

p16INK4A inactivation mechanisms in non-small-cell lung cancer patients occupationally exposed to asbestos

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Abstract

Epidemiological studies have shown that asbestos fibers constitute the major occupational risk factor and that asbestos acts synergistically with tobacco smoking to induce lung cancer. Although some somatic gene alterations in lung cancer have been linked to tobacco smoke, few data are available on the role of asbestos fibers. P16/CDKN2A is an important tumor suppressor gene that is frequently altered in lung cancer via promoter 5'-CpG island hypermethylation and homozygous deletion, and rarely via point mutation. Many studies suggest that tobacco smoking produces P16/CDKN2A promoter hypermethylation in lung cancer, but the status of this gene in relation to asbestos exposure has yet to be determined. The purpose of this study was to investigate the mechanism of P16/CDKN2A alterations in lung cancer in asbestos-exposed patients. P16/CDKN2A gene status was studied in 75 human non-small-cell lung cancer (NSCLC) cases with well-defined smoking habits, and detailed assessment of asbestos exposure, based on occupational questionnaire and determination of asbestos bodies in lung tissue. The results of this study confirm published data on the effect of tobacco smoke on P16/CDKN2A gene alterations, characterized by significantly higher P16/CDKN2A promoter hypermethylation in heavy smokers (more than 40 Pack-Years (P-Y)) than in smokers of less than 40 P-Y. These results also demonstrate a higher incidence of loss of heterozygosity and homozygous deletion in asbestos-exposed cases, after adjustment for age and cumulative tobacco consumption, than in unexposed cases (P = 0.0062). This study suggests that P16/CDKN2A gene inactivation in asbestos-exposed NSCLC cases mainly occurs via deletion, a feature also found in malignant mesothelioma, a tumor independent of tobacco smoking but associated with asbestos exposure, suggesting a possible relationship with an effect of asbestos fibers.

MESH Keywords Aged ; Asbestos ; toxicity ; Carcinogens ; toxicity ; Carcinoma, Non-Small-Cell Lung ; chemically induced ; genetics ; metabolism ; Cyclin-Dependent Kinase Inhibitor p16 ; antagonists & inhibitors ; genetics ; metabolism ; DNA Methylation ; Female ; Gene Deletion ; Humans ; Loss of Heterozygosity ; Lung Neoplasms ; chemically induced ; genetics ; metabolism ; Male ; Middle Aged ; Occupational Exposure ; Promoter Regions, Genetic ; drug effects ; Smoking

Author Keywords Lung cancer ; Asbestos ; Occupation ; Tobacco smoking ; P16/CDKN2A ; INK4A ; Deletion ; Hypermethylation

Introduction

Lung cancer is still the leading cause of cancer-related death in the world [1]. Environmental and occupational factors, and genetic susceptibility interact to influence lung carcinogenesis [2]. About 90% of lung cancer risks are attributable to tobacco smoking [3]. Other environmental, occupational and genetic factors also contribute to the development of lung cancer. Epidemiological studies have shown that asbestos fibers constitute the major occupational risk factor and that asbestos acts synergistically with tobacco smoking to induce lung cancer [4–6]. Molecular analyses of lung cancer cells have demonstrated that some alterations in oncogenes and tumor suppressor genes can be associated with the risk factor, especially tobacco smoke. In contrast, other molecular changes were not associated with exposure to certain risk factors. For instance, mutations in TP53 and KRAS genes and P16/CDKN2A promoter 5'-CpG island hypermethylation in lung cancer were associated with tobacco smoking, while EGFR mutations were mainly found in lung adenocarcinoma in never smokers [7–9]. In this context, it has been suggested that some gene alterations could be markers of exposure to specific carcinogenic factors. The nature of genetic alterations attributable to exposure to asbestos fibers remains to be defined in lung cancer.

