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Aberrant Promoter Hypermethylation of the *Death-Associated Protein Kinase* Gene Is Early and Frequent in Murine Lung Tumors Induced by Cigarette Smoke and Tobacco Carcinogens

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ABSTRACT

Loss of expression of the *death-associated protein* (*DAP*)-*kinase* gene by aberrant promoter methylation may play an important role in cancer development and progression. The purpose of this investigation was to determine the commonality for inactivation of the *DAP-kinase* gene in adenocarcinomas induced in mice by chronic exposure to mainstream cigarette smoke, the tobacco carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and vinyl carbamate, and the occupational carcinogen methylene chloride. The timing for inactivation was also determined in alveolar hyperplasias that arise in lung cancer induced in the A/J mouse by NNK. The *DAP-kinase* gene was not expressed in three of five NNK-induced lung tumor-derived cell lines or in a spontaneously arising lung tumor-derived cell line. Treatment with 5-aza-2′-deoxycytidine restored expression; dense methylation throughout the *DAP-kinase* CpG island detected by bisulfite sequencing supported methylation as the inactivating event in these cell lines. Methylation-specific PCR detected inactivation of the *DAP-kinase* gene in 43% of tumors associated with cigarette smoke, a frequency similar to those reported in human non-small cell lung cancer. In addition, *DAP-kinase* methylation was detected in 52%, 60%, and 50% of tumors associated with NNK, vinyl carbamate, and methylene chloride, respectively. Methylation was observed at similar prevalence in both NNK-induced hyperplasias and adenocarcinomas (46% *versus* 52%), suggesting that inactivation of this gene is one pathway for tumor development in the mouse lung. Bisulfite sequencing of both premalignant and malignant lesions revealed dense methylation, substantiating that this gene is functionally inactivated at the earliest histological stages of adenocarcinoma development. This study is the first to use a murine model of cigarette smoke-induced lung cancer and demonstrate commonality for inactivation by promoter hypermethylation of a gene implicated in the development of this disease in humans.

INTRODUCTION

Loss of expression of death-associated protein (DAP)-kinase is common in human cancers. *DAP-kinase* is a Ca^{+}/calmodulin-regulated serine-threonine kinase involved in IFN-γ-, tumor necrosis factor α-, Fas-, transforming growth factor β-, and ceramide-induced apoptosis (1–6). *DAP-kinase* also is involved in apoptosis triggered by the detachment of cells from the extracellular matrix (7). Reintroduction of the *DAP-kinase* gene into highly metastatic mouse lung carcinoma cells delayed tumor growth and strongly reduced their metastatic capacity (8). *DAP-kinase* also suppresses c-*myc*- and E2F-induced oncogenic transformation by activating the *p53* apoptotic pathway in a $p19^{ARF}$-dependent manner (1). Thus, loss of this gene at the earliest stages of tumor formation would provide a selective growth advantage, whereas loss at later stages could also facilitate tumor progression and metastasis.

Inactivation of *DAP-kinase* occurs largely through transcriptional silencing by DNA hypermethylation. Methylation of the *DAP-kinase* promoter has been observed in bladder, renal, and breast cancer cell lines as well as in head and neck, gastric, and colorectal carcinomas (9–13). Additionally, a very high frequency of *DAP-kinase* promoter methylation has been detected in B-cell malignancies, including 100% of Burkitt's lymphomas and 84% of B-cell lymphomas (14). Finally, methylation prevalences ranging from 23% to 44% have been observed in non-small cell lung cancer (NSCLC; Refs. 15–17). The common targeting for inactivation of this gene in many solid tumors reinforces its importance in apoptosis and tumor suppression.

