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Gene Promoter Hypermethylation in Mouse Lung Tumors

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Abstract

The mouse is a good model for evaluating the efficacy of chemopreventive agents for lung cancer. Gene silencing by promoter hypermethylation is a critical component for the development and progression of lung cancer and an emerging target for preventive intervention by demethylating agents. Genes methylated in mouse lung tumors could serve as biomarkers to evaluate the effectiveness of demethylating agents for preventing lung cancer and causing gene reexpression *in vivo*. The purpose of the current study was to evaluate a panel of genes inactivated by promoter hypermethylation in human lung cancer for silencing by this epigenetic mechanism in murine lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), cigarette smoke, or arising spontaneously. Cadherin-13, estrogen receptor-α, progesterone receptor, and runt-related transcription factor-3 were frequently methylated in mouse lung tumor-derived cell lines, whereas cadherin-1 and suppressor of cytokine signaling-1 were not. Methylation within these four genes was associated with lack of expression that could be restored after treatment with 5-aza-2′-deoxycytidine and with methylation within the CpG island of each gene. Methylation-specific PCR revealed that methylation of these four genes occurred at prevalences of 24% to 69% in primary lung tumors arising spontaneously or induced by exposure to cigarette smoke or NNK. Estrogen receptor-α methylation was more frequent in spontaneously occurring lung cancer than cigarette smoke–induced or NNK-induced lung cancer, whereas runt-related transcription factor-3 showed the opposite relationship. Thus, genes can be targeted for inactivation by methylation, depending on exposure history. This study indicates that methylation events frequently observed in human lung cancer are recapitulated in the mouse model and identifies four potential biomarkers for assessing intervention approaches for reversing epigenetically mediated gene silencing. (Mol Cancer Res 2006;4(4):267–73)

Introduction

Lung cancer is the leading cause of cancer mortality among men and women in the United States (1). Smoking cessation remains the best approach to reduce cancer risk in smokers. Although cessation of smoking is associated with a decrease in lung cancer risk, the cumulative risk for lung cancer by age 75 for a person who quits smoking at age 50 is still six times greater than for an individual who has never smoked (2). The effect of this continued risk is seen in the clinics, where half of the lung cancers diagnosed are in former smokers (3). These statistics substantiate the need to develop approaches for early detection and preventive interventions to impede lung cancer development.

The A/J mouse lung has proven to be a good model for studying pathways involved in the development of adenocarcinomas and for testing the efficacy of chemopreventive agents following exposure to individual tobacco carcinogens (4). One of the difficulties in evaluating the efficacy of a chemopreventive agent is the lack of biomarkers that could ultimately be assessed not only in the animal model but also in human clinical trials. Although most chemopreventives act by affecting multiple signaling pathways to cause growth arrest and/or apoptosis, we and others have asked whether agents that cause the reexpression of tumor suppressor genes silenced by methylation could be an effective approach for reversing preinvasive disease or blocking cancer progression (5). Silencing of genes by promoter hypermethylation is now recognized as a crucial component in lung cancer initiation and progression, making it an attractive target for prevention (6, 7). Combining the demethylating agent 5-aza-2′-deoxycytidine (DAC) with the histone deacetylase inhibitor sodium phenylbutyrate reduced lung tumor development by 50% in A/J mice treated with the tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; ref. 8). This finding suggests that blocking and/or reversing epigenetically mediated gene silencing might provide a novel clinical strategy to prevent lung cancer. Validation of this approach would be facilitated by the identification of genes methylated in the mouse lung that could serve as biomarkers to assess the effectiveness of demethylating agents and inhibitors of histone deacetylation against preinvasive cancer.