P16/CDKN2A is an important tumor suppressor gene showing both genetic or epigenetic alterations frequently found in cancers. In lung cancer, P16/CDKN2A is inactivated via promoter 5'-CpG island hypermethylation and homozygous deletion, and rarely via point mutations [10–14]. According to published data, P16/CDKN2A promoter hypermethylation in NSCLC is observed in about 20 to 40% of cases [15–20]. Gene deletion and point mutations also contribute to loss of p16^{INK4A} expression, as these alterations have been reported to occur with a frequency of 10 to 30% and less than 5%, respectively [21–23].

The relationship between P16/CDKN2A alteration and tobacco smoking has been largely investigated. According to Kim et al. (2001), who studied a large series of 185 non-small-cell lung cancer (NSCLC) cases, P16/CDKN2A promoter hypermethylation was more likely found in current smokers than in non-smokers, and was associated with pack-years (P-Y) and duration of smoking, suggesting that the mechanism of action of tobacco smoke involves P16/CDKN2A promoter hypermethylation [16]. Similarly, P16/CDKN2A promoter hypermethylation was statistically associated with tobacco smoking in a series of 51 NSCLC cases [22]. In another study, allelic loss at chromosome 9p21 was associated with tobacco smoking in 47 NSCLC, but no association was observed between tobacco smoking and P16/CDKN2A homozygous deletion or promoter hypermethylation [24]. In contrast, a link between homozygous deletion and never smoking status was reported by Kraunz et al. [25].

In contrast with the numerous studies on tobacco smoking, few data are available on P16/CDKN2A gene status in lung cancer in asbestos-exposed subjects. No statistically significant difference was observed between asbestos-exposed and unexposed patients in four studies investigating P16/CDKN2A gene inactivation [13, 16, 18, 25]. Nevertheless, one study found that P16/CDKN2A promoter hypermethylation was significantly enhanced in asbestos-exposed cases compared to unexposed cases [26]. More details on P16/CDKN2A gene status are available in malignant mesothelioma, a cancer related to asbestos exposure but not to tobacco smoking. In this disease, P16/CDKN2A gene is generally altered via homozygous deletion [27–29].

The purpose of this study was to investigate the mechanism of P16/CDKN2A alterations in lung cancer of asbestos-exposed patients. P16/CDKN2A gene status was studied in 75 cases of human NSCLC with well-defined smoking habits, and detailed assessment of asbestos exposure, based on both occupational questionnaire and determination of asbestos bodies in lung tissue. After adjustment for age and cumulative tobacco consumption, the results showed higher P16/CDKN2A promoter hypermethylation in unexposed cases and loss of heterozygosity (LOH) and homozygous deletion (HD) of P16/CDKN2A in asbestos-exposed cases.

PATIENTS AND METHODS

NSCLC population

Patients

Patients were selected from consecutive cases of primary NSCLC after surgical resection at 4 hospitals (Centre Hospitalier Intercommunal, Créteil; Hôpital Européen Georges Pompidou, Paris; Centre Hospitalier Universitaire, Caen; Centre Chirurgique Marie Lannelongue, Plessis-Robinson) from January 1994 through June 1999. The study was approved by the local Ethics Committee and all patients provided written informed consent. The following eligibility criteria were required: (a) lung tumor histology; (b) absence of neoadjuvant chemotherapy or radiotherapy; (c) both normal and tumor lung tissue available; (d) data on asbestos exposure history including quantification of asbestos bodies (AB) in lung tissue and interviewer-administered questionnaire; and (e) data on smoking habits. A total of 75 patients, 34 asbestos-exposed and 41 unexposed, were selected.

Tissue samples were snap-frozen in liquid nitrogen after surgical resection and stored at -80°C until use. Detailed information describing the tumors was obtained from pathology reports.

Smoking habits

Information regarding smoking status was obtained from an interviewer-administered questionnaire: smoking class, i.e never smokers, current smokers and former smokers (quitting smoking at least 1 year before diagnosis); age at onset of smoking, smoking duration and tobacco consumption (cigarettes, cigars and pipes), expressed as pack-years (P-Y). Heavy smokers were defined as patients who had smoked more than 40 P-Y. This value represents the median of the overall study population.

