$p16^{INK4A}$ inactivation mechanisms in non small-cell lung cancer patients occupationally exposed to asbestos

Pascal Andujar[a-c], Jinhui Wang[d], Alexis Descatha[e-g], Françoise Galateau-Sallé[h,i], Issam Abd-Alsamad[j], Marie-Annick Billon-Galland[k], Hélène Blons[l,m], Bénédicte Clin[h,n], Claire Danel[o,p], Bruno Housset[a-c], Pierre Laurent-Puig[l,m], Françoise Le Pimpec-Barthes[q,r], Marc Letourneux[h,n], Isabelle Monnet[a], Jean-François Régnard[s], Annie Renier[p,r], Jessica Zucman-Rossi[p,r], Jean-Claude Pairon[a-c], Marie-Claude Jaurand[p,r*].

[a]Hôpital Intercommunal de Créteil, Service de Pneumologie et de Pathologie Professionnelle, Créteil, F-94010, France

[b]INSERM, U955, Equipe 4, Créteil, F-94010, France

[c]Université Paris 12, Faculté de Médecine, Créteil, F-94010, France

[d]INSERM, E0337, Créteil, F-94010, France

[e]AP-HP, GHU Ouest, Hôpital Poincaré, Unité de Pathologie Professionnelle, Environnementale et d'Insertion, Garches, F-92380, France

[f]INSERM, U687, Villejuif, F-94807, France

[g]Université de Versailles Saint-Quentin-en-Yvelines, Faculté de médecine Paris-Ile-de-France-Ouest, Guyancourt, F-78280, France

[h]INSERM, ERI 3, Caen, F-14000, France

[i]CHU Caen, Service d'Anatomo-Pathologie, Caen, F-14033, France

[j]Hôpital Intercommunal de Créteil, Service d'Anatomo-Pathologie, F-94010, Créteil, France

[k]Laboratoire d'Etude des Particules Inhalées, Paris, F-75013, France

[l]INSERM, UMR-S775, Paris, F-75006, France

[m]Université Paris Descartes, Paris, F-75005, France

[n]CHU Caen, Service de Médecine du Travail et Pathologie Professionnelle, Caen, F-14033, France

[o]AP-HP, GHU Ouest, Hôpital Européen Georges Pompidou, Service d'Anatomo-Pathologie, Paris, F-75015, France

[p]Université Paris 7, Paris, F-75005, France

[q]AP-HP, GHU Ouest, Hôpital Européen Georges Pompidou, Service de Chirurgie Thoracique, Paris, F-75015, France

[r]INSERM, U674, Fondation Jean Dausset-CEPH, IFR105, Paris, F-75010, France

[s]Département de Chirurgie Thoracique et Vasculaire, et de Transplantation Cœur-Poumon, Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson, F-92350, France

*Corresponding author: Phone: +33 1 53 72 51 88; Fax: +33 1 53 72 51 92

Email address: jaurand@cephb.fr (MC. Jaurand)

Present addresses:

J. Wang: Division of Biology, Beckman Research Institute, City of Hope National Medical Center, Duarte, CA 91010, California, United States of America.

C. Danel: AP-HP, GHU Nord, Hôpital Bichat-Claude Bernard, Service d'Anatomie et de Cytologie Pathologiques, 46 rue Henri Huchard, F-75018 Paris, France.

JF Régnard: AP-HP, GHU Ouest, Hôpital Hôtel-Dieu, Service de Chirurgie Thoracique, 1 place du Parvis de Notre-Dame, F-75004 Paris, France.

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.

Key words

Lung cancer; asbestos; occupation; tobacco smoking; *P16/CDKN2A*; *INK4A*; deletion; hypermethylation.

1. 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 *P16CDKN2A* 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.

2. PATIENTS AND METHODS

2.1. 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 Chirurgical 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.

2.2. $p16^{INK4A}$ 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, Heildelberg, 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.

