according to the cDNA synthesis protocol from Invitrogen. Expression of *SYNE1, AKAP12*, and *IL20RA* was evaluated using RT-PCR and electrophoresis in 3% agarose gels. In total, five pairs of RT-PCR primers were used. SYNE1-F1/R1 was used to amplify the longest (L1) and longer (L2) SYNE1 transcripts and SYNE1-F2/R2 to amplify all four SYNE1 transcripts: L1, L2, α (A), and β (B). AKAP12-F1/R1 and AKAP12-F2/R2 amplified the A and B transcripts of AKAP12, respectively, whereas IL20RA-F1/R1 amplified the IL20RA transcript. Large introns were included in the RT-PCR amplification regions and isolated RNA was treated with DNase to exclude PCR products from contaminating DNA. RT-PCR primers and amplification conditions are described in Supplementary Table S4.

Data analysis. Patient characteristics, including age, gender, smoking status, and race, and gene methylation are summarized with mean and SD for continuous variables and proportions for categorical variables. Fisher's exact test was used to assess the association between methylation and patient characteristics and the association between pairs of genes. Methylation of genes and patient characteristics were assessed for association with survival. Survival time was calculated from time of diagnosis until death or last follow-up visit. Individual genes and variables

Table 1. Characteristics of the CpG islands analyzed

No	Gene	Location	CpG island size (bp)	CpG island from 5′-gene (bp)	No. CpG	% CpG	No. GpC	% GpC	% C+G	Obs/Exp CpG*	CpG/GpC ratio	No. *Bst*UI and/or *Taq*I sites †	Selection method
1	*SMAP1*	*6q12-q13*	793	Within	95	24.0	113	28.5	73.9	0.88	0.84	3	CGH
2	*TTK*	*6q13-q21*	419	Within	39	18.6	44	21.0	63.2	0.94	0.89	1	CGH
3	*RRAGD*	*6q15-q16*	1,469	Within	169	23.0	177	24.1	74.1	0.85	0.95	4	Microarray
4	*MAP3K7*	*6q16.1-q16.3*	723	Within	67	18.5	58	16.0	64.0	0.91	1.16	4	CGH
5	*AIM1*	*6q21*	1,221	35	119	19.5	142	23.3	69.1	0.82	0.84	5	Microarray
6	*CCNC*	*6q21*	706	Within	61	17.3	59	16.7	62.7	0.88	1.03	6	Microarray
7	*CDC40*	*6q21*	941	−657	104	22.1	112	23.8	70.1	0.91	0.93	6	Microarray
8	*NR2E1*	*6q21*	4,868	Within	328	13.5	465	19.1	60.6	0.74	0.71	6	Microarray
9	*REV3L*	*6q21*	1,952	Within	224	23.0	255	26.1	70.1	0.94	0.88	9	*In silico*
10	*PEX7*	*6q21-q22.2*	810	Within	91	22.5	97	24.0	68.6	0.96	0.94	8	*In silico*
11	*KIAA1919*	*6q22*	524	Within	51	19.5	67	25.6	67.9	0.85	0.76	2	CGH
12	*MYB*	*6q22-q23*	2,257	Within	212	18.8	275	24.4	65.8	0.87	0.77	12	Microarray