Despite numerous studies demonstrating frequent methylation of *DAP-kinase* in many human cancers, the commonality for this inactivation in cell lines or tumors from other species has not been examined. Furthermore, the timing of *DAP-kinase* inactivation by methylation during tumor development has not been established. Our group has used the mouse lung to study mechanisms involved in the development and progression of lung cancer induced by defined exposure to environmental and occupational carcinogens (18, 19). The fact that genetic and epigenetic alterations found in human lung adenocarcinomas are also common in murine tumors makes the mouse lung an ideal model for examining pathways involved in lung cancer induced by specific carcinogens (20). For example, activation of the *K-ras* oncogene is an early and frequent event in both human lung adenocarcinoma and in spontaneous and chemically induced lung tumors in mice (21–25). Furthermore, both human adenocarcinomas associated with smoking and mouse lung tumors induced with the tobacco carcinogen benzo(*a*)pyrene contain codon 12 G to T mutations in the *K-ras* oncogene indicative of base mis-pairing associated with the benzo(*a*)pyrene 7,8-diolexpoxide DNA adduct (21, 22). The frequency for methylation of the *estrogen receptor* α gene is also similar in murine and human lung tumors (26). Finally, the $p16^{INK4a}$ gene is a common target for inactivation in both human and mouse lung tumors. Interestingly, this gene is silenced primarily by methylation in human tumors, but is both methylated and deleted in murine tumors (27, 28).

The purpose of this investigation was to determine whether adenocarcinomas induced in the B6C3F1 mouse by chronic exposure to mainstream cigarette smoke arise in part through inactivation of the *DAP-kinase* gene. These studies were extended to compare the prevalence for *DAP-kinase* methylation in tumors induced by the tobacco carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and vinyl carbamate and the occupational carcinogen methylene chloride. Finally, the timing for inactivation of this gene was determined in alveolar hyperplasias that arise in our well-characterized model of lung cancer induced in the A/J mouse by NNK.

MATERIALS AND METHODS

Tumor Induction. Female A/J mice (6 weeks of age) were treated with a single dose (100 mg/kg, i.p.) of NNK and sacrificed at 4-week intervals

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starting at 14 weeks after carcinogen treatment out to 54 weeks (29). Female B6C3F1 mice (6–7 weeks old) were exposed to mainstream cigarette smoke for up to 30 months. 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 (Hazelton Systems, Inc., Aberdeen, MD). The mass concentration of cigarette smoke total particulate material was determined by gravimeteric analysis of the filter samples taken during the exposure periods and determined to be 250 mg total particulate matter/m^3 (30). Female B6C3F1 mice (8–9 weeks of age) were exposed to 2000 ppm methylene chloride delivered to H-2000 whole-body exposure chambers for 6 h/day, 5 days/week, up to 104 weeks (31). Female C3A mice were treated with a single dose of vinyl carbamate (60 mg/kg, i.p.) and sacrificed approximately 1 year after treatment (32). All tumors were classified as adenocarcinomas by histological characteristics. Hyperplasias induced by NNK treatment were distinguished by a proliferation of type II epithelial cells along intact alveolar septae (29).

Cell Culture and 5-Aza-2′-deoxycytidine (DAC) Reexpression Experiments. The CL13, CL20, CL25, CL30, and IO33 cell lines were developed from A/J mouse lung tumors induced by NNK (33). Cell lines J303, P212, and P261 were developed from B6C3F1 mouse lung tumors induced by cigarette smoke. The SPON4 cell line was developed from a spontaneous lung tumor in the A/J mouse lung. Cell lines CL13, CL20, and IO33 were grown in RPMI 1640 supplemented with 10% fetal bovine serum; cell lines CL25, CL30, SPON4, J303, P212, and P261 were grown in ITRI-1 medium (33). Cells were maintained at 37°C in a humid atmosphere containing 5% CO_2. The cell lines were treated with 1 μM DAC for 4 days, with media changes and fresh DAC added every 24 h. After 96 h, cells were trypsinized and pelleted for RNA isolation.

Nucleic Acid Isolation. Total cellular RNA was extracted from the murine cell lines using TRI reagent (Sigma, St. Louis, MO). Contaminating DNA was removed by digestion with DNase I, followed by phenol extraction and ethanol precipitation. DNA from NNK-induced tumors, methylene chloride-induced tumors, vinyl carbamate-induced tumors, and approximately half of the cigarette smoke-induced tumors was obtained from frozen tumors. DNA from NNK-induced hyperplasias and additional cigarette smoke-induced lung tumors was recovered by microdissection as follows: sequential sections were prepared from tumors and hyperplasias; deparaffinzed; and stained with toluidine blue to facilitate dissection. A 25-gauge needle attached to a tuberculin syringe was used to remove the lesions under a dissecting microscope. Due to the small size of the alveolar hyperplasias, microdissection was used to enrich the samples. Thus, the inclusion of normal-appearing cells was necessary to ensure that enough sample remained to conduct the methylation-specific PCR (MSP) assay after bisulfite modification and column clean-up of the DNA template. Because the goal of the study was to determine whether *DAP-kinase* methylation was present in these lesions and not to quantitate methylation levels, microdissection was used to enrich the samples. DNA was isolated from microdissected lesions and frozen tumors by overnight digestion with Pronase (1%), followed by standard phenol-chloroform extraction and ethanol precipitation (25).