The extrapolation of findings from mouse to human lung cancer would be strengthened by the ability to study gene methylation in murine tumors induced by tobacco smoke. A robust mouse model for studying tobacco-induced lung cancer had been lacking until a recent study by Hutt et al. (9) showed

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that lifetime, whole-body exposure of B6C3F1 mice to mainstream cigarette smoke induced neoplasms in ~45% of mice. The prevalence for methylation of two genes implicated in human lung cancer, retinoic acid receptor-β (RAR-β) and death-associated protein kinase (DAPK), was determined in tumors induced by cigarette smoke, NNK, vinyl carbamate, and methylene chloride (10, 11). RAR-β was methylated in 90% of lung tumors induced by exposure to NNK or cigarette smoke but in only 60% and 56% of tumors induced by vinyl carbamate and methylene chloride, respectively (10). In contrast, DAPK was methylated in ~60% of tumors induced by the four different carcinogens, with no difference in frequency between exposures (11). Bisulfite sequencing of the CpG islands of these two genes revealed a marked contrast in the density and distribution of methylation. DAPK showed complete methylation throughout a dense CpG island, whereas RAR-β showed relatively few methylated CpGs at the 3′ end of a sparse island. These two studies show how genes can be targeted differentially by carcinogen exposure and also display marked differences in density of CpG methylation associated with their silencing.

The purpose of this investigation was to extend these studies to develop a more comprehensive gene methylation profile in mouse lung tumors induced by cigarette smoke or NNK and compare those gene methylation prevalences with that found in tumors arising spontaneously. Candidate genes analyzed for methylation were chosen based on diverse functions and previous demonstration of methylation in human lung cancer (12-20). The genes examined were cadherin-1 and cadherin-13 (CDH1 and CDH-13), the mouse homologues of human E-cadherin and H-cadherin, estrogen receptor-α (ER-α), progesterone receptor (PGR), runt-related transcription factor-3 (RUNX-3), and suppressor of cytokine signaling-1. The cadherins are a family of cell-surface glycoproteins responsible for cell recognition and adhesion. Because of its role in adhesion, loss of cadherin function could play a role in local tumor invasion through the basement membrane (21). The ER and PGR are two members of the hormone receptor superfamily that modulate cell growth through communication with nuclear receptors (22). RUNX-3 is a member of a family of transcription factors that regulate transforming growth factor-β signaling pathways to regulate cell cycle, differentiation, and apoptosis (23). Finally, suppressor of cytokine signaling-1 is a negative regulator of the Janus-activated kinase/signal transducers and activators of transcription pathway whose activation by cytokines and growth factors results in the transactivation of target genes, such as c-fos (24). Thus, the inactivation of these genes by methylation would contribute to malignant transformation in the mouse lung. To gain additional insight into the mechanisms of gene silencing by methylation, the density and location of methylated CpGs within the 5′ promoter region of each candidate gene were determined.

Results

Methylation of Candidate Genes in Mouse Lung Tumor-Derived Cell Lines

Combined bisulfite restriction analysis (COBRA) was used to screen for methylation of genes in cell lines derived from mouse lung tumors (Fig. 1A). The CDH-13, ER-α, PGR, and RUNX-3 loci were frequently methylated (4/9, 7/9, 5/9, and 6/9,