13	*TBPL1*	*6q22.1-q22.3*	1,534	Within	116	15.1	119	15.5	60.6	0.83	0.97	7	CGH
14	*PTPRK*	*6q22.2-23.1*	1,968	Within	173	17.6	207	21.0	65.5	0.82	0.84	8	*In silico*
15	*MAP3K5*	*6q22.33*	1,690	Within	187	22.1	236	27.9	70.5	0.90	0.79	11	*In silico*
16	*TNFAIP3*	*6q23*	1,316	Within	121	18.4	149	22.6	65.4	0.86	0.81	5	Microarray
17	*CTGF*	*6q23.1*	740	Within	103	27.8	114	30.8	75.1	1.01	0.90	7	*In silico*
18	*IL20RA*	*6q23.3*	827	172	104	25.2	128	31.0	75.7	0.88	0.81	10	Microarray
19	*PEX3*	*6q23-q24*	474	−32	47	19.8	54	22.8	64.8	0.95	0.87	6	Microarray
20	*TCF21*	*6q23-q24*	579	363	51	17.6	69	23.8	68.0	0.76	0.74	8	*In silico*
21	*PERP*	*6q24*	628	Within	87	27.7	99	31.5	74.7	1.00	0.88	8	*In silico*
22	*FUCA2*	*6q24*	554	Within	55	19.9	69	24.9	68.8	0.84	0.80	9	*In silico*
23	*GPR126*	*6q24.1*	870	Within	91	20.9	113	26.0	72.2	0.80	0.81	5	Microarray
24	*AIG1*	*6q24.2*	474	0	52	21.9	54	22.8	70.5	0.90	0.96	9	*In silico*
25	*RAB32*	*6q24.3*	945	Within	106	22.4	127	26.9	73.7	0.83	0.83	6	Microarray
26	*SASH1*	*6q24.3*	1,044	Within	103	19.7	118	22.6	65.2	0.93	0.87	15	*In silico*
27	*PLAGL1*	*6q24-q25*	931	Within	118	25.3	138	29.6	74.0	0.93	0.86	4	Microarray
28	*LATS1*	*6q24-q25.1*	776	Within	84	21.6	106	27.3	72.6	0.83	0.79	3	*In silico*
29	*SYNE1*	*6q25*	662	Within	74	22.4	90	27.2	74.3	0.82	0.82	9	Microarray
30	*AKAP12*	*6q25.1*	1,267	Within	132	20.8	172	27.2	69.8	0.86	0.77	7	Microarray
31	*MTHFD1L*	*6q25.1*	1,365	57	173	25.3	206	30.2	76.2	0.87	0.84	5	Microarray
32	*RBM16*	*6q25.1-q25.3*	1,217	Within	140	23.0	162	26.6	73.0	0.86	0.86	6	CGH
33	*CNKSR3*	*6q25.2*	1,441	Within	141	19.6	171	23.7	69.5	0.82	0.82	4	*In silico*
34	*PNLDC1*	*6q25.3*	347	Within	37	21.3	46	26.5	68.9	0.93	0.80	7	CGH, Microarray
35	*SOD2*	*6q25.3*	1,306	Within	135	20.7	182	27.9	71.3	0.82	0.74	6	CGH
36	*SYNJ2*	*6q25.3*	1,372	Within	156	22.7	183	26.7	72.5	0.87	0.85	6	CGH, Microarray
37	*SYTL3*	*6q25.3*	603	−6,115	73	24.2	83	27.5	74.5	0.87	0.88	6	Microarray
38	*ACAT2*	*6q25.3-q26*	1,341	655	125	18.6	153	22.8	66.0	0.86	0.82	8	CGH, Microarray
39	*WTAP*	*6q25-q27*	1,336	Within	149	22.3	175	26.2	71.0	0.89	0.85	6	CGH, Microarray
40	*MAP3K4*	*6q26*	1,187	Within	143	24.1	149	25.1	66.6	1.12	0.96	8	CGH
41	*DACT2*	*6q27*	814	Within	91	22.4	114	28.0	71.0	0.90	0.80	11	Microarray
42	*PDCD2*	*6q27*	639	Within	77	24.1	105	32.9	74.2	0.88	0.73	5	*In silico*
43	*PHF10*	*6q27*	1,615	Within	255	31.6	279	34.6	80.7	1.00	0.91	9	CGH