Asbestos exposure

A questionnaire comprising complete job history, including past occupational, domestic, and environmental exposure to asbestos was completed by face-to-face interview. On the basis of these data, occupational exposure to asbestos was evaluated by consensus between two occupational hygienists not informed about asbestos body (AB) counts. Asbestos exposure was ascertained if the questionnaire concluded on definite occupational exposure to asbestos for more than 10 years, and/or when the AB count was higher than 1000 per gram of dry lung tissue, a value indicative of non-trivial (usually occupational) asbestos exposure [30, 31]. Unexposed subjects were those with no occupational or environmental exposure identified from assessment of the questionnaire and with an AB count less than 1000 per gram of dry lung tissue.

p16^{INK4a} immunohistochemistry (IHC)

Deparaffinized tissue sections were labeled with specific antibodies directed against p16^{INK4a}. The monoclonal antibody p16^{INK4a} (CINtec histology kit based on a primary monoclonal mouse antibody clone E6H4[®] directed against human p16^{INK4a} protein) was used (mtm laboratories AG, Heidelberg, Germany). After using peroxidase blocking reagent to cover the specimen, the slide were covered with 200 µL of primary antibody (monoclonal mouse antibody clone E6H4[®] directed against human p16^{INK4a} protein) incubated for 30 min, and placed in a fresh buffer bath for 5 min (mtm laboratories AG). The specimen was then covered with 200 µL of visualization reagent, incubated for 30 min, and placed twice in a fresh buffer bath for 5 min. The specimen was covered with 200 µL of substrate–chromogen solution DAB, incubated for 10 min and rinsed with distilled water. Finally, the slides were immersed in a hematoxylin bath for 5 min and mounted with non-aqueous, permanent mounting medium. Negative and positive controls were performed. Results were expressed as percentage of positive cell detection for the marker. Tumor cell labeling was scored according to the percentage of tumor cells showing positive immunoreactivity. Score 1 was attributed when less than 25% of tumor cells showed positive nuclear staining, score 2 when between 25 and 75% of tumor cells were positive and score 3 when more than 75% of cells showed nuclear staining. Negative controls were obtained after incubation of the sections with primary antibodies, but without secondary antibodies. Positive nuclear staining of endothelial cells in each carcinoma lung tissue section was used as internal positive control. Only nuclear staining was considered to be positive.

Genomic DNA extraction and methylation-specific PCR

Genomic DNA from tumor and normal tissue samples was isolated by sodium dodecyl sulfate (SDS)-proteinase K digestion followed by phenol and chloroform extraction. Methylation-specific PCR was performed according to the method of Herman et al (1996) [32]. Briefly, one µg DNA in a volume of 50 µl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. Thirty µl of 10 mM hydroquinone (Sigma, Sophia-Antipolis, France) and 520 µl of 3 M sodium bisulfite (Sigma) at pH 5.0, both freshly prepared, were added and mixed, and samples were incubated at 50°C for 16 hours. Modified DNA was desalted using Wizard DNA purification resin (Promega, Charbonnières, France) according to the manufacturer's recommendations and diluted in 50 µl of water. DNA modification was completed by treatment with 0.3 M NaOH (final concentration) for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at -20°C. Bisulfite-modified DNA (100 ng) was amplified with specific primer sets corresponding to methylated and unmethylated sequences (20 µM of each primer) (Table 1), in a total volume of 25 µl of GeneAmp PCR Gold Taq Buffer (Applied Biosystems, Courtaboeuf, France) containing 1.0 mM MgCl₂, [22], 0.2 mM dNTPs, 4% dimethylsulfoxide and 1 unit of Taq polymerase Ampli Taq Gold DNA Polymerase (Applied Biosystems). After an initial denaturation step at 95°C for 10 min, PCR reactions were carried out in a Gene Amp 9700 apparatus (Perkin-Elmer) for 35 cycles including a denaturation step at 95°C for 15 sec, an annealing step at 58°C for methylation primer set and 60°C for unmethylation primer set for 15 sec and an extension step at 72°C for 30 sec, followed by a final extension step at 72°C for 4 min. Methylated (97 bp) and unmethylated (151 bp) PCR amplification products were analyzed on 2.5% agarose gel electrophoresis using ethidium bromide staining. Water was used as negative control and normal lung tissue was used as positive control for each subject.