Reverse Transcription-PCR. First-strand cDNA was generated at 42°C from 3 μg of total RNA using the SuperScript II kit (Invitrogen, San Diego, CA). PCR primer sequences were 5′-TCAGGGCGACGAAGCGACA-3′ (sense) and 5′-CTGCCTGAACACAG TCATGAT-3′ (antisense), and they were designed to cross an exon splice junction. After an initial denaturation at 94°C for 10 min, amplification was carried out for 40 cycles, which consisted of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. This was followed by a final extension at 72° for 5 min to generate a 157-bp product. The *β-actin* gene was amplified as a control for RNA integrity. Products were visualized on 2% agarose gels.

MSP. The methylation status of the *DAP-kinase* promoter in all murine cell lines, hyperplasias, and tumors was determined using a nested, two-stage MSP assay as described previously (34). The two-stage MSP was used throughout the study because of difficulty in amplifying products from formalin-fixed tissue where DNA is degraded. This enabled results obtained from frozen and fixed tumors to be combined. Stage 1 primers were located at −267 and +93 (+1 was the transcriptional start site) and amplified a region that included a portion of the CpG-rich promoter. Primer sequences were 5′-GATTTTT-GGGTTTATATTTTGAGAGG-3′ (sense) and 5′-CTCCCRCTCCTCCCAC-TACCTA-3 prime (antisense; R = G + A). After amplification by stage 1 primers, two different stage 2 PCRs were performed: one amplified unmethylated DNA; and the other amplified methylated DNA. The stage 2 unmethylated and methylated primers were located at −112 and +49 and −108 and +48, respectively. Primer sequences were as follows: stage 2 unmethylated, 5′-TGGGAGGAGTTGTGA GTGT-3′ (sense) and 5′-ACAACTATCACTT-CATACACC-3′ (antisense); and stage 2 methylated, 5′-AGGAGTCGCGA-GCGTAGC-3′ (sense) and 5′-CAACTATCGCTTCGTACGC-3′ (antisense). Normal A/J mouse lung and a murine cell line positive for *DAP-kinase* methylation (IO33) served as negative and positive controls, respectively. The products were visualized on 2% agarose gels.

Bisulfite Sequencing. Stage 1 MSP primers were used to amplify the *DAP-kinase* gene from DNA of A/J mouse normal lung and four cell lines (CL25, CL13, CL30, and IO33). DNA from normal A/J mouse lung, mouse lung hyperplasias derived from exposure to NNK, and mouse lung tumors derived from exposure to NNK and cigarette smoke were amplified using either unmethylated or methylation-specific stage 2 primers. The methylation-specific primers recognize a sequence in which CpGs are methylated; therefore, only methylated alleles are amplified. The 360-bp (stage 1) and 156-bp (stage 2) PCR products were ligated into the PCR II vector using the TA cloning kit (Invitrogen). Four to six clones from each sample were commercially sequenced in both directions [University of New Mexico Center for Genetics in Medicine (Albuquerque, NM) and Sequetech (Mountain View, CA)].

RESULTS

The Murine *DAP-Kinase* CpG Island. The CpG island within the murine *DAP-kinase* promoter comprises approximately 84 CpGs over 880 bp that extend through the transcriptional start site (GenBank accession number XM127345). The density of CpGs within this region is similar to that seen in the analogous region in the human *DAP-kinase* promoter (46 CpGs within 590 bp). In the human promoter, an additional 125 CpGs are located 1000 bp upstream of the initial 590 bp, whereas in the mouse promoter, the density of CpGs is reduced greatly beyond the initial 500 bp. The deduced amino acid sequence of the *DAP-kinase* gene was 90% homologous to the human sequence present in GenBank (accession number AC007847). Thus, there is good conservation between human and mouse with respect to both amino acid sequence and density of CpGs around the transcriptional start site of the *DAP-kinase* gene.

