Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis

(*p16* methylation/sputum/NNK/squamous cell carcinoma)

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ABSTRACT The $p16^{INK4a}$ (*p16*) tumor suppressor gene can be inactivated by promoter region hypermethylation in many tumor types including lung cancer, the leading cause of cancer-related deaths in the U.S. We have determined the timing of this event in an animal model of lung carcinogenesis and in human squamous cell carcinomas (SCCs). In the rat, 94% of adenocarcinomas induced by the tobacco specific carcinogen 4-methylnitrosamino-1-(3-pyridyl)-1-butanone were hypermethylated at the *p16* gene promoter; most important, this methylation change was frequently detected in precursor lesions to the tumors: adenomas, and hyperplastic lesions. The timing for *p16* methylation was recapitulated in human SCCs where the *p16* gene was coordinately methylated in 75% of carcinoma *in situ* lesions adjacent to SCCs harboring this change. Moreover, the frequency of this event increased during disease progression from basal cell hyperplasia (17%) to squamous metaplasia (24%) to carcinoma *in situ* (50%) lesions. Methylation of *p16* was associated with loss of expression in both tumors and precursor lesions indicating that both alleles were functionally inactivated. The potential of using assays for aberrant *p16* methylation to identify disease and/or risk was validated by detection of this change in sputum from three of seven patients with cancer and 5 of 26 cancer-free individuals at high risk. These studies show for the first time that an epigenetic alteration, aberrant methylation of the *p16* gene, can be an early event in lung cancer and may constitute a new biomarker for early detection and monitoring of prevention trials.

The $p16^{INK4a}$ (*p16*) tumor suppressor gene that maps to chromosome band 9p21, is inactivated in >70% of cell lines derived from all histologic types of human nonsmall cell lung cancers (NSCLCs) (1, 2) predominantly through homozygous deletion (1) or in association with aberrant promoter region hypermethylation (3). These inactivating events are conserved across species, with homozygous deletion and aberrant methylation accounting for loss of *p16* expression in 40% and 45%, respectively, of cell lines derived from rat lung tumors (4). Moreover, the methylated phenotype seen in the rat cell lines showed an absolute correlation with the detection of methylation in primary tumors and the aberrant promoter region methylation was also detected in four of eight primary tumors from which the derived cell line had homozygous deletion of *p16* (4). Thus, the methylation change may precede genetic instability within the CpG island of this gene.

Several genetic abnormalities frequently present in human lung cancer have now been found throughout the respiratory tract of smokers (5–7). These include allelic loss, but not homozygous deletion, involving 9p21 in premalignant lesions from cancer and cancer-free patients (6, 7). This finding suggests that inactivation of the *p16* gene by aberrant methylation could represent a critical step in the genesis of NSCLC by allowing the uncontrolled clonal expansion of some of these premalignant lesions to cancer. The present investigation addresses this issue by defining when aberrant methylation of the *p16* gene occurs during the development of lung carcinoma in an animal model, then extends these studies to the development of human squamous cell carcinoma (SCC). The findings link aberrant methylation of *p16* to the earliest stages of respiratory carcinogenesis and demonstrate the potential use of this epigenetic change as a biomarker to identify persons at high risk or with lung neoplasia.

MATERIALS AND METHODS

Human and Rodent Tissue Samples. SCC and precursor lesions that included carcinoma *in situ* (CIS), squamous cell metaplasia with varying degree of atypia, and basal cell hyperplasia were obtained from patients at either Johns Hopkins Medical Institutions (Baltimore, MD) or St. Mary's Hospital (Grand Junction, CO). In addition, biopsy samples were obtained through bronchoscopy of patients at St. Mary's Hospital being evaluated for lung cancer. Sputum was collected by standardized procedures (8) from patients at Johns Hopkins Medical Institutions being evaluated for possible lung cancer through referral from their primary care physician. Sputum was considered unsatisfactory for evaluation if alveolar lung macrophages were absent or if a marked inflammatory component was present that diluted the concentration of pulmonary epithelial cells. Adenocarcinomas and precursor lesions, adenomas and alveolar hyperplasias, were induced in F344/N rats by treatment with 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) 3 times a week (50 mg/kg, i.p.) for 20 weeks (9). Alveolar type II cells (purity >80%) were isolated from vehicle-treated rats by protease digestion and centrifugal elutriation (9).

