Hypermethylation of the $p16^{Ink4a}$ promoter in B6C3F1 mouse primary lung adenocarcinomas and mouse lung cell lines

Arti C.Patel1,4, Colleen H.Anna1, Julie F.Foley2, Patricia S.Stockton3, Frederick L.Tyson3, J.Carl Barrett1 and Theodora R.Devereux1,5

1Laboratory of Molecular Carcinogenesis, 2Laboratory of Experimental Pathology and 3Division of Extramural Research and Training, Chemical Exposures and Molecular Biology Branch, National Institute of Environmental Health Sciences, PO Box 12233, Mail Drop D4-04, Research Triangle Park, NC 27709, USA and 4Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

5To whom correspondence should be addressed
Email: devereux@niehs.nih.gov

Primary lung tumors from B6C3F1 mice and mouse lung cell lines were examined to investigate the role of transcriptional silencing of the $p16^{Ink4a}$ tumor suppressor gene by DNA hypermethylation during mouse lung carcinogenesis. Hypermethylation (>50% methylation at two or more of the CpG sites examined) of the $p16^{Ink4a}$ promoter region was detected in DNA from 12 of 17 (70%) of the B6C3F1 primary mouse lung adenocarcinomas examined, whereas hypermethylation was not detected in normal B6C3F1, C57BL/6 and C3H/He mouse lung tissues. Immunohistochemistry performed on the B6C3F1 adenocarcinomas revealed heterogeneous expression of the p16 protein within and among the tumors. Laser capture microdissection was employed to collect cells from immunostained sections of four tumors displaying areas of relatively high and low p16 expression. The methylation status of the microdissected samples was assessed by sodium bisulfite genomic sequencing. The pattern of p16 expression correlated inversely with the DNA methylation pattern at promoter CpG sites in nine of 11 (82%) of the microdissected areas displaying variable p16 expression. To provide further evidence that hypermethylation is involved in the loss of $p16^{Ink4a}$ gene expression, three mouse lung tumor cell lines (C10, sp6c and CMT64) displaying complete methylation at seven promoter CpG sites and no $p16^{Ink4a}$ expression were treated with the demethylating agent, 5-aza-2'-deoxycytidine. Reexpression of $p16^{Ink4a}$ and partial demethylation of the $p16^{Ink4a}$ promoter were observed in two cell lines (C10 and sp6c) following treatment. These are the first reported studies to provide strong evidence that DNA methylation is a mechanism for p16 inactivation in mouse lung tumors.

Introduction

Mouse lung adenocarcinomas share many histological and molecular characteristics with human lung adenocarcinomas (1). Both human adenocarcinomas associated with smoking and mouse lung tumors induced with carcinogens, such as benzo[a]pyrene present in cigarette smoke, possess character-istic codon 12 mutations in the K-ras proto-oncogene (2). Tumors from both species also exhibit loss of heterozygosity (LOH) in homologous regions of the genome that contain putative tumor suppressor genes (1). As a result of these similarities, mouse models for lung carcinogenesis are being used to identify the steps in tumor progression and the relative contributions of genetic, epigenetic and environmental factors in the development of lung cancer (1,3–5).

Numerous studies report LOH of chromosome 9p in a high frequency of primary non-small cell lung cancers (NSCLCs) (6–11). The $p16^{Ink4a}$ tumor suppressor, which maps to this region of loss, acts as an inhibitor of CDK4/CDK6 and blocks the G1 to S transition by preventing the phosphorylation of pRb (12). The $p16^{Ink4a}$ gene is inactivated in human lung cancer cell lines and primary tumors by homozygous deletion, mutation or hypermethylation (13–18). The frequency of homozygous deletions and point mutations in $p16^{Ink4a}$ is lower in lung cancers than some other malignancies (19), although analyses of NSCLCs reveal low and often undetectable expression of the $p16^{Ink4a}$ gene (19). However, methylation of CpG sites within the $p16^{Ink4a}$ promoter region appears to play an important role in transcriptional repression of $p16^{Ink4a}$ in NSCLC (17,19–21). The role for DNA methylation in lung cancer is supported by studies with human lung cancer cell lines showing that treatment with the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) restores expression of $p16^{Ink4a}$ (17,18).

Evidence from several studies also supports the involvement of $p16^{Ink4a}$ aberrations in mouse lung carcinogenesis. Mouse lung carcinomas show LOH on chromosome 4 in an area that is syntenic to human chromosome 9p21 and corresponds to the location of the mouse tumor suppressor genes $p15^{ink4b}$ and $p16^{Ink4a}$ (22–24). Furthermore, one study reports decreased expression and homozygous codeletion of $p15^{INK4B}$, $p16^{INK4A}$ and $p19^{Arf}$ in some mouse lung tumor cell lines (23). This study also found that several mouse lung tumor cell lines possess the $p16^{INK4A}$ gene but lack the $p16^{INK4A}$ transcript (23). Additionally, variable expression of $p16^{INK4A}$ is found by RT–PCR in A/J mouse lung tumors that do not display point mutations in the coding region (25). Immunohistochemistry of A/J mouse lung tumors revealed focal areas that lack $p16$ staining and RT–PCR showed variable expression of $p16^{INK4A}$ (26). However, homozygous deletions of chromosome 4 and point mutations of $p16^{INK4A}$ are infrequent (22,23,25–27). One study reported that 2/28 (7%) lung tumors from mice treated transplacentally with 3-methylcholanthrene exhibit point mutations in exon 2 of $p16^{INK4A}$ (27). Based on these observations, it seems likely that hypermethylation of promoter region CpG sites is a mechanism for p16 inactivation in mouse lung tumors. Methylation, like coding region mutations, can lead to the inactivation of tumor suppressor genes, thus providing a selective growth advantage for the affected cells (20).