Fluorescence in situ hybridization (FISH) for analysis of P16/CDKN2A zygosity

Touch preparations were fixed in cool methanol/acetic acid (3:1 v/v) for 5 min, then air dried. The preparations were treated with RNase A (0.1 mg/ml) and pepsin (50 µg/ml). DNA denaturation was performed by treating slides with 70% formamide in 2× standard saline citrate (SSC) at 72°C for 1 min, followed by immediate immersion in ice-cooled 2 × SSC, and dehydration in 70%, 85% and 100% ethanol. Ten µl of denatured commercially available P16/CDKN2A specific probe (PONC0921: P16/CDKN2A direct-labeled with Rhodamine, and chromosome 9 classical satellite direct-labeled with Fluorescein, Q.BIOgene, Illkirch, France) were applied on the

sections, then covered with a 20 × 20 mm coverslip and incubated in a humid chamber at 37°C for 16 h. The slides were washed with 1× washing buffer (0.5 × SSC/0.1% SDS) at 65°C for 5 min, immersed in 1 × PBS for 5 min at room temperature according to the manufacturer's recommendations, counterstained with HOECHST 33342 Molecular probes (Invitrogen, Cergy-Pontoise, France), and mounted with Vectashield (Vector Laboratories, Paris, France). The slides were observed under a fluorescent microscope (LEICA HC) with appropriate filters. One hundred to 200 nuclei were analyzed to score the number of fluorescent signals. The presence of a deletion was confirmed when the percentage of nuclei containing either LOH or homozygous deletion of P16/CDKN2A and showing two chromosome 9 signals was equal to or greater than 50%.

Homozygous deletion of P16/CDKN2A gene

Homozygous deletion of P16/CDKN2A gene was determined on tumor DNA by real-time quantitative PCR according to the $\Delta\Delta C_t$ quantitative method using Human Serum Albumin gene (HSA) as reference gene [33, 34]. Positive controls were normal human lymphocyte DNA and negative controls were water without DNA. Tumor DNA, positive controls and no-template controls were run in triplicate for P16/CDKN2A and HSA genes. Primers were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and mgb TaqMan[®] probes from Applied Biosystems (Table 1). The PCR mix consisted of ABsolute[™] QPCR MIX 1× (ABgene, Courtaboeuf, France), 300 nM primers, 200 nM probe, H₂O and 20 ng of DNA template in a final volume of 10 μ l. Real-time quantitative PCR was performed using an ABI Prism 7900 HT sequence detection system with Sequence Detection System 2.0 software (Applied Biosystems). After an initial denaturation step at 95°C for 15 min, PCR reactions were carried out for 40 cycles including a denaturation step at 95°C for 15 sec, and an annealing step at 60°C for 15 sec. The normalized amount of P16/CDKN2A gene in tumor samples was determined ΔC_t by the average of C_t of non-tumor tissues as calibrator. Quantification was performed by normalizing ΔC_t of P16/CDKN2A gene results to those of HSA gene in the same sample. The value $2 \times 2^{-\Delta\Delta C_t}$ represents an estimation of the gene copy number in tumor tissue. The cut-off value was $2 \times 2^{-\Delta\Delta C_t} \leq 0.60$ for homozygous deletion [33].