*Obs/Exp = (CpG * N) / (C * G).
†Restriction sites within the COBRA-analyzed regions.

were assessed with Kaplan-Meier plots and the log-rank test. Proportional hazards models were used to simultaneously examine multiple genes while adjusting for patient characteristics. All analyses were conducted using Statistical Analysis System version 9.1.3.

Results

Gross copy number alteration of chromosome 6 in lung cancer. Four lung cancer cell lines (H1435, H1568, H2009, and Calu-6) were examined by array CGH analysis to identify specific loci with copy number alterations. The X chromosome of each cell line was evaluated to verify the quality of array performance and for data normalization (Fig. 1*A*). The array CGH profile of H1435 (adenocarcinoma cell line from a never smoker) revealed a copy number alteration of an ~70 Mbp segment in the region between *6p12.1* and *6q22.3* (Fig. 1*B*). Similarly, a single deletion of ~15.41 Mbp segment in the region between *6p22.1* and *6p12.2* and *6q25-27* was observed in the Calu-6 cell line (data not shown). Chromosome 6 of the other two cell lines, H1568 and H2009, showed no significant copy number alteration (data not shown). Genes within *6q12-22.3* and *6q25-27* that showed significant copy number

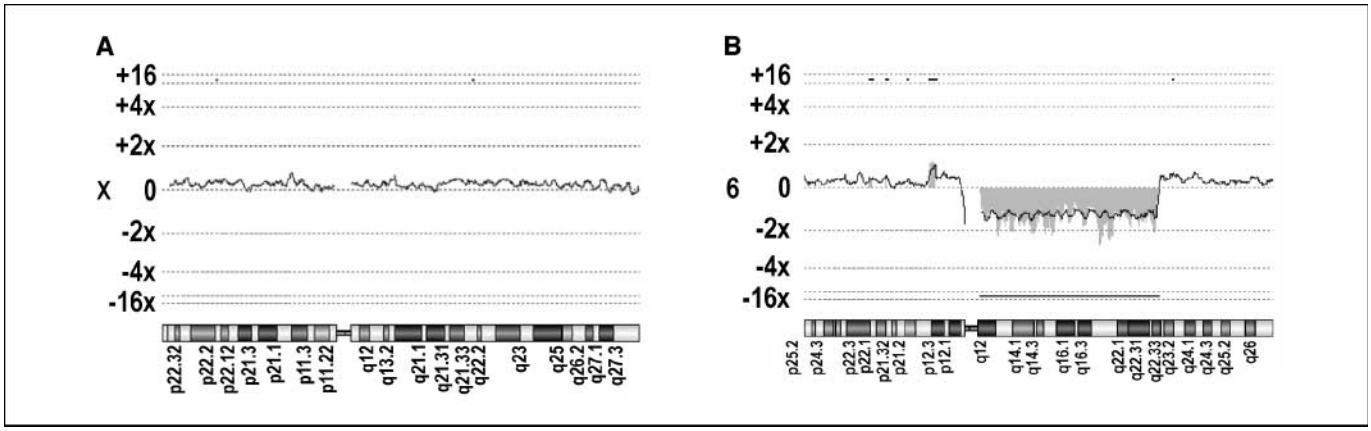

Figure 1. Array CGH analysis of H1435, a lung adenocarcinoma cell line from a never smoker. *A,* results from the X chromosome were used to verify that the quality of array performance and data normalization were good (Window = 2.0 Mb; Z-score = 1.5). *B,* a large single-copy deletion between *6p12.1* and *6q22.33* was found within chromosome 6 (Window = 2.0 Mb; Z-score = 2.5).

alteration and harbor CpG islands in their promoter regions were selected for assessment of methylation (Table 1).

Promoter hypermethylation of candidate genes within chromosome *6q*. A total of 43 genes with CpG-rich promoters that met the CpG island criteria of Takai and Jones (23) were selected from *6q* for methylation analysis (Table 1). Thirteen genes were identified through array CGH analysis of H1435 and Calu-6 that localized to the region of LOH in *6q*. Twenty-one genes were obtained from the transcriptome array, and 4 of these were detected by both assays. The remaining 13 genes were selected *in silico* based on biological function. The COBRA analysis revealed that 31 genes (72.1%) were devoid of promoter methylation (0 of 12) in lung cancer cell lines (Fig. 2). Two genes, *PNLDC1* and *PLAGL1*, that showed a high prevalence for methylation in the lung cancer cell lines (100% and 83.3%, respectively) also were methylated in 100% of normal HBECs and PBMCs. *PNLDC1* was completely methylated (no unmethylated signal) in all cell lines and control samples (Fig. 3*A*). In contrast, *PLAGL1* was partially methylated (approximately 1:1 methylated and unmethylated alleles) in 75% (9 of 12) of the lung cancer cell lines and all normal HBECs and PBMCs (Fig. 3*B*). The *PLAGL1* promoter CpG island analyzed in this study is located within the differentially methylated region (DMR) observed in various human tissue (24). However, 25% (3 of 12) of lung cancer cell lines showed loss of differential (allele specific) methylation of the DMR (Fig. 3*C*). Two of the cell lines (SKLU-1 and SW-900) were completely unmethylated, whereas the third cell line (H358) was completely methylated (biallelic methylation; Fig. 3*C*). Two genes, *NR2E1* and *DACT2*, were methylated in 67% and 83% of the lung cancer cell lines, respectively, and 100% of the PBMCs but not in normal HBEC (Fig. 2; Table 2). The remaining eight genes (*TCF21, IL20RA, SYNE1, AKAP12, ACAT2, LATS1, AIM1*, and *SASH1*) showed tumor-specific