To gain a better understanding of mouse lung tumorigenesis and the role of DNA methylation, we analyzed primary mouse
lungs and mouse lung cell lines for the methylation status of the p16\textsubscript{ink4a} promoter region. Expression of p16\textsubscript{ink4a} by RT–PCR and immunohistochemistry was compared with DNA methylation status. Laser capture microdissection (LCM) and bisulfite genomic sequencing were performed to determine the methylation status of the p16\textsubscript{ink4a} promoter in tumor areas that express relatively low or high levels of the p16\textsubscript{ink4a} transcript. In addition, three mouse lung cell lines (C10, sp6c and CMT64), displaying complete methylation at seven promoter region CpG sites adjacent to the translational start site and lacking p16\textsubscript{ink4a} transcript expression, were treated with the demethylating agent 5-Aza-CdR to determine if demethylation would result in re-expression of the p16\textsubscript{ink4a} transcript.

Materials and methods

Tumor samples

Lung tumors were induced in female B6C3F1 mice by inhalation exposure to 2000 p.p.m. methylene chloride for 6 h/day, 5 days/week. Both chemically induced and spontaneous lung adenocarcinomas were collected over a period of 27 months as described previously (28). At necropsy, sections of the tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. The remaining tumor tissue was frozen and stored at –70°C.

Cell lines

Cell lines, LM1, CMT64, CMT167, CL201.1, CL20H, sp6c, NNK30pp, sp10 and sp10pp (derived from mouse lung adenocarcinomas), cell line E9 (arising in culture) and cell line C10 (immortalized and non-transformed) were cultured in culture) and cell line C10 (immortalized and non-transformed) were cultured as described previously (23).

DNA isolation

Lung tumors and cells were digested overnight at 37°C in TNE buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA disodium salt pH 7.5), 1% SDS and 10 µg/ml Pronase. Isolation of DNA was accomplished through successive phenol/chloroform extractions. DNA was ethanol precipitated and resuspended in TE (1 mM Tris, 0.1 mM EDTA pH 7.5).

Loss of heterozygosity (LOH) analysis of primary mouse lung tumors

Microsatellite markers D4Mi77 (Research Genetics, Huntsville, AL) and Mts1 were used to amplify polymorphic regions between strain C57BL/6 and C3H/He mice to identify portions of tumors with LOH at the p16\textsubscript{ink4a} locus. The Mts1 forward (5'-GA TTT CTA CGG AAA GCC CTG-3') and reverse (5'-TAT TGT GCA TTT GTG TGT CTG G-3') primers were located 2395 and 2172 bp upstream of the translational start site, respectively. PCR amplification with and without [\(\alpha\]33P]dATP (Amersham, Piscataway, NJ) was performed on 14 methylene-chloride-induced and three spontaneous B6C3F1 lung tumors or mouse lung tumor cell lines was restricted with EcoRI at 37°C overnight in a total volume of 40 µl and 20 µl, respectively. The digested DNA was denatured at 75°C for 15 min in either (tumor samples) or 2 µl (cell line samples) of 3 M NaOH. For the bisulfite modification, 500 µl of 4.8 M sodium bisulfite and 28 µl of 20 mM hydroquinone were added to the tumor samples, whereas 250 µl of 4.8 M sodium bisulfite and 14 µl of 20 mM hydroquinone were added to the cell line samples. Both sets of samples were overlayed with light mineral oil and incubated at 55°C for 5 h. The modified DNA was purified using Centricron 30 filter units (Millipore Corporation, Bedford, MA) as directed by the manufacturer, and the final volume was adjusted to 100 µl with dH\(_2\)O. Samples were desulfonated with 4.5 µl of 3 M NaOH and neutralized with 28 µl of 5 M ammonium acetate. Glycogen (1 µg) was added to the DNA isolated from the cell lines, but not the tumor samples. The DNA was ethanol precipitated overnight in 3 vol of 100% ethanol at –20°C. The DNA pellet was washed with 70% ethanol, dried and resuspended in 20 µl dH\(_2\)O.

Bisulfite modification of primary mouse lung tumor and cell line DNA

Methylation of the p16\textsubscript{ink4a} promoter was evaluated using sodium bisulfite genomic sequencing (31,32). An aliquot of 5 µg of DNA isolated from frozen B6C3F1 lung tumors or mouse lung tumor cell lines was restricted with EcoRI at 37°C overnight in a total volume of 40 and 20 µl, respectively. The digested DNA was denatured at 75°C for 15 min in either (tumor samples) or 2 µl (cell line samples) of 3 M NaOH. For the bisulfite modification, 500 µl of 4.8 M sodium bisulfite and 28 µl of 20 mM hydroquinone were added to the tumor samples, whereas 250 µl of 4.8 M sodium bisulfite and 14 µl of 20 mM hydroquinone were added to the cell line samples. Both sets of samples were overlayed with light mineral oil and incubated at 55°C for 5 h. The modified DNA was purified using Centricron 30 filter units (Millipore Corporation, Bedford, MA) as directed by the manufacturer, and the final volume was adjusted to 100 µl with dH\(_2\)O. Samples were desulfonated with 4.5 µl of 3 M NaOH and neutralized with 28 µl of 5 M ammonium acetate. Glycogen (1 µg) was added to the DNA isolated from the cell lines, but not the tumor samples. The DNA was ethanol precipitated overnight in 3 vol of 100% ethanol at –20°C. The DNA pellet was washed with 70% ethanol, dried and resuspended in 20 µl dH\(_2\)O.

