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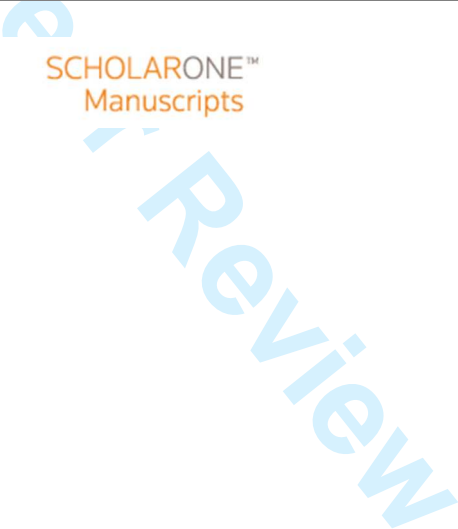
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Differential mutation profiles and similar intronic *TP53* polymorphisms in asbestos-related lung cancer and pleural mesothelioma

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ABSTRACT

Given the interest to define 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 on mesothelial carcinogenesis, we compared genetic alterations in relevant cancer genes between lung cancer, 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 alterations analyses were performed in 100 non-small cell lung cancers (NSCLC) from 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 bodies quantification in lung tissue. Genetic analyses were also performed in 34 MPM.

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 to 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., SNPs 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 defining cell-dependent mechanisms of action of asbestos, and identifying susceptibility factors to asbestos.

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RUNNING TITLE

Genetic aspects of asbestos-related thoracic tumours

KEYWORDS

Occupational exposure; asbestos; thoracic neoplasm; mutation; single nucleotide polymorphism.

For Peer Review

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, 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).

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

113

114 PATIENTS AND METHODS

115 NSCLC patients

116 Subjects were selected from consecutive primary NSCLC cases after surgical resection in 5
117 French hospitals (Centre Hospitalier Intercommunal, Créteil; Hôpital Européen Georges
118 Pompidou, Paris; Institut Mutualiste Montsouris, Paris; Centre Hospitalier Universitaire, Caen;
119 Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson) from 1994 through 2007. NSCLC
120 tissue samples were snap-frozen in liquid nitrogen after surgical resection and stored at -80°C
121 until use. The study was approved by the local Ethics Committee and all patients provided
122 written informed consent. Detailed information describing the tumors was obtained from
123 pathology reports. The eligibility criteria were: (a) lung tumour histology; (b) absence of
124 neoadjuvant chemotherapy or radiotherapy; (c) availability of both normal and neoplastic lung
125 tissue; (d) data on asbestos exposure history including quantification of asbestos bodies (AB) in
126 lung tissue and interviewer-administered questionnaire; and (e) data on smoking habits (11-13).
127 From these four eligibility criteria, 350 NSCLC cases were recruited. These cases were
128 representative of the French lung cancer population in terms of age, gender, histology and
129 tobacco smoking characteristics (14).

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131 MPM primary cell cultures

132 Thirty four human cell cultures were obtained from surgical resection, pleural biopsies or
133 malignant pleural fluid of confirmed MPM cases, provided by 4 French hospitals (Centre
134 Hospitalier Intercommunal, Créteil; Hôpital Européen Georges Pompidou, Paris; Centre
135 Hospitalier Universitaire, Caen; Centre Hospitalier Universitaire, Marseille) from 1985 through
136 2007, as previously described (15). Detailed information describing MPM histology was
137 obtained from pathology reports. An additional series of 25 MPM was studied for analysis of
138 SNPs 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 (PBS, 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 1,000 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 1,000 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

164 years-old and over, who were matched together. For tobacco consumption, 3 classes were
165 defined (low: 0 to 5 P-Y; medium to high: 6 to 60 P-Y (± 10 P-Y); very high: over 61 P-Y).

166

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

174

175 **Mutational analysis of *TP53*, *NF2*, *KRAS* and *EGFR* genes**

176 Mutations of *TP53* exons 2 to 11, *NF2* exons 1 to 17, *KRAS* exons 1 and 2 and *EGFR* exons 18
177 to 21 were screened by DNA sequencing. Genomic DNA was extracted from frozen NSCLC
178 tumors and MPM primary cell cultures using a standard phenol-chloroform extraction
179 procedure. DNA amplification was performed by PCR with a combination of forward and
180 reverse primers located in the introns surrounding the sequenced exon (supplementary Table
181 1s) and Taq polymerase Hot Star (Qiagen, Courtaboeuf, France) on a Gene Amp 9700
182 apparatus (Perkin-Elmer, Courtaboeuf, France). PCR products were purified through Millipore
183 genomic columns (Prolabo, Paris, France), checked for quality and quantified prior to
184 sequencing. Sequencing PCR was performed on purified PCR products and Big Dye
185 Terminator v1.1 cycle sequencing kit (Applied Biosystems, Courtaboeuf, France) on a Gene
186 Amp 9700 apparatus (Perkin-Elmer). PCR reactions were carried out for 25 cycles including
187 denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and extension at 60°C for 4 min.
188 Sequencing PCR products were purified through Sephadex G50 fine resin (GE Healthcare

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189 Biosciences AB, Uppsala, Sweden) in Multiscreen Millipore columns (Prolabo) and analyzed
190 on an ABI PRISM 3100 Genetic analyser (Applied Biosystems). Mutational analysis was
191 performed using Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA) and
192 verified by independent amplification and sequencing. Genetic alterations were validated by an
193 independent sequencing experiment in the DNA complementary strand.

194

195 **Statistical analyses**

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

205

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 eighteen MPM cases (53.0%), moderate in six cases (17.6%) and low in five cases (14.7%), while no evidence of asbestos exposure was found in five cases (14.7%).

EGFR DNA sequencing analysis

EGFR mutations were found in 8% of the whole NSCLC population (Table II). 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 II). No *EGFR* mutation was detected among MPM cases.

231 *KRAS* DNA sequencing analysis

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

243 *NF2* DNA sequencing analysis

244 No *NF2* mutation was detected in either exposed or unexposed NSCLC patients, while 38.2%
245 (13/34) of MPM cases showed *NF2* gene mutations (supplementary Table 3s). The mutations
246 consisted of large deletions of one or several exons and point mutations. Deletions were found
247 in 10 (29.4%) MPM cases and point mutations were found in 4 (11.8%) cases. One deletion and
248 one point mutation were both present in one MPM. In NSCLC patients, no significant
249 difference was observed considering gender; age and histological subtypes. A significant link
250 between *NF2* mutations and asbestos exposure was detected ($P=0.043$), but it was not
251 confirmed in a largest series of 59 MPM cases ($P=0.372$).

253 *TP53* DNA sequencing analysis

254 *TP53* mutations were found in 39% of the overall NSCLC population (Table II). No significant
255 difference was observed between males and females. In contrast, a statistically significant

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3 256 difference was found between histological types of NSCLC. *TP53* mutations were more
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5 257 frequent in squamous cell carcinomas (52.3%) than in lung adenocarcinomas (29.3%)
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7 258 ($P=0.02$). Almost all mutations (36/39) were detected in former or current smokers (more than
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9 259 10 P-Y). The frequencies of *TP53* mutations according to smoking status were different
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11 260 between non-smokers (15.4%) versus former and current smokers (42.5%), but this difference
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13 261 was at the limit of statistical significance ($P=0.061$). No significant difference was observed
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15 262 according to asbestos exposure: 19 mutations (38%) were found in asbestos-exposed cases and
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17 263 20 mutations (40%) were found in unexposed cases (Table II). This lack of significance
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19 264 persisted after stratification for gender, histological type and smoking status.
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21 265 Sequencing analysis revealed different types of mutations. The spectrum of *TP53* mutations is
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23 266 reported in Table III, Figure 1 and supplementary Table 2s. Transversion was the most frequent
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25 267 type of mutation in both asbestos-exposed and unexposed patients (Figure 1). The frequency of
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27 268 G:C to T:A transversions over the total *TP53* mutations were more frequent in NSCLC from
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29 269 asbestos-exposed patients (59%) of than from unexposed patients (33%) ($P=0.138$) (Figure 1).
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31 270 The percentage of cases with G:C to T:A transversions was not significantly different in the
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33 271 asbestos-exposed group (26%) compared to the unexposed group (14%) ($P=0.134$) taking into
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35 272 account the total number of cases (Table III). However, when we only considered mutated
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37 273 cases, the number of patients with at least one G:C to T:A transversion in *TP53* was
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39 274 significantly higher in the asbestos-exposed NSCLC group than in the unexposed NSCLC
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41 275 group: 13/19 (68%) and 7/20 (35%) cases, respectively ($P=0.037$) (Table III). Moreover, more
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43 276 than 90% of G to T transversions occurred in the non-transcribed DNA strand, and
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45 277 transversions in this strand were also more frequent in the asbestos-exposed NSCLC group than
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47 278 in the unexposed NSCLC group: 12/19 (63%) and 6/20 (30%) cases, respectively ($P=0.038$).
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49 279 Four *TP53* mutations (11.8%) were found in the MPM population. All were point mutations:
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51 280 one transition in exon 5, at codon 173 (517G>A,V173M), changing the encoded valine into
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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 3s).