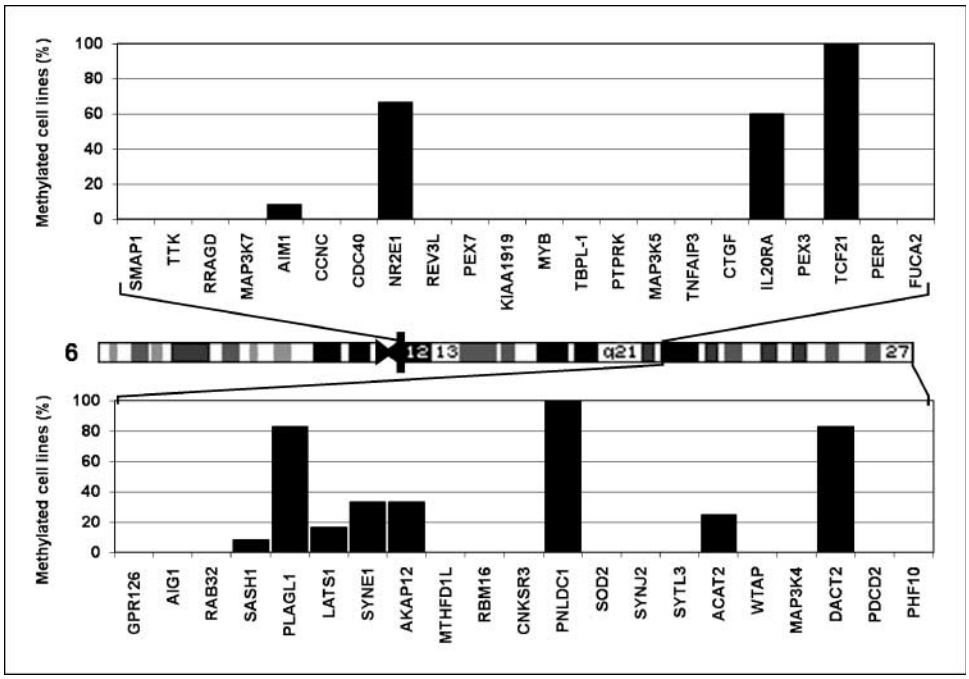

Figure 2. DNA methylation profile of selected chromosome 6 genes in lung cancer cell lines. Promoter regions of 43 genes located within the long arm of chromosome 6 (*6q*) were selected based on the presence of a major copy number alteration, significant increase in gene expression after DAC treatment, and presence of CpG island and candidate tumor suppressor function. DNA methylation profile of these CpG islands was screened in 12 lung cancer cell lines using COBRA, and the percentage of cell lines with methylation of a specific promoter region is shown for each of the 43 CpG islands.

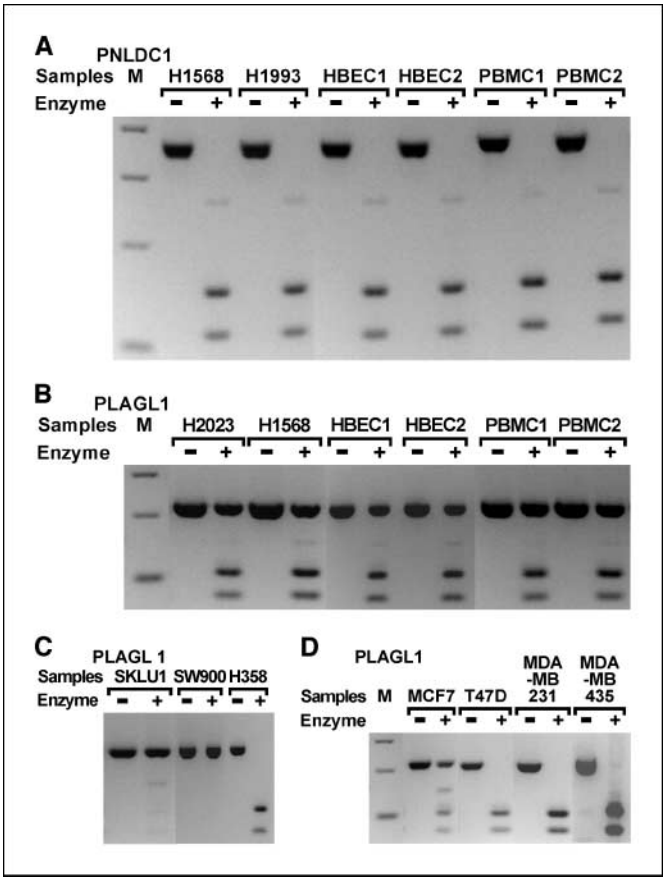

Figure 3. Hypermethylation of *PNLDC1* promoter and *PLAGL1* DMR in normal and malignant cells. *A,* COBRA using the *Bst*UI restriction enzyme that recognizes only methylated CGCG sites revealed complete methylation (no unmethylated DNA) of *PNLDC1* promoter in lung cancer cell lines as well as normal HBECs and PBMCs. *B,* similar analysis of the *PLAGL1* DMR with *Bst*UI showed partial digestion [a roughly 1:1 mixture of methylated (cut) and unmethylated (uncut) DNA] in all normal HBECs and PBMCs as well as 75% of the lung cancer cell lines (two examples of each sample type are shown). *C,* three lung cancer cell lines (25%) have lost the normal differential (partial) methylation. Whereas SKLU-1 and SW-900 were completely unmethylated, H358 was completely methylated. *D,* loss of differential methylation (complete methylation) of the *PLAGL1* DMR was seen in three of four (75%) breast cancer cell lines tested.

methylation [i.e., no methylation in normal HBECs (0 of 20) and PBMCs (0 of 20); Fig. 2; Table 2]. Five of these eight genes were methylated at prevalence of >20% in cell lines and thereby selected for further analysis in primary adenocarcinomas.