DAP-Kinase Expression Correlates with Methylation State in Murine Lung Tumor-Derived Cell Lines. The *DAP-kinase* gene was not expressed in three of five NNK-induced mouse lung tumor-derived cell lines (IO33, CL13, and CL30) or in a spontaneously arising mouse lung tumor-derived cell line [SPON4 (Fig. 1)]. Expression of this gene was detected in the CL25 and CL20 cell lines as well as in lung tissue from the A/J mouse. Treatment with DAC, a demethylating agent that inhibits cytosine DNA-methyltransferases, restored expression of *DAP-kinase* in all four cell lines showing no endogenous expression and increased the level of expression in the CL20 cell line (Fig. 1).

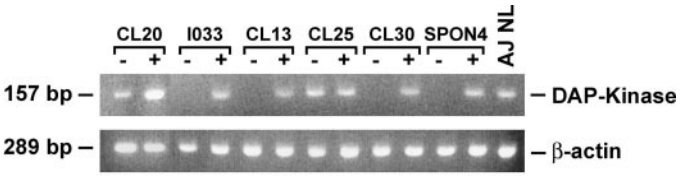

Fig. 1. Reexpression of the *death-associated protein* (*DAP*)*-kinase* gene in lung tumor-derived cell lines after treatment with 5-aza-2′-deoxycytidine (DAC). Cells were grown for 4 days in either 1 μM DAC (+) or media without DAC (−). Treatment with DAC restored expression of *DAP-kinase* in three lung tumor-derived cell lines induced by NNK (IO33, CL13, and CL30) and in one spontaneously arising lung tumor cell line (SPON4) and increased expression of *DAP-kinase* in CL20, as shown by the presence of a 157-bp product. CL25 showed normal *DAP-kinase* expression with and without DAC treatment. A/J normal lung (*AJ NL*) served as a positive control.

Table 1 *Frequency of DAP[a]-kinase methylation in murine lung tumor-derived cell lines, hyperplasias, and primary tumors*

Sample type	Exposure	Mouse strain	Methylation frequency (%)
Cell lines			
	NNK	A/J	4/5 (80%)
	Spontaneous	A/J	1/1 (100%)
	Cigarette smoke	B6C3F1	1/3 (33%)
Hyperplasias			
	NNK	A/J	6/13 (46%)
Primary tumors			
	NNK	A/J	13/25 (52%)
	Cigarette smoke	B6C3F1	9/21 (43%)
	Methylene chloride	B6C3F1	9/18 (50%)
	Vinyl carbamate	C3A	9/15 (60%)

[a] DAP, death-associated protein; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

MSP analysis of the cell lines verified results observed with the DAC reexpression experiment (Table 1). Each of the four cell lines exhibiting strong reexpression by treatment with DAC was positive for *DAP-kinase* methylation, as was CL20, which showed increased expression with DAC treatment. Three additional cell lines, J303, P212, and P261 (all derived from cigarette smoke-induced lung tumors), were also analyzed by MSP. One of the three cell lines, P212, was positive for *DAP-kinase* methylation. Of the cell lines positive for *DAP-kinase* methylation, unmethylated alleles were only detected for CL20 (data not shown). This corroborates the expression profile seen for this cell line with and without DAC treatment and indicates either an allelic difference or cell heterogeneity for methylation.

Bisulfite sequencing was used to more precisely map and determine the density of methylation throughout the *DAP-kinase* promoter. A 360-bp region of the *DAP-kinase* promoter from −267 to +93 (+1 was the translational start site) encompassing 50 CpGs was amplified by MSP. Sequencing of three cell lines positive for *DAP-kinase* methylation by MSP revealed dense methylation of the amplified region (Fig. 2). Methylation densities ranged from 70% to 94% within the promoter region of the CL13, CL30, and IO33 cell lines. CL25, the cell line that was negative for *DAP-kinase* methylation by MSP, was completely unmethylated, as was normal mouse lung (Fig. 2, *AJ NL*).