***TP53* polymorphisms**

TP53 DNA sequencing in NSCLC detected single nucleotide polymorphisms (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* (rs12947788: 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 9 cases (supplementary Table 2s). 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 in unexposed patients ($P=0.01$ and $P=0.07$, respectively). We next

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3 306 analyzed the *TP53* intron 7 polymorphisms in additional 25 MPM characterized for asbestos
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5 307 exposure: high CEI (40.0%), moderate (16.0%), low (20.0%) and no exposure (24.0%) (Figure
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7 308 2). Considering all 59 MPM cases, the presence of the rs12947788 and rs1295105 SNP minor
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9 309 alleles was observed in 39.3% and 25.0% of asbestos-exposed MPM patients with high CEI,
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11 310 but not in patients with moderate/low CEI and unexposed patients ($P<0.001$ and $P=0.012$,
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13 311 respectively) (Figure 2). For both SNPs, the minor alleles (A for rs12947788 and C for
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15 312 rs12951053) were either associated with the major alleles (heterozygous patients) or present
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17 313 alone (homozygous or hemizygous patients). The minor allele of rs12951053 SNP was always
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19 314 associated with the minor allele of rs12947788 SNP (supplementary Table 3s).
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316 **DISCUSSION**

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

322 Evaluation of asbestos exposure differs between NSCLC and MPM. As NSCLC is a
323 multifactorial disease, mainly linked to tobacco smoking and as asbestos-related lung cancer
324 are generally related to high level of exposure, it is generally recommended to evaluate
325 cumulative asbestos exposure by two complementary approaches: (i) a specific questionnaire
326 carried out by an occupational hygienist or by the use of job/exposure matrices; (ii) a
327 mineralogical analysis of biological samples (bronchoalveolar lavage or lung tissue) (12). It
328 should be noted that a negative result of a mineralogical quantification would not definitively
329 exclude a past asbestos exposure. In contrast, MPM is a disease mainly linked to asbestos
330 exposure even at low levels of exposure. Consequently, mineralogical analyses are not required
331 in the MPM clinical management (16-17). Consequently, cumulative asbestos exposure
332 evaluation is often less accurate in MPM. Moreover, in MPM, lung tissue is not often available
333 for mineralogical analyses, as MPM diagnosis or treatment very rarely includes a surgical lung
334 resection. In our study, detailed occupational information was available in clinical reports, and
335 was estimated to be sufficient for occupational hygienist's evaluation in order to classify the
336 MPM cases in four cumulative exposure index (null, low, moderate and high) taking into
337 account probability, frequency, intensity and duration of exposure.

338 So far, few or conflicting data have been published concerning identification of specific gene
339 mutations, which might be the signature of asbestos in thoracic cancers. Limited data have been

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3 340 reported on *KRAS* mutations in asbestos-exposed NSCLC patients. One study reported that
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5 341 *KRAS* mutations were associated with asbestos exposure in a histological subtype, as the
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7 342 prevalence of mutations in lung adenocarcinomas was higher in asbestos-exposed patients than
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9 343 in unexposed patients (18). However, in another study, an increased probability of *KRAS*
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11 344 mutations was observed in occupationally exposed cases, but was not significant after
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13 345 adjustment for smoking and histology (19). Concerning *TP53*, contradictory results were
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15 346 reported on the link between *TP53* mutations and asbestos exposure (19-20). For other genes,
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17 347 no data are available on *EGFR* mutations linked to asbestos-related lung cancer. In contrast,
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19 348 *P16/CDKN2A* gene inactivation in asbestos-exposed NSCLC mainly occurred via deletion, a
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21 349 feature also found in MPM, suggesting a link with the mechanism of action of asbestos fibres,
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23 350 while tobacco smoke induced promoter hypermethylation in lung cancer (11). In MPM, few
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25 351 signatures were reported in the literature, except chromosomal abnormalities which were more
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27 352 frequent in chromosome 1, 4 and 14 in asbestos-exposed patients (21-22).

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32 353 We did not find *EGFR* mutations associated with asbestos exposure in NSCLC, but confirm
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34 354 that the mutation rate in *EGFR* was higher in non-smokers than in smokers. Similar absence of
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36 355 link between *EGFR* mutation and asbestos is also suggested from our data using 34 MPM
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38 356 primary cell cultures. In MPM, the *EGFR* mutational status has been mainly investigated in
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40 357 primary tumours and no mutation was detected (23-24). This result shows that primary cell
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42 358 cultures are a useful tool to investigate somatic mutations in MPM. *EGFR* is known to be
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44 359 overexpressed in 44 to 97% of MPM cases, as demonstrated by immunohistochemical studies
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46 360 (25). Our data show that *EGFR* mutation is not a characteristic of MPM, and that
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48 361 overexpression could be due to transcriptional, translational, or posttranslational deregulation,
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50 362 such as recycling defects or degradation failure.
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363 Our results based on 100 NSCLC cases are consistent with the absence of relationship between
364 *KRAS* mutations and asbestos-exposure, independently of histological subtype. No significant
365 difference was observed after stratification for gender, histological type and smoking status
366 between asbestos-exposed and unexposed patients. As for *EGFR*, the absence of *KRAS*
367 mutations in MPM is consistent with no link with asbestos.

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

377 In the present study, the frequency of *TP53* gene mutations in NSCLC was similar between
378 asbestos-exposed and unexposed NSCLC cases, matched for age, gender, histological type and
379 smoking habits. However, a higher frequency of G:C to T:A transversions over the total
380 number of *TP53* mutations was observed in asbestos-exposed NSCLC cases than in unexposed
381 NSCLC cases, as these transversions represented 59% and 33%, respectively. The G:C to T:A
382 frequency of transversions in the unexposed NSCLC cases is consistent with the reported
383 frequency of 29% in the IARC database (<http://www-p53.iarc.fr/>) of over 2,860 lung cancer
384 cases. In the literature G:C to T:A transversions are thought to be a signature of tobacco smoke
385 in lung cancer, and derived from mutagenic agents present in tobacco smoke, particularly
386 PAHs compounds, although reactive oxygen species (ROS) could also be involved (9,29-30).

387 In one previous study, an increased frequency of G:C to T:A transversions due to asbestos
388 exposure was previously hypothesized (20). Our data show that G:C to T:A can be linked to
389 asbestos. They show both an increased frequency of G:C to T:A transversions associated with
390 asbestos exposure, and that G to T transversions in the non-transcribed DNA strand occurred
391 more frequently in the asbestos-exposed than in the unexposed NSCLC group ($P=0.038$). This
392 is consistent with an effect of exogenous or environmental carcinogens that preferentially
393 induce mutations in the non-transcribed DNA strand (31). The significant enhancement of G:C
394 to T:A transversions in lung cancer from asbestos-exposed patients is consistent with a
395 synergistic role of asbestos with tobacco smoking in lung cancer (32). The mechanism is not
396 clear but it has been suggested that asbestos fibres could serve as a vehicle to deliver
397 concentrated doses of tobacco carcinogens to target cells (33).