Microdissection of Tumors and Precursor Lesions. Human SCCs and precursor lesions were obtained by three different sampling strategies: (*i*) CIS lesions sharing the same airway and adjacent to the SCC; (*ii*) CIS, squamous metaplasia with varying degree of cellular atypia, and basal cell hyperplasia found in either different airways or at different bronchial generations from the primary SCC at the time of lobe resection; and (*iii*) CIS, squamous metaplasia, and basal cell hyperplasia obtained by bronchoscopy independent of any tu-

Abbreviations: SCC, squamous cell carcinoma; CIS, carcinoma *in situ*; *p16*, $p16^{INK4a}$; SCLC, small cell lung cancer; NSCLC, nonsmall cell lung cancer; NNK, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone; *Rb*, retinoblastoma; NFP, neutral buffered formalin; MSP, methylation-specific PCR.
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mor. Lung lobes obtained at resection and biopsies were fixed by perfusion or immersion in neutral buffered formalin (NFB), respectively. A portion of each lung tumor induced by NNK was frozen in liquid nitrogen and the remaining tumor and lung tissue fixed in NBF. All lung lobes from the treated rats were perfused with NBF, embedded in paraffin; 5-μm sections were cut, and stained with hematoxylin and eosin to identify precursor lesions. Lung lobes obtained from patients undergoing resection were sampled as follows. The lung tissue surrounding the tumor was sampled extending in all directions toward the periphery of the lung lobe. Approximately eight separate pieces of tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin to identify precursor lesions. Lung lesions were classified based on World Health Organization criteria. Sequential sections from biopsies and lesions identified in resections were cut (5–10 μm), deparaffinized, 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. Because of the extensive contamination of some lesions with normal tissue (e.g., SCC, adenoma, alveolar hyperplasia) or the small size of some lesions, <0.001 mm^3, it was essential to include normal appearing cells to ensure that after bisulfite modification and column clean-up of the DNA template enough sample remained to conduct the methylation-specific PCR (MSP) assay as described below. Occasionally where the lesion was pure, of substantial size (>500 cells), and easily dissected from the basement membrane (e.g., CIS), it was possible to microdissect only the lesion itself. Thus, because the goal of the study was to determine whether *p16* methylation was present in these lesions and not to quantitate methylation levels, microdissection was used to enrich the samples being examined for tumor or premalignant cells. Adenocarcinomas induced by NNK were not microdissected from fixed tissue, rather the frozen tissue that was comprised of tumor cells and normal appearing cells was used for analysis.

Nucleic Acid Isolation and Methylation-Specific PCR. DNA was isolated from frozen tumors and sputum by digestion with Pronase in 1% SDS, followed by standard phenol-chloroform extraction and ethanol precipitation. DNA was prepared from microdissected tissue by the method of Levi *et al.* (10).

The methylation state of the p16 gene was determined by the recently developed method of MSP (4, 11). Briefly, in MSP, genomic DNA is modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracil, then to thymidine during the subsequent PCR step. Before PCR, the modified DNA is purified with the Wizard DNA purification system (Promega), desulfonated with NaOH, precipitated with ethanol, and finally resuspended in a Tris-EDTA buffer. Two sets of primers are used to amplify each region of interest: one pair recognizes a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognizes a sequence in which CpG sites are methylated (unmodified by bisulfite). Primers are localized to regions containing frequent cytosines (to distinguish unmodified from modified DNA), and CpG pairs near the 3′ end of the primers provide maximal discrimination in the PCR reaction between methylated and unmethylated DNA. Primer sequences have been described (4, 11) and were localized to regions in and around the transcription start site of the *p16* gene, a region shown to correlate with loss of gene expression (4, 11). PCR amplification was performed by using ≈150–300 ng of treated DNA as the template. Two sets of primers were used at the same position, one set specific for DNA methylated at CpG sites, and one for fully unmethylated DNA. After bisulfite treatment, double-stranded DNA is no longer complementary, so the primer pairs designed amplified only the modified sense strand. PCR conditions have been described (4, 11). Forty cycles of amplification were used in analysis of modified DNA from microdissected samples for *p16* methylation. Negative controls were

performed by using both sets of modified primers with untreated DNA, to confirm that unmodified DNA could not be amplified in the event of incomplete bisulfite reactions. Products were visualized on 2.0% agarose gels or 6% nondenaturing acrylamide gels. When the amount of DNA was not limiting (tumors and sputum samples), assays were repeated beginning with the bisulfite step. Otherwise, PCR reactions were generally conducted in duplicate on each modified DNA template.

The MSP method has several advantages over that of conventional Southern hybridization. (*i*) Small amounts of DNA template can be used (<200 ng) compared with μg quantities of DNA needed for Southern hybridization and is thus amenable to using DNA obtained from fixed tissue. (*ii*) Because primers are designed that recognize either methylated or unmethylated alleles, contaminating normal tissue does not interfere with the ability to detect methylation. (*iii*) The sensitivity of this assay to detect methylated alleles in a background of unmethylated alleles is $>10^{-4}$ (11) that makes it ideal for identifying cells containing methylated alleles in a heterogeneous population of cells like those present in sputum. However, it is important to recognize that the MSP method is not intended to provide quantitative information on methylation of a particular gene, but is a extremely sensitive assay for detecting methylation in the presence of contaminating normal tissue.