2.3. 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 $MgCl_2$, [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

238 electrophoresis using ethidium bromide staining. Water was used as negative
239 control and normal lung tissue was used as positive control for each subject.
240
241 2.4. Fluorescence in situ hybridization (FISH) for analysis of *P16/CDKN2A*
242 zygosity
243
244 Touch preparations were fixed in cool methanol/acetic acid (3:1 v/v) for 5 min,
245 then air dried. The preparations were treated with RNase A (0.1 mg/ml) and
246 pepsin (50 µg/ml). DNA denaturation was performed by treating slides with 70%
247 formamide in 2× standard saline citrate (SSC) at 72°C for 1 min, followed by
248 immediate immersion in ice-cooled 2 × SSC, and dehydration in 70%, 85% and
249 100% ethanol. Ten µl of denatured commercially available *P16/CDKN2A* specific
250 probe (PONC0921: *P16/CDKN2A* direct-labeled with Rhodamine, and
251 chromosome 9 classical satellite direct-labeled with Fluorescein, Q.BIOgene,
252 Illkirch, France) were applied on the sections, then covered with a 20 × 20 mm
253 coverslip and incubated in a humid chamber at 37°C for 16 h. The slides were
254 washed with 1× washing buffer (0.5 × SSC/ 0.1% SDS) at 65°C for 5 min,
255 immersed in 1 × PBS for 5 min at room temperature according to the
256 manufacturer's recommendations, counterstained with HOECHST 33342
257 Molecular probes (Invitrogen, Cergy-Pontoise, France), and mounted with
258 Vectashield (Vector Laboratories, Paris, France). The slides were observed
259 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%.

2.5. 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 ΔΔCt 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/CKN2A* 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, H_2O and 20 ng of DNA template in a final volume of 10 µl. 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 ΔCt by the average of Ct of non-tumor tissues as calibrator. Quantification was performed by normalizing ΔCt of *P16/CDKN2A* gene results to those of *HAS* gene in the same sample. The value $2 \times 2^{-\Delta\Delta Ct}$ represents an estimation of the gene copy number in tumor tissue. The cut-off value was $2 \times 2^{-\Delta\Delta Ct} \leq 0.60$ for homozygous deletion [33].

2.6. 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).

2.7. 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.

3. RESULTS

3.1. 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.

3.2. $p16^{INK4A}$ 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.

3.3. *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.

3.3.1. *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%)

371 unexposed cases (Odds ratio: 4.43 [95% Confidence Interval: 1.48 – 13.31]).
372 Notably, among samples with a score of 1 for $p16^{INK4A}$ expression, a significantly
373 lower rate of *P16/CDKN2A* methylation was found in the asbestos-exposed
374 group than in the unexposed group (16.7% vs 59.3%; $P = 0.0019$) (Table 3).
375
376 3.3.2. Homozygous deletion and loss of heterozygosity of *P16/CDKN2A* gene
377
378 FISH was performed when sufficient tissue sample was available. Nineteen
379 samples with $p16^{INK4A}$ IHC scores of 1 or 2 and no promoter hypermethylation
380 were studied. This group included 13 asbestos-exposed and 6 unexposed
381 patients. FISH analyses detected 4 homozygous deletions and 15 LOH (Fig. 3).
382 There were homozygous deletions in 2 asbestos-exposed subjects and 2
383 unexposed subjects, and LOH in 11 asbestos-exposed subjects and 4
384 unexposed subjects, respectively. Quantitative PCR analysis confirmed FISH
385 deletion results. After adjustment for age and cumulative tobacco consumption,
386 homozygous deletion and LOH were significantly higher in asbestos-exposed
387 cases (n = 17, 50.0%) than in unexposed cases (n = 10, 24.4%) ($P = 0.0172$;
388 Odds ratio: 3.58 [95% Confidence Interval: 1.25 – 10.23]). Notably, in samples
389 with $p16^{INK4A}$ IHC score 1, a significantly higher rate of *P16/CDKN2A* deletion
390 was found in the asbestos-exposed group than in the unexposed group (66.7%
391 vs 29.2%; $P = 0.0082$). *P16/CDKN2A* deletion was not significantly different
392 between heavy smokers and other subjects ($P = 0.0754$; Odds ratio: 2.59 [95%

