Differential mutation profiles and similar intronic TP53 polymorphisms in asbestos-related lung cancer and pleural mesothelioma

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Given the interest in defining biomarkers of asbestos exposure and to provide insights into asbestos-related and cell-specific mechanisms of neoplasia, the identification of gene alterations in asbestos-related cancers can help to a better understanding of exposure risk. To understand the aetiology of asbestos-induced malignancies and to increase our knowledge of mesothelial carcinogenesis, we compared genetic alterations in relevant cancer genes between lung cancers, induced by asbestos and tobacco smoke, and malignant pleural mesothelioma (MPM), a cancer related to asbestos, but not to tobacco smoke. TP53, KRAS, EGFR and NF2 gene alteration analyses were performed in 90 non-small cell lung cancer (NSCLC) patients, 50 asbestos-exposed and 50 unexposed patients, matched for age, gender, histology and smoking habits. Detailed assessment of asbestos exposure was based on both specific questionnaires and asbestos body quantification in lung tissue. Genetic analyses were also performed in 34 MPM patients. TP53, EGFR and KRAS mutations were found in NSCLC with no link with asbestos exposure. NF2 was only altered in MPM. Significant enhancement of TP53 G:C to T:A transversions was found in NSCLC from asbestos-exposed patients when compared with unexposed patients (P = 0.037). Interestingly, TP53 polymorphisms in intron 7 (rs12947788 and rs12951053) were more frequently identified in asbestos-exposed NSCLC (P = 0.046) and MPM patients than in unexposed patients (P < 0.001 and P = 0.012, respectively). These results emphasize distinct genetic alterations between asbestos-related thoracic tumours, but identify common potential susceptibility factors, i.e. single nucleotide polymorphisms in intron 7 of TP53. While genetic changes in NSCLC are dominated by the effects of tobacco smoke, the increase of transversions in TP53 gene is consistent with a synergistic effect of asbestos. These results may help to define cell-dependent mechanisms of action of asbestos and identify susceptibility factors to asbestos.

Introduction

Asbestos is a well-known carcinogen and an important risk factor for lung cancer and malignant pleural mesothelioma (MPM) (1). Recently, the International Agency for Research on Cancer considered that there is also sufficient evidence that asbestos fibre exposure is a risk factor in laryngeal and ovarian cancer (2,3). Although asbestos was banned several years ago in most developed countries, it remains largely used in other countries and continues to pose health problems due to the long latency period (20–40 years). Concerns about environmental exposure have been raised (2,4). While occupational exposure to asbestos is associated with an increased risk of lung cancer, both occupational and environmental exposures are associated with a risk of MPM (5).

Knowledge of genetic alterations in human cancer has several impacts by identifying biomarkers of exposure and susceptibility factors and guiding the development of targeted cancer therapeutic strategies. The definition of biomarkers of asbestos exposure is of interest to provide insights into asbestos-related and cell-specific mechanisms of neoplasia, and the identification of gene alterations in asbestos-related cancers can help to a better understanding of exposure risk. Genetic analyses of lung cancer have shown a relationship between specific gene mutations and various environmental and occupational factors, and asbestos-related gene alterations in human cancers have been identified in lung cancer and MPM (6,7).

Epidemiological studies have shown that the fraction of lung cancer risk attributable to occupational asbestos exposure and tobacco smoking is about 5 and 90%, respectively (4,8). Genetic susceptibility and other environmental and occupational factors also contribute to the development of lung cancer, such as radon, hexavalent chromium, arsenic and polycyclic aromatic hydrocarbons (PAHs) (2,9). While tobacco smoking is a major risk factor for lung cancer and asbestos fibres a lower risk factor, asbestos fibres constitute the...
major occupational risk factor for human MPM, with no link with tobacco smoking (10).

As the genes and biological pathways altered in cancer cells depend on both the carcinogen and the cell type, comparison between gene alterations in lung cancer from asbestos-exposed patients and MPM patients would provide insight into asbestos-related and cell-specific mechanisms of neoplasia. The purpose of this study was to characterise molecular alterations related to asbestos exposure, in order to more clearly understand the mechanism of asbestos oncogenesis and to identify new biomarkers in non-small cell lung cancers (NSCLC). KRAS, EGFR, NF2 and TP53 genes were studied in 100 NSCLC patients with well-defined smoking habits, detailed assessment of asbestos exposure based on both occupational questionnaire and determination of asbestos bodies (AB) in lung tissue and in 34 MPM patients.