Bisulfite modification of macroscopic micrdissected samples

The LCM samples (10 µl) were restricted with EcoRI at 37°C overnight in a total volume of 20 µl and samples were treated as outlined above for the cell lines, and as described previously (32). Following bisulfite modification, the samples were purified using the Prep-A-Gene DNA Purification System (Bio-Rad, Hercules, CA) as described in the instruction manual. The desulfonation, neutralization and ethanol precipitation steps were carried out as described above including glycerogen as a carrier. DNA was resuspended in 30 µl TE.

PCR amplification

The bisulfite modified DNA was amplified in nested PCR amplification reactions with modified primers designed using the published sequence for the 5′-untranslated region of p16\textsubscript{ink4a} (GenBank accession no. U70181). The modified primers, with the exception of primer RM4 (see sequence below), contained no CpG sites. All cytosines in the primer sequences were replaced by thymines to enable amplification of sodium bisulfite modified DNA. The underlined bases in the primer sequences represent cytosines that are ultimately converted to thymines following bisulfite treatment. The outer PCR reaction was performed using primers FM1 (5′-GTT GTG TAT AGA ATT TTA GTA TGT-3′) and RM2 (5′-CCA CCC TTA CCA ATC TAT CTA GAT C-3′) located 753 and 5 bp upstream of the translational start site, respectively. The PCR reaction was performed with 1 µl DNA template in a 20 µl reaction subjected to hot start at 85°C and 24 cycles (denaturation at 94°C for 1 min, annealing at 56°C for 1 min) or 35 cycles at 94°C for 1 min and 50°C for 35 s anneal, 72°C for 1 min and 1 cycle extension). Three different primer sets were used for the inner PCR reaction so that CpG sites –1 to –9 and –12 to –20 could be examined for methylation status. The three primer sets included: (i) set 1 (allowed examination of sites –12 to –20): FM3 (5′-TTT TAA TTA TTT GGT GTT GTA TGT-3′), located 506 bp upstream of the translational start site, and RM4 (5′-AC CCA AAC TAC AAA AAA AAT ACA-3′), located 272 bp upstream of the translational start site; (ii) set 2 (allowed examination...
of sites –8 to –9): 20FM (5’-GGT GTA TAA TTT ATG TTA TAT TTA-3’), located 206 bp upstream of the translational start site, and RM2 (sequence given above); (iii) set 3 (allowed examination of sites –1 to –7): FM6 (5’-TTT TTA GAG GAA GGA AGG AGG GAT TT-3’), located 107 bp upstream of the translational start site, and RM2 (sequence given above). For the paraffin-embedded samples, primer set 2 (20FM and RM2) was used for the outer PCR reaction, and primer set 3 (42FM and 11RM) was used for the inner PCR reaction. DNA from normal lung and no-DNA controls were included in all PCR amplification steps.

**Cycle sequencing**

PCR products were electrophoresed on a 1.5% low melting point agarose gel and visualized using ethidium bromide. The desired bands were excised and gel purified using Quagen purification columns (Quagen Inc., Valencia, CA). The PCR products were sequenced using 33P Thermosequenase Cycle Sequencing Kit (US Biochemical/Amersham, Cleveland, OH) and primers RM1, RM4 and FM5 (sequence given above) were used as the sequencing primers. Normal lung and no-DNA controls were included in the cycle sequencing reactions. The sequencing reactions were performed in duplicate to verify results.

**Southern analysis of cell lines**

High molecular weight DNA (10 µg) was either digested with HindIII alone or digested sequentially with both HindIII and HpaII (New England Biolabs). HpaII is a methylation-sensitive restriction enzyme that does not cut if either cytosine in the CCGG sequence is methylated. Digested DNA samples were loaded onto 2% agarose gels and separated at 45 V for 17 h. The DNA was transferred to GeneScreen Plus® hybridization transfer membranes (NEN Research Products, Boston, MA) and crosslinked. The filters were hybridized overnight at 42°C in 10 ml Hybridization Buffer (Oncor, Gaithersburg, MD) and probed with a 862 bp gel purified and 32P-labeled PCR product generated with p16\textsuperscript{INK4a} primers mp16-7F (5’-GTG TAC GAA ATC CTA GCA CTG-3’) and mp16-7R (5’-CGA GGG CAT CCC GAA-3’), located 750 bp upstream of the translational start site and mp16-13F (5’-GTA CGA CCG AAA GAG TTC G-3’), located 112 bp downstream of the transcription start site within exon 1t. This region included three HpaII restriction sites and no HindIII restriction sites. Filters were washed twice in 2X SSC/0.1% SDS at room temperature for 10 min each followed by subsequent washes in 0.1X SSC/0.1% SDS at 50°C. The filters were exposed to Biomax®MR film (Eastman Kodak Company, Rochester, NY) for 3 days at –80°C.