Mutational analysis of P16/CDKN2A gene

Mutations in P16/CDKN2A exons 1 to 3 were screened by DNA sequencing. Genomic DNA was extracted from cell cultures using a standard phenol-chloroform extraction procedure. DNA amplification was performed by PCR with a combination of forward and reverse primers (Table 1) and Taq polymerase Hot Star (Qiagen, Courtaboeuf, France). PCR was carried out with a Gene Amp 9700 apparatus (Perkin-Elmer). After an initial denaturation step at 95°C for 10 min, PCR reactions were carried out for 40 cycles including a denaturation step at 95°C for 30 sec, an annealing step at 58°C for 30 sec, and an extension step at 72°C for 30 sec. Extension during the final step was continued for 10 min. PCR products were purified with distilled water through Millipore genomics columns (Prolabo, Paris, France), checked for quality and quantified prior to sequencing. Sequencing PCR was performed on purified PCR products using forward or reverse primer located in the exon (Table 1) and Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) on a Gene Amp 9700 apparatus (Perkin-Elmer). PCR reactions were carried out for 25 cycles including denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and extension at 60°C for 4 min. Sequencing PCR products were purified with distilled water through Sephadex G50 fine resin (GE Healthcare Biosciences AB, Uppsala, Sweden) in Multiscreen Millipore columns (Prolabo) and analyzed on an ABI PRISM 3100 Genetic analyser (Applied Biosystems). Analyses were performed using Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical analyses

Clinicopathologic characteristics of NSCLC cases were compared using Chi² and Fisher's exact tests, as appropriate. Wilcoxon nonparametric test was used to compare the rank of CA between asbestos-exposed and unexposed NSCLC cases. Using Chi² and Fisher's exact tests, asbestos-exposed NSCLC cases were compared to unexposed NSCLC cases based on p16^{INK4a} IHC status, such as smokers (including former smokers) compared to never smokers. Similar analyses were performed after stratification according to the various p16^{INK4a} IHC subtypes. Multivariate analyses were also performed using logistic models on P16/CDKN2A promoter hypermethylation and deletion, taking into account cumulative tobacco consumption, age and asbestos exposure. Statistical analyses were performed using "Statistical Analysis System" software (SAS v9.1 Inc, Cary, NC, USA). A P value less than 0.05 was considered statistically significant.

RESULTS

Population

Clinicopathologic characteristics of 34 asbestos-exposed and 41 unexposed NSCLC cases are shown in Table 2. No significant difference was observed between asbestos-exposed and unexposed groups, for age, histological NSCLC subtype and smoking habits (smoking status, age at onset, smoking duration and cumulative tobacco consumption), while gender was at the limit of statistical significance. About half of the population was current smokers and never smokers did not exceed 10%. The most common histological subtypes of lung cancer were squamous carcinoma and adenocarcinoma.

p16^{INK4a} protein expression

Lack of p16^{INK4A} expression (score 1) was found in 68.0% of the overall population with 24 (70.6%) cases and 27 (65.8%) cases in the asbestos-exposed and unexposed groups, respectively (Table 3). This difference was not statistically significant. Figure 1 represents a positive (A) and a negative (B) immunostaining.

P16/CDKN2A gene alteration

P16/CDKN2A gene alterations by promoter hypermethylation, deletion or point mutations were found in 66.7% of all cases. Gene alterations were found in 79.4% and 68.3% of cases in the asbestos-exposed and unexposed groups, respectively. This difference was not statistically significant.

P16/CDKN2A methylation-specific PCR

Twenty eight (37.3%) cases demonstrated P16/CDKN2A promoter hypermethylation in bisulfite-treated genomic DNA. Matched normal tissue samples were analyzed to check the specificity of promoter methylation in tumor tissue. P16/CDKN2A promoter hypermethylation was not observed in these samples (Fig. 2).

Heavy smokers with more than 40 P-Y had significantly higher P16/CDKN2A promoter hypermethylation than other smokers (less than 40 P-Y; excluding never smokers) (P = 0.0295) (Odds ratio: 3.37 [95% Confidence Interval: 1.13 – 10.06] after adjustment for age and asbestos exposure).