Patients and methods

NSCLC patients

Subjects were selected from consecutive primary NSCLC cases after surgical resection in five French hospitals (Centre Hospitalier Intercommunal, Créteil; Hôpital Européen Georges Pompidou, Paris; Institut Mutualiste Montsouris, Paris; Centre Hospitalier Universitaire, Caen and Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson) from 1994 through 2007. NSCLC tissue samples were snap-frozen in liquid nitrogen after surgical resection and stored at −80°C until use. The study was approved by the local Ethics Committee and all patients provided written informed consent. Detailed information describing the tumours was obtained from pathology reports. The eligibility criteria were (i) lung tumour histology, (ii) absence of neoadjuvant chemotherapy or radiotherapy, (iii) availability of both normal and neoplastic lung tissue, (iv) data on asbestos exposure history including quantification of AB in lung tissue and interviewer-administered questionnaire and (v) data on smoking habits (11–13). From these five eligibility criteria, 350 NSCLC cases were recruited. These cases were representative of the French lung cancer population in terms of age, gender, histology and tobacco smoking characteristics (10).

MPM primary cell cultures

Thirty-four human cell cultures were obtained from surgical resections, pleural biopsies or malignant pleural fluids of confirmed MPM cases, provided by four French hospitals (Centre Hospitalier Intercommunal, Créteil; Hôpital Européen Georges Pompidou, Paris; Centre Hospitalier Universitaire, Caen and Centre Hospitalier Universitaire, Marseille) from 1985 through 2007, as previously described (15). Detailed information describing MPM histology was obtained from pathology reports. An additional series of 25 MPM in TP53 intron 7 and NF2 mutations. Cells were grown in RPMI 1640 medium, as described elsewhere (15). Prior to DNA extraction, confluent cultures between 7 and 12 passages were washed with phosphate-buffered saline (Invitrogen, Cergy-Pontoise, France) and stored at −80°C.

Smoking habits and asbestos exposure evaluation

In NSCLC patients, information regarding smoking status was obtained from an interviewer-administered questionnaire to assess smoking classification, 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, expressed as pack-years (P-Y).

Asbestos exposure was evaluated by an interviewer-administered specific questionnaire and by AB quantification in the lung parenchyma for NSCLC patients. The questionnaire comprised complete job history to determine past occupational exposure and included queries to estimate domestic and environmental exposure, 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 AB counts. AB quantification was performed as previously described (11–13). Definite occupational exposure to asbestos was ascertained if the questionnaire concluded on occupational exposure for more than 10 years, 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 (12,13). Finally, only NSCLC cases with definite occupational exposure to asbestos were selected and, consequently, 50 asbestos-exposed NSCLC cases (E+) were retained. Unexposed subjects (139) 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. Fifty unexposed NSCLC cases (E−) were matched to the asbestos-exposed NSCLC cases for age, gender, histological type and cumulative tobacco consumption. For age, the range was ±6 years, except for patients aged 70 years or older, who were matched together. For tobacco consumption, three classes were defined: low: 0–5 P-Y; medium to high: 6–60 P-Y (±10 P-Y); very high: >61 P-Y.

The level of asbestos exposure in MPM patients was based on clinical reports. An asbestos cumulative exposure index (CEI) was estimated for each subject by an occupational hygienist. Four levels of CEI were defined for each subject and classified into four classes: high (defined as definite or probable, continuous or discontinuous and high or moderate occupational exposure for at least 10 years), moderate (all other occupational exposure), low (possible, sporadic and low exposure for at least 10 years, or passive exposure) and null (no exposure).

Mutational analysis of TP53, NF2, KRAS and EGFR genes

Mutations of TP53 exons 2–11, NF2 exons 1–17, KRAS exons 1 and 2 and EGFR exons 18–21 were screened by DNA sequencing. Genomic DNA was extracted from frozen NSCLC tumours and MPM primary cell cultures using a standard phenol–chloroform extraction procedure. DNA amplification was primed by polymerase chain reaction (PCR) with a combination of forward and reverse primers located in the introns surrounding the sequenced exon (supplementary Table 1, available at Mutagenesis Online) and Taq polymerase Hot Start (Qiagen, Courtaboeuf, France) on a Gene Amp 9700 apparatus (Perkin-Elmer, Courtaboeuf, France). PCR products were purified through Millipore genomic columns (Prolabo, Paris, France), checked for quality and quantified prior to sequencing. Sequencing PCR was performed on purified PCR products and Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Courtaboeuf, France) on a Gene Amp 9700 apparatus (Perkin-Elmer). PCR reactions were carried out for 25 cycles including denaturation at 96°C for 40 s, annealing at 55°C for 5 s and extension at 60°C for 4 min. Sequencing PCR products were purified through Sephadex G50 fine resin (GE Healthcare Biosciences AB, Uppsala, Sweden) in Multiscreen Millipore columns (Prolabo) and analysed on an ABI PRISM 3100 Genetic analyser (Applied Biosystems). Mutational analysis was performed using Sequencer 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA) and verified by independent amplification and sequencing. Genetic alterations were validated by an independent sequencing experiment in the DNA complementary strand.