**Treatment of cell lines with 5-Aza-CdR**

C10 and sp6c cell lines were plated at a density of 5 × 10\textsuperscript{5} cells and allowed to attach overnight. Cells were then treated with either 0.1 µM 5-Aza-CdR, 1 µM 5-Aza-CdR, 30 nM Trichostatin A (TSA), 0.1 µM 5-Aza-CdR/30 nMT SA or 1 µM 5-Aza-CdR/30 nMT TSA for 24 h. Media were changed every 24 h and the cells were harvested on day 3 following treatment. Pellets were frozen and stored at –80°C for subsequent DNA and RNA isolation. The CMTr64 cells were cultured in the same manner as described above, except that these cells were treated with TSA following a 24 h treatment with 0.1 µM 5-Aza-CdR and 1 µM 5-Aza-CdR.

**RNA isolation and RT-PCR of tumors and cell lines**

Tumors were homogenized in lysin buffer using a Polytron and cells were lysed using the QiAshredder (Qiagen Inc.). The RNA was isolated from both the tumors and cells using RNeasy spin columns (Qiagen Inc.) according to the manufacturer’s instructions. First-strand cDNA was generated from 5 µg total RNA using the SuperScript Kit (Life Technologies). The PCR reaction was performed using promoter- and exon-specific primers for amplification of p16\textsuperscript{INK4a}, p16\textsuperscript{INKB}, p19\textsuperscript{Arf} and β-actin in separate reactions. Conditions were optimized for individual primer sets to use the lowest number of amplification cycles. Fourteen methylene chloride-induced and three spontaneous B6C3F1 mouse lung adenocarcinomas were screened for genetic aberrations in p16\textsuperscript{INK4a} using LOH and SSCP analyses. LOH of the C3H/He allele was observed in 4/14 (28%) of the chemically induced tumors and 0/3 of the spontaneous tumors examined at two microsatellite markers near and within the p16\textsuperscript{INK4a} gene (data not shown).

**Methylation of the p16ink4a promoter in primary mouse lung tumors**

Because few mutations or homozygous deletions of p16\textsuperscript{INK4a} were detected in our mouse lung tumors, we decided to investigate by bisulfite genomic sequencing the possible role of p16 inactivation by DNA methylation in mouse lung tumorigenesis. The DNA was modified by treatment with sodium bisulfite, which deaminates unmethylated cytosine residues to uracils that are later replaced by thymines during the PCR amplification process. This method leaves the methylated cytosines intact, allowing site-specific and region-specific methylation to be determined. Direct sequencing of sodium-bisulfite-treated DNA was used to assess the methylation status of Cpg sites –1 to –9 (nucleotides –23 to –172 relative to the translational start site, respectively) and –12 to –19 (nucleotides –307 to –433 relative to the translational start site, respectively) in 14 methylene chloride-induced adenocarcinomas, three spontaneous B6C3F1 mouse lung adenocarcinomas, and normal C57BL/6, C3H and B6C3F1 mouse lungs. The methylation status of individual Cpg sites in the p16\textsuperscript{INK4a} promoter region was scored visually according to the following categories: U, unmethylated/undetectable methylation; P, partially methylated, <50% methylation; and M, methylated, ≥50% methylation (Table 1). All of the tumors possessed at

**Results**

**LOH and mutation analysis in B6C3F1 primary mouse lung tumors**

Characterization of LOH and p16\textsuperscript{INK4a} mutations in the tumor set used in this study was performed prior to beginning an assessment of p16\textsuperscript{INK4a} methylation status. Five of the methylenchloride-induced tumors in this study were examined previously for LOH by Hegi et al. (22). The remaining 11 tumors were generated in the same carcinogen bioassay. Previous studies showed that LOH in the chromosome 4 region surrounding the p16\textsuperscript{INK4a} gene is common in mouse lung tumors (22,24,34,35). Fourteen methylene chloride-induced and three spontaneous B6C3F1 primary mouse lung adenocarcinomas were screened for genetic aberrations in p16\textsuperscript{INK4a} using LOH and SSCP analyses. LOH of the C3H/He allele was observed in 4/14 (28%) of the chemically induced tumors and 0/3 of the spontaneous tumors examined at two microsatellite markers near and within the p16\textsuperscript{INK4a} gene (data not shown). SSCP analysis of p16\textsuperscript{INK4a} exons 1 and 2 revealed no point mutations in any of the 17 B6C3F1 tumors analyzed in our study.
least six partially methylated sites, while 12/17 (70%) of the tumors displayed ≥50% methylation at two or more CpG sites. The overall pattern of methylation appeared random, and site-specific methylation was not observed. Sites −1 to −7 that are closest to the translational start site were methylated more frequently and to a greater degree than the other CpG sites assessed. One CpG site was partially methylated in 1/5 normal lung tissues examined.

Expression of p16ink4a by RT–PCR and immunohistochemistry

Similar to results obtained by Belinsky et al. (25), analysis of p16ink4a expression by RT–PCR in the 17 B6C3F1 mouse lung tumors and three normal lungs revealed variable expression of the p16ink4a transcript (data not shown). Due to a polymorphism in exon 1α of p16ink4a between the C57BL/6 and C3H/He mice, it was possible to assess allele-specific expression by SSCP analysis following RT–PCR. Overall differential expression between the C57BL/6 and C3H/He alleles was not observed in these samples (Figure 1), although one tumor (MC667) exhibited LOH of the C57BL/6 allele. LOH of another region of this tumor sample was not detected in our preliminary study, possibly due to tissue heterogeneity.