Hypermethylation of *6q* genes in primary lung adenocarcinoma. Primary lung adenocarcinomas from 100 smokers (37 current, 59 former, and 4 unclassified) and 75 never smokers were screened for methylation. The prevalence for promoter hypermethylation of *TCF21, SYNE1, AKAP12, IL20RA*, and *ACAT2* was 81%, 50%, 39%, 26%, and 14%, respectively (Table 2). None of the genes showed significant difference in prevalence of methylation between tumors from smokers versus never smokers and did not vary by age of cancer diagnosis. The methylation status of each CpG located in the promoter regions of *SYNE1, AKAP12*, and *IL20RA* was characterized further by bisulfite sequencing. The *TCF21* promoter was not sequenced because dense methylation of this gene has already been described in NSCLC and head and neck tumors (16). *SYNE1* and *AKAP12* gene promoters showed virtually no methylation (0 of 205 and 1 of 165 CpGs, respectively) in normal HBECs, whereas *IL20RA* showed minimal methylation (12 of 150 CpGs; Fig. 4*A*). In contrast, the promoter CpG islands of *SYNE1, AKAP12*, and *IL20RA* showed dense methylation in the primary adenocarcinomas (80%, 68%, and 43% of the CpGs) and lung cancer cell lines (88%, 95%, and 89% of the CpGs), respectively (Fig. 4*A*). The slightly lower density of methylation in the primary tumor for *SYNE1* and *AKAP12* compared with the cancer cell lines is likely due to the presence of normal inflammatory and stromal cells, whereas some heterogeneity for methylation is likely present in the promoter regions of *IL20RA* in the sequenced tumor specimen.

Promoter methylation is associated with silencing of gene expression. The relationship between promoter hypermethylation and gene expression of *SYNE1, AKAP12*, and *IL20RA* was further characterized by determining the levels of transcription of these genes in cell lines. For *SYNE1* and *AKAP12*, two different primer pairs (SYNE1-F1/R1 and SYNE1-F2/R2, and AKAP12-F1/R1 and AKAP12-F2/R2) were used to measure the expression of the different transcripts. Robust expression of the different transcripts of the three genes was seen in H2228, consistent with the lack of methylation seen for these genes in this cell line (Fig. 4*B–D*). In contrast, cell lines such as Calu-6, where the promoter regions of the three genes were hypermethylated, all four transcripts of *SYNE1*, both A and B transcripts of *AKAP12*, as well as the *IL20RA* transcript were not expressed (Fig. 4*B–D*).

DAC treatment restores expression of silenced genes. Cell lines with methylated and unmethylated promoters for each of the three genes were treated with DAC to verify the cause and effect relationship between promoter CpG island hypermethylation and loss of gene expression. In Calu-6 cells, the expression of *SYNE1-α, SYNE1-β, AKAP12-A, AKAP12-B*, and *IL20RA* transcripts was completely silenced by promoter hypermethylation but was restored to normal levels (levels seen in cell lines with unmethylated promoter) after DAC treatment (Fig. 4*B–D*). The longer and longest transcripts of *SYNE1* (*SYNE1 L*) were only partially reexpressed after DAC treatment (Fig. 4*B*). In contrast, the expression of these genes in the H2228 cell line where the promoter CpG islands were unmethylated was not altered by the DAC treatment (Fig. 4*B–D*). Treatment with TSA did not affect the expression of these genes.

Survival and the association of methylation between genes. As expected, patients with advanced-stage lung adenocarcinoma (stages II–IV) had significantly shorter survival than those with early-stage (stage I) disease (P = 0.001; hazard ratio, 2.24). No significant association was seen between survival and promoter hypermethylation for any of the five genes individually or in combination. The association of methylation of the five genes was assessed in tumors independent of smoking status. A positive association was found in early-stage adenocarcinomas between methylation of *SYNE1* and *TCF21* (P = 0.02), *SYNE1* and *AKAP12* (P < 0.001), and *AKAP12* and *IL20RA* (P = 0.045). Strikingly, 88% of tumors methylated for *SYNE1* were methylated for *TCF21*, whereas 74% of tumors positive for methylation of *AKAP12* were also methylated for *SYNE1*. Furthermore, the methylation status of *SYNE1* was associated with the methylation of three or more of the other four genes (P < 0.001). A similar scenario was also seen for *AKAP12* (P < 0.002).