Statistical analyses

Clinicopathological characteristics of asbestos-exposed and unexposed NSCLC cases were compared using Wilcoxon test for quantitative variables, and Pearson’s chi-square test for qualitative variables (Fisher’s exact test was used for qualitative variables where chi-square test was not a valid test, i.e. expected cell count was <5). Comparisons were also based on mutational analysis and polymorphism status, after stratification according to the various relevant clinicopathological subtypes. Multivariate analyses were also performed using logistic models on TP53 SNPs. A P value less than 0.05 was considered statistically significant. Statistical analyses were performed using ‘Statistical Analysis System’ software (SAS v9.1 Inc., Cary, NC, USA).

Results

NSCLC and MPM populations

Clinicopathological data of NSCLC patients are reported in Table I. As both populations were matched, no significant differences were observed between asbestos-exposed and unexposed NSCLC groups for age, gender, histological subtype and smoking habits (smoking status, age at onset of tobacco consumption, duration of smoking and cumulative tobacco consumption). MPM cases were mainly males (88.2%) with a mean age of 68.6±5.9 years in females and 61.2±11.6 years in males. The most common histological subtype of MPM was epithelioid (79.4%), while biphasic, sarcomatoid and desmoplastic forms represented 11.8, 5.9 and 2.9% of MPM cases, respectively. Asbestos CEI was high in 18 MPM cases.
Genetic aspects of asbestos-related thoracic tumours

No KRAS mutation was detected among MPM cases. No mutation was detected in non-smokers (7.7%), while no evidence of asbestos exposure was found in 5 cases (14.7%), while moderate in 6 cases (17.6%) and low in 5 cases (14.7%), while moderate in 6 cases (17.6%) and low in 5 cases (14.7%), while moderate in 6 cases (17.6%) and low in 5 cases (14.7%).

EGFR DNA sequencing analysis

EGFR mutations were found in 8% of the whole NSCLC population (Table I). The mutation rate was higher in women than in men: 25% (3/12) and 5.7% (5/88), respectively, at the LIMIT of statistical significance (P = 0.053). When smoking was considered, the percentage of EGFR mutations was significantly higher in non-smokers than in current plus former smokers: 30.8% (4/13) and 4.6% (4/87), respectively (P = 0.009). In contrast, no significant difference was observed according to histological subtype or asbestos exposure. EGFR mutations were detected in both asbestos-exposed and unexposed cases, in two and six cases, respectively. However, after stratification for gender, a higher EGFR mutation rate was found in female asbestos-unexposed cases, all of whom being non-smokers (Table I). No EGFR mutation was detected among MPM cases.

NF2 DNA sequencing analysis

No NF2 mutation was detected in either exposed or unexposed NSCLC patients, while 38.2% (13/34) of MPM cases showed NF2 gene mutations (supplementary Table III, available at Mutagenesis Online). The mutations consisted of large deletions of one or several exons and point mutations. Deletions were found in 10 (29.4%) MPM cases and point mutations were found in 4 (11.8%) cases. One deletion and one point mutation were both present in one MPM. In MPM patients, no significant difference was observed considering gender, age and histological subtypes. A significant link between NF2 mutations and asbestos exposure was detected (P = 0.043), but it was not confirmed in a largest series of 59 MPM cases (P = 0.372).