DAP-Kinase Methylation in Murine Lung Hyperplasias and Tumors. The frequency of *DAP-kinase* methylation was examined by MSP in primary murine lung adenocarcinomas associated with exposure to mainstream cigarette smoke ($n = 21$) and compared with two tobacco carcinogens, the tobacco-specific nitrosamine NNK ($n = 25$) and vinyl carbamate ($n = 15$). To investigate whether methylation of *DAP-kinase* is a general target of lung carcinogens, additional tumors associated with the occupational carcinogen methylene chloride ($n = 18$) were examined. The *DAP-kinase* gene was methylated in 43% of tumors associated with cigarette smoke. The prevalence for methylation in tumors associated with exposure to NNK and vinyl carbamate was similar to that seen in the cigarette smoke-induced tumors (Fig. 3; Table 1). Similarly, the *DAP-kinase* gene was inactivated in half of the methylene chloride-induced tumors (Table 1). Unmethylated alleles were detected in all tumor samples due to the presence of normal stromal and inflammatory cells

To establish the timing for inactivation of the *DAP-kinase* gene by methylation, 13 alveolar hyperplasias derived from exposure to NNK were examined by MSP. Methylation was observed in 46% of the hyperplasias (Table 1), a prevalence similar to that observed in the primary tumors.

Finally, to compare methylation densities between premalignant and malignant lesions, bisulfite sequencing was performed on DNA from two alveolar hyperplasias induced by NNK and four adenocarcinomas associated with exposure to either cigarette smoke or NNK. All lesions selected for sequencing were scored as methylated by the MSP assay. DNA was amplified using stage 2 MSP primers that amplified a 156-bp region ranging from −108 to +48 (+1 was the translational start site) and encompassing 16 CpGs. Sequencing of the two hyperplasias revealed dense methylation throughout the region examined, with methylation densities ranging from 63% to 75% (Fig. 4). Sequencing of tumors associated with exposure to NNK showed

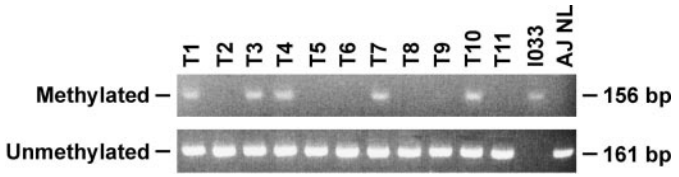

Fig. 3. Methylation-specific PCR analysis of *death-associated protein* (*DAP*)-*kinase* in murine lung tumors induced by exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Tumors positive for *DAP-kinase* methylation (*T1*, *T3*, *T4*, *T7*, and *T10*) are indicated by the presence of a 156-bp product. A murine cell line positive for DAP-kinase methylation (IO33) and normal A/J mouse lung (*AJ NL*) served as a positive control and a negative control, respectively. Unmethylated alleles were detected in all tumor samples and in the mouse normal lung, as depicted by the presence of a 161-bp band. Unmethylated alleles were not detected in IO33.