393 Confidence Interval: 0.91 – 7.40] after adjustment for age and asbestos
394 exposure).
395
396 3.3.3. *P16/CDKN2A* DNA sequencing analysis
397
398 A total of 5 (6.7%) mutations was found in the whole series, including 2
399 asbestos-exposed cases and 3 unexposed cases, and all mutations were
400 detected in former or current smokers (more than 20 P-Y). All mutations were
401 point mutations resulting in amino acid changes. One mutation was identified in
402 exon 1α, at codon 42 (124A→T,N42Y), changing the encoded asparagine into
403 tyrosine. Four mutations were identified in exon 2, at codon 57 (170C→T,A57V),
404 changing the encoded alanine into valine, at codon 80 (238C→T,R80X),
405 changing arginine into a stop codon, at codon 85 (253G→C,A85P), changing
406 alanine into proline, and at codon 85 (322G→T,D108Y), changing aspartic acid
407 into tyrosine.

4. 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 *P/16CDKN2A* 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*

453 promoter hypermethylation was associated with tobacco smoking, and has been
454 found in some studies to be linearly related to tobacco smoking [16, 19, 37-39].
455 In human malignant mesothelioma, a disease mostly related to asbestos
456 exposure, inactivation of *P16/CDKN2A* mainly occurs by homozygous deletion
457 suggesting a possible relationship with the effect of asbestos fibers [29, 40, 41].
458 Moreover, asbestos fibers are known to produce chromosomal losses and
459 rearrangements in epithelial cells [42-44] and the major type of alteration of the
460 orthologous *p16/Cdkn2a* gene is deletion in asbestos-induced murine
461 mesotheliomas [45, 46]. These results suggest that asbestos fiber exposure
462 could be linked to this type of genetic damage.
463 In conclusion, this study confirms that *P16/CDKN2A* gene inactivation is a
464 recurrent alteration in lung cancer and is the first to emphasize a specific type of
465 damage in relation to the type of carcinogen exposure by studying well
466 characterized populations. While the net frequency of *P16/CDKN2A* alterations
467 was similar in asbestos-exposed and unexposed lung cancer cases, a
468 significantly higher frequency of deletions was found in asbestos-exposed cases
469 compared to unexposed cases. *P16/CDKN2A* hypermethylation was not
470 different between the two groups, but was enhanced as a result of tobacco
471 consumption. These results provide new data in the field of biomarkers of
472 exposure to carcinogens.
473

CONFLICT OF INTEREST STATEMENT

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

ACKNOWLEDGMENTS

Grant support: This work was supported by funds from INSERM, Chancellerie des Universités de Paris (Legs POIX) and University of Paris 12, grants from Ministère de l'Emploi et de la Solidarité, Agence National de la Recherche (n° 05 9 31/ANR) and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET) (n° RD-2004-015).

Jinhui Wang was a fellow from University of Paris 12.

The authors would like to thank Thérèse de Gasté for her technical assistance, Drs Elisabeth Dulmet and Vincent De Montpreville (Centre Chirurgical Marie Lannelongue), Dr Redha Souilamas and Prof. Marc Riquet (Hôpital Européen Georges Pompidou), Xavier Janson (Laboratoire d'Etude des Particules Inhalées) and Soizick Chamming's (Institut Interuniversitaire de Médecine du Travail de Paris – Ile de France) for their participation in the study.

REFERENCES

1. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008; 359: 1367-1380.

2. Boffetta P. Human cancer from environmental pollutants: the epidemiological evidence. Mutat Res 2006; 608: 157-162.

3. Hecht SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 1999; 91: 1194-1210.

4. Axelson O. Alternative for estimating the burden of lung cancer from occupational exposures--some calculations based on data from Swedish men. Scand J Work Environ Health 2002; 28: 58-63.

5. Nurminen M, Karjalainen A. Epidemiologic estimate of the proportion of fatalities related to occupational factors in Finland. Scand J Work Environ Health 2001; 27: 161-213.