TP53 DNA sequencing analysis

TP53 mutations were found in 39% of the overall NSCLC population (Table I). No significant difference was observed between males and females. In contrast, a statistically significant difference was found between histological types of NSCLC. TP53 mutations were more frequent in squamous cell carcinomas (52.3%) than in lung adenocarcinomas (29.3%) (P = 0.02). Almost all mutations (36/39) were detected in former or current smokers (more than 10 P-Y). The frequencies of TP53 mutations according to smoking status were different between non-smokers (15.4%) versus former and current smokers (42.5%), but this difference was at the limit of statistical significance (P = 0.061). No significant difference was observed according to asbestos exposure: 19 mutations (38%) were found in asbestos-exposed cases and 20 mutations (40%) were found in unexposed cases (Table I). This lack of significance persisted after stratification for gender, histological type and smoking status.

Sequencing analysis revealed different types of mutations. The spectrum of TP53 mutations is reported in Table I.

<table>
<thead>
<tr>
<th>Table I. Clinicopathological characteristics of NSCLC cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSCLC cases</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>All cases, n</td>
</tr>
<tr>
<td>Mean age (years ± SD)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Histology, n (%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
</tr>
<tr>
<td>Current smokers</td>
</tr>
<tr>
<td>Former smokers</td>
</tr>
<tr>
<td>Never smokers</td>
</tr>
<tr>
<td>Cumulative tobacco consumption (P-Y ± SD)</td>
</tr>
<tr>
<td>Age at onset (years ± SD)</td>
</tr>
<tr>
<td>Duration (years ± SD)</td>
</tr>
<tr>
<td>Asbestos exposure Questionnaire (n)</td>
</tr>
<tr>
<td>AB per gram of dry lung tissue, median (min–max)</td>
</tr>
</tbody>
</table>

AB: asbestos bodies; max: maximum; min: minimum; ns: not significant, i.e. P ≥ 0.05; NSCLC: non-small cell lung cancer; P-Y: pack-years; SD: standard deviation.

*Definite occupational exposure to asbestos of at least 10 years.

*No occupational, domestic and environmental exposure to asbestos.

*Wilcoxon, Pearson’s chi-square and Fisher’s exact tests as appropriate.

(53.0%), moderate in 6 cases (17.6%) and low in 5 cases (14.7%), while moderate in 6 cases (17.6%) and low in 5 cases (14.7%), while moderate in 6 cases (17.6%) and low in 5 cases (14.7%).

KRAS DNA sequencing analysis

KRAS mutations were found in 13% of the NSCLC population (Table I): 12 in codon 12 and 1 in codon 19. No significant difference was observed between men and women. A significantly higher rate of KRAS mutations was found in adenocarcinoma (19%; 11/58) compared with squamous cell carcinoma (4.8%; 2/42) (P < 0.037). Regarding smoking status, only 1 (7.7%) KRAS mutation was found in non-smokers compared with 12 (13.8%) in current and former smokers, but the difference was not significant. No significant difference was observed according to asbestos exposure. Five KRAS mutations were detected in asbestos-exposed cases versus eight in unexposed cases. Stratification for gender, histological type and smoking status did not modify the statistical result (Table I). No KRAS mutation was detected among MPM cases.

NF2 DNA sequencing analysis

No NF2 mutation was detected in either exposed or unexposed NSCLC patients, while 38.2% (13/34) of MPM cases showed NF2 gene mutations (supplementary Table III, available at Mutagenesis Online). The mutations consisted of large deletions of one or several exons and point mutations. Deletions were found in 10 (29.4%) MPM cases and point mutations were found in 4 (11.8%) cases. One deletion and one point mutation were both present in one MPM. In MPM patients, no significant difference was observed considering gender, age and histological subtypes. A significant link between NF2 mutations and asbestos exposure was detected (P = 0.043), but it was not confirmed in a largest series of 59 MPM cases (P = 0.372).

TP53 DNA sequencing analysis

TP53 mutations were found in 39% of the overall NSCLC population (Table I). No significant difference was observed between males and females. In contrast, a statistically significant difference was found between histological types of NSCLC. TP53 mutations were more frequent in squamous cell carcinomas (52.3%) than in lung adenocarcinomas (29.3%) (P = 0.02). Almost all mutations (36/39) were detected in former or current smokers (more than 10 P-Y). The frequencies of TP53 mutations according to smoking status were different between non-smokers (15.4%) versus former and current smokers (42.5%), but this difference was at the limit of statistical significance (P = 0.061). No significant difference was observed according to asbestos exposure: 19 mutations (38%) were found in asbestos-exposed cases and 20 mutations (40%) were found in unexposed cases (Table I). This lack of significance persisted after stratification for gender, histological type and smoking status.