Immunohistochemistry for Detecting p16 Protein. Tissue sections (4–5 microns) were deparaffinized and incubated in Antigen Retrieval Citra (BioGenex Laboratories, San Ramon, CA) at the recommended dilution and heated by steam for 30 min. Slides were incubated overnight at 4°C with mouse monoclonal anti-p16 (Neomarkers, Union City, CA) diluted to a final concentration of 4 μg/ml. Slides were then rinsed and incubated with universal secondary antibody containing goat anti-mouse IgG (Ventana Medical Systems, Tucson, AZ) for 30 min, developed with diaminobenzidine (Ventana Medical Systems) for 20 min, and counterstained with hematoxylin for 1 min.

RESULTS

Methylation of p16 in NNK-Induced Lung Lesions. The development of pulmonary adenocarcinomas induced by NNK progress from focal hyperplasias of type II alveolar epithelial cells to adenomas and finally to carcinomas (9). The current study used a new sensitive PCR procedure (11), MSP, to examine the methylation state of the *p16* gene in whole lung and type II cells from sham-exposed rats and in hyperplasias, adenomas, and carcinomas from rats treated with NNK. Only unmethylated *p16* alleles were detected by MSP in whole lung and alveolar type II cells (Fig. 1) from sham-exposed rats. Methylation of the *p16* gene was detected in 15 of 16 adenocarcinomas, 4 of 6 adenomas, and 17 of 20 hyperplasias (see Fig. 1 for representative results). Unmethylated alleles were also simultaneously detected in these samples, because tumors and adenomas were contaminated with stromal and inflammatory cells, or normal cells in the case of the epithelial hyperplasias. Photomicrographs (Fig. 2 *A–C*) illustrate the morphologic features of the precursor lesions containing methylated *p16* alleles. The adenomas exhibited minimal atypia and contained large numbers of neutrophils and macrophages within their luminal spaces and stroma. Epithelial hyperplasias were focal, consisted of a single layer of epithelial cells without atypia lining the septa, and did not disrupt the normal alveolar architecture.

Methylation of p16 Is an Early Event in the Development of Human SCC. Examination of SCCs obtained by biopsy or through lobectomy revealed a marked propensity toward methylation of the *p16* gene, with 11 of 18 of these randomly selected neoplasms showing this change (see Fig. 3*B* for
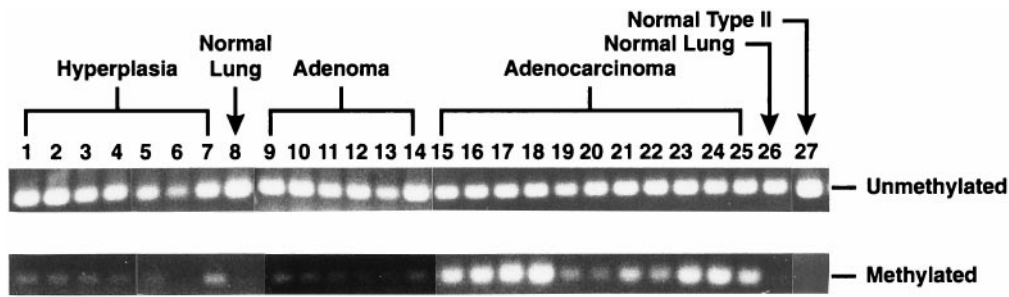
FIG. 1.   Detection of *p16* methylation in hyperplasias, adenomas, and adenocarcinomas induced by NNK in the rat. Methylation is evident in hyperplasias from lanes 1–5 and 7, in adenomas from lanes 9–11 and 14, and in adenocarcinomas from lanes 15–25. Unmethylated *p16* alleles were present in all lesions due to contaminating normal tissue and in normal lung and alveolar type II cells. No methylated alleles are detected in modified DNA from normal lung (lanes 8 and 26) or alveolar type II cells (lane 27).

representative results). Thus, the high incidence of *p16* hypermethylation makes this histologic form of NSCLC ideally suited for defining the timing for this change in the progression of human respiratory carcinogenesis. Human SCC (12) is thought to arise through a series of morphologic changes beginning with basal cell hyperplasia, leading to squamous metaplasia characterized by increasing severity of cellular atypia (dyplasia) to CIS, and ultimately to SCC. The first series of samples examined for *p16* methylation were CIS lesions adjacent to SCCs. Ten of twelve sample pairs examined showed an absolute concordance, being either both methylated (six pairs) or unmethylated (four pairs) at the *p16* locus (Fig. 3*A*). Moreover, in two pairs where the CIS lesion was precisely microdissected excluding the basal lamina and normal appear-

ing cells, only methylated *p16* alleles were detected by MSP (Fig. 3*A*) substantiating the clonal nature of the change. The normal appearing bronchus adjacent to one of these SCCs also contained methylated *p16* alleles, while normal lymphocytes within the field did not. In the two pairs with discordant results, one CIS lesion was methylated and the adjacent SCC unmethylated, while the other CIS lesion was unmethylated and the adjacent SCC methylated. These latter two pairs illustrate that some SCCs can arise via pathways that either exclude the *p16* gene or inactivate this gene via homozygous deletion.