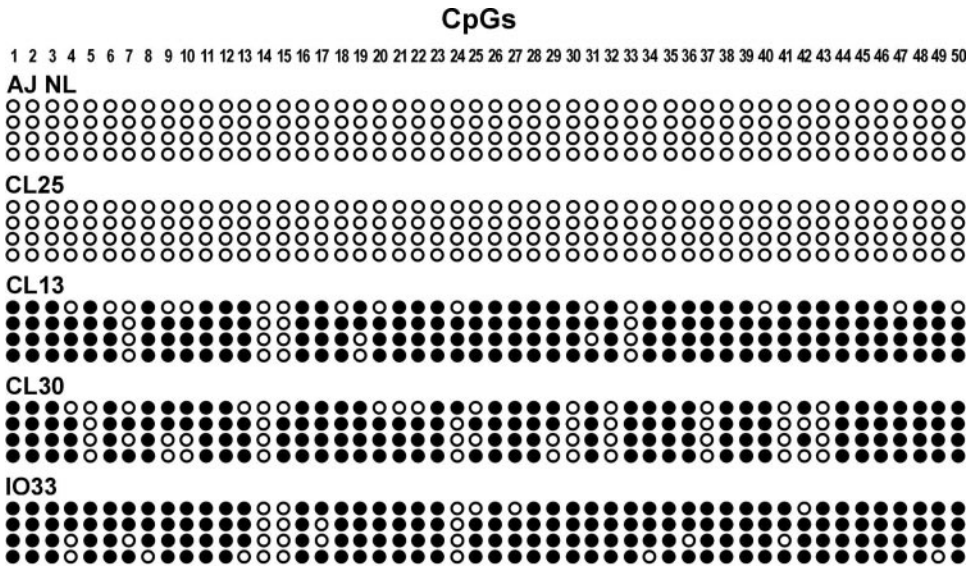

Fig. 2. Density of methylation within the *death-associated protein* (*DAP*)-*kinase* CpG island in murine lung cancer cell lines and normal mouse lung. Bisulfite-modified DNA was amplified using stage 1 MSP primers specific to the mouse *DAP-kinase* promoter. The primers used recognized a bisulfite modified template but did not discriminate between methylated and unmethylated alleles. The primers were located at −267 and +93 (+1 was the translational start site). Amplified PCR products were cloned and sequenced to determine the methylation status of the 50 CpGs within the amplified region. *Open* and *filled circles* represent unmethylated CpGs and methylated CpGs, respectively. Each *row* represents one clone.

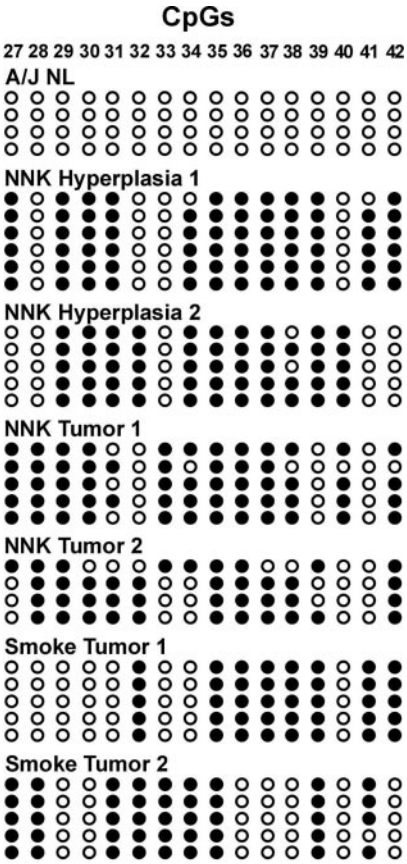

Fig. 4. Density of methylation within the *death-associated protein* (*DAP*)-*kinase* CpG island in murine lung hyperplasias and lung tumors. Stage 2 methylation-specific PCR primers were used to amplify the *DAP-kinase* gene in murine hyperplasias derived from exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and murine lung tumors derived from exposure to NNK or cigarette smoke. Stage 2 unmethylated primers were used to amplify the normal A/J mouse lung. Primers were located at −112 and +49 (unmethylated) and −108 and +48 (methylated), and +1 was the translational start site. Amplified PCR products were cloned, and four to six individual clones were sequenced to determine the methylation status of the 16 CpGs within the amplified region. *CpGs numbered 27 through 42* correspond to the same numbered CpGs depicted in Fig. 2. *Open circles* and *filled circles* represent unmethylated CpGs and methylated CpGs, respectively. Each *row* represents one clone.

densities ranging from 56% to 81%, whereas the density of methylation in cigarette smoke-associated tumors was somewhat lower, at 50–56%. Clones from the same tumor or hyperplasia showed a high degree of homology with respect to methylation density, and although methylation densities varied somewhat between tumors and exposures, similar patterns of methylation were observed in almost all tumors and hyperplasias examined. This pattern consisted of regions of dense CpG methylation, followed by short regions (1–5 CpGs) of little or no methylation. This type of methylation pattern has been seen in other genes, most notably, *O^6-methylguanine-DNA methyltransferase*. The methylation pattern in colon and cervical tumor-derived cell lines revealed dense regions of methylation within the *O^6-methylguanine-DNA methyltransferase* gene in which nearly every CpG within a small region was methylated, interspersed with regions of very infrequent methylation (35).

DISCUSSION

This investigation demonstrates that aberrant promoter methylation of the *DAP-kinase* gene, a frequent event in NSCLC, is recapitulated in a murine model of cigarette smoke-induced lung cancer. In addition, this study links aberrant methylation of *DAP-kinase* to the earliest histological stage of adenocarcinoma. Methylation of the *DAP-kinase* gene was also common in murine lung tumor-derived cell lines and primary tumors induced by environmental and occupational carcinogens. The density of methylation within the *DAP-kinase* promoter in both premalignant and malignant tumors was extensive in an area of the gene that has been shown to correlate with loss of gene transcription in human cancer cell lines (14).