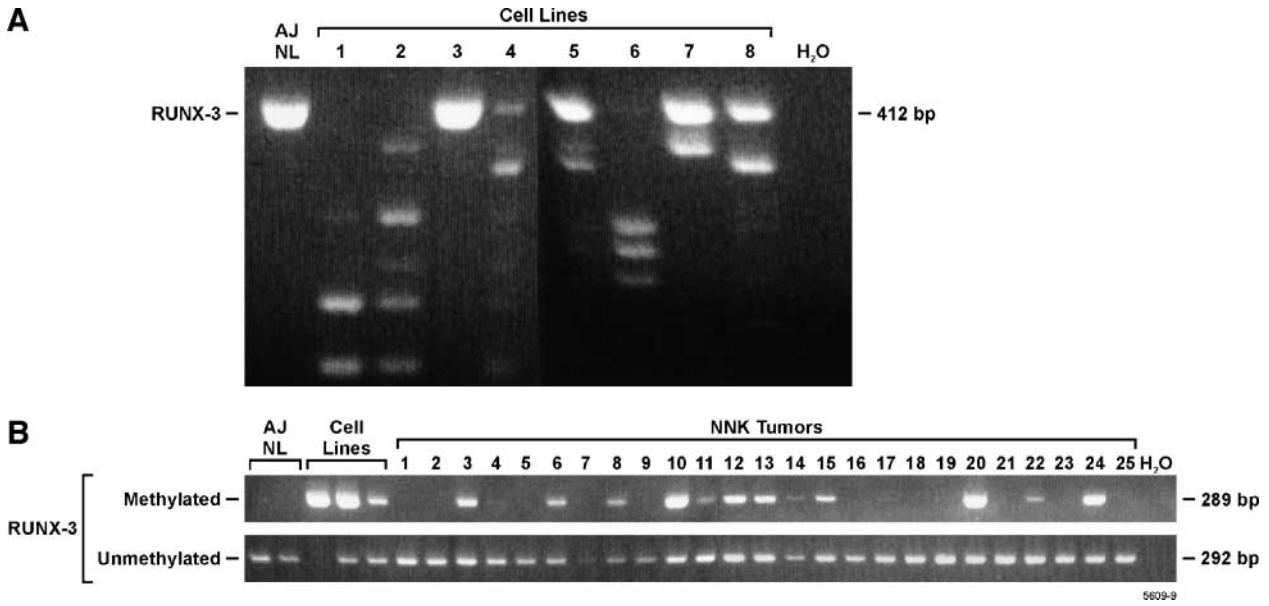

FIGURE 1. **A.** Methylation in cell lines derived from mouse lung tumors determined by COBRA. Bisulfite-modified DNA was amplified with primers specific to promoter CpG islands of the genes of interest containing *Bst*UI sites. PCR products were digested with *Bst*UI. Representative experiment for the RUNX-3 gene. The presence of a 412-bp band indicates no methylation (*lane 3*), whereas a 412-bp band plus smaller bands indicates partial or heterozygous methylation (*lanes 4, 5, 7*, and *8*). The absence of the 412-bp band and presence of lower molecular weight bands indicates complete methylation (*lanes 1, 2*, and *6*). The normal A/J mouse lung was completely unmethylated. **B.** MSP analysis of murine cell lines and tumors induced by NNK. Representative experiment for RUNX-3 gene. Tumors and cell lines positive for RUNX-3 methylation show the presence of a 289-bp methylated PCR product. Unmethylated alleles were detected in all tumor samples due to the presence of stromal and inflammatory cells and in some cell lines due to heterogeneity for methylation. Only unmethylated alleles were detected in normal A/J mouse lung. AJNL, A/J normal lung.

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respectively). Restriction patterns revealed fully methylated PCR products (*lane 1*), partially methylated products (*lane 7*), or unmethylated products (*lane 3*). No methylation was detected in DNA from six normal AJ mouse lungs (Fig. 1A and data not shown). CDH-1 and suppressor of cytokine signaling-1 were unmethylated in all mouse lung tumor-derived cell lines and excluded from further study (data not shown).

Expression of Methylated Candidate Genes in Mouse Lung Tumor-Derived Cell Lines Is Restored by DAC Treatment

The expression of genes methylated based on COBRA was determined in six cell lines treated and untreated with DAC (Fig. 2). The CDH-13 transcript was not detected in two of six cell lines; expression was restored in both after DAC treatment. ER-α was not expressed in four of six cell lines, all of which reexpressed after DAC treatment. PGR was not expressed in five of six cell lines, four of which reexpressed after DAC. RUNX-3 transcript was not detected in three of six cell lines; expression was restored in the three cell lines after treatment with the demethylating agent. With the exception of the PGR gene for the J303 cell line, there was a perfect correlation between methylation detected by COBRA and gene expression.