Immunohistochemical staining of 13 samples, MC478, MC512, MC667, MC995, MC69, MC717, MC599, MC242, MC1339A, and MC1339B, revealed focal areas of relatively high and low expression within each tumor, demonstrating tissue heterogeneity (Figure 2). Sufficient tumor tissue was not available to perform immunohistochemical analysis on the remaining samples.

Methylation of p16ink4a in laser capture microdissected samples

In order to demonstrate that the methylation status affected p16 expression, the LCM technique was used to microdissect areas of relatively high versus low p16 staining in tumor samples MC152, MC512, MC667 and MC995 (Figure 2E and F). Following DNA isolation and sodium bisulphite treatment, methylation was examined at CpG sites −1 to −7. In 9/11 (82%) of the microdissected areas from the four tumors, the pattern of methylation correlated inversely with the pattern of expression (Figure 3; Table II). For example, in tumor sample

---

### Table I. LOH and methylation patterns at p16 ink4a promoter CpG sites in B6C3F1 mouse lung tumors

<table>
<thead>
<tr>
<th>Sample</th>
<th>LOH</th>
<th>Methylated sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC 69</td>
<td>−C3H</td>
<td>U U U P P U U U M M M U</td>
</tr>
<tr>
<td>MC 989</td>
<td>−C3H</td>
<td>M M M M P M M M M M</td>
</tr>
<tr>
<td>MC 795</td>
<td>−C3H</td>
<td>M M M M P M M M M M</td>
</tr>
<tr>
<td>MC 692</td>
<td>−C3H</td>
<td>M M M P M U U U P</td>
</tr>
<tr>
<td>MC 59</td>
<td>−C3H</td>
<td>U P U U U U U U P P U U P</td>
</tr>
<tr>
<td>MC 1399</td>
<td>−C3H</td>
<td>U M U U U U U U P M M M</td>
</tr>
<tr>
<td>MC 512</td>
<td>−C3H</td>
<td>U U U U U U P U U M M M M</td>
</tr>
<tr>
<td>MC 1009</td>
<td>−C3H</td>
<td>U P M M P U U U U M M M M</td>
</tr>
<tr>
<td>MC 599</td>
<td>−C3H</td>
<td>U U U P P U U U P P P</td>
</tr>
<tr>
<td>MC 717</td>
<td>−C3H</td>
<td>U U U U M U U U U M M M M</td>
</tr>
<tr>
<td>MC 242</td>
<td>−C3H</td>
<td>U U U U U U U U P P P P</td>
</tr>
<tr>
<td>MC 667</td>
<td>−C3H</td>
<td>U U U M M M M</td>
</tr>
<tr>
<td>MC 1339A</td>
<td>−C3H</td>
<td>U U P U M M M</td>
</tr>
<tr>
<td>Normal C3H</td>
<td>U U U U U U U U U U</td>
<td></td>
</tr>
<tr>
<td>Normal B6C3F1</td>
<td>U U U U U U U U U U</td>
<td></td>
</tr>
</tbody>
</table>

---

*The promoter CpG sites are numbered in reference to the translational start site, with site −1 immediately upstream of the start codon.

*The location (bp upstream) of each CpG site relative to the translational start site.

*Putative transcription factor (TF) binding sites containing CpG sites identified using MatInspector (48).

*Samples with detectable LOH. The designations −C3H and −C57 indicate loss of the C3H/He or the C57BL/6 alleles, respectively.

*The methylation status of a particular site is given by the following key: M, ≥50% methylated CpG site; P, <50% methylated CpG site; U, unmethylated CpG site. Blanks indicate that methylation was not determined.
Methylation of the mouse p16\textsuperscript{Ink4a} promoter

Fig. 2. Immunohistochemical analysis of p16\textsuperscript{Ink4a} expression in B6C3F1 mouse lung adenocarcinomas and subsequent laser capture microdissection of these samples. (A) Tumor sample MC152 incubated with p16\textsuperscript{Ink4a} antibody (100×). Areas of tumor with relatively high and low p16 expression are designated with arrows. (B) Tumor sample MC152 incubated with non-immune mouse IgG (100×). Note the lack of staining in this section. (C) Tumor sample MC995 incubated with p16\textsuperscript{Ink4a} antibody (40×). Note that within the tumor are populations of cells showing high or low expression of the p16 protein (see arrows). (D) Higher magnification of tumor sample MC995 incubated with p16\textsuperscript{Ink4a} antibody (200×). Note that some cells are strongly positive for the p16 protein while others have low levels of the p16 protein (see arrows). (E) Serial section of tumor sample MC995 which corresponds to (C) and shows the areas sampled by LCM and areas of high and low p16 expression (see arrows). (F) Captured cells from LCM procedure representing neoplastic cells with high p16 expression (MC995-2) and low p16 expression (MC995-3) (see arrows).

MC152-4 (Figure 3; Table II) all seven CpG sites examined in DNA from a region of cells with a low level of p16 expression were completely methylated (no T band in the lane corresponding to the methylated C band), while only one site was partially methylated in DNA from tumor sample MC152-2 in an area expressing a high level of p16 (Figure 3; Table II). An exception was tumor MC995 in which one area of high p16 expression exhibited high promoter methylation and another area with low expression displayed low methylation (Table II). A semi-quantitative analysis of the number of sites displaying ≥50% methylation (designated by M in Table II) demonstrates that 31% (11/35) of the sites analyzed are methylated in tumor areas expressing high levels of p16 and 64% (27/42) of the sites analyzed are methylated in tumor areas in expressing low levels of p16 (Table II).