Sequencing analysis revealed different types of mutations. The spectrum of TP53 mutations is reported in Table I.
Table II. TP53, EGFR and KRAS gene mutation rates according to gender, histological characteristics, smoking habits and asbestos exposure in NSCLC

<table>
<thead>
<tr>
<th>TP53 gene mutations</th>
<th>EGFR gene mutations</th>
<th>KRAS gene mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Asbestos-exposed cases</td>
</tr>
<tr>
<td>All cases (n = 100)</td>
<td>39/100</td>
<td>19/50</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 88)</td>
<td>36/88</td>
<td>17/44</td>
</tr>
<tr>
<td>Female (n = 12)</td>
<td>3/12</td>
<td>2/6</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC (n = 42)</td>
<td>22/42</td>
<td>11/21</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers (n = 13)</td>
<td>2/13*</td>
<td>1/7</td>
</tr>
<tr>
<td>Current smokers</td>
<td>21/45</td>
<td>7/17</td>
</tr>
<tr>
<td>(n = 45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former smokers</td>
<td>16/42</td>
<td>11/26</td>
</tr>
</tbody>
</table>

ADC: adenocarcinoma; SCC: squamous cell carcinoma.

*Pearson’s chi-square and Fisher’s exact tests as appropriate, ns: not significant, i.e. P ≥ 0.05.

Table III. Spectrum of TP53 mutations in NSCLC

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Asbestos-exposed NSCLC cases</th>
<th>Unexposed NSCLC cases</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with TP53 mutations</td>
<td>19*</td>
<td>20*</td>
<td>ns</td>
</tr>
<tr>
<td>Type of TP53 mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Point mutations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transversion</td>
<td>14</td>
<td>11</td>
<td>ns</td>
</tr>
<tr>
<td>G:C to T:A</td>
<td>13</td>
<td>7</td>
<td>0.037</td>
</tr>
<tr>
<td>G:C to T:A in non-transcribed DNA strand</td>
<td>12</td>
<td>6</td>
<td>0.038</td>
</tr>
<tr>
<td>Transition</td>
<td>4</td>
<td>5</td>
<td>ns</td>
</tr>
<tr>
<td>Deletion or insertion</td>
<td>3</td>
<td>5</td>
<td>ns</td>
</tr>
</tbody>
</table>

NSCLC: non-small cell lung cancer.

Two different types of mutations were found in the same case: ‘one patient had a deletion and a transversion and another had a transversion and a transition; ‘one patient had an insertion and a transversion.

*Pearson’s chi-square and Fisher’s exact tests as appropriate, ns: not significant, i.e. P ≥ 0.05.

III. Figure 1 and supplementary Table IIIs, available at Mutagenesis Online. Transversion was the most frequent type of mutation in both asbestos-exposed and unexposed patients (Figure 1). The frequency of G:C to T:A transversions over the total TP53 mutations was more frequent in NSCLC from asbestos-exposed patients (59%) than from unexposed patients (33%) (P = 0.138) (Figure 1). The percentage of cases with G:C to T:A transversions was not significantly different in the asbestos-exposed group (26%) compared with the unexposed group (14%) (P = 0.134) taking into account the total number of cases (Table III). However, when we only considered mutated cases, the number of patients with at least one G:C to T:A transversion in TP53 was significantly higher in the asbestos-exposed NSCLC group than in the unexposed NSCLC group: 13/19 (68%) and 7/20 (35%) cases, respectively (P = 0.037) (Table III). Moreover, more than 90% of G to T transversions occurred in the non-transcribed DNA strand, and transversions in this strand were also more frequent in the asbestos-exposed NSCLC group than in the unexposed NSCLC group: 12/19 (63%) and 6/20 (30%) cases, respectively (P = 0.038).

Four TP53 mutations (11.8%) were found in the MPM population. All were point mutations: one transition in exon 5, at codon 173 (517G>A,V173M), changing the encoded valine into methionine; two transitions in exon 7, at codon 248 (742C>T,R248W) and (743G>A,R248Q), changing arginine into tryptophan and glutamine, respectively and one transversion at codon 249 (747G>T,R249S), changing arginine into serine (supplementary Table IIIs, available at Mutagenesis Online).