6. Selikoff IJ, Hammond EC. Asbestos and smoking. JAMA 1979; 242: 458-459.

7. Le Calvez F, Mukeria A, Hunt JD, Kelm O, Hung RJ, Taniere P, Brennan P, Boffetta P, Zaridze DG, Hainaut P. TP53 and KRAS mutation load and types in

509 lung cancers in relation to tobacco smoke: distinct patterns in never, former, and
510 current smokers. Cancer Res 2005; 65: 5076-5083.

511 8. Mounawar M, Mukeria A, Le Calvez F, Hung RJ, Renard H, Cortot A, Bollart
512 C, Zaridze D, Brennan P, Boffetta P, Brambilla E, Hainaut P. Patterns of EGFR,
513 HER2, TP53, and KRAS mutations of p14arf expression in non-small cell lung
514 cancers in relation to smoking history. Cancer Res 2007; 67: 5667-5672.

515 9. Subramanian J, Govindan R. Molecular genetics of lung cancer in people
516 who have never smoked. Lancet Oncol 2008; 9: 676-682.

517 10. Gazzeri S, Gouyer V, Vour'ch C, Brambilla C, Brambilla E. Mechanisms of
518 p16INK4A inactivation in non small-cell lung cancers. Oncogene 1998; 16:
519 497-504.

520 11. Hommura F, Dosaka-Akita H, Kinoshita I, Mishina T, Hiroumi H, Ogura S,
521 Katoh H, Kawakami Y. Predictive value of expression of p16INK4A,
522 retinoblastoma and p53 proteins for the prognosis of non-small-cell lung cancers.
523 Br J Cancer 1999; 81: 696-701.

524 12. Geradts J, Fong KM, Zimmerman PV, Maynard R, Minna JD. Correlation of
525 abnormal RB, p16ink4a, and p53 expression with 3p loss of heterozygosity,
526 other genetic abnormalities, and clinical features in 103 primary non-small cell

527 lung cancers. Clin Cancer Res 1999; 5: 791-800.

528 13. Lamy A, Sesboue R, Bourguignon J, Dautreaux B, Metayer J, Frebourg T,
529 Thiberville L. Aberrant methylation of the CDKN2a/p16INK4a gene promoter
530 region in preinvasive bronchial lesions: a prospective study in high-risk patients
531 without invasive cancer. Int J Cancer 2002; 100: 189-193.

532 14. Liu Y, An Q, Li L, Zhang D, Huang J, Feng X, Cheng S, Gao Y.
533 Hypermethylation of p16INK4a in Chinese lung cancer patients: biological and
534 clinical implications. Carcinogenesis 2003; 24: 1897-1901.

535 15. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile
536 of human cancer. Cancer Res 2001; 61: 3225-3229.

537 16. Kim DH, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, Mark EJ,
538 Kelsey KT. p16(INK4a) and histology-specific methylation of CpG islands by
539 exposure to tobacco smoke in non-small cell lung cancer. Cancer Res 2001; 61:
540 3419-3424.

541 17. Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation
542 analysis: a powerful new tool for lung cancer diagnosis. Oncogene 2002; 21:
543 5450-5461.

544 18. Jarmalaite S, Kannio A, Anttila S, Lazutka JR, Husgafvel-Pursiainen K.

545 Aberrant p16 promoter methylation in smokers and former smokers with
546 nonsmall cell lung cancer. Int J Cancer 2003; 106: 913-918.

547 19. Gu J, Berman D, Lu C, Wistuba, II, Roth JA, Frazier M, Spitz MR, Wu X.
548 Aberrant promoter methylation profile and association with survival in patients
549 with non-small cell lung cancer. Clin Cancer Res 2006; 12: 7329-7338.

550 20. Nakata S, Sugio K, Uramoto H, Oyama T, Hanagiri T, Morita M, Yasumoto K.
551 The methylation status and protein expression of CDH1, p16(INK4A), and fragile
552 histidine triad in nonsmall cell lung carcinoma: epigenetic silencing, clinical
553 features, and prognostic significance. Cancer 2006; 106: 2190-2199.

554 21. Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its
555 relatives. Biochim Biophys Acta 1998; 1378: F115-177.