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

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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: 22/06/2012) (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://variantgps.nci.nih.gov/cgfseq/pages/home.do>; last access: 22/06/2012). 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 introns 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

436 susceptibility factor to develop asbestos-related thoracic cancer. Studies of normal tissues
437 would be necessary to confirm this hypothesis.

438 The role of these SNPs in *TP53* function remains unknown. Polymorphism in introns could
439 alter transcription or mRNA splicing (44-45). However, an unpublished transcriptomic
440 microarray study carried out on our MPM primary cell culture collection shows that *TP53*
441 mRNA expression is independent of intron 7 SNPs polymorphism (data not shown). PCR
442 analyses, performed on MPM cDNA using several sets of primers located between exons 5 and
443 11, failed to detect any specific splice variants (data not shown). Moreover, using several
444 available web tools, *in silico* analysis of the sequence of the *TP53* intron 7 did not identify
445 DNA sequences for non-coding mRNA or binding sites for transcription factor which could
446 indicate the presence of a putative transcriptional enhancer. Nevertheless, the lack of an
447 identified functional role of these SNPs cannot be excluded. They could be associated with a
448 haplotype containing another SNP, crucial for susceptibility to asbestos. In two studies, perfect
449 linkage disequilibrium between both SNPs was described and it was suggested that rs12951053
450 is in weak linkage disequilibrium with SNPs affecting transcription factor binding sites (46-47).
451 Analysis of our data using haploview software and the SNPStats program
452 (<http://bioinfo.iconcologia.net/SNPStats>) confirmed this linkage disequilibrium, but haplotype
453 analysis did not identify a haplotype significantly associated with asbestos exposure (data not
454 shown).

455 In conclusion, our results demonstrate the specificities of human malignant thoracic tumours
456 linked to asbestos exposure. In NSCLC, effects of tobacco smoke dominate the genetic
457 changes, and genetic alterations of *TP53* are consistent with a synergistic effect of asbestos.
458 Concerning genetic alterations, the current hypothesis is that *NF2* mutations observed in MPM
459 are linked to cell specificity and a particular function of *NF2* protein in mesothelial cells.

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3 460 Interestingly, similar intronic *TP53* polymorphisms were found, for the first time, in both
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5 461 asbestos-related NSCLC and MPM. Although the significance of these polymorphisms is
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7 462 unknown, future work in this area must examine their involvement, as susceptibility factors to
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9 463 develop asbestos-related thoracic cancer.

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48 480 **CONFLICT OF INTEREST STATEMENT**

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50 481 The authors declare they have no actual or potential competing financial interests.

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55 483 **LIST OF ABBREVIATION:**

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3 484 AB: asbestos bodies; CEI: cumulative exposure index; E-: unexposed NSCLC cases; E+:
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5 485 asbestos-exposed NSCLC cases; EGFR: Epidermal growth factor receptor; KRAS: Kirsten rat
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7 486 sarcoma; MPM: Malignant pleural mesothelioma; NF2: Neurofibromatosis type 2; NSCLC:
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9 487 Non-small cell lung cancer; PAHs: polycyclic aromatic hydrocarbons; P-Y: pack-years; ROS:
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11 488 reactive oxygen species; SNPs: single nucleotide polymorphisms; TP53: Tumour protein 53.
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For Peer Review

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For Peer Review

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7 624 **Fig. 1.** Types of *TP53* gene alteration in asbestos-exposed and unexposed NSCLC cases.
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10 625 Percentages were based on the total number of mutations in each group.

11 626 **Fig. 2.** Single nucleotide polymorphisms in *TP53* intron 7 in non-small-cell lung cancer and
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14 627 malignant pleural mesothelioma populations according to asbestos exposure. (A) rs12947788;
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16 628 (B) rs12951053.
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Differential mutation profiles and similar intronic *TP53* polymorphisms in asbestos-related lung cancer and pleural mesothelioma

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47 **ABSTRACT**

48 Given the interest to define biomarkers of asbestos exposure and to provide insights into
49 asbestos-related and cell-specific mechanisms of neoplasia, the identification of gene
50 alterations in asbestos-related cancers can help to a better understanding of exposure risk.

51 To understand the aetiology of asbestos-induced malignancies and to increase our knowledge
52 on mesothelial carcinogenesis, we compared genetic alterations in relevant cancer genes
53 between lung cancer, induced by asbestos and tobacco smoke, and malignant pleural
54 mesothelioma (MPM), a cancer related to asbestos, but not to tobacco smoke.

55 *TP53*, *KRAS*, *EGFR* and *NF2* gene alterations analyses were performed in 100 non-small cell
56 lung cancers (NSCLC) from 50 asbestos-exposed and 50 unexposed patients matched for age,
57 gender, histology and smoking habits. Detailed assessment of asbestos exposure was based on
58 both specific questionnaires and asbestos bodies quantification in lung tissue. Genetic analyses
59 were also performed in 34 MPM.

60 *TP53*, *EGFR* and *KRAS* mutations were found in NSCLC with no link with asbestos exposure.
61 *NF2* was only altered in MPM. Significant enhancement of *TP53* G:C to T:A transversions was
62 found in NSCLC from asbestos-exposed patients when compared to unexposed patients
63 ($P=0.037$). Interestingly, *TP53* polymorphisms in intron 7 (rs12947788 and rs12951053) were
64 more frequently identified in asbestos-exposed NSCLC ($P=0.046$) and MPM patients than in
65 unexposed patients ($P<0.001$ and $P=0.012$, respectively). These results emphasize distinct
66 genetic alterations between asbestos-related thoracic tumours, but identify common potential
67 susceptibility factors i.e., SNPs in intron 7 of *TP53*.

68 While genetic changes in NSCLC are dominated by the effects of tobacco smoke, the increase
69 of transversions in *TP53* gene is consistent with a synergistic effect of asbestos. These results
70 may help defining cell-dependent mechanisms of action of asbestos, and identifying
71 susceptibility factors to asbestos.

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5 73 **RUNNING TITLE**
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7 74 Genetic aspects of asbestos-related thoracic tumours
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11 76 **KEYWORDS**
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14 77 Occupational exposure; asbestos; thoracic neoplasm; mutation; single nucleotide
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16 78 polymorphism.
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79 **INTRODUCTION**

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

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

97 Epidemiological studies have shown that the fraction of lung cancer risk attributable to
98 occupational asbestos exposure and tobacco smoking is about 5% and 90%, respectively (4,8).

99 Genetic susceptibility and other environmental and occupational factors also contribute to the
100 development of lung cancer, such as radon, hexavalent chromium, arsenic, polycyclic aromatic
101 hydrocarbons (PAHs) (2,9). While tobacco smoking is a major risk factor for lung cancer and
102 asbestos fibres a lower risk factor, asbestos fibres constitute the major occupational risk factor
103 for human MPM, with no link with tobacco smoking (10).

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3 104 As the genes and biological pathways altered in cancer cells depend on both the carcinogen and
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5 105 the cell type, comparison between gene alterations in lung cancer from asbestos-exposed
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7 106 patients and MPM patients would provide insight into asbestos-related and cell-specific
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9 107 mechanisms of neoplasia. The purpose of this study was to characterize molecular alterations
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11 108 related to asbestos exposure, in order to more clearly understand the mechanism of asbestos
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13 109 oncogenesis and to identify new biomarkers in non-small cell lung cancers (NSCLC). *KRAS*,
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15 110 *EGFR*, *NF2* and *TP53* genes were studied in 100 NSCLC patients with well-defined smoking
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17 111 habits, detailed assessment of asbestos exposure based on both occupational questionnaire and
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19 112 determination of asbestos bodies in lung tissue, and in 34 MPM patients.
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PATIENTS AND METHODS

NSCLC patients

Subjects were selected from consecutive primary NSCLC cases after surgical resection in 5 French hospitals (Centre Hospitalier Intercommunal, Créteil; Hôpital Européen Georges Pompidou, Paris; Institut Mutualiste Montsouris, Paris; Centre Hospitalier Universitaire, Caen; 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 tumors was obtained from pathology reports. The eligibility criteria were: (a) lung tumour histology; (b) absence of neoadjuvant chemotherapy or radiotherapy; (c) availability of both normal and neoplastic lung tissue; (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 (11-13). From these four 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 (14).