Discussion

Chromosomal regions with recurrent LOH in malignancies, such as the long arm of chromosome 6 in lung cancer, have been predicted to contain one or more tumor suppressor genes. Genetic,

Table 2. Prevalence of promoter hypermethylation of chromosome *6q* genes in primary lung adenocarcinoma samples from smokers and never smokers

Gene	NHBEC (n = 20), no. positive (%)	PBMC (n = 20), no. positive (%)	Lung cancer cell lines (n = 12), no. positive (%)	Primary adenocarcinoma, no. positive (%)		
				Total (n = 175)	Smokers (n = 100)	Never smokers (n = 75)
TCF21	0 (0)	0 (0)	12 (100)	142 (81)	77 (77)	65 (87)
SYNE1	0 (0)	0 (0)	4 (33)	88 (50)	48 (48)	40 (53)
AKAP12	0 (0)	0 (0)	4 (33)	67 (39)	41 (41)	26 (35)
IL20RA	0 (0)	0 (0)	8 (67)	45 (26)	24 (24)	21 (28)
ACAT2	0 (0)	0 (0)	3 (25)	21 (14)	12 (12)	13 (18)
DACT2	0 (0)	20 (100)	10 (83)	ND	ND	ND
NR2E1	0 (0)	20 (100)	8 (67)	ND	ND	ND
PLAGL1	20 (100)	20 (100)	10 (83)	ND	ND	ND
PNLDC1	20 (100)	20 (100)	12 (100)	ND	ND	ND

Abbreviation: ND, not done.

epigenetic, and gene expression assays were used in this study to identify frequent promoter hypermethylation–mediated inactivation of five genes (*TCF21, SYNE1, AKAP12, IL20RA*, and *ACAT2*) in primary lung adenocarcinomas from smokers and never smokers. All five genes are candidate tumor suppressor genes located in and around the candidate *6q23-25* lung cancer susceptibility locus and, with the exception of *TCF21*, have not been shown to be epigenetically silenced in lung cancer. Promoter hypermethylation of *TCF21* has been described in a limited number of aerodigestive tumors, primarily with squamous cell histology (16). Overall, 12 of the 43 promoter CpG islands screened (28%) showed methylation in one or more lung cancer cell lines. Four (9%) were also methylated in normal HBEC and PBMC, corroborating the recently conducted comprehensive CpG island methylation screening (256 CpG islands were analyzed from chromosome 6 alone) where ~12% of the promoter CpG islands were methylated in normal human tissue (13).

The genes selectively methylated in lung adenocarcinomas have diverse functions that when abrogated could contribute to neoplastic transformation. *TCF21* is a basic helix-loop-helix transcription factor that is critical for lung development. *TCF21*-null mice are born alive but die shortly after birth due to severely underdeveloped lungs and