TP53 polymorphisms
TP53 DNA sequencing in NSCLC detected SNPs at six different sites in TP53, described in NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/snp). Only two SNPs found in intron 7 of TP53 (rs129477788: 979+72C>T and rs12951053: 979+92T>G) showed significantly different frequency between asbestos-exposed and unexposed NSCLC cases. Minor alleles (A for rs12947788 and C for rs12951053) were associated in nine cases (supplementary Table IIIs, available at Mutagenesis Online). The minor allele was always associated with the major allele for both SNP (heterozygous patients).
The minor allele for rs12947788 SNP was detected in 10% of NSCLC cases, predominantly in asbestos-exposed cases (16%) in comparison with unexposed cases (4%) \( (P = 0.046) \) (Figure 2). After adjustment for age, gender, tobacco status and histological type, the difference remained statistically significant \( (P = 0.046) \).

Similarly, the minor allele for rs12951053 SNP was found in 10% of NSCLC cases, predominantly in asbestos-exposed cases (16%) in comparison with unexposed cases (4%) \( (P = 0.046) \) (Figure 2). As the logistic model failed to converge in rs12951053 SNP analysis, data could not be adjusted for age, gender, tobacco status and histological type. However, adjusting for age and histology, the association between rs12951053 SNP and asbestos-exposed NSCLC cases was significant \( (P = 0.048) \).

Interestingly, two SNPs similar to those observed in NSCLC were frequently observed in intron 7 of \( TP53 \) in the series of the 34 MPM. The rs12947788 and rs12951053 SNP minor alleles were observed only in asbestos-exposed MPM patients with high CEI, but not in patients with moderate/low CEI and unexposed patients \( (P = 0.01 \) and \( P = 0.07 \), respectively) (Figure 2). For both SNPs, the minor alleles (A for rs12947788 and C for rs12951053) were either associated with the major alleles (heterozygous patients) or present alone (homozygous or hemizygous patients). The minor allele of rs12951053 SNP was always associated with the minor allele of rs12947788 SNP (supplementary Table IIIs).

### Discussion

The purpose of this study was to characterise molecular alterations and biomarkers related to asbestos exposure. High frequencies of \( TP53 \), \( EGFR \) and \( KRAS \) mutations were found in NSCLC but not in MPM, while \( NF2 \) was only altered in MPM. Here, we show that two \( TP53 \) polymorphisms located in intron 7 were identified in both MPM and asbestos-exposed NSCLC.

Evaluation of asbestos exposure differs between NSCLC and MPM. As NSCLC is a multifactorial disease, mainly linked to tobacco smoking and as asbestos-related lung cancer
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A. rs12947788 single nucleotide polymorphism

![Graph showing the distribution of SNP rs12947788 in different exposure groups.]

B. rs12951053 single nucleotide polymorphism

![Graph showing the distribution of SNP rs12951053 in different exposure groups.]

Fig. 2. SNPs in *TP53* intron 7 in NSCLC and MPM populations according to asbestos exposure. (A) rs12947788 and (B) rs12951053.

are generally related to high level of exposure, it is generally recommended to evaluate cumulative asbestos exposure by two complementary approaches: (i) a specific questionnaire carried out by an occupational hygienist or by the use of job/exposure matrices and (ii) a mineralogical analysis of biological samples (bronchoalveolar lavage or lung tissue) (12). It should be noted that a negative result of a mineralogical quantification would not definitively exclude a past asbestos exposure. In contrast, MPM is a disease mainly linked to asbestos exposure even at low levels of exposure. Consequently, mineralogical analyses are not required in the MPM clinical management (16,17). Consequently, cumulative asbestos exposure evaluation is often less accurate in MPM. Moreover, in MPM, lung tissue is not often available for mineralogical analyses, as MPM diagnosis or treatment very rarely includes a surgical lung resection. In our study, detailed occupational information was available in clinical reports and was estimated to be sufficient for occupational hygienist’s evaluation in order to classify the MPM cases in four CEI (null, low, moderate and high) taking into account probability, frequency, intensity and duration of exposure.

So far, few or conflicting data have been published concerning identification of specific gene mutations, which might be the signature of asbestos in thoracic cancers. Limited data have been reported on *KRAS* mutations in asbestos-exposed NSCLC patients. One study reported that *KRAS* mutations were associated with asbestos exposure in a histological subtype, as the prevalence of mutations in lung adenocarcinomas was higher in asbestos-exposed patients than in unexposed patients (18). However, in another study, an increased probability of *KRAS* mutations was observed in occupationally exposed cases, but was not significant after adjustment for smoking and histology (19). Concerning *TP53*, contradictory results were reported on the link between *TP53* mutations and asbestos exposure (19,20). For other genes, no data are available on *EGFR* mutations linked to asbestos-related lung cancer. In contrast, *P16* /
CDKN2A gene inactivation in asbestos-exposed NSCLC mainly occurred via deletion, a feature also found in MPM, suggesting a link with the mechanism of action of asbestos fibres, while tobacco smoke induced promoter hypermethylation in lung cancer (11). In MPM, few signatures were reported in the literature, except chromosomal abnormalities that were more frequent in chromosomes 1, 4 and 14 in asbestos-exposed patients (21,22).