The status of *p16* was further characterized in lesions in airways from lobectomy cases or in biopsies obtained through bronchoscopy from persons undergoing evaluation for lung cancer. The eight biopsies collected were independent from any malignancy that was ultimately diagnosed and were comprised of CIS, squamous metaplasia with varying degree of atypia, and hyperplastic epithelium. Thirteen SCCs, six of which harbored the *p16* methylation change, premalignant lesions, and CIS were also obtained from 13 persons undergoing lobectomies. In these surgical specimens, most premalignant lesions examined were from either different airways or at different bronchial generations from those harboring the primary SCC identified at the time of lobe resection. Three of the lesions arose from the same airway and same generation as
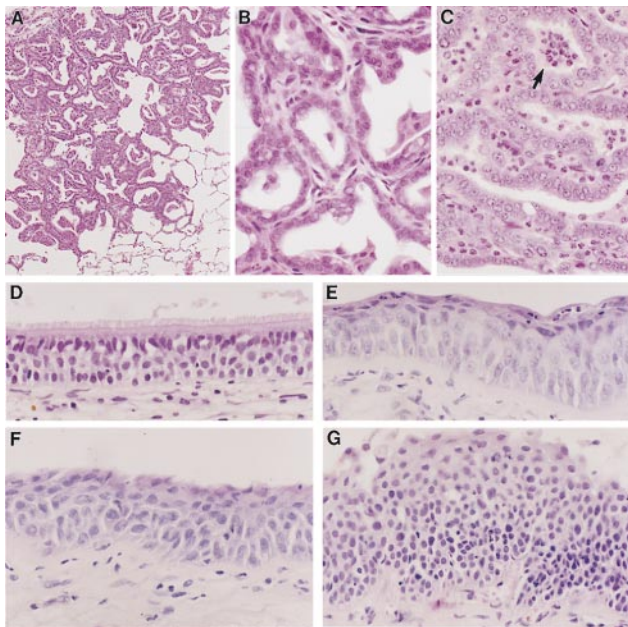

FIG. 2.   Rat and human lesions with *p16* methylation. (*A*) Focal epithelial hyperplasia in the lung of a rat treated with NNK. Note the small size of the lesion and the lack of disruption of the normal alveolar architecture. (*B*) Higher magnification of the lesion in *A*. (*C*) Adenoma in the lung of an NNK-treated rat. The epithelial cells exhibit minimal atypia. Note the large number of neutrophils and macrophages within the luminal spaces and in the stroma (arrow points to one luminal aggregate of inflammatory cells). (*D*) Minimal basal cell hyperplasia of the bronchial epithelium in a human biopsy specimen. Note the normal appearing ciliated surface. (*E*) Squamous metaplasia with minimal atypia of the bronchial epithelium in a section obtained at lobectomy. This patient had a SCC distant from this site at the time of lobectomy. (*F*) Squamous metaplasia with moderate atypia of the bronchial epithelium obtained at lobectomy. This patient also had a SCC distant from this site at the time of lobectomy. (*G*) CIS of the bronchial epithelium in a human biopsy specimen.
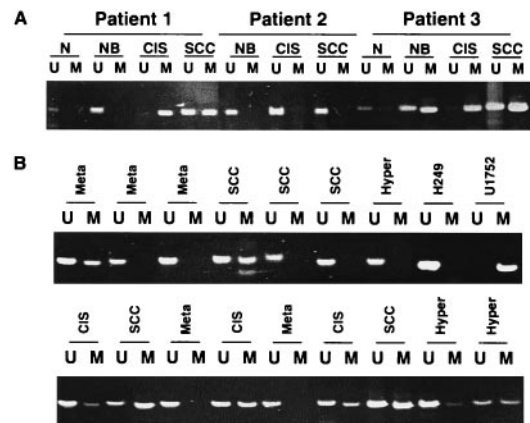

FIG. 3.   Methylation of *p16* in premalignant lesions, CIS, and SCC. (*A*) Lymphocytes microdissected from in and around the SCC constituted the normal (N) tissue for analysis. Other areas analyzed included normal appearing bronchus (NB) within the cancer field, adjacent CIS, and SCC obtained from three patients. (*B*) Precursor lesions and SCCs obtained through biopsy or lobectomy from several different cases. Lesions microdissected included basal cell hyperplasia (Hyper), squamous metaplasia (Meta), CIS, and SCC. Cell lines H249 and U172 serve as positive controls for detecting unmethylated (U) and methylated (M) *p16* alleles, respectively by MSP. A PCR product of the appropriate molecular weight (151 bp for U, 150 bp for M) indicates the presence of unmethylated and/or methylated *p16* alleles in that sample.

the SCC. Thirty-five premalignant or CIS lesions were examined from the two collections. Methylation of *p16* (see Fig. 3*B* for representative results and Fig. 4 for summary of all results) was detected in 3 of 6 CISs (50%) and in 4 of 17 (24%) squamous metaplasias with minimal to moderate atypia (Fig. 2 *D–G*). The frequency of *p16* methylation appeared less frequent in lower grade lesions being present in 2 of 12 (17%) lesions exhibiting minimal basal cell hyperplasia with a normal appearing ciliated surface (Fig. 2*D* and 3*B*). In contrast to the findings for CIS adjacent to established SCC, there was no apparent relationship between the methylation status of the cancers and the precursor lesions within the lobectomy cases. In total, at least one lesion was methylated at the *p16* locus in 10 of the 13 lobectomies examined.