Prevalence for Gene Methylation in Primary Lung Tumors Varies with Exposure History

Methylation prevalence was determined in primary mouse lung tumors by methylation-specific PCR (MSP; Fig. 1B). Tumors were induced by exposure to cigarette smoke ($n = 45$), NNK ($n = 25$), or occurred spontaneously ($n = 16$). The results are summarized in Table 1. The incidence of methylation for the CDH-13 gene did not differ across exposures (cigarette smoke, 64%; NNK, 64%; spontaneous, 69%). In contrast, a significantly higher prevalence for methylation of the ER-α gene was seen in spontaneously occurring tumors than in tumors induced by exposure to cigarette smoke or NNK (63% versus 27% and 32%, respectively; $P < 0.05$). The prevalence for methylation of PGR was greater in lung tumors induced by cigarette smoke (61%) or arising spontaneously (58%) than in tumors induced by NNK (24%; $P < 0.01$). A trend for a higher prevalence of methylation in cigarette smoke–induced and NNK-induced tumors than in spontaneously occurring tumors was seen for the RUNX-3 gene (39% and 44% versus 25%, respectively; $P = 0.07$). No methylation was detected in any of the genes in DNA from the six vehicle-treated A/J and B6C3F1 mouse lungs (Fig. 1B and data not shown). Unmethylated alleles for all four genes were detected in all tumors due to the presence of normal stromal and inflammatory cells and possibly tumor heterogeneity. Methylation of genes detected in cell lines by COBRA was corroborated by the MSP assay.

Table 1. Prevalence for Gene Promoter Hypermethylation in Lung Tumors Induced by Cigarette Smoke, NNK, or Arising Spontaneously

Gene	Exposure (%)		
	Cigarette smoke*	NNK	Spontaneous
CDH-13	29/45 (64)	16/25 (64)	11/16 (69)
ER-α	12/45 (27)	8/25 (32)	10/16 (63)†
PGR	22/36 (61)	6/25 (24)‡	9/16 (56)
RUNX-3	14/36 (39)	11/25 (44)	4/16 (25)§

*Only 36 of 45 tumors could be analyzed for methylation of PGR and RUNX-3 because of insufficient tissue.
† $P < 0.05$, compared with spontaneously arising or cigarette smoke induced.
‡ $P < 0.001$, compared with cigarette smoke or NNK induced.
§ $P = 0.08$, compared with cigarette smoke or NNK induced.

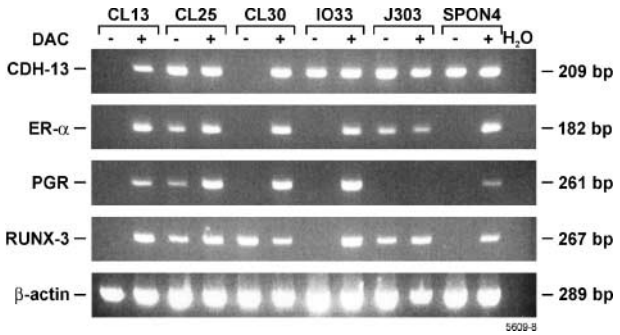

FIGURE 2. Reexpression of the CDH-13, ER-α, PGR, and RUNX-3 genes in lung tumor-derived cell lines after treatment with DAC. Cells were grown for 4 days in either 1 μm DAC (+) or medium without DAC (–). Total cellular RNA was reverse transcribed and amplified with primers specific to transcripts of indicated genes. β-Actin was amplified as a loading control. Treatment with DAC restored expression in cell lines with no basal gene expression.

Association between Methylation of Genes within a Single Tumor

The association between methylation of these four genes as well as RAR-β and DAPK from our previous studies (10, 11) was assessed in tumors independent of exposure due to limited sample size for individual exposures (Fig. 3). A positive association was seen between methylation of RAR-β and RUNX-3 ($P = 0.006$) and that of CDH-13 and ER-α ($P = 0.05$).

Density and Pattern of CpG Methylation

Sequencing of bisulfite modified DNA from the 5′ promoter region of the CDH-13, ER-α, PGR, and RUNX-3 genes was done to determine the density and location of methylated CpGs (Fig. 4). Cell lines containing methylated genes and unmethylated normal lung from the A/J mouse were sequenced. A region spanning 312 bp within the CDH-13 gene and containing 19 CpGs was sequenced. Approximately 50% of CpGs were methylated in the CL13 cell line. Individual clones displayed either 100% or 0% methylation, strongly suggesting monoallelic methylation of CDH-13 in this cell line. However, the unmethylated allele was also not expressed in this cell line (Fig. 2). CL25, IO33, and A/J normal lung, all unmethylated by MSP, showed 0% methylated CpGs in the CDH-13 promoter. A 257-bp region containing 13 CpGs in the promoter region of ER-α showed 0% methylation for A/J normal lung. CL13, IO33, and SPON4 cell lines were methylated at 88%, 60%, and 88% of CpGs, respectively. A 434-bp region containing 17 CpGs in the promoter of the PGR gene was completely unmethylated in A/J normal lung, whereas 49%, 26%, and 47% of CpG sites were methylated in the CL13, P212, and SPON4