MPM primary cell cultures

Thirty four human cell cultures were obtained from surgical resection, pleural biopsies or malignant pleural fluid of confirmed MPM cases, provided by 4 French hospitals (Centre Hospitalier Intercommunal, Créteil; Hôpital Européen Georges Pompidou, Paris; Centre Hospitalier Universitaire, Caen; 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 was studied for analysis of SNPs 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 (PBS, 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 1,000 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 1,000 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

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164 years-old and over, who were matched together. For tobacco consumption, 3 classes were
165 defined (low: 0 to 5 P-Y; medium to high: 6 to 60 P-Y (\pm 10 P-Y); very high: over 61 P-Y).

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

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175 **Mutational analysis of *TP53*, *NF2*, *KRAS* and *EGFR* genes**

176 Mutations of *TP53* exons 2 to 11, *NF2* exons 1 to 17, *KRAS* exons 1 and 2 and *EGFR* exons 18
177 to 21 were screened by DNA sequencing. Genomic DNA was extracted from frozen NSCLC
178 tumors and MPM primary cell cultures using a standard phenol-chloroform extraction
179 procedure. DNA amplification was performed by PCR with a combination of forward and
180 reverse primers located in the introns surrounding the sequenced exon (supplementary Table
181 1s) and Taq polymerase Hot Star (Qiagen, Courtaboeuf, France) on a Gene Amp 9700
182 apparatus (Perkin-Elmer, Courtaboeuf, France). PCR products were purified through Millipore
183 genomic columns (Prolabo, Paris, France), checked for quality and quantified prior to
184 sequencing. Sequencing PCR was performed on purified PCR products and Big Dye
185 Terminator v1.1 cycle sequencing kit (Applied Biosystems, Courtaboeuf, France) on a Gene
186 Amp 9700 apparatus (Perkin-Elmer). PCR reactions were carried out for 25 cycles including
187 denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and extension at 60°C for 4 min.
188 Sequencing PCR products were purified through Sephadex G50 fine resin (GE Healthcare

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3 189 Biosciences AB, Uppsala, Sweden) in Multiscreen Millipore columns (Prolabo) and analyzed
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5 190 on an ABI PRISM 3100 Genetic analyser (Applied Biosystems). Mutational analysis was
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7 191 performed using Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA) and
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9 192 verified by independent amplification and sequencing. Genetic alterations were validated by an
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11 193 independent sequencing experiment in the DNA complementary strand.
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16 **Statistical analyses**

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18 196 Clinicopathological characteristics of asbestos-exposed and unexposed NSCLC cases were
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20 197 compared using Wilcoxon test for quantitative variables, and Pearson's Chi-square test for
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22 198 qualitative variables (Fisher's exact test was used for qualitative variables where Chi-square
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24 199 test was not a valid test, i.e. expected cell count was <5). Comparisons were also based on
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26 200 mutational analysis and polymorphism status, after stratification according to the various
27
28 201 relevant clinicopathological subtypes. Multivariate analyses were also performed using logistic
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30 202 models on *TP53* SNPs. A *P* value less than 0.05 was considered statistically significant.
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32 203 Statistical analyses were performed using "Statistical Analysis System" software (SAS v9.1
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34 204 Inc, Cary, NC, USA).
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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 eighteen MPM cases (53.0%), moderate in six cases (17.6%) and low in five cases (14.7%), while no evidence of asbestos exposure was found in five cases (14.7%).

***EGFR* DNA sequencing analysis**

EGFR mutations were found in 8% of the whole NSCLC population (Table II). 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 II). No *EGFR* mutation was detected among MPM cases.

***KRAS* DNA sequencing analysis**

KRAS mutations were found in 13% of the NSCLC population (Table II): twelve in codon 12 and one 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 to squamous cell carcinoma (4.8%; 2/42) ($p<0.037$). Regarding smoking status, only one (7.7%) *KRAS* mutation was found in non-smokers compared to 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 II). 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 3s). 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 NSCLC 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 II). No significant difference was observed between males and females. In contrast, a statistically significant

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256 difference was found between histological types of NSCLC. *TP53* mutations were more
257 frequent in squamous cell carcinomas (52.3%) than in lung adenocarcinomas (29.3%)
258 ($P=0.02$). Almost all mutations (36/39) were detected in former or current smokers (more than
259 10 P-Y). The frequencies of *TP53* mutations according to smoking status were different
260 between non-smokers (15.4%) versus former and current smokers (42.5%), but this difference
261 was at the limit of statistical significance ($P=0.061$). No significant difference was observed
262 according to asbestos exposure: 19 mutations (38%) were found in asbestos-exposed cases and
263 20 mutations (40%) were found in unexposed cases (Table II). This lack of significance
264 persisted after stratification for gender, histological type and smoking status.

265 Sequencing analysis revealed different types of mutations. The spectrum of *TP53* mutations is
266 reported in Table III, Figure 1 and supplementary Table 2s. Transversion was the most frequent
267 type of mutation in both asbestos-exposed and unexposed patients (Figure 1). The frequency of
268 G:C to T:A transversions over the total *TP53* mutations were more frequent in NSCLC from
269 asbestos-exposed patients (59%) of than from unexposed patients (33%) ($P=0.138$) (Figure 1).

270 The percentage of cases with G:C to T:A transversions was not significantly different in the
271 asbestos-exposed group (26%) compared to the unexposed group (14%) ($P=0.134$) taking into
272 account the total number of cases (Table III). However, when we only considered mutated
273 cases, the number of patients with at least one G:C to T:A transversion in *TP53* was
274 significantly higher in the asbestos-exposed NSCLC group than in the unexposed NSCLC
275 group: 13/19 (68%) and 7/20 (35%) cases, respectively ($P=0.037$) (Table III). Moreover, more
276 than 90% of G to T transversions occurred in the non-transcribed DNA strand, and
277 transversions in this strand were also more frequent in the asbestos-exposed NSCLC group than
278 in the unexposed NSCLC group: 12/19 (63%) and 6/20 (30%) cases, respectively ($P=0.038$).

279 Four *TP53* mutations (11.8%) were found in the MPM population. All were point mutations:
280 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 3s).

***TP53* polymorphisms**

TP53 DNA sequencing in NSCLC detected single nucleotide polymorphisms (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* (rs12947788: 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 9 cases (supplementary Table 2s). 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 in unexposed patients ($P=0.01$ and $P=0.07$, respectively). We next

analyzed the *TP53* intron 7 polymorphisms in additional 25 MPM characterized for asbestos exposure: high CEI (40.0%), moderate (16.0%), low (20.0%) and no exposure (24.0%) (Figure 2). Considering all 59 MPM cases, the presence of the rs12947788 and rs1295105 SNP minor alleles was observed in 39.3% and 25.0% of asbestos-exposed MPM patients with high CEI, but not in patients with moderate/low CEI and unexposed patients ($P<0.001$ and $P=0.012$, 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 3s).

DISCUSSION

The purpose of this study was to characterize 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 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; (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 cumulative exposure index (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

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340 reported on *KRAS* mutations in asbestos-exposed NSCLC patients. One study reported that
341 *KRAS* mutations were associated with asbestos exposure in a histological subtype, as the
342 prevalence of mutations in lung adenocarcinomas was higher in asbestos-exposed patients than
343 in unexposed patients (18). However, in another study, an increased probability of *KRAS*
344 mutations was observed in occupationally exposed cases, but was not significant after
345 adjustment for smoking and histology (19). Concerning *TP53*, contradictory results were
346 reported on the link between *TP53* mutations and asbestos exposure (19-20). For other genes,
347 no data are available on *EGFR* mutations linked to asbestos-related lung cancer. In contrast,
348 *P16/CDKN2A* gene inactivation in asbestos-exposed NSCLC mainly occurred via deletion, a
349 feature also found in MPM, suggesting a link with the mechanism of action of asbestos fibres,
350 while tobacco smoke induced promoter hypermethylation in lung cancer (11). In MPM, few
351 signatures were reported in the literature, except chromosomal abnormalities which were more
352 frequent in chromosome 1, 4 and 14 in asbestos-exposed patients (21-22).