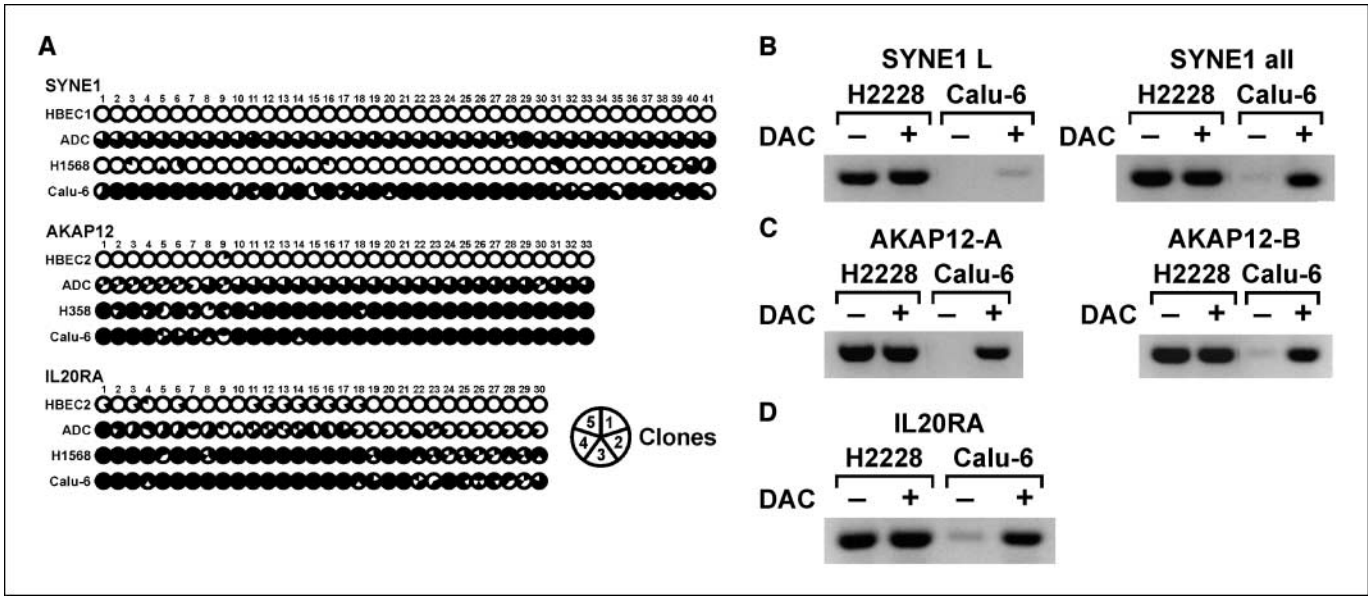

Figure 4. Dense methylation of *SYNE1, AKAP12*, and *IL20RA* promoters silences expression of these genes in lung adenocarcinoma. *A,* a total of 41, 33, and 30 CpG sites within the promoter CpG islands of *SYNE1, AKAP12*, and *IL20RA*, respectively, were amplified with the COBRA primers that do not discriminate between methylated and unmethylated DNA, and five clones were sequenced per sample. As shown by the key, each *circle* is divided into five equal parts, representing the methylation status of one clone for the specified CpG. *Filled regions,* methylated clones; *open regions,* unmethylated clones. *B,* in the Calu-6 cell line where the promoter region of *SYNE1* was methylated, expression of the longer and longest *SYNE1* transcripts (*SYNE1 L*) as well as all four *SYNE1* transcripts (*SYNE1 all*) was silenced. Expression could be restored after DAC treatment. Similarly, *AKAP12-A* and *AKAP12-B* transcripts (*C*) as well as the *IL20RA* transcript (*D*) were silenced in Calu-6 cell lines where the promoter regions of the two genes were methylated. Expression of these genes in Calu-6 was restored after DAC treatment. In contrast, robust expression of each gene was observed in the H2228 cell line where the promoter regions of all three genes were devoid of methylation, and this expression pattern in H2228 was not altered after DAC treatment.

kidneys that lack alveoli and mature glomeruli, indicating a critical role of *TCF21* in lung development (25). Promoter hypermethylation of *TCF21* silences gene expression, and its reexpression through retroviral transfection as well as DAC treatment reverses neoplastic properties (16). The high prevalence (81%) for inactivation of this gene in primary lung adenocarcinomas shown in this study was also seen in squamous cell carcinomas (16), making it one of the most common targets in NSCLC.

The *SYNE1* gene spans 0.5 Mb of genomic DNA, comprising 147 exons and encoding four different transcripts through alternative splicing (26). It is a multifunctional gene involved in cytokinesis, nuclear organization and the structural integrity, and function of the Golgi apparatus (27, 28). Hypermethylation in the promoter CpG island and mutation in the coding region of *SYNE1* were recently reported in approximately 95% and 10% of colorectal cancer cases, respectively, indicating a predominant role of epigenetic regulation of *SYNE1* in this cancer (19, 29). Hypermethylation of *SYNE1* associated with the silencing of all four transcripts was seen in 50% of lung adenocarcinoma samples from smokers and never smokers. The size of this gene prohibited sequencing of unmethylated cell lines and tumors; however, the low prevalence for mutation in colon tumors suggests that methylation is the dominant mechanism for altering function of this gene.

AKAP12/Gravin is one of the A-kinase anchoring proteins (AKAP) that regulates mitogenesis by anchoring key signaling proteins (such as protein kinase A and protein kinase C) and modulating the expression of genes involved in cell cycle and apoptosis (30). It suppresses tumor cell viability and growth by inducing apoptosis via caspase-3, up-regulation of Bax, and down-regulation of Bcl-2 expression (30). AKAP12 also induces cell cycle arrest by increasing the expression of cell cycle checkpoint proteins Cip1/p21 and Kip1/p27 and reducing the cyclin D1 expression (30). AKAP12 also is implicated in decreasing vascular endothelial growth factor expression and vascular growth that is critical for tumor growth and metastasis (31). Overexpression of *SSeCKS* (rodent orthologue of human *AKAP12*) in rats reduces metastasis of prostate cancer cells to the lungs (31). The tumor suppressor function of AKAP12 is strongly suppressed in various cancers, including gastric, prostate, ovarian, and breast cancer, and promoter hypermethylation–mediated inactivation of *AKAP12* has been shown in gastric carcinoma and childhood myeloid malignancies (32, 33). These studies, coupled with our findings of methylation of *AKAP12* in primary lung adenocarcinoma, support the premise that this gene represents another common target for silencing in cancer.