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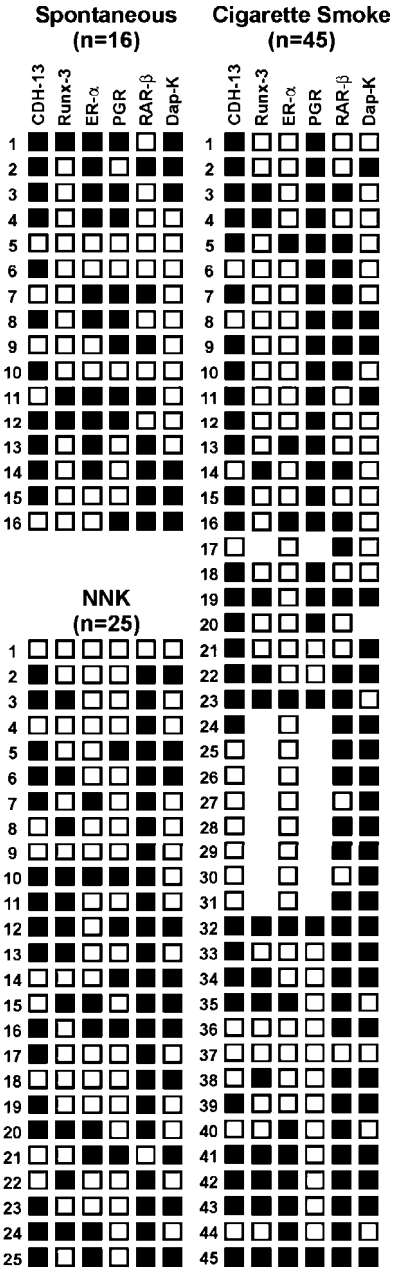

FIGURE 3. Map showing the methylation profile in individual spontaneous, NNK-induced, and cigarette smoke–induced mouse lung tumors. Numbers, individual tumors and methylation state of the CDH-13, RUNX-3, ER-α, PGR, RAR-β, and DAPK genes in each column. Filled and open boxes, methylated and unmethylated tumor specimens, respectively, for the gene.

cell lines, respectively. The RUNX-3 CpG island contains 89 CpGs over 893 bp. Only 1% of CpG sites was methylated in normal lung tissue, whereas 65% and 74% of CpGs were methylated in the CL13 and IO33 cell lines, respectively.

Discussion

This investigation shows that four epigenetic alterations in human lung cancer, methylation of the H-cadherin (CDH-13), ER-α, PGR, and RUNX-3 genes, occur in mouse lung tumors induced through exposure to cigarette smoke or NNK. The transcriptional silencing of each gene was correlated with hypermethylation based on reexpression by DAC and methylation throughout each CpG island in cell lines. In contrast, methylation of E-cadherin (CDH-1) and suppressor of cytokine signaling-1, which occurs in 20% and 55%, respectively, of human lung tumors, was not observed in any mouse lung tumors (13, 15, 16). Similar species differences have been seen previously for the p16 gene, which is commonly methylated in human lung tumors but shows diminished expression associated with loss of heterozygosity in NNK-induced mouse tumors and is deleted in established cell lines (7, 25, 26).