353 We did not find *EGFR* mutations associated with asbestos exposure in NSCLC, but confirm
354 that the mutation rate in *EGFR* was higher in non-smokers than in smokers. Similar absence of
355 link between *EGFR* mutation and asbestos is also suggested from our data using 34 MPM
356 primary cell cultures. In MPM, the *EGFR* mutational status has been mainly investigated in
357 primary tumours and no mutation was detected (23-24). This result shows that primary cell
358 cultures are a useful tool to investigate somatic mutations in MPM. *EGFR* is known to be
359 overexpressed in 44 to 97% of MPM cases, as demonstrated by immunohistochemical studies
360 (25). Our data show that *EGFR* mutation is not a characteristic of MPM, and that
361 overexpression could be due to transcriptional, translational, or posttranslational deregulation,
362 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 2,860 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).

387 In one previous study, an increased frequency of G:C to T:A transversions due to asbestos
388 exposure was previously hypothesized (20). Our data show that G:C to T:A can be linked to
389 asbestos. They show both an increased frequency of G:C to T:A transversions associated with
390 asbestos exposure, and that G to T transversions in the non-transcribed DNA strand occurred
391 more frequently in the asbestos-exposed than in the unexposed NSCLC group ($P=0.038$). This
392 is consistent with an effect of exogenous or environmental carcinogens that preferentially
393 induce mutations in the non-transcribed DNA strand (31). The significant enhancement of G:C
394 to T:A transversions in lung cancer from asbestos-exposed patients is consistent with a
395 synergistic role of asbestos with tobacco smoking in lung cancer (32). The mechanism is not
396 clear but it has been suggested that asbestos fibres could serve as a vehicle to deliver
397 concentrated doses of tobacco carcinogens to target cells (33).

398 The four *TP53* mutations in MPM cases are distributed without relation to asbestos exposure.
399 The lower percentage of *TP53* mutations in MPM than in NSCLC, and the different type of
400 mutations are consistent with the lack of association between smoking and MPM, in contrast to
401 lung cancer (10,34), G:C to T:A transversions due to ROS production would have been
402 expected on the basis of the mechanism of asbestos-induced DNA damage (35), but only one
403 of four mutation was G:C to T:A transversion in our series of MPM. This result suggests that
404 the induction of transversions by ROS does not dominate the mechanism of mesothelial cell
405 transformation or that repair mechanisms of ROS-induced DNA damage are efficient.
406 However, some studies in MPM suggested that several different DNA repair systems are
407 affected by the presence of SNP or by deregulated genes expression of DNA repair enzymes
408 (36). Other mechanisms could play a role, as physical interference with mitosis (tangled
409 hypothesis) (37). *TP53* mutations detected in MPM might be due to other factors. Black spots
410 described in the human pleura attest that several sorts of pollutants could be present in this
411 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: 22/06/2012) (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://variantgps.nci.nih.gov/cgfseq/pages/home.do>; last access: 22/06/2012). 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 introns 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

436 susceptibility factor to develop asbestos-related thoracic cancer. Studies of normal tissues
437 would be necessary to confirm this hypothesis.

438 The role of these SNPs in *TP53* function remains unknown. Polymorphism in introns could
439 alter transcription or mRNA splicing (44-45). However, an unpublished transcriptomic
440 microarray study carried out on our MPM primary cell culture collection shows that *TP53*
441 mRNA expression is independent of intron 7 SNPs polymorphism (data not shown). PCR
442 analyses, performed on MPM cDNA using several sets of primers located between exons 5 and
443 11, failed to detect any specific splice variants (data not shown). Moreover, using several
444 available web tools, *in silico* analysis of the sequence of the *TP53* intron 7 did not identify
445 DNA sequences for non-coding mRNA or binding sites for transcription factor which could
446 indicate the presence of a putative transcriptional enhancer. Nevertheless, the lack of an
447 identified functional role of these SNPs cannot be excluded. They could be associated with a
448 haplotype containing another SNP, crucial for susceptibility to asbestos. In two studies, perfect
449 linkage disequilibrium between both SNPs was described and it was suggested that rs12951053
450 is in weak linkage disequilibrium with SNPs affecting transcription factor binding sites (46-47).

451 Analysis of our data using haploview software and the SNPStats program
452 (<http://bioinfo.iconcologia.net/SNPStats>) confirmed this linkage disequilibrium, but haplotype
453 analysis did not identify a haplotype significantly associated with asbestos exposure (data not
454 shown).

455 In conclusion, our results demonstrate the specificities of human malignant thoracic tumours
456 linked to asbestos exposure. In NSCLC, effects of tobacco smoke dominate the genetic
457 changes, and genetic alterations of *TP53* are consistent with a synergistic effect of asbestos.
458 Concerning genetic alterations, the current hypothesis is that *NF2* mutations observed in MPM
459 are linked to cell specificity and a particular function of *NF2* protein in mesothelial cells.

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3 460 Interestingly, similar intronic *TP53* polymorphisms were found, for the first time, in both
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5 461 asbestos-related NSCLC and MPM. Although the significance of these polymorphisms is
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7 462 unknown, future work in this area must examine their involvement, as susceptibility factors to
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9 463 develop asbestos-related thoracic cancer.

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44 45 46 47 48 480 **CONFLICT OF INTEREST STATEMENT**

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50 481 The authors declare they have no actual or potential competing financial interests.

51 52 53 54 55 483 **LIST OF ABBREVIATION:**

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3 484 AB: asbestos bodies; CEI: cumulative exposure index; E-: unexposed NSCLC cases; E+:
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5 485 asbestos-exposed NSCLC cases; EGFR: Epidermal growth factor receptor; KRAS: Kirsten rat
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7 486 sarcoma; MPM: Malignant pleural mesothelioma; NF2: Neurofibromatosis type 2; NSCLC:
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9 487 Non-small cell lung cancer; PAHs: polycyclic aromatic hydrocarbons; P-Y: pack-years; ROS:
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11 488 reactive oxygen species; SNPs: single nucleotide polymorphisms; TP53: Tumour protein 53.
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For Peer Review

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622 **LEGEND TO FIGURE**

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624 **Fig. 1.** Types of *TP53* gene alteration in asbestos-exposed and unexposed NSCLC cases.

625 Percentages were based on the total number of mutations in each group.

626 **Fig. 2.** Single nucleotide polymorphisms in *TP53* intron 7 in non-small-cell lung cancer and
627 malignant pleural mesothelioma populations according to asbestos exposure. (A) rs12947788;
628 (B) rs12951053.

For Peer Review

Table I. Clinicopathological characteristics of non-small cell lung cancer cases

	NSCLC cases		<i>P</i> *
	Asbestos-exposed subjects	Unexposed subjects	
All cases n	50	50	ns
Mean age (years \pm SD)	65.9 \pm 9.6	64.8 \pm 8.8	ns
Gender n (%)			
Female	6 (12)	6 (12)	ns
Male	44 (88)	44 (88)	
Histology n (%)			
Squamous cell carcinoma	21 (42)	21 (42)	ns
Adenocarcinoma	29 (58)	29 (58)	
Smoking status n (%)			
Current smokers	17 (34)	28 (56)	
Former smokers	26 (52)	16 (32)	ns
Never smokers	7 (14)	6 (12)	
Cumulative tobacco consumption (P-Y \pm SD)	35.9 \pm 21.7	36.6 \pm 23.7	ns
Age at onset (years \pm SD)	17.8 \pm 3.6	19.3 \pm 4.8	ns
Duration (years \pm SD)	38.8 \pm 10.7	40.9 \pm 10.6	ns
Asbestos exposure			
Questionnaire (n)	50 ^s	0 ^f	
AB/ g dry lung tissue (median) [Min – Max]	1,870 [204 – 737,941]	49 [0 – 161]	< 10 ⁻³

AB: Asbestos bodies; Max: Maximum; Min: Minimum; ns: not significant, ie $P \geq 0.05$; NSCLC: non-small cell lung cancer; P-Y: Pack-Years; SD: Standard Deviation.