P16/CDKN2A promoter hypermethylation was found in 8 asbestos-exposed patients (23.5%) and in 20 unexposed cases (48.8%). This difference was statistically significant (P = 0.0244). After adjustment for age and cumulative tobacco consumption (excluding 6 never smokers, 4 in the unexposed group and 2 in the asbestos-exposed group), the difference remained statistically significant (P = 0.0079) in 8 (24.2%) asbestos-exposed patients and 18 (48.7%) unexposed cases (Odds ratio: 4.43 [95% Confidence Interval: 1.48 – 13.31]). Notably, among samples with a score of 1 for p16^{INK4A} expression, a significantly lower rate of P16/CDKN2A methylation was found in the asbestos-exposed group than in the unexposed group (16.7% vs 59.3%; P = 0.0019) (Table 3).

Homozygous deletion and loss of heterozygosity of P16/CDKN2A gene

FISH was performed when sufficient tissue sample was available. Nineteen samples with p16^{INK4A} IHC scores of 1 or 2 and no promoter hypermethylation were studied. This group included 13 asbestos-exposed and 6 unexposed patients. FISH analyses detected 4 homozygous deletions and 15 LOH (Fig. 3). There were homozygous deletions in 2 asbestos-exposed subjects and 2 unexposed subjects, and LOH in 11 asbestos-exposed subjects and 4 unexposed subjects, respectively. Quantitative PCR analysis confirmed FISH deletion results. After adjustment for age and cumulative tobacco consumption, homozygous deletion and LOH were significantly higher in asbestos-exposed cases (n = 17, 50.0%) than in unexposed cases (n = 10, 24.4%) (P = 0.0172; Odds ratio: 3.58 [95% Confidence Interval: 1.25 – 10.23]). Notably, in samples with p16^{INK4A} IHC score 1, a significantly higher rate of P16/CDKN2A deletion was found in the asbestos-exposed group than in the unexposed group (66.7% vs 29.2%; P = 0.0082). P16/CDKN2A deletion was not significantly different between heavy smokers and other subjects (P = 0.0754; Odds ratio: 2.59 [95% Confidence Interval: 0.91 – 7.40] after adjustment for age and asbestos exposure).

P16/CDKN2A DNA sequencing analysis

A total of 5 (6.7%) mutations was found in the whole series, including 2 asbestos-exposed cases and 3 unexposed cases, and all mutations were detected in former or current smokers (more than 20 P-Y). All mutations were point mutations resulting in amino acid changes. One mutation was identified in exon 1α, at codon 42 (124A→T,N42Y), changing the encoded asparagine into tyrosine. Four mutations were identified in exon 2, at codon 57 (170C→T,A57V), changing the encoded alanine into valine, at codon 80 (238C→T,R80X), changing arginine into a stop codon, at codon 85 (253G→C,A85P), changing alanine into proline, and at codon 85 (322G→T,D108Y), changing aspartic acid into tyrosine.

DISCUSSION

P16/CDKN2A is a tumor suppressor gene located in 9p21, inactivated in many types of human cancer. Gene promoter hypermethylation and homozygous deletion are the main mechanisms of P16/CDKN2A gene inactivation in NSCLC, while point mutations are less frequent [21 , 25 , 35].

In lung cancer, promoter hypermethylation of P16/CDKN2A gene seems to be associated with the main risk factor, tobacco smoke. Asbestos fibers represent a well known lung carcinogen but their mode of action has yet to be determined. This study investigated P16/CDKN2A status in asbestos-exposed and unexposed patients with NSCLC matched for age, gender, histological type and smoking habits to avoid bias. Asbestos exposure was well characterized on the basis of an occupational questionnaire and AB count in lung tissue.

Lack of p16^{INK4A} expression and alteration of P16/CDKN2A gene were detected in about two-thirds of all NSCLC cases. This result is consistent with other reports and indicates a major role of P16/CDKN2A inactivation in NSCLC carcinogenesis [10 , 36].

No differences in the frequency of p16^{INK4A} protein expression or P16/CDKN2A gene alteration were observed between asbestos-exposed and unexposed NSCLC cases. However, the mechanism of P16/CDKN2A inactivation appears to be different between asbestos-exposed and unexposed groups.