The prevalence of *DAP-kinase* methylation was approximately 50% in murine lung tumors associated with cigarette smoke and in two tobacco-related carcinogen exposures, NNK and vinyl carbamate. This prevalence approximates results reported for primary NSCLCs associated with exposure to tobacco (17). Two of the most likely candidates responsible for the carcinogenicity of cigarette smoke are the polyaromatic hydrocarbons and the tobacco-specific nitrosamines, such as NNK (36, 37). The primary mode of action of NNK in carcinogenesis is mediated through the formation of alkylating or pyridyloxobutylating promutagenic DNA adducts (38). Vinyl carbamate, a potent carcinogen in the mouse lung, is an intermediate formed by the bioactivation of the tobacco carcinogen ethyl carbamate (38, 39). The carcinogenicity of vinyl carbamate is also derived from its ability to form promutagenic adducts in DNA (18, 40). In addition to the tobacco-related compounds, the frequency of *DAP-kinase* methylation was also high in tumors induced by exposure to the occupational carcinogen methylene chloride. Although methylene chloride is associated with the development of lung tumors in mice, the mechanism by which it induces these tumors is uncertain. Some studies suggest it is a genotoxic carcinogen causing sister chromatid exchange or loss of heterozygosity (41), whereas others fail to demonstrate direct interactions with DNA, and no specific DNA adducts have been identified (42, 43). Thus, carcinogens inducing lung cancer through distinct effects on DNA show a similar propensity to arise in part through inactivation of the *DAP-kinase* gene. Currently, the mechanisms by which carcinogen exposure leads to aberrant gene-specific promoter hypermethylation are unknown. The presence of DNA adducts has been shown to block transcription factor binding (44). The disruption of replication timing that would follow such inhibition has been proposed as a mechanism by which normally unmethylated regions of DNA become aberrantly methylated (45).

NNK-induced lung cancer in the A/J mouse is characterized by the initial development of focal proliferation of type II cells along the alveolar septae that progress to adenomas and finally to carcinomas. These alveolar hyperplasias exhibit a very high rate of conversion to carcinomas, making them ideal for establishing the timing for genetic and epigenetic changes identified in malignant tumors (46). The timing for inactivation of the *DAP-kinase* gene has not been clearly defined in human lung cancer. Previously, we reported a low frequency of *DAP-kinase* methylation in nonmalignant bronchial epithelial cells from the airways of smokers (47). Our current study indicates that inactivation of this gene is common in alveolar hyperplasias. The fact that methylation was observed at a similar frequency (46% *versus* 52%) in NNK-induced hyperplasias and adenocarcinomas suggests that one pathway for tumor progression in the mouse lung involves the clonal selection of cells with inactivation of the *DAP-kinase* gene. The similar pattern for methylation seen within the promoter region by bisulfite sequencing between these preneoplastic lesions and tumors supports the argument that inactivation of this gene is occurring at the earliest histological stage for development of adenocarcinoma. The importance of this gene in tumor development has been supported by its association with reduced survival in persons diagnosed with stage I NSCLC (17). Furthermore, because *DAP-kinase* is an integral component of several apoptotic pathways, its silencing should facilitate expansion of premalignant clones of cells.

Lung cancer is currently the leading cause of cancer death in both men and women in the United States with a 2-year survival of 12% for

persons with unresectable disease (48). A recent comparison of four chemotherapy regimens considered the "best approach" revealed no difference in survival, leading the investigators to conclude that chemotherapy for NSCLC has reached a therapeutic plateau (49). The development of effective preventive interventions could prove invaluable for reducing the high mortality associated with this disease. One promising molecular strategy for the treatment of lung cancer involves the reactivation of genes that have been silenced by aberrant methylation. Previous *in vitro* studies have demonstrated that treatment with low doses of the demethylating agent DAC in combination with a histone deacetylase inhibitor can effectively cause reexpression of genes silenced by aberrant promoter methylation (50). Recently, we extended these findings to an *in vivo* system in which treatment of mice after exposure to NNK with a low dose of DAC in combination with the histone deacetylase inhibitor sodium phenylbutyrate dramatically decreased tumor multiplicity.[3] Reversing methylation of *the DAP-kinase* gene in hyperplasias and adenomas within the mouse lung will be a valuable biomarker for establishing the efficacy of this promising preventive approach.

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[3] S. A. Belinsky, unpublished observations.

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