^sDefinite occupational exposure to asbestos of at least 10 years; ^fNo occupational, domestic and environmental exposure to asbestos; *Wilcoxon, Pearson's Chi-square and Fisher's exact tests as appropriate.

Table II. *TP53*, *EGFR* and *KRAS* gene mutation rates according to gender, histological characteristics, smoking habits and asbestos exposure in non-small cell lung cancer

	<i>TP53</i> gene mutations				<i>EGFR</i> gene mutations				<i>KRAS</i> gene mutations			
	Total	Asbestos-exposed cases	Unexposed cases	<i>P</i> ^a	Total	Asbestos-exposed cases	Unexposed cases	<i>P</i> ^a	Total	Asbestos-exposed cases	Unexposed cases	<i>P</i> ^a
All cases (n=100)	39/100	19/50	20/50	ns	8/100	2/50	6/50	ns	13/100	5/50	8/50	ns
Gender												
Male (n = 88)	36/88	17/44	19/44	ns	5/88 ^f	2/44	3/44	ns	12/88	5/44	7/44	ns
Female (n = 12)	3/12	2/6	1/6	ns	3/12 ^f	0/6	3/6	ns	1/12	0/6	1/6	ns
Histology												
SCC (n = 42)	22/42 ^b	11/21	11/21	ns	3/42	0/21	3/21	ns	2/42 ^c	1/21	1/21	ns
ADC (n = 58)	17/58 [†]	8/29	9/29	ns	5/58	2/29	3/29	ns	11/58 ^c	4/29	7/29	ns
Smoking status												
Non-smokers (n = 13)	2/13 ^d	1/7	1/6	ns	4/13 ^e	1/7	3/6 ^g	ns	1/13	0/7	1/6	ns
Current smokers (n = 45)	21/45 ^d	7/17	14/28	ns	2/45 ^e	0/17	2/28 ^g	ns	4/45	1/17	3/28	ns
Former smokers (n = 42)	16/42 ^d	11/26	5/16	ns	2/42 ^e	1/26	1/16 ^g	ns	8/42	4/26	4/16	ns

ADC: Adenocarcinoma; SCC: Squamous cell carcinoma.

^aPearson's Chi-square and Fisher's exact tests as appropriate, ns: not significant, *i.e.* $P \geq 0.05$;

^b: $P = 0.020$; ^c: $P = 0.037$ (comparison of mutations between histological types);

^d: $P = 0.061$; ^e: $P = 0.009$ (comparison of mutations between non smokers, and current and former smokers);

^f: $P = 0.053$ (comparison of mutations between gender);

^g: $P = 0.017$ (comparison of mutations between non-smokers, and current and former smokers in the asbestos-unexposed group).

Table III. Spectrum of *TP53* mutations in non-small cell lung cancer

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

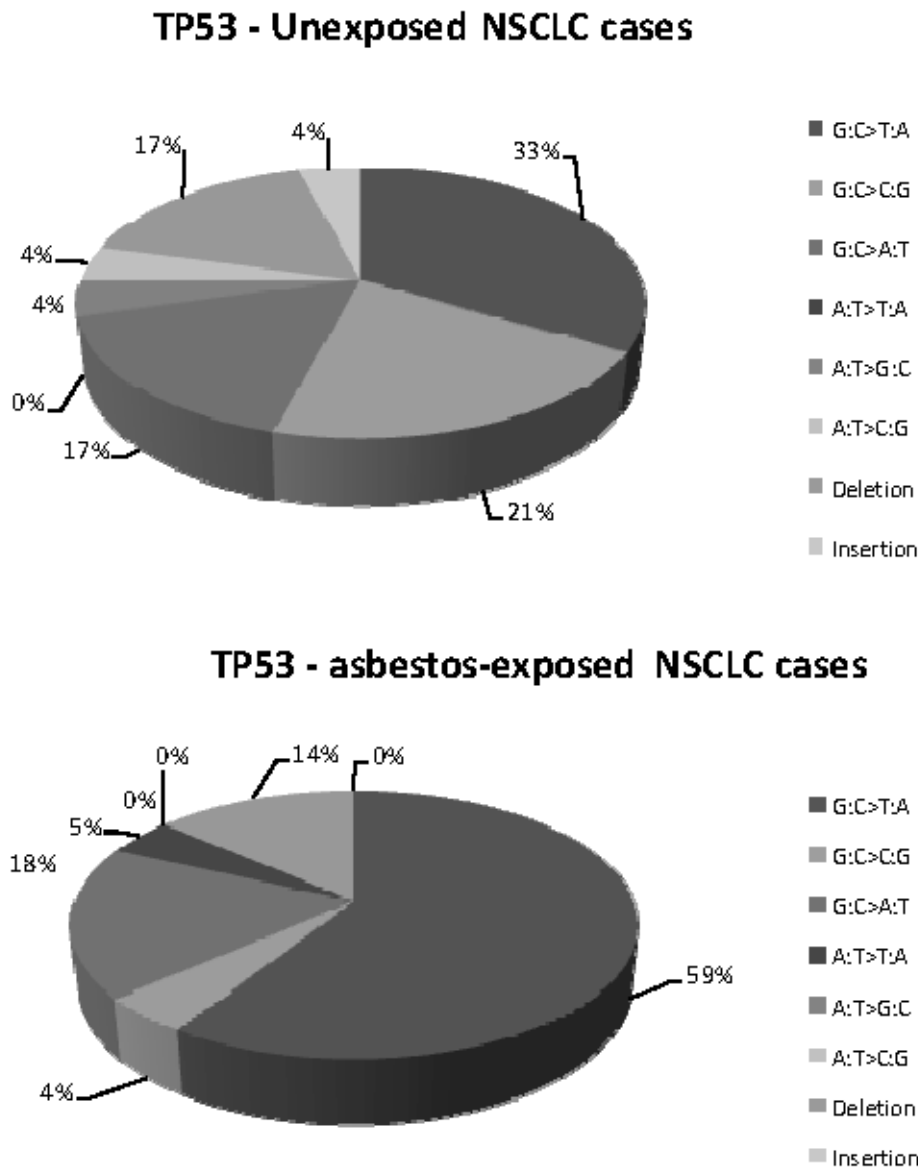
NSCLC: non-small-cell lung cancer.

*Pearson's Chi-square and Fisher's exact tests as appropriate, ns: not significant ie, $P \geq 0.05$;

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.

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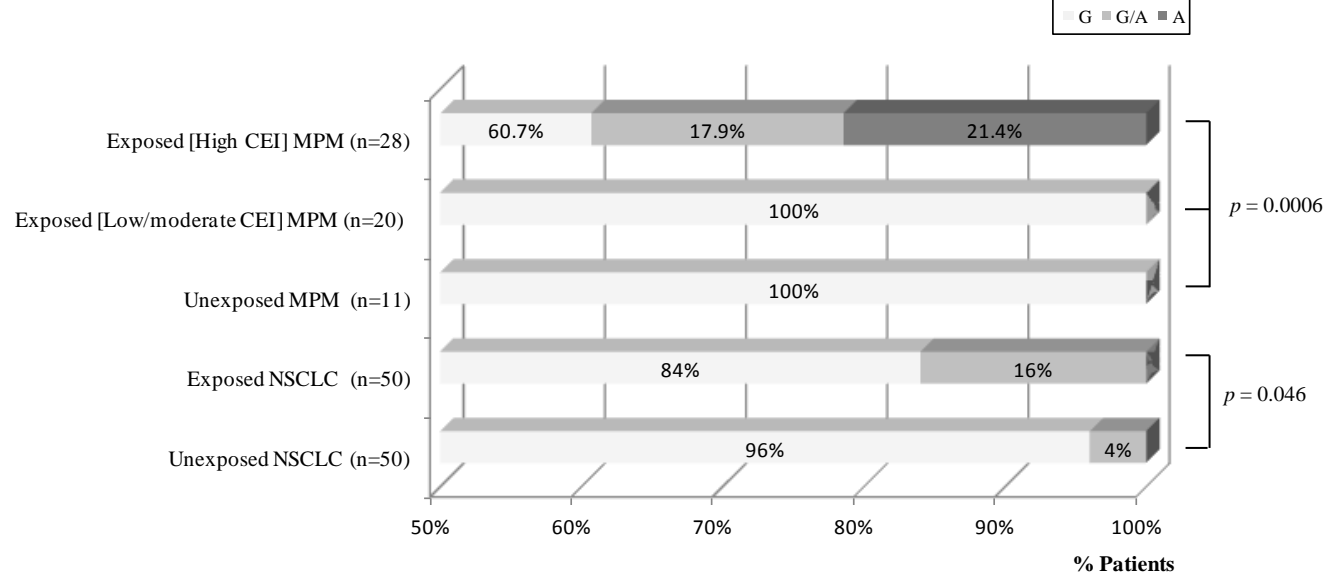
Figure 1: *TP53* gene alteration type in asbestos-exposed and unexposed NSCLC cases