Methylation of p16\textsuperscript{Ink4a} in mouse lung cell lines

Cell lines E9, LM1, CMT64, CMT167, C10, CL20I.1, CL20H, sp6c, NNK30pp, sp10 and sp10pp, which were previously shown to possess the p16\textsuperscript{Ink4a} gene but lack p16\textsuperscript{Ink4a} expression by RT–PCR (23), were examined for hypermethylation of the p16\textsuperscript{Ink4a} promoter by both Southern analysis and bisulfite genomic sequencing (Table III). By Southern analysis either complete or partial methylation was observed in cell lines
individual methylated CpG sites. Sites –10 and –11 were not assessed by bisulfite genomic sequencing because primer design in this region was not optimal.

**Treatment of mouse lung cell lines with 5-Aza-CdR**

To provide further evidence that methylation of promoter region CpG sites affected the expression of the p16\(^{\text{ink4a}}\) transcript in mouse lung carcinogenesis, three cell lines (C10, sp6c and CMT64) that displayed complete methylation at sites –1 to –7 by bisulfite genomic sequencing and undetectable p16\(^{\text{ink4a}}\) expression prior to treatment were exposed to the demethylating agent, 5-Aza-CdR. The expression of p15\(^{\text{ink4b}}\), p16\(^{\text{ink4a}}\), p19\(^{\text{Arf}}\) and actin transcripts was assessed by RT–PCR (Figure 5). Re-expression of p16\(^{\text{ink4a}}\) was observed in the C10 and sp6c cell lines following treatment with 1 µM 5-Aza-CdR. Evidence from previous studies (36) has shown that treatment with TSA, a histone deacetylase inhibitor, in conjunction with 5-Aza-CdR enhances the expression of some genes. However, treatment with 0.1 µM 5-Aza-CdR or 1 µM TSA, or a combination of the two, did not have an observable effect on p16\(^{\text{ink4a}}\) expression in these cells. Expression of p15\(^{\text{ink4b}}\) and p19\(^{\text{Arf}}\) was consistent and unchanged in both cell lines with all treatments (Figure 5). Bisulfite genomic sequencing of DNA from treated C10 and sp6c cell lines displayed partial methylation in cells treated with 1 µM 5-Aza-CdR in comparison with untreated cells that were methylated completely (data not shown). DNA from all other treatments displayed complete methylation of sites –1 to –7. Interestingly, in the treated and untreated CMT64 cells low levels of p16\(^{\text{ink4a}}\) expression were observed but no p15\(^{\text{ink4b}}\) and p19\(^{\text{Arf}}\) transcripts were detected in the untreated CMT64 cells. Enhanced expression of p16 and re-expression of the p15\(^{\text{ink4b}}\) and p19\(^{\text{Arf}}\) transcripts were observed upon treatment with either 0.1 or 1 µM 5-Aza-CdR (data not shown).

**Effect of 5-Aza-CdR treatment on cell proliferation**

Treatment of the C10 and sp6c cells with either 0.1 or 1 µM 5-Aza-CdR decreased cell growth when compared with the untreated cells. In addition, treatment with the 1 µM 6-Aza-C, which is not a demethylating agent like 5-Aza-CdR, did not adversely affect cell growth (Figure 6). These results are similar to those observed in a study by Otterson et al. (17).

**Discussion**

The detection of methylated CpG sites within the p16\(^{\text{ink4a}}\) promoter region in B6C3F1 primary mouse lung tumors and mouse lung cell lines provides the first definitive evidence that DNA hypermethylation and subsequent downregulation of p16\(^{\text{ink4a}}\) expression play a role in mouse lung carcinogenesis. The patterns of p16\(^{\text{ink4a}}\) expression observed in this study corroborate previous studies with A/J mouse lung tumors, which reported variable expression of both p16\(^{\text{ink4a}}\) mRNA and protein (25,26). Studies with mouse lung tumor cell lines (23) and primary A/J mouse lung tumors (25) showed that p16\(^{\text{ink4a}}\) may become inactivated by homozygous deletion, although there are some cell lines that lack the p16\(^{\text{ink4a}}\) transcript but possess the gene. In general, point mutations in p16\(^{\text{ink4a}}\) have not been observed in chemically induced or spontaneous mouse lung tumors (25,26), except in one study that detected a small percentage of point mutations in 3-methylcholanthrene transplacentally induced DBA/2 lung tumors (27). Collectively, these studies provide support for a role for p16 inactivation in mouse lung carcinogenesis, and
our study indicates that another mechanism of p16 inactivation in mouse lung tumors is promoter DNA methylation.

Although hypermethylation of CpG islands has been shown to transcriptionally inactivate several tumor suppressor genes, including p16\(^{INK4a}\), in human cancers (12), the role of DNA methylation in mouse lung carcinogenesis has not been extensively investigated. One study reported no detectable methylation of p16\(^{INK4a}\) in A/J mouse lung tumors (25). However, that study only examined methylation at one Thal restriction site in the promoter and one HpaII restriction site in exon 1α using Southern analysis and methylation-sensitive restriction enzyme digests (25). Our study used bisulfite genomic sequencing, a more sensitive PCR-based method, to assess the methylation status of multiple CpG sites in the p16\(^{INK4a}\) promoter. In contrast to normal lung tissue, we found at least partial methylation of p16\(^{INK4a}\) in all of the chemically induced and spontaneous mouse lung adenocarcinomas examined.