Methylation of these genes was also common in tumors arising spontaneously, with only the RUNX-3 gene occurring at a prevalence of <30%. The only significant difference in methylation profiles between spontaneous and tobacco-induced tumors was for ER-α. The higher prevalence for methylation of this gene in spontaneous tumors from the B6C3F1 mouse parallels our previous findings in spontaneous lung tumors from the A/J mouse (20). This finding suggests that silencing of the ER-α gene is important for the development of tumors arising spontaneously in the mouse lung possibly through effects on cell proliferation (27, 28). Few studies have examined methylation of this gene in human lung tumors. Consistent with studies in mice, we observed that the prevalence for methylation of ER-α was greater in lung tumors from never-smokers than smokers (20). In contrast, Lai et al. (29) found differences between gender, but not smokers and never-smokers, for methylation of this gene in lung tumors from patients in Taiwan. The reason for the discordance between these results is not apparent but could be due to ethnicity-related and/or geography-related differences. The etiology for lung cancer in the never-smoker may be due to in part to exposure to environmental tobacco smoke and other environmental and occupational carcinogens (e.g., radon). Although these exposures cannot account for spontaneous tumors in the mouse lung, it is interesting that these tumors develop in part through inactivation of genes, such as ER-α, DAPK, RAR-β, and H-cadherin, which are also silenced in adenocarcinomas from the never-smoker (9, 20, 30, 31).

The positive associations seen between methylation of RAR-β and RUNX-3 as well as CDH-13 and ER-α could reflect a specific methylation phenotype or suggest an interaction by these genes to regulate cell function. RUNX-3 is a RUNX that interacts with transforming growth factor-β–activated SMADs to mediate transforming growth factor-β signaling (23). There have been no direct studies to examine the interaction of RAR-β and transforming growth factor-β in lung cells; however, these two complex signaling pathways could converge downstream to modify the activity of key growth factors. For example, mouse skin models have shown that the application of retinoic acid can induce transforming growth factor-β in the epidermis and in the follicular epithelium (32). Similarly, the cadherins comprise a group of transmembrance proteins whose interaction with the catenins mediate cell-cell interactions in many different cell types. Tyrosine phosphorylation of catenins has been proposed as one mechanism to mediate interaction of these proteins with the cadherins (33). Epidermal growth factor can induce tyrosine phosphorylation of β-catenin that in turn leads to the association of autophosphorylated ER-α with the cadherin-catenin complex

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(34). Thus, loss of function of ER-α and H-cadherin through methylation would disrupt the ability to regulate cell adhesion.

Our earlier studies on methylation of the mouse RAR-β and DAPK genes (10, 11) revealed two paradigms for gene silencing by methylation. RAR-β was sparsely methylated over a short stretch of the 5′ promoter region extending into exon 2 containing 13 CpGs over 310 bp. By contrast, DAPK was densely methylated at 50 CpGs over 360 bp in the 5′ promoter region. These results suggested that DAPK silencing by methylation occurs in a density-dependent fashion, whereas RAR-β silencing occurs through methylation at key CpGs, such as those contained within Sp1 sites. Bisulfite sequencing revealed that the RUNX-3 gene is silenced in a density-dependent manner based on dense methylation of CpGs over an 893-bp CpG island. In contrast, methylation profiles for the CDH-13, ER-α, and PGR genes are more consistent with silencing through site-specific methylation.

This study has identified four additional genes that can serve as biomarkers in the mouse lung to evaluate the ability of demethylating agents and inhibitors of histone deacetylation to reverse or impede the progression of premalignancy. The potential mutagenicity and side effects from DAC, the most potent demethylating agent known, will preclude its clinical application in cancer-free, high-risk persons for lung cancer. Rather, the efficacy of less potent demethylating agents that are either natural compounds, such as the tea polyphenol (−)-epigallocatechin-3-gallate, or drugs used to treat nonneoplastic conditions, such as the antihypertensive agent hydralazine, needs to be evaluated in the mouse model (35, 36). Genes methylated in mouse lung tumors will be valuable biomarkers to determine whether these demethylating agents can cause reexpression of genes with dense and extensive methylated CpG islands or are more efficacious toward genes whose silencing is mediated through site-specific methylation that may encompass critical transcription factors.