P16/CDKN2A promoter hypermethylation was found to be significantly lower in the asbestos-exposed NSCLC group than in the asbestos unexposed group, after adjustment for age and smoking status, as assessed by methylation-specific PCR. In contrast, deletions were statistically more frequent in the asbestos-exposed group, as assessed by FISH and quantitative PCR analyses, than in the unexposed group.

These results contrast with data previously published by other authors who failed to demonstrate any statistically significant difference in the frequency of promoter hypermethylation or homozygous deletion of P16/CDKN2A gene between cases with asbestos exposure and unexposed cases [13 , 16 , 18 , 25], or who reported higher P16/CDKN2A promoter hypermethylation in the asbestos-exposed group [26].

This discordance with published data could be attributed to the number of asbestos-exposed NSCLC cases, a different population or the method used to assess asbestos exposure [13 , 16 , 18 , 25], as a smaller or unbalanced number of asbestos-exposed and unexposed NSCLC cases were investigated in some studies [13 , 18 , 25]. In some studies, asbestos exposure was only based on interviewer-administered questionnaire [13 , 16 , 25]. In addition, tobacco smoking could be a confounding factor. In the study reporting higher P16/CDKN2A promoter hypermethylation in the asbestos-exposed group, this alteration was not related to tobacco smoking [26]. This finding contrasts with most of the published data, including the present results, where P16/CDKN2A promoter hypermethylation was associated with tobacco smoking, and has been found in some studies to be linearly related to tobacco smoking [16 , 19 , 37 –39].

In human malignant mesothelioma, a disease mostly related to asbestos exposure, inactivation of P16/CDKN2A mainly occurs by homozygous deletion suggesting a possible relationship with the effect of asbestos fibers [29 , 40 , 41]. Moreover, asbestos fibers are known to produce chromosomal losses and rearrangements in epithelial cells [42 –44] and the major type of alteration of the orthologous p16/Cdkn2a gene is deletion in asbestos-induced murine mesotheliomas [45 , 46]. These results suggest that asbestos fiber exposure could be linked to this type of genetic damage.

In conclusion, this study confirms that P16/CDKN2A gene inactivation is a recurrent alteration in lung cancer and is the first to emphasize a specific type of damage in relation to the type of carcinogen exposure by studying well characterized populations. While the net frequency of P16/CDKN2A alterations was similar in asbestos-exposed and unexposed lung cancer cases, a significantly higher frequency of deletions was found in asbestos-exposed cases compared to unexposed cases. P16/CDKN2A hypermethylation was not different between the two groups, but was enhanced as a result of tobacco consumption. These results provide new data in the field of biomarkers of exposure to carcinogens.

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Footnotes:

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflict of interest.

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Figure 1

Immunodetection of p16^{INK4A} by immunohistochemistry in NSCLC patients

A: Positive control of p16^{INK4A} B: Positive immunostaining of p16^{INK4A} in a lung adenocarcinoma case (× 200) C: Lack of p16^{INK4A} expression in a lung adenocarcinoma case (× 200)