Protein Levels for p16 Correlate with Methylation Status in SCCs and Precursor Lesions. The relationship between *p16* methylation and expression was assessed through immunohistochemical analysis of 13 SCCs and 7 precursor lesions. There was concordance between methylation status and the detection of protein in 18 of 20 samples. The two disconcordant samples, a SCC and adjacent CIS, were both unmethylated, but did not express any p16 protein. This lack of expression is most likely due to homozygous deletion of the *p16* gene, a mechanism observed in primary lung tumors (13) and derived cell lines (1). Thus, in all other samples, methylation of the *p16* gene was associated with loss of p16 protein, while the opposite was observed in unmethylated lesions. Fig. 5 depicts some of the staining patterns observed. In a squamous metaplasia that was unmethylated at the p16 locus (Fig. 5*A*), ≈60% of the epithelial cell nuclei were positive for p16 protein. In contrast, complete lack of p16 staining was observed in a squamous metaplasia (Fig. 5*B*) that was methylated. All five of the SCCs in which no methylation of the *p16* gene was detected showed abundant expression of the protein (Fig. 5*C*), a finding consistent with our previous studies demonstrating an apparent overexpression of this gene in tumors (14) or cell lines (4) where no methylation was detected. In four of seven methylated SCCs there was a complete absence of the p16 protein (Fig. 5*D*). However, in some tumors, small focal areas of cells stained for the protein, a pattern also observed by Kratze *et al.* (15).

Detection of Aberrant p16 Methylation in Exfoliated Cells Within Sputum. Premalignant cells shed from the airways or malignant cells from carcinomas can be recovered after spu-

FIG. 5.   Immunohistochemistry to detect p16 protein in human lung lesions. (*A*) Immunohistochemical detection of p16 protein (brown nuclear staining) in an area of bronchiolar squamous metaplasia. (*B*) Lack of detectable p16 protein in an area of bronchiolar squamous metaplasia with marked atypia. (*C*) Strong nuclear p16 immunostaining in a SCC. (*D*) Lack of p16 protein in a SCC.

tum induction. Thus, detecting aberrant methylation of *p16* in these cells from radiographically cancer-free persons could be a biomarker for impending disease. This hypothesis was tested by analysis of sputum collected without prior knowledge of their medical history from 33 persons who smoked. Methylated *p16* alleles were detected in DNA isolated from sputum of eight people (see Fig. 6 for representative results and Fig. 4 for summary of results). In contrast, no methylated *p16* alleles were present in DNA from normal human bronchial epithelial cells obtained through bronchoscopy from three never smokers (see Fig. 6 for representative results). Three persons positive for *p16* methylation were diagnosed with lung cancer at the time of sputum collection. The three tumors were classified as SCC, NSCLC (histology not specified), and a small cell lung cancer (SCLC). Cytologic examination of the sputum from the SCC and NSCLC cases revealed SCC cells and atypical cells, respectively, and the intensity of the signal for methylated *p16* alleles was greatest from these two samples (one is shown in Fig. 4) compared with all other positive sputum samples. The marked difference in intensity of the methylated product between the eight positive sputum samples was not unexpected due to the fact that tumors located in the airways should exfoliate a ''relatively high'' number of cells, while the number of cells recovered in sputum from premalignant lesions harboring methylated alleles will vary considerably depending on the size and location of the lesion(s) within the lung. Cytology of the sputum from the patient with SCLC was ruled unsatisfactory due to the absence of alveolar macrophages; the fact that this person was diagnosed with a second tumor (NSCLC) 2 years after collection of the sputum analyzed was significant. In the five cancer-free cases that were positive for *p16* methylation, cytology was classified as: (*i*) moderate atypia, (*ii*) marked atypical cells and suspicious for malignancy, (*iii*) negative, (*iv*) unsatisfactory, and (*v*) squamous metaplasia. The person with unsatisfactory cytology developed a SCLC 1 year later.

Four persons whose sputum was negative for *p16* methylation had lung cancer at the time of sputum induction. However, only one of these tumors, a SCC, was diagnosed by sputum cytology. The 21 remaining persons whose sputum was negative for *p16* methylation were classified by sputum cytology as negative in 14 persons, unsatisfactory in 5 persons, and
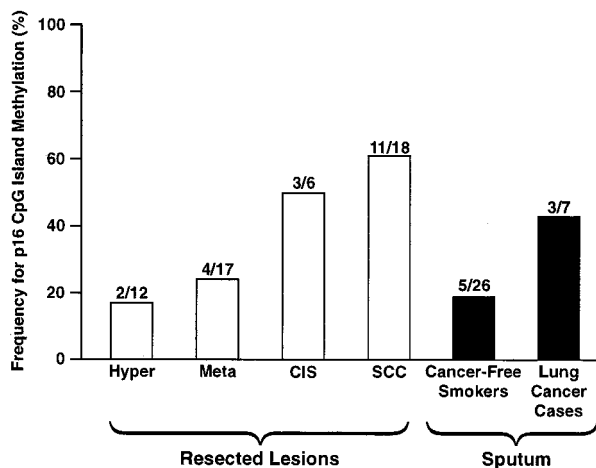