Materials and Methods

Tumor Induction

Female A/J mice (6 weeks old) were treated with a single dose (100 mg/kg i.p.) of NNK and sacrificed 50 weeks after treatment (37). Female B6C3F1 mice (6-7 weeks old) were exposed to mainstream cigarette smoke for up to 30 months. Details of the exposure system have been described (9). Briefly, two 70-cm^2 puffs/min from research cigarettes (type 2R1, Tobacco Health Research Institute, Lexington, KY) were generated by smoking machines (type 1300, AMESA Electronics, Geneva, Switzerland). Cigarette smoke was diluted with filtered air and delivered to H-2000 whole-body exposure chambers (Hazleton Systems, Inc., Aberdeen, MD). The mass concentration of cigarette smoke total particulate material, determined by gravimetric analysis of the filter samples taken during the exposure periods, was determined to be 250 mg total particulate matter/m^3 (38). Spontaneous, cigarette smoke–induced and NNK-induced tumors were classified as adenomas or adenocarcinomas according to histologic characteristics.

Cell Culture and DAC Treatment

The CL13, CL25, CL30, and IO33 cell lines were derived from lung tumors induced in AJ mice by treatment with NNK (37); the J303 and P212 cell lines were derived from cigarette smoke–induced B6C3F1 lung tumors (10). CL13, CL30, and IO33 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum; CL25, J303, and P212 cells were grown in ITRI-1 medium (39). The cell lines were treated with DAC (1 μm) for 4 days, with medium changes and fresh DAC added every 24 hours. Cells were harvested for isolation of DNA and RNA.

Nucleic Acid Isolation

Genomic DNA was extracted from cell pellets and tumors by digestion with proteinase K, RNase A, and RNase T1 followed

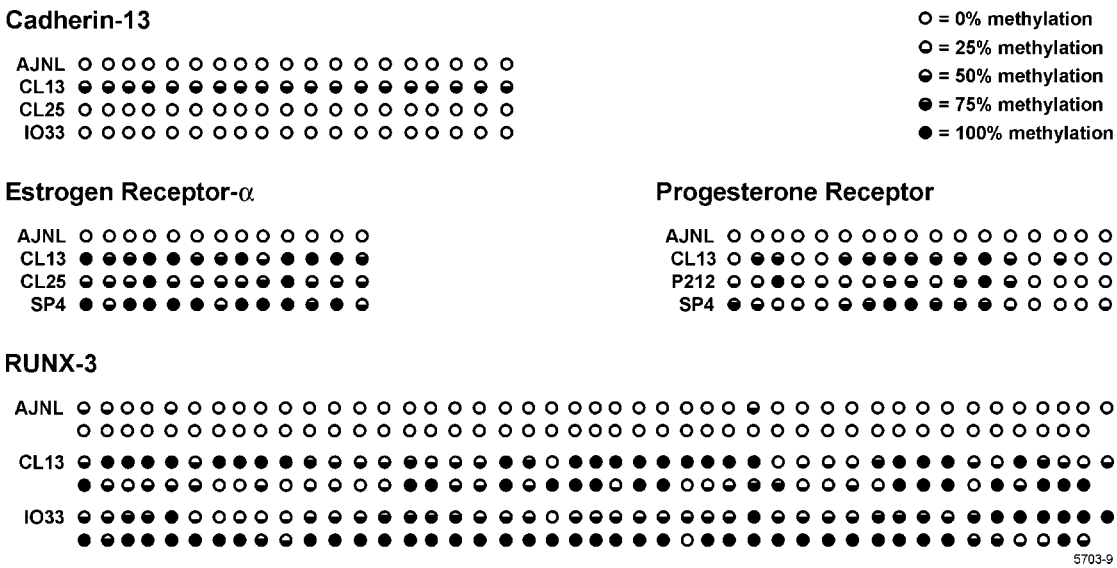

FIGURE 4. Density and distribution of CpG island methylation of the CDH-13, ER-α, PGR, and RUNX-3 genes in mouse lung tumor-derived cell lines and normal lung. Bisulfite-modified DNA was amplified with primers specific to the promoter CpG islands of the indicated genes. For each gene/cell line combination, eight individual clones were sequenced. The fraction of clones methylated at each individual CpG is shown. AJNL, A/J normal lung.