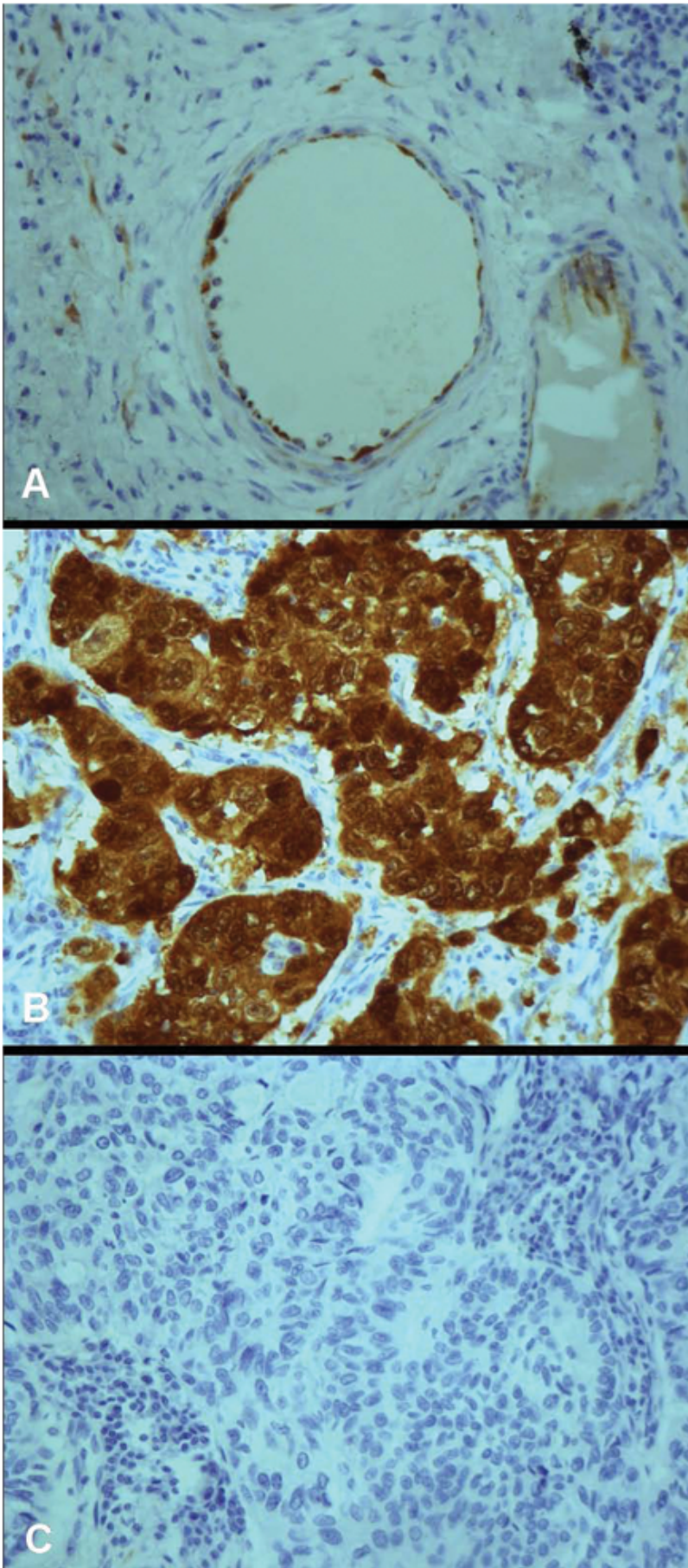
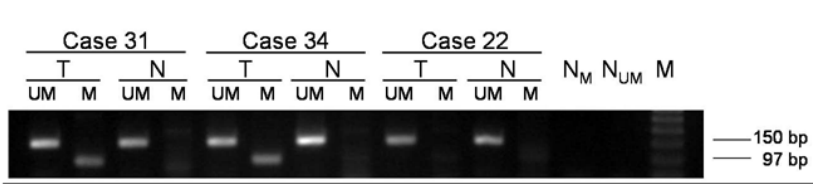


Figure 2

Representative patterns of aberrant methylation promoter of P16/CDKN2A gene in NSCLC patients

Primer sets used for amplification are designated as unmethylated (UM) or methylated (M). Twenty μ l of PCR product were run on 2.5% agarose gel stained with ethidium bromide, and visualized under UV illumination. T: tumor tissue; N: normal lung tissue; M: DNA 50 bp ladder; N_M: negative control with methylated sequence specific primer; N_{UM}: negative control with unmethylated sequence specific primer.

**Figure 3**

FISH hybridization

Dual-color FISH with PONC0921 probe. (P16/CDKN2A specific DNA probe is direct-labeled with rhodamine and the chromosome 9 classical satellite probe is direct-labeled with fluorescein). **A** : Normal lung tissue used as positive control showed two red signals and two green signals. **B** : NSCLC case showing two green signals and one red signal indicating a loss of heterozygosity of P16/CDKN2A gene. **C** : NSCLC case showing two green signals and no red signal, indicating a homozygous deletion of P16/CDKN2A gene.