We did not find EGFR mutations associated with asbestos exposure in NSCLC, but confirm that the mutation rate in EGFR was higher in non-smokers than in smokers. Similar absence of link between EGFR mutation and asbestos is also suggested from our data using 34 MPM primary cell cultures. In MPM, the EGFR mutational status has been mainly investigated in primary tumours and no mutation was detected (23,24). This result shows that primary cell cultures are a useful tool to investigate somatic mutations in MPM. EGFR is known to be overexpressed in 44–97% of MPM cases, as demonstrated by immunohistochemical studies (25). Our data show that EGFR mutation is not a characteristic of MPM and that overexpression could be due to transcriptional, translational or posttranslational deregulation, such as recycling defects or degradation failure.

Our results based on 100 NSCLC cases are consistent with the absence of relationship between KRAS mutations and asbestos exposure, independently of histological subtype. No significant difference was observed after stratification for gender, histological type and smoking status between asbestos-exposed and unexposed patients. As for EGFR, the absence of KRAS mutations in MPM is consistent with no link with asbestos.

To the best of our knowledge, only one study has investigated NF2 mutations in 75 lung cancer cell lines by single-strand conformation polymorphism analysis of 8 out of 17 coding exons, but no mutation was detected (26). Our data demonstrate the absence of mutation by sequencing the whole gene, which is essential, as mutations in other cancers have been found in all exons. The NF2 gene status in NSCLC contrasts with that in MPM, in which a high rate of NF2 mutations was found, in agreement with the data of the literature (7,26–28). This finding suggests that the NF2 gene plays an important role in mesothelial cell homeostasis and is likely an important tumour suppressor gene accounting for asbestos-induced mesothelial cell neoplasia.

In the present study, the frequency of TP53 gene mutations in NSCLC was similar between asbestos-exposed and unexposed NSCLC cases, matched for age, gender, histological type and smoking habits. However, a higher frequency of G:C to T:A transversions over the total number of TP53 mutations was observed in asbestos-exposed NSCLC cases than in unexposed NSCLC cases, as these transversions represented 59 and 33%, respectively. The G:C to T:A frequency of transversions in the unexposed NSCLC cases is consistent with the reported frequency of 29% in the IARC database (http://www-p53.iarc.fr/) of over 2860 lung cancer cases. In the literature, G:C to T:A transversions are thought to be a signature of tobacco smoke in lung cancer and derived from mutagenic agents present in tobacco smoke, particularly PAHs compounds, although reactive oxygen species (ROS) could also be involved (9,29,30). In one previous study, an increased frequency of G:C to T:A transversions due to asbestos exposure was previously hypothesised (20). Our data show that G:C to T:A can be linked to asbestos. They show both an increased frequency of G:C to T:A transversions associated with asbestos exposure and that G to T transversions in the non-transcribed DNA strand occurred more frequently in the asbestos-exposed than in the unexposed NSCLC group (P = 0.038). This is consistent with an effect of exogenous or environmental carcinogens that preferentially induce mutations in the non-transcribed DNA strand (31). The significant enhancement of G:C to T:A transversions in lung cancer from asbestos-exposed patients is consistent with a synergistic role of asbestos with tobacco smoking in lung cancer (32). The mechanism is not clear but it has been suggested that asbestos fibres could serve as a vehicle to deliver concentrated doses of tobacco carcinogens to target cells (33).

The four TP53 mutations in MPM cases are distributed without relation to asbestos exposure. The lower percentage of TP53 mutations in MPM than in NSCLC and the different type of mutations are consistent with the lack of association between smoking and MPM, in contrast to lung cancer (10,34). G:C to T:A transversions due to ROS production would have been expected on the basis of the mechanism of asbestos-induced DNA damage (35), but only one of four mutation was G:C to T:A transversion in our series of MPM. This result suggests that the induction of transversions by ROS does not dominate the mechanism of mesothelial cell transformation or that repair mechanisms of ROS-induced DNA damage are efficient. However, some studies in MPM suggested that several different DNA repair systems are affected by the presence of SNP or by deregulated expression of DNA repair enzymes (36). Other mechanisms could play a role, as physical interference with mitosis (tangled hypothesis) (37). TP53 mutations detected in MPM might be due to other factors. Black spots described in the human pleura attest that several sorts of pollutants could be present in this tissue (38).