General patterns of DNA methylation, which include critical site, multiple elements and methylation density models, are compatible with the concept that methylation is a dynamic, stochastic, partial and regional process (20). Unlike some studies that demonstrate abrogation of transcription factor binding sites by critical site methylation (37,38), our study shows that decreased gene expression of p16\(^{INK4a}\) correlates best with a density-dependent methylation model. Evidence from work with p15\(^{INK4b}\) in primary acute leukemia demonstrated that the density of methylation at CpG sites strongly correlated with gene silencing (39). In that case, 30–40% of the methylation at CpG sites was associated with transcriptional repression (39). Another study using human bladder cancer cell lines provided evidence that density-dependent methylation is one mechanism of p16\(^{INK4a}\) promoter methylation that results in a decrease in gene expression (40). That study (40) and ours found a higher degree of methylation in sites near the 3′ end of the promoter. Thus, the pattern of p16\(^{INK4a}\) methylation observed in the mouse lung adenocarcinomas and cell lines in our study is similar to methylation patterns observed for human cell cycle regulatory genes, including p16\(^{INK4a}\).

Discrepancies between expression and methylation in a few samples can be explained by several scenarios. Tumor areas which possess low or no expression and low methylation may have homozygous deletions of p16\(^{INK4a}\). Tumor areas that displayed apparent high expression and high methylation of p16\(^{INK4a}\) are more difficult to explain. Levels of the p16 protein

---

**Table III. Methylation status and characteristics of mouse lung cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Background</th>
<th>Methylation status</th>
<th>Cell line characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMT64</td>
<td>C57BL/6</td>
<td>+</td>
<td>Tumorigenic, metastatic murine lung adenocarcinoma cell line</td>
</tr>
<tr>
<td>CMT167</td>
<td>C57BL/6</td>
<td>+</td>
<td>Tumorigenic, metastatic murine lung adenocarcinoma cell line derived from CMT64</td>
</tr>
<tr>
<td>C9</td>
<td>BALB/c</td>
<td>+</td>
<td>Immortalized murine epithelial lung cell line, type 2 cells, non-tumorigenic</td>
</tr>
<tr>
<td>C10</td>
<td>BALB/c</td>
<td>+</td>
<td>Urethane-induced lung carcinoma</td>
</tr>
<tr>
<td>LM1</td>
<td>A/J</td>
<td>–</td>
<td>DMBA-induced murine lung adenocarcinoma cell line</td>
</tr>
<tr>
<td>CL20L1</td>
<td>A/J</td>
<td>–</td>
<td>DMBA-induced murine lung adenocarcinoma cell line, high invasive potential</td>
</tr>
<tr>
<td>CL20H</td>
<td>A/J</td>
<td>–</td>
<td>DMBA-induced murine lung adenocarcinoma cell line, high invasive potential</td>
</tr>
<tr>
<td>Sp6c</td>
<td>A/J</td>
<td>+</td>
<td>Murine lung adenocarcinoma cell line derived from spontaneously occurring tumor</td>
</tr>
<tr>
<td>NKK30pp</td>
<td>A/J</td>
<td>ND(^c)</td>
<td>Murine lung adenocarcinoma cell line derived from spontaneously occurring tumor</td>
</tr>
<tr>
<td>Sp10</td>
<td>A/J</td>
<td>+</td>
<td>Murine lung adenocarcinoma cell line derived from spontaneously occurring tumor</td>
</tr>
<tr>
<td>Sp10pp</td>
<td>A/J</td>
<td>–</td>
<td>Murine lung adenocarcinoma cell line derived from spontaneously occurring tumor</td>
</tr>
</tbody>
</table>

\(^a\)Methylation status based on Southern analysis according to the following key: +, methylated sites detected; –, no methylated sites detected.

\(^b\)Methylation status based on sodium bisulfite genomic sequencing according to the following key: M, all seven CpG sites examined were completely methylated with no band in the corresponding T lane; P, partial methylation of individual CpG sites.

\(^c\)ND, p16\(^{INK4a}\) DNA was not detected in sample NNN30pp.

\(^d\)NA, p16\(^{INK4a}\) DNA did not amplify from cell line NNN30pp.

---

**Fig. 4.** Southern blot hybridization of DNA from mouse lung cell lines for methylation of p16\(^{INK4a}\). Genomic DNA from 11 mouse lung tumor cell lines was restricted with HindIII alone (lane a) or HindIII/HpaII (lane b) to evaluate the methylation status in the promoter region and exon 1α of p16\(^{INK4a}\). This region contained three HpaII sites. A 7.3 kb product is detected in samples digested with only HindIII. Samples are indicated above each lane. Normal 1, DNA isolated from A/J lung; normal 2, DNA isolated from C57BL/6 lung; normal 3, DNA isolated from BALB/c lung.
A.C.Patel et al.