FIG. 4.   Summary of the frequency for *p16* methylation in premalignant lesions, CIS, SCC, and sputum samples. The frequency (expressed as a percentage) for detecting *p16* methylation is depicted for basal cell hyperplasia (Hyper), squamous metaplasia (Meta), CIS, SCC, and in sputum from cancer-free smokers and lung cancers. Above the bar graphs the total number of positive samples per total sample population for a specific lesion or population is indicated.
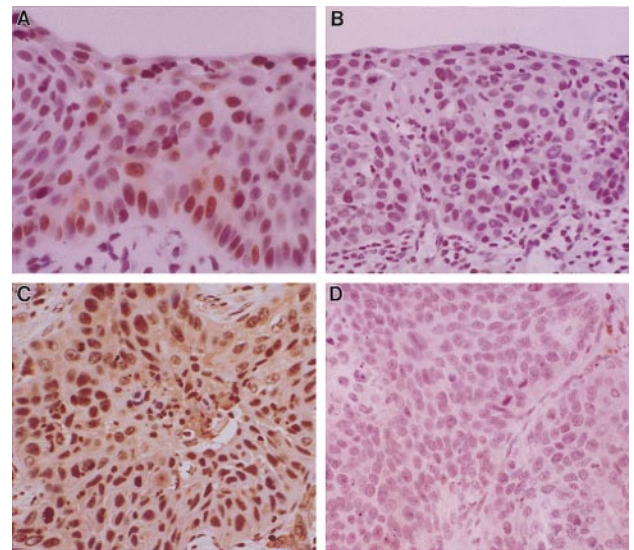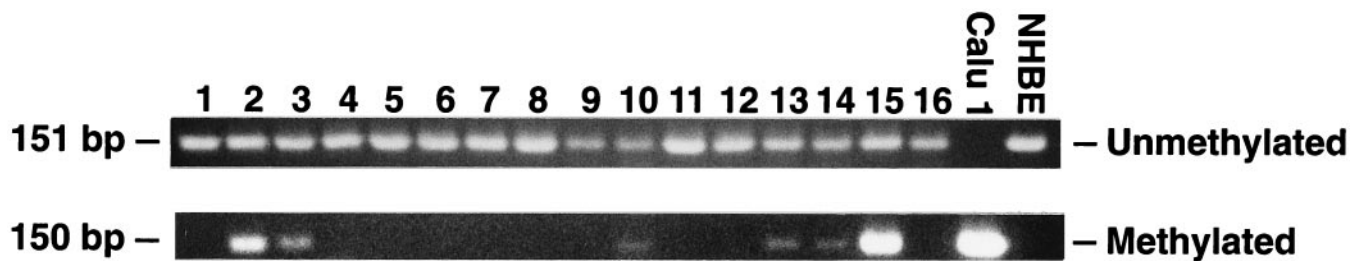
FIG. 6.   Methylation of *p16* in sputum from smokers. Bisulfite modified DNA was amplified by primers specific to methylated or unmethylated alleles of the *p16* gene as described. Unmethylated *p16* alleles were detected in all 16 sputum samples and in DNA from normal human bronchial epithelial (NHBE) cells obtained from a never-smoker. The Calu1 cell line (positive control for *p16* methylation) is completely methylated. Methylated alleles of *p16* were observed in DNA from 4 cancer-free persons (lanes 3, 10, 13, and 14), 1 person diagnosed at the time of sputum induction with a SCLC (lane 2) who developed a NSCLC 2 years later, 1 smoker (lane 15) who was positive for lung cancer at the time of sputum induction, and in the positive control (Calu1). No methylation was observed in the negative control normal human bronchial epithelial cells. Samples negative for *p16* methylation are shown in lanes 1, 4–9, 11–12, and 16.

marked atypia in 2 persons. Thus, classifying persons as cancer free was based on sputum cytology that appeared negative for malignant cells and a negative chest x-ray. Based on current hospital records, no additional lung tumors have been seen in this group of subjects over a follow-up period of ≈3 years since sputum collection.

DISCUSSION

This investigation demonstrates for the first time that inactivation of the *p16* tumor suppressor gene by aberrant methylation is an early and likely critical event in the development of NSCLC. Furthermore, consistent with the timing of this epigenetic alteration in the genesis of NSCLC, *p16* methylation was detected in epithelial cells shed from the airways of some persons at risk for lung cancer. Thus, the detection of *p16* methylation in sputum could represent a new biomarker for early detection of respiratory neoplasia and a target for intervention strategies.