Two SNPs in TP53 intron 7 (rs12947788 and rs12951053), all heterozygous, were found in NSCLC. Their frequencies were significantly higher in the asbestos-exposed group and exceeded those reported in databases for similar ethnic populations (native European subjects), suggesting a predisposition linkage. Lower rates, dependent on ethnicity, are generally reported in the literature (http://www.ncbi.nlm.nih.gov/projects/SNP/; last access: June 22, 2012) (39). More recent data on SNP frequencies in European populations have shown rates of 4.3 and 6.5% for rs12947788 and rs12951053 heterozygous SNPs, respectively, among 23 European subjects of the SNP500Cancer controls database of the National Cancer Institute. Another database of the Human Diversity Panel found 6.1% for rs12951053 heterozygous SNPs among 66 European subjects, including 29 French subjects (http://vari-antgps.ncbi.nlm.nih.gov/cgfseq/pages/home.do; last access: June 22, 2012). Previously, in Singaporean Chinese population, it was suggested that these SNPs could be a predisposition factor to lung cancer, but asbestos exposure was not taken into account (40).

Multiple SNPs have been identified in the tumour suppressor gene TP53, but the significance of most of these SNPs is still unclear. Some studies have reported that intronic variants of the TP53 gene (especially a 16 bp insertion/duplication in intron 3) are associated with increased risk for several types of cancer, and a poorer prognosis in NSCLC, possibly linked to a low level of TP53 expression (41,42). In addition, a meta-analysis found limited evidence in support of the hypothesis that some polymorphisms in TP53, as in codon 72 or in intron 2, 3 or 6, could represent risk factors for lung cancer (43).
For the first time, we report rs12947788 and rs12951053 SNPs in MPM. Interestingly, these SNPs are found at higher rates in asbestos-exposed MPM with high CEI than those observed in asbestos-exposed NSCLC. Associated with our results in NSCLC, this finding reinforces the hypothesis that these polymorphisms might enhance the asbestos risk and may act as susceptibility factor to develop asbestos-related thoracic cancer. Studies of normal tissues would be necessary to confirm this hypothesis.

The role of these SNPs in TP53 function remains unknown. Polymorphism in introns could alter transcription or mRNA splicing (44,45). However, an unpublished transcriptomic microarray study carried out on our MPM primary cell culture collection shows that TP53 mRNA expression is independent of intron 7 SNPs polymorphism (data not shown). PCR analyses performed on MPM cDNA using several sets of primers located between exons 5 and 11, failed to detect any specific splice variants (data not shown). Moreover, using several available web tools, in silico analysis of the sequence of the TP53 intron 7 did not identify DNA sequences for non-coding mRNA or binding sites for transcription factor, which could indicate the presence of a putative transcriptional enhancer. Nevertheless, the lack of an identified functional role of these SNPs cannot be excluded. They could be associated with a haplotype containing another SNP, crucial for susceptibility to asbestos. In two studies, perfect linkage disequilibrium between both SNPs was described and it was suggested that rs12951053 is in weak linkage disequilibrium with SNPs affecting transcription factor binding sites (46,47). Analysis of our data using haplotype software and the SNPsStats program (http://bioinfo.iconcologia.net/SNPsStats) confirmed this linkage disequilibrium, but haplotype analysis did not identify a haplotype significantly associated with asbestos exposure (data not shown).

In conclusion, our results demonstrate the specificities of human malignant thoracic tumours linked to asbestos exposure. In NSCLC, effects of tobacco smoke dominate the genetic changes, and genetic alterations of TP53 are consistent with a synergistic effect of asbestos. Concerning genetic alterations, the current hypothesis is that NF2 mutations observed in MPM are linked to cell specificity and a particular function of NF2 protein in mesothelial cells. Interestingly, similar intronic TP53 polymorphisms were found, for the first time, in both asbestos-related NSCLC and MPM. Although the significance of these polymorphisms is unknown, future work in this area must examine their involvement, as susceptibility factors to develop asbestos-related thoracic cancer.

Supplementary data
Supplementary Tables Is–IIIs are available at Mutagenesis Online

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