556 22. Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y, Motoyama T.
557 Frequent epigenetic silencing of the p16 gene in non-small cell lung cancers of
558 tobacco smokers. Jpn J Cancer Res 2002; 93: 1107-1113.

559 23. Iwakawa R, Kohno T, Anami Y, Noguchi M, Suzuki K, Matsuno Y, Mishima K,
560 Nishikawa R, Tashiro F, Yokota J. Association of p16 homozygous deletions with
561 clinicopathologic characteristics and EGFR/KRAS/p53 mutations in lung
562 adenocarcinoma. Clin Cancer Res 2008; 14: 3746-3753.

563 24. Sanchez-Cespedes M, Decker PA, Doffek KM, Esteller M, Westra WH, Alawi
564 EA, Herman JG, Demeure MJ, Sidransky D, Ahrendt SA. Increased loss of
565 chromosome 9p21 but not p16 inactivation in primary non-small cell lung cancer
566 from smokers. Cancer Res 2001; 61: 2092-2096.

567 25. Kraunz KS, Nelson HH, Lemos M, Godleski JJ, Wiencke JK, Kelsey KT.
568 Homozygous deletion of p16INK4a and tobacco carcinogen exposure in
569 nonsmall cell lung cancer. Int J Cancer 2006; 118: 1364-1369.

570 26. Dammann R, Strunnikova M, Schagdarsurengin U, Rastetter M, Papritz M,
571 Hattenhorst UE, Hofmann HS, Silber RE, Burdach S, Hansen G. CpG island
572 methylation and expression of tumour-associated genes in lung carcinoma. Eur
573 J Cancer 2005; 41: 1223-1236.

574 27. Lee WC, Testa JR. Somatic genetic alterations in human malignant
575 mesothelioma (review). Int J Oncol 1999; 14: 181-188.

576 28. Hirao T, Bueno R, Chen CJ, Gordon GJ, Heilig E, Kelsey KT. Alterations of
577 the p16(INK4) locus in human malignant mesothelial tumors. Carcinogenesis
578 2002; 23: 1127-1130.

579 29. Ladanyi M. Implications of P16/CDKN2A deletion in pleural mesotheliomas.
580 Lung Cancer 2005; 49 Suppl 1: S95-98.

581 30. Pairon JC, Orlowski E, Iwatsubo Y, Billon-Galland MA, Dufour G,
582 Chamming's S, Archambault C, Bignon J, Brochard P. Pleural mesothelioma and
583 exposure to asbestos: evaluation from work histories and analysis of asbestos
584 bodies in bronchoalveolar lavage fluid or lung tissue in 131 patients. Occup
585 Environ Med 1994; 51: 244-249.

586 31. De Vuyst P, Karjalainen A, Dumortier P, Pairon JC, Monso E, Brochard P,
587 Teschler H, Tossavainen A, Gibbs A. Guidelines for mineral fibre analyses in
588 biological samples: report of the ERS Working Group. European Respiratory
589 Society. Eur Respir J 1998; 11: 1416-1426.

590 32. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB.
591 Methylation-specific PCR: a novel PCR assay for methylation status of CpG
592 islands. Proc Natl Acad Sci U S A 1996; 93: 9821-9826.

593 33. Blons H, Pallier K, Le Corre D, Danel C, Tremblay-Gravel M, Houdayer C,
594 Fabre-Guillevin E, Riquet M, Dessen P, Laurent-Puig P. Genome wide SNP
595 comparative analysis between EGFR and KRAS mutated NSCLC and
596 characterization of two models of oncogenic cooperation in non-small cell lung
597 carcinoma. BMC Med Genomics 2008; 1: 25.

598 34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using
599 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;

600 25: 402-408.

601 35. Kohno T, Yokota J. How many tumor suppressor genes are involved in
602 human lung carcinogenesis? Carcinogenesis 1999; 20: 1403-1410.

603 36. Kashiwabara K, Oyama T, Sano T, Fukuda T, Nakajima T. Correlation
604 between methylation status of the p16/CDKN2 gene and the expression of p16
605 and Rb proteins in primary non-small cell lung cancers. Int J Cancer 1998; 79:
606 215-220.