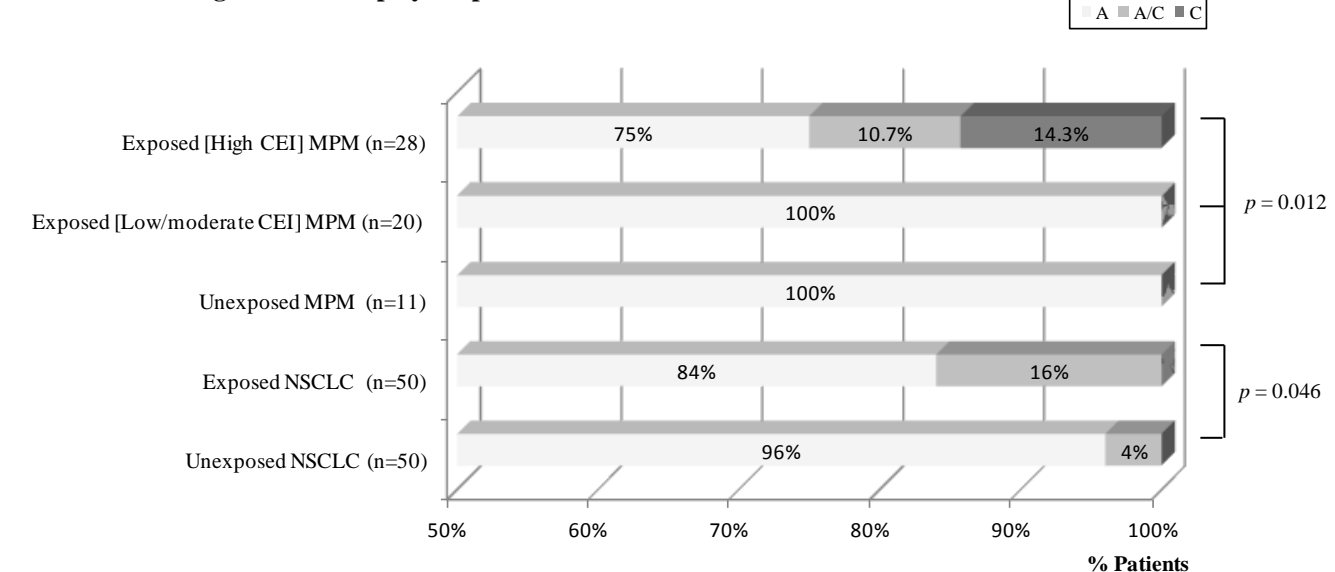
NSCLC: non-small cell lung cancer.

Figure 2. Single nucleotide polymorphisms in *TP53* intron 7 in non-small-cell lung cancer and malignant pleural mesothelioma populations according to asbestos exposure. (A) rs12947788; (B) rs12951053.

A. rs12947788 single nucleotide polymorphism



B. rs12951053 single nucleotide polymorphism



MPM: Malignant pleural mesothelioma; NSCLC: Non-small-cell cancer lung; CEI: Cumulative exposure index to asbestos.

Supplementary Table Is: Primer sets used for *KRAS*, *EGFR*, *TP53* and *NF2* for PCR analyses

Gene	Exon	Primer name	Primer sequence 5'→3'	T _m (°C)	Product size (bp)
<i>KRAS</i>	Exon 1	KRASEx1F KRASEx1R	CTGGTGGAGTATTTGATAGTG CATATGATGTCACAATACCAAG	56	555
	Exon 2	KRASEx2F KRASEx2R	GTATGTGGTAGCATCTCATGG GTACAGAAGGCTGTGGAGTC	56	667
<i>EGFR</i>	Exon 18	EGFREx18F EGFREx18R	CAAATGAGCTGGCAAGTGCCGTGTC GAGTTTCCCAAACACTCAGTGAAAC	58	400
	Exon 19	EGFREx19F EGFREx19R	GCAATATCAGCCTTAGGTGCGGCTC CATAGAAAGTGAACATTTAGGATGTG	58	372
	Exon 20	EGFREx20F EGFREx20R	CCATGAGTACGTATTTTGAAACTC CATATCCCATGGCAAACCTCTTGC	58	408
	Exon 21	EGFREx21F EGFREx21R	CTAACGTTCCGCCAGCCATAAGTCC GCTGCGAGCTCACCCAGAATGTCTGG	58	415
<i>TP53</i>	Exons 2-3	P53Ex2F P53Ex3R	GATCCCCACTTTTCCTCTTG GTCCCCAGCCCAACCCTTGT	64	288
	Exon 4	P53Ex4F P53Ex4R	CGTTCTGGTAAGGACAAGGG GGAATCCCAAAGTTCCAAAC	64	484
	Exons 5-6	P53Ex5F P53Ex6R	CTAGTGGGTTGCAGGAGGTG TCATGGGGTTATAGGGAGGTC	64	614
	Exons 7-8-9	P53Ex7F P53Ex9R	CCTCCCCTGCTTGCCACA ATGCCCAATTGCAGGTAAAACA	64	921
	Exon 10	P53Ex10F P53Ex10R	TCAAACAATTGTAACCTGAACCATC GGCAGGATGAGAATGGAATC	64	269
	Exon 11	P53Ex11F P53Ex11R	AGCCTTAGGCCCTTCAAAGC AAAACCCAAAATGGCAGGGGAG	64	250
<i>NF2</i>	Exon 1	NF2ex1F NF2ex1R	CTGAGGCCTGTGCAGCAAC TTCTGCCCGTCTCTAGCTAC	64	295
	Exon 2	NF2ex2F NF2ex2R	CAGTCCTCATCAGCTGTCTT TACTATACAGCTACAGCGCC	64	354
	Exon 3	NF2ex3F NF2ex3R	CCTCTTGCTATAGGTGACTTTG ACAGAACCAAAGGGGCTGACC	64	438
	Exon 4	NF2ex4F NF2ex4R	CCAGAGCTGCTCTTGACACT ACAGTGTTCTTCAAGTCCAC	64	567
	Exon 5	NF2ex5F NF2ex5R	TCCAGCTCTGTTCAAGAAATGG TAGACCACATATCTGCTATGTC	64	190
	Exon 6	NF2ex6F NF2ex6R	ACTGCCAGATGAGGGTTGCA AGCATGTCCTAGTTTTCAGTT	64	405
	Exon 7	NF2ex7F NF2ex7R	GAATGCTTGATTTGGTGCCC GGGGGGAGAAGTACTTACAAG	64	495

Exon 8	NF2ex8F NF2ex8R	GGACCTGCTGAACTTGTCA CCATCTGCAGTACACACATGT	64	274
Exon 9	NF2ex9F NF2ex9R	GGCATAACTTCATGCTGGTC ACAAGATGTCACCTCTGATATCCT	64	281
Exon 10	NF2ex10F NF2ex10R	TCTGAGGCGCCTTCATTTTG GTAGGCATCGGCAAATGAAG	64	483
Exon 11	NF2ex11F NF2ex11R	AAAGGTCCCAAAGGGGAGA AGGAGACCAAGCTCCAGAAA	64	302
Exon 12	NF2ex12F NF2ex12R	CATCTCAGTGTTCAAGGCAG CTTGAGGACAACCTGCTGTAG	64	392
Exon 13	NF2ex13F NF2ex13R	AGACGCAGTTCTTGCACATTC TTTGCTGAATGGTCACCCT	64	627
Exon 14	NF2ex14F NF2ex14R	GATCGGTTGTCAACACAGTAG AGGGCACAGGGGGCTACA	64	225
Exon 15	NF2ex15F NF2ex15R	TCAAACCCTAGATCGCACAC TGAGGAAACCAGATGCCAAC	64	346
Exon 16	NF2ex16F NF2ex16R	CACTCATCACGATTTTCAGGC CATCACCACATAGAGAGTACC	64	190
Exon 17	NF2ex17F NF2ex17R	TAAAATAGGCAGGGATCTCCG CAGGTCAGAGAACTAGAACG	64	576