IL20RA encodes a receptor for interleukin (IL)-20 and IL-24 (34). IL-24 (MDA-7) induces G_2-M cell cycle arrest and apoptotic cell death through up-regulation of proapoptotic proteins (Bax and Bak) and down-regulation of antiapoptotic proteins (Bcl-2 and BCL-xL; refs. 35, 36). *IL-24* gene transfer kills various cancer cells, including lung and breast cancer, by inhibition of Wnt/phosphatidylinositol 3-kinase signaling pathways (37). This cytotoxicity of IL-24 could be abolished by anti–IL-24 as well as anti-IL20R antibodies, indicating that the bystander tumor-specific cytotoxicity of IL-24 is mediated via IL-24 binding to IL-20R1/IL-20R2 heterodimeric receptor complex (38). Therefore, silencing of *IL20RA* by promoter hypermethylation represents another survival strategy of lung cancer cells by blocking cell cycle arrest and apoptotic signals from *IL-24*. Although *IL20RA* methylation was observed in a portion (26%) of lung adenocarcinomas, it was significantly associated with methylation of *AKAP12*. Interestingly, methylation of the *SYNE1* promoter was also significantly associated with methylation of *TCF21* and *AKAP12* genes. Taken together, these findings indicate a coordinated silencing of multiple growth-regulatory genes within chromosome *6q* in lung cancer.

Our screen of *6q* for methylation also identified a partial (allele specific) methylation of *PLAGL1* in normal HBECs and PBMCs, indicating epigenetic-mediated regulation of this gene in adult human lung and peripheral blood cells. PLAGL1 is a maternally imprinted zinc finger protein with functional similarities to p53. It interacts physically and functionally with cyclic AMP-responsive element binding protein–binding protein and p300 and induces cell cycle arrest and apoptosis (39–41). *PLAGL1* is differentially methylated and imprinted in normal human peripheral blood lymphocytes, skin fibroblasts, ovary, placenta, and some human fetal tissues including fetal lung (24, 42–44). Down-regulation of *PLAGL1* has been reported in lung, breast, and ovarian cancers, and in ovarian cancers, it correlates with hypermethylation of the DMR (43, 45). In this study, 25% of lung cancer cell lines showed loss of differential methylation that could be indicative of loss of imprinting. However, PCR-based assays could not be used to assess *PLAGL1* methylation in primary lung tumors due to the presence of lymphocytic infiltration that would contain normal monoallelic methylation. Interestingly, three of four breast cancer cell lines showed complete methylation, suggesting that abrogation of function of this gene could be a contributing factor in breast cancer development (Fig. 3*D*). In addition to *PLAGL1* (*6q24*), the region between *6q24* and *6q27* also harbors several imprinted genes that include *HYMAI* (*6q24.1-2*), *IGF2R* (*6q25.3*), *SLC22A2* (*6q26*), and *SLC22A3* (*6q26-27*; reviewed in ref. 46). Normal allele-specific expression of imprinted genes that are located in close proximity as well as their deregulation in cancer is often coordinately regulated, making this an interesting locus for future investigation.

This study also identified cell- and tumor-specific methylation of the *NR2E1* and *DACT2* promoters. Methylation of these genes was seen in all normal PBMCs and the majority of lung tumor–derived cell lines that are free of PBMC but not in HBECs. In contrast, *PNLDC1* was methylated in all normal PBMCs and HBECs evaluated. The function of these genes is not well described; however, the differential methylation patterns seen in the cell types suggest that promoter methylation–mediated regulation may be occurring at the embryonic stage and/or in response to differentiation.

A final key question from this study is the contribution of the identified genes silenced in adenocarcinoma to familial susceptibility for lung cancer. It is clear for early-onset cancers (such as breast cancers associated with germ-line BRCA1/2 mutations) that the second hit in the affected locus plays a major role in the genesis of the disease. However, the three methylated genes (*IL20RA, SYNE1*, and *AKAP12*) within the *6q23-25* locus where LOH is frequent in susceptible families did not show an increased prevalence for silencing in early-onset (<50 years) lung cancer patients. Thus, although the genes discovered in this study probably do not predispose one for lung cancer, their high prevalence for silencing likely contributes to the development of sporadic lung cancer in smokers and never smokers.

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Conflict of interest: S.A. Belinsky is a consultant to Oncomethylome Sciences. Under a licensing agreement between Lovelace Respiratory Research Institute and Oncomethylome Sciences, nested MSP was licensed to Oncomethylome Sciences, and the author is entitled to a share of the royalties received by the Institute from sales of the licensed technology. The Institute, in accordance with its conflict-of-interest

policies, is managing the terms of these arrangements. The commercial rights to standard MSP also belong to Oncomethylome Sciences. S.B. Baylin is a consultant to Oncomethylome Sciences and is entitled to royalties from any commercial use of this procedure.

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