607 37. Soria JC, Rodriguez M, Liu DD, Lee JJ, Hong WK, Mao L. Aberrant promoter
608 methylation of multiple genes in bronchial brush samples from former cigarette
609 smokers. Cancer Res 2002; 62: 351-355.

610 38. Belinsky SA, Palmisano WA, Gilliland FD, Crooks LA, Divine KK, Winters SA,
611 Grimes MJ, Harms HJ, Tellez CS, Smith TM, Moots PP, Lechner JF, Stidley CA,
612 Crowell RE. Aberrant promoter methylation in bronchial epithelium and sputum
613 from current and former smokers. Cancer Res 2002; 62: 2370-2377.

614 39. Toyooka S, Suzuki M, Tsuda T, Toyooka KO, Maruyama R, Tsukuda K,
615 Fukuyama Y, Iizasa T, Fujisawa T, Shimizu N, Minna JD, Gazdar AF. Dose effect
616 of smoking on aberrant methylation in non-small cell lung cancers. Int J Cancer
617 2004; 110: 462-464.

618 40. Illei PB, Rusch VW, Zakowski MF, Ladanyi M. Homozygous deletion of
619 CDKN2A and codeletion of the methylthioadenosine phosphorylase gene in the
620 majority of pleural mesotheliomas. Clin Cancer Res 2003; 9: 2108-2113.

621 41. Musti M, Kettunen E, Dragonieri S, Lindholm P, Cavone D, Serio G, Knuutila
622 S. Cytogenetic and molecular genetic changes in malignant mesothelioma.
623 Cancer Genet Cytogenet 2006; 170: 9-15.

624 42. Yegles M, Janson X, Dong HY, Renier A, Jaurand MC. Role of fibre
625 characteristics on cytotoxicity and induction of anaphase/telophase aberrations
626 in rat pleural mesothelial cells in vitro: correlations with in vivo animal findings.
627 Carcinogenesis 1995; 16: 2751-2758.

628 43. Murthy SS, Testa JR. Asbestos, chromosomal deletions, and tumor
629 suppressor gene alterations in human malignant mesothelioma. J Cell Physiol
630 1999; 180: 150-157.

631 44. Dusinska M, Collins A, Kazimirova A, Barancokova M, Harrington V,
632 Volkovova K, Staruchova M, Horska A, Wsolova L, Kocan A, Petrik J, Machata M,
633 Ratcliffe B, Kyrtopoulos S. Genotoxic effects of asbestos in humans. Mutat Res
634 2004; 553: 91-102.

635 45. Lecomte C, Andujar P, Renier A, Kheuang L, Abramowski V, Mellottee L,

636 Fleury-Feith J, Zucman-Rossi J, Giovannini M, Jaurand MC. Similar tumor
637 suppressor gene alteration profiles in asbestos-induced murine and human
638 mesothelioma. Cell Cycle 2005; 4: 1862-1869.

639 46. Altomare DA, Vaslet CA, Skele KL, De Rienzo A, Devarajan K, Jhanwar SC,
640 McClatchey AI, Kane AB, Testa JR. A mouse model recapitulating molecular
641 features of human mesothelioma. Cancer Res 2005; 65: 8090-8095.

642 47. Soufir N, Avril MF, Chompret A, Demenais F, Bombled J, Spatz A,
643 Stoppa-Lyonnet D, Benard J, Bressac-de Paillerets B. Prevalence of p16 and
644 CDK4 germline mutations in 48 melanoma-prone families in France. The French
645 Familial Melanoma Study Group. Hum Mol Genet 1998; 7: 209-216.

646 48. Soufir N, Lacapere JJ, Bertrand G, Matichard E, Meziani R, Mirebeau D,
647 Descamps V, Gerard B, Archimbaud A, Ollivaud L, Bouscarat F, Baccard M,
648 Lanternier G, Saiag P, Lebbe C, Basset-Seguin N, Crickx B, Cave H,
649 Grandchamp B. Germline mutations of the INK4a-ARF gene in patients with
650 suspected genetic predisposition to melanoma. Br J Cancer 2004; 90: 503-509.
651