The fact that hypermethylation of the *p16* gene is likely an essential step for the evolution of many NSCLCs is substantiated by our results and others. Functional inactivation of the *p16* gene in both tumors and precursor lesions was evident based on loss of p16 protein. Moreover, in the majority of samples examined, loss of expression appeared to be homogenous, substantiating the clonal nature of the methylation event. Our studies did not attempt to elucidate whether loss of *p16* function was due to methylation of both alleles or a combination of methylation and allelic loss. Both mechanisms have been documented (3). Several studies have corroborated our findings in tumors that methylation of the *p16* gene is clearly associated with loss of gene transcription (3, 4, 16). Furthermore, the selective advantage of this epigenetic event for the loss of function of the cyclin D-retinoblastoma pathway in lung tumors appears to be identical to that for either homozygous deletion of *p16* or retinoblastoma mutations. Thus, retinoblastoma mutations are common in SCLC, while *p16* mutations or hypermethylation are rare; the opposite is true in NSCLC (2, 3, 17).

A pivotal role for *p16* methylation in the earliest stages of lung cancer is further substantiated by our finding of a high frequency for this change in alveolar hyperplasias and adenomas from NNK-treated rats. These precursor lesions have an extremely high conversion rate to adenocarcinomas in this carcinogenesis model (9). Our findings of *p16* methylation in the putative stages for progression of human SCC also support a critical role for this molecular change in human cancer. The frequency for *p16* methylation increased from the lowest to highest grade precursor lesions to SCC. Strikingly, the 50% incidence of *p16* methylation in CIS, the precursor lesion thought to have the strongest likelihood of developing into SCC, paralleled findings in established SCCs. The strong

concordance between SCCs and adjacent CIS lesions for *p16* methylation is also consistent with an important role for *p16* inactivation in tumor progression within a single site. The lack of concordance for *p16* status between the SCCs and premalignant lesions at sites distant to the tumor is consistent with the epigenetic and genetic autonomy of multiple tumors in the same patient (18).

The detection of *p16* methylation in exfoliated cells within sputum from cancer-free smokers reaffirms the involvement of this genetic change in early respiratory carcinogenesis. Hypermethylation of *p16* has not been reported in normal tissue from unexposed individuals (3) and was not evident in normal human bronchial epithelial cells or peripheral lymphocytes (19), two of the major cell types within sputum. Direct comparison of sputum from current or former smokers to never smokers is technically difficult because never smokers produce very little sputum and the composition of the sample could also differ from that of a current or former smoker. The best control for our study was in fact sputum samples negative for *p16* methylation. If a low background for methylation of this gene existed in normal lung tissue, it should have been detected by MSP in the majority of the sputum samples, and this was not the case. Further support for the lack of *p16* alterations in never smokers is provided by studies of loss of heterozygosity by Wistuba *et al.* (6) and Mao *et al.* (7). These studies examined >100 biopsies from 30 never smokers and found no evidence for loss of heterozygosity at the 9p21 locus where the *p16* gene is located. This was in marked contrast to the frequent detection of allelic loss at this locus in biopsies from current and former smokers. Thus, it is likely that exfoliated cells from premalignant lesions or from established tumors within airways of persons at high risk for NSCLC account for the presence of the hypermethylated alleles in the positive sputum samples. The finding in one individual diagnosed with SCLC and positive for *p16* hypermethylation in the sputum at the time of diagnosis is particularly interesting because the *p16* gene is rarely inactivated in this tumor type (2, 3, 17). However, this individual was diagnosed with a separate NSCLC 2 years later, thus the methylation detected in sputum likely stemmed from cells exfoliated by the NSCLC or the precursor lesion from which it arose.

The association of *p16* gene hypermethylation with early stages of NSCLC and the detection of this change in sputum of patients at high risk for developing lung cancer create some exciting possibilities for approaching risk assessment, early detection, and prevention of this disease. However, several important relationships must be clarified in future studies. The exact relationship between the presence of the *p16* methylation change and the risk for lung cancer must be established. After smoking cessation, molecular markers presumably indicative of high risk, such as loss of heterozygosity at chromosome loci commonly found in lung cancers (6, 7), are still detected at high

frequency in people with putative precursor lesions. Individuals with such changes do remain at an elevated risk for development of lung cancer (20). However, it is apparent that many lesions dispersed throughout the lungs of current smokers regress after smoking cessation (21). It will be critical to determine the relationship between *p16* hypermethylation and the reversibility of these premalignant lesions. This information may emerge from monitoring individuals having *p16* methylation changes in sputum, but who stop smoking. If reversible, the detection of *p16* methylation in sputum could be an excellent intermediate biomarker to monitor the efficacy of intervention therapy in high-risk, cancer-free persons.