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by phenol/chloroform extraction and ethanol precipitation. DNA from NNK-induced and approximately half of the cigarette smoke–induced tumors was obtained from frozen tissues. DNA from spontaneously arising tumors and additional cigarette smoke–induced tumors was obtained by microdissection from paraffin-embedded lung tissue. All tumors were from different mice. Sequential sections were prepared from tumors and deparaffinized. A 25-gauge needle attached to a tuberculin syringe was used to remove the lesions under a dissecting microscope. Total cellular RNA was extracted from cell pellets using Tri Reagent (Sigma, St. Louis, MO) and treated with DNase I to destroy any contaminating DNA followed by phenol extraction and ethanol precipitation.

Reverse Transcription-PCR

Total cellular RNA (3 μg) was reverse transcribed using a SuperScript II kit (Invitrogen, Carlsbad, CA). cDNA was treated with RNase H to destroy any residual RNA. PCR was done on 2 μL cDNA using primers specific for the gene being analyzed. Primer sequences and PCR conditions are available upon request. Primers specific for amplification of the β-actin gene were used as a template quality control as described previously (40). Products were visualized on agarose gels.

Bisulfite Sequencing

Genomic DNA was treated with sodium bisulfite as described previously (41) to modify unmethylated cytosine residues to uracil and then to cytosine during subsequent PCR. Modified DNA was amplified using primers specific for the CpG island of the gene being sequenced. Primer sequences and PCR conditions are available upon request. PCR products were separated on 3% agarose/Tris-borate EDTA gels and the band was excised. Products were purified using a GeneClean kit (Bio 101, Qbiogene, Irvine, CA) and cloned into the pCR II vector using a TA Cloning kit (Invitrogen). Individual clones were grown, DNA recovered using a Qiaspin kit (Qiagen, Valencia, CA), and then sequenced with an automated sequencer (Department of Biochemistry, University of New Mexico Sequencing Facility, Albuquerque, NM). Eight clones were sequenced for each gene. Data were pooled, and the fraction of clones methylated at each particular CpG site was determined.

Combined Bisulfite Restriction Analysis

COBRA was used to evaluate DNA isolated from cell lines for the initial methylation screening (42). Primary PCR primers were designed flanking a region of the 5′ CpG island of each candidate gene. Both forward and reverse primers contain no CpG dinucleotides, so that they recognize and amplify methylated and unmethylated templates equally. The primers were designed to incorporate multiple *Bst*UI recognition sequences, CG′CG, which would be retained after bisulfite treatment only in methylated DNA. Primer sequences and PCR conditions are available upon request. Bisulfite modified DNA was amplified and digested with *Bst*UI, and results were analyzed on 3% agarose/Tris-borate EDTA gels. Digestion of the PCR products into lower molecular weight products is indicative of methylation.

Methylation-Specific PCR

Bisulfite-modified cell lines and tumor DNA were assayed by MSP to analyze the methylation status of each gene. A nested, two-stage approach was used (30) to increase the efficiency for gene amplification, thereby allowing the determination of methylation state in samples whose DNA has been degraded through formalin fixation and/or long-term storage in paraffin. Stage 1 primers amplify both methylated and unmethylated alleles of the genes being analyzed. Two sets of nested primers were used for the stage 2 PCR. One primer pair recognizes alleles containing methylated CpG sites, whereas the other recognizes unmethylated alleles. Primers are located within regions containing frequent CpG dinucleotides to distinguish between methylated and unmethylated DNA. DNA (~150 ng) was used for stage 1 PCR. Stage 1 products were diluted 50-fold, and 5 μL were used for stage 2 PCR. Primer sequences and PCR conditions are available upon request. Age-matched normal AJ and B6C3F1 mouse lung DNAs were used as a control in all PCRs, and products were visualized on agarose gels.

Data Analysis

The Wilcoxon rank sum test was used for pair-wise comparisons of methylation between different exposure groups. The Fisher's exact test was used to determine associations between methylation events in a single tumor. All tests were two-sided, with significance set at $P < 0.05$.

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