Fig. 5. Expression of p15\textsuperscript{ink4b}, p16\textsuperscript{ink4a}, p19\textsuperscript{arf} and actin by RT–PCR in C10 and sp6C mouse lung cell lines following treatment with 5-Aza-CdR. Following reverse transcription of total RNA, cDNA amplification was performed in separate reactions, and then the PCR products were resolved on a 6% acrylamide/urea/formamide gel. The PCR products from the C10 and sp6c cell lines are in lanes 1–6 and 7–12, respectively. The specific treatments are noted on the figure. Lanes 3, 6, 9 and 12, re-expression of p16\textsuperscript{ink4a} following treatment with 5-Aza-CdR; lane 13, normal cDNA from C57BL/6 lung; lanes 14–17, sample 3 with PCR products loaded in individual lanes. (**Note that actin and p15\textsuperscript{ink4b} aliquots were not loaded in lane 8.)

Fig 6. Cell lines C10 and sp6c were subjected to four treatments: (i) control, no treatment; (ii) 1 µM 6-Aza-C; (iii) 0.1 µM 5-Aza-CdR; (iv) 1 µM 5-Aza-CdR. Cells were plated at a density of 2×10\textsuperscript{5} cells and allowed to attach overnight. The cells were treated for 24 h, washed with PBS, and incubated in fresh media for an additional 48 h. At the end of the incubation period, the cells were trypsinized and counted using a hemocytometer. The cell number was calculated and recorded as percent of the control.

increase in response to DNA damage (41), and this may occur early in lung carcinogenesis, possibly as a result of mutations in oncogenes such as K-ras (42). This could help to explain the variable expression of p16\textsuperscript{ink4a} among tumors and within tumors, especially after chemical treatment. Similar to the regulation of expression of the human telomerase reverse transcriptase (h\textit{hERT}) gene (43), expression of p16 could be regulated by both methylation-dependent and independent mechanisms. It is possible that methylation increases early in neoplastic progression even while cells are subjected to DNA damage and levels of p16 are high (44). Hypermethylation of p16\textsuperscript{ink4a} has been detected in the preinvasive lesions of individuals with established lung carcinoma (20,44). Thus, increased random CpG methylation of the p16\textsuperscript{ink4a} promoter that represses gene expression in a progressive (40) manner could be manifested at later stages in tumor development after methylation accumulates and reaches a critical threshold in different parts of the tumor (40). Furthermore, based on the evidence from our study, showing some methylation of the p16\textsuperscript{ink4a} promoter in all tumors examined, p16\textsuperscript{ink4a} hypermethylation is likely to precede LOH, which was found in 24% of the tumors in this study and has previously been documented in carcinomas but not adenomas (22). The question of whether methylation exists in mouse lung adenomas remains to be answered since only adenocarcinomas were examined in this study.

The experiments with the 5-Aza-CdR-treated mouse lung cell lines further supports the involvement of p16 in mouse lung carcinogenesis and duplicates observations from studies with human cell lines (17,18). Treatment of the methylated C10 and sp6c cell lines, which lack p16\textsuperscript{ink4a} expression, with the demethylating agent, 5-Aza-CdR, induced re-expression of the p16\textsuperscript{ink4a} transcript. Changes in chromatin conformation did not seem to be important, because treatment with the histone deacetylase inhibitor TSA, either alone or in combination with 5-Aza-CdR, had no observable effect on p16\textsuperscript{ink4a} expression. In previous studies of human cell lines, TSA treatment in the presence of low doses of 5-Aza-CdR, but not high doses, resulted in significant re-expression of MLH1, TIMP3 and p16\textsuperscript{ink4a} (36). In our study, re-expression of p16\textsuperscript{ink4a} correlated with a decrease in the methylation status of the p16\textsuperscript{ink4a} promoter in the sp6c and C10 cell lines treated with 1 µM 5-Aza-CdR, supporting the role for DNA methylation in the inactivation of p16 in mouse lung carcinogenesis.

Interestingly, treatment of the CMT64 cell line, which expressed low levels of p16 and no detectable p19 or p15, with 5-Aza-CdR induced re-expression of p19, a component of the p53 pathway, and p15. This observation provides
evidence of a role for p15 and p19 in mouse lung carcinogenesis and is supported by observations that p15Ink4b and p19Arf are frequently inactivated in mouse lung tumor cell lines (23). Together these studies emphasize the importance of not only p16 but also p15 and p19 in mouse lung tumor development.

Overall our study demonstrates an additional similarity between mouse models of lung carcinogenesis and the development of human lung cancer. Hypermethylation of the p16INK4a promoter at CpG sites has been demonstrated in a variety of primary human tumors, including NSCLC, with the effect of decreased expression of the p16INK4a transcript (19,21,45,46), thus providing a selective growth advantage for the affected cells (20). Until recently, the paradigm for the inactivation of tumor suppressor genes has involved primarily intragenic mutation and loss of chromosomal material via loss of heterozygosity or homozygous deletion (47). However, the emergence of DNA methylation as a mechanism in carcinogenesis not only adds another pathway that should be considered when attempting to identify mechanisms of gene inactivation (47) in both mouse and human lung carcinogenesis, but also underscores the importance of epigenetic mechanisms in the development of cancer.

Acknowledgements

We thank Catherine M.White for her technical assistance and Drs Roger Wiseman and Robert Sills for their critical review of the manuscript. We also acknowledge Astrid Haugen-Strano for developing the Mts1 primers used for PCR and LOH analyses of the microsatellite sequence in the p16INK4a promoter.

References


Received January 14, 2000; revised May 18, 2000; accepted May 24, 2000.