P16 methylation changes in sputum could also prove invaluable as a marker for early detection, risk assessment, and monitoring clinical course. The MSP assay used in the present study detects methylation of *p16* in 1 in 10^5 cells by primers designed to amplify only methylated alleles (9). Sputum cytology requires a skilled cytopathologist, while immunohistochemical methods to detect other markers (22, 23) rely on subtle differences in nuclear staining of cells often obscured by mucous. In fact, in the present study, three cases are described where cytology was either negative or unsatisfactory, yet *p16* hypermethylation was still detected. The impact of detecting *p16* methylation in exfoliated cells from cancer-free smokers as a prognostic indicator will only be realized in prospective studies, but clearly this molecular change could provide a key biomarker for early detection and clinical trials in lung cancer.

1. Kamb, A., Guis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K. Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E. & Skolnick, M. H. (1994) *Science* **8,** 436–440.
2. Otterson, G. A., Kratze, R. A., Coxon, A., Kim, Y. W. & Kaye, F. J. (1994) *Oncogene* **9,** 3375–3378.
3. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B. & Sidransky, D. (1995) *Nat. Med.* **1(7),** 686–692.
4. Swafford, D. S., Middleton, S. K., Palmisano, W. A., Nikula, K. J., Tesfaigzi, J., Baylin, S. B., Herman, J. G. & Belinsky, S. A. (1997) *Mol. Cell. Biol.* **17,** 1366–1374.
5. Crowell, R. E., Gilliland, F. D., Temes, R. T., Harms, H. J., Neft, R. E., Heaphy, E., Auckley, D. H., Crooks, L. A., Jordan, S. W., Samet, J. M., *et al.* (1996) *Cancer Epidemiol. Biomarkers Prev.* **5,** 631–637.
6. Wistuba, I. I., Lam, S., Behrens, C., Virmani, A. K., Fong, K. M., LeRiche, J., Samet, J. M., Srivastava, S., Minna, J. D. & Gazdar, A. F. (1997) *J. Natl. Cancer Inst.* **89(18),** 1366–1373.
7. Mao, L., Lee, J. S., Kurie, J. M., Fan, Y. H., Lippman, S. M., Lee, J. J., Ro, J. Y., Broxson, A., Yu, R., Morice, R. C., *et al.* (1997) *J. Natl. Cancer Inst.* **89,** 857–862.
8. Saccomano, G., Archer, V., Auerbach, O., Saunders, R. P. & Bennan, L. M. (1974) *Cancer* **33,** 256–270.
9. Belinsky, S. A., Foley, J. F., White, C. M., Anderson, M. W. & Maronpot, R. R. (1990) *Cancer Res.* **50,** 3772–3780.
10. Levi, S., Urgano-Ispizua, A., Gill, R., Thomas, D. M., Gilbertson, T. J., Foster, C. & Marshall, C. J. (1991) *Cancer Res.* **51,** 3497–3502.
11. Herman, J. G., Graff, J. R., Myohannen, S., Nelkin, B. D. & Baylin, S. B. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9821–9826.
12. Koss, L. G. (1992) *Diagnostic Cytology and Its Histopathologic Bases*. (Philadelphia: Lippincott), 4th Ed., Vol.1.
13. Xiao, S., Daizong, L., Corson, J. M., Vijg, J. & Fletcher, J. A. (1995) *Cancer Res.* **55,** 2968–2971.
14. Belinsky, S. A., Swafford, D. S., Middelton, S. K., Kennedy, C. H. & Tesfaigzi, J. (1997) *Carcinogenesis* **18,** 115–120.
15. Kratze, R. A., Greaten, T. M., Rubies, J. B., Maddaus, D. E., Niewoehner, G. A., Niehans & Geradts, J. (1996) *Cancer Res.* **56,** 3415–3420.
16. Otterson, G. A., Khleif, S. N., Chen, W., Coxon, A. B. & Kaye, F. J. (1995) *Oncogene* **11,** 1211–1216.
17. Shapiro, G. I., Edwards, C. D., Kobzik, L., Godleski, J., Richards, W., Sugarbaker, D. & Rollins, B. J. (1995) *Cancer Res.* **55,** 505–509.
18. Harpole, D. H., Herndon, J. E., Wolfe, W. G., Iglehart, J. D. & Marks, J. R. (1995) *Cancer Res.* **55,** 51–56.
19. Herman, J. G., Civin, C. I., Issa, J.-P. J., Collector, M. I., Sharkis, S. J. & Baylin, S. B. (1997) *Cancer Res.* **57,** 837–841.
20. Tong, L., Spitz, M. R., Fueger, J. J. & Amos, C. I. (1996) *Cancer* **78,** 1004–1010.
21. Auerbach, O., Stout, A. P., Hammond, E. C. & Garfinkel, L. (1962) *N. Eng. J. Med.* **267,** 119–125.
22. Anderson, M., Sladon, S., Michels, R., Davidson, L., Conwell, K., II, Lechner, J., Franklin, W., Saccomanno, G. & Wiest, J. (1996) *J. Cell Biochem.* **64,** 185–190.
23. Zhou, J., Jensen, S. M., Steinberg, S. M., Mulshine, J. L. & Linnoila, R. I. (1996) *Lung Cancer* **14,** 85–97.