F: forward; R: reverse; Tm: melting temperature

Supplementary Table IIs : Mutations in non-small cell lung cancer

NSCLC cases	Gender	Age (year)	Histology	Asbestos exposure	Asbestos bodies per g dry lung tissue	Smoking status	P-Y	Smoking age at onset (year)	Cumulative duration smoking (year)	EGFR mutations (NM_005228.3)	KRAS mutations (NM_033360.2)	NF2 mutations (NM_000268.3)	TP53 mutations (NM_000546.4)	TP53 SNP rs12947788	TP53 SNP rs12951053
1008	Male	59.3	SCC	E	10592	2	20	18	11	-	-	-	-	G	A
1009	Male	66.7	SCC	U	160	2	20	16	42	-	-	-	-	G	A
1012	Male	62	SCC	E	1231	1	50	14	47	-	-	-	-	G	A
1027	Male	68.4	SCC	U	104	2	44	19	41	-	-	-	-	G	A
1031	Male	47.9	ADC	E	4260	2	3	16	30	-	-	-	-	G/A	A/C
1045	Male	70	ADC	U	0	1	55	11	59	-	-	-	569C>T, P190L	G	A
1050	Male	68.8	SCC	U	0	1	45	19	50	-	-	-	-	G	A
1069	Male	52	ADC	E*	685	2	28	18	29	-	-	-	-	G/A	A/C
1072	Male	79	ADC	U	57	2	6	14	61	-	-	-	-	G	A
1073	Male	67.5	SCC	E	3346	2	42	18	32	-	-	-	-	G	A
1074	Male	71.8	SCC	E	13121	2	39	20	44	-	-	-	-	G	A
1076	Male	66.8	ADC	E	3552	1	40	15	51	-	-	-	-	G	A
1079	Male	65.6	ADC	E	1034	2	63	14	42	-	-	-	-	G	A
1081	Female	77.2	ADC	U	67	0	0	-	-	747-753 delins	-	-	-	G	A
1083	Male	72.9	SCC	E	1323	2	14	32	29	-	-	-	841C>A, D281E	G	A
1085	Male	70.8	SCC	E	28621	2	35	21	35	-	-	-	445 del (T), S149FsX169	G	A
2023	Female	61.6	ADC	E	1865	1	71	nd	nd	-	-	-	-	G	A
2029	Female	77.4	ADC	E	1041	0	0	-	-	-	-	-	-	G	A
2031	Female	77.1	ADC	E	1045	0	0	-	-	-	-	-	-	G	A
2034	Male	62.3	ADC	U	127	2	35	17	36	-	-	-	-	G/A	A/C
2036	Male	66.9	ADC	U	143	2	24	17	26	-	-	-	-	G	A
2045	Male	58.4	SCC	U	44	1	55	32	27	-	-	-	517G>A, V173M	G	A
2051	Male	67.3	SCC	E	737941	2	24	20	24	-	-	-	465G>T, V157F	G	A
2054	Female	73.3	ADC	E	1895	0	0	-	-	-	-	-	-	G	A
2057	Male	77	SCC	E	1688	2	59	14	59	-	-	-	-	G	A
2059	Male	70.8	ADC	E	1111	2	38	22	39	-	35G>T, G12V	-	-	G/A	A/C
2063	Female	76.8	ADC	U	0	0	0	-	-	-	35G>T, G12V	-	775G>T,D259Y	G	A
3019	Male	82.9	ADC	E	1436	2	30	20	43	-	-	-	-	G	A
3021	Male	53.4	ADC	E*	620	2	54	16	26	-	34G>T, G12C	-	-	G	A
3030	Male	67.5	SCC	E	1818	2	39	13	39	-	-	-	438G>A, W146X	G	A
3039	Male	75.1	SCC	E	1082	1	56	16	56	-	-	-	-	G	A
3040	Male	77.5	SCC	U	44	2	43	16	58	-	-	-	-	G	A
3048	Male	57.5	SCC	U	0	1	74	15	42	1658C>T; P553L	-	-	902 del (C), P301fsX344	G	A

NSCLC cases	Gender	Age (year)	Histology	Asbestos exposure	Asbestos bodies per g dry lung tissue	Smoking status	P-Y	Smoking age at onset (year)	Cumulative duration smoking (year)	EGFR mutations (NM_005228.3)	KRAS mutations (NM_033360.2)	NF2 mutations (NM_000268.3)	TP53 mutations (NM_000546.4)	TP53 SNP rs12947788	TP53 SNP rs12951053
3064	Male	68.6	SCC	E	2328	2	40	24	40	-	-	-	375G>T, T125T	G	A
3068	Male	73.5	SCC	E	2143	0	0	-	-	-	-	-	-	G	A
3071	Male	85.3	ADC	E	1190	0	0	-	-	-	-	-	-	G	A
3077	Male	53.8	ADC	E	1314	2	37	14	30	-	-	-	734G>T, G245V	G/A	A/C
3081	Male	79.6	SCC	E	1659	1	28	17	21	-	-	-	-	G	A
3083	Male	65.4	ADC	E	4433	0	0	-	-	747-753 delins	-	-	-	G	A
3091	Male	78.9	ADC	U	78	1	115	14	65	-	-	-	-	G	A
3093	Male	54.7	ADC	U	0	1	35	20	35	-	-	-	-	G	A
3096	Male	57.3	ADC	U	0	1	34	20	35	-	34G>T, G12C	-	-	G	A
3103	Male	76.5	SCC	U	51	1	30	21	55	-	-	-	-	G	A
3110	Male	55.4	ADC	U	0	1	29	22	34	-	-	-	-	G	A
3113	Male	46.1	ADC	U	32	2	22	10	30	-	-	-	-	G	A
3114	Female	69.2	SCC	U	95	1	39	25	44	-	-	-	-	G	A
3121	Female	62.7	ADC	U	142	1	60	17	46	-	-	-	-	G	A
4005	Male	62.8	ADC	E	2751	2	38	20	38	-	-	-	-	G	A
4022	Male	78.9	SCC	E	14402	2	33	18	32	-	-	-	919+1 G>T	G	A
4032	Male	62.5	ADC	E	1991	1	43	20	42	-	-	-	473G>T, R158L	G	A
4035	Male	51.8	ADC	E	2027	1	32	20	32	-	-	-	-	G	A
5023	Male	75.1	ADC	U	97	0	0	-	-	-	-	-	-	G	A
5027	Male	67.5	ADC	U	86	2	61	23	41	-	57G>T, L19F	-	486 del (C), I162FsX169	G	A
5054	Female	60.2	ADC	U	131	0	0	-	-	2573T>G, L858R	-	-	-	G	A
5068	Male	58.5	SCC	U	60	1	40	20	38	2508C>T, R836R	-	-	841G>C, D281H	G	A
5073	Male	60.1	SCC	U	0	1	60	18	42	-	35G>T, G12V	-	699 Ins (A), H233FsX240 ;	G	A
5075	Male	49	ADC	U	150	1	25	20	29	-	-	-	-	G	A
5078	Male	74.2	SCC	U	0	1	20	24	50	-	-	-	465G>T, V157F	G	A
5081	Female	74.5	SCC	E	2767	2	60	20	53	-	-	-	742C>T, R248W ; 743G>T,	G	A
5082	Male	55.1	ADC	U	0	1	45	15	40	-	-	-	-	G	A
5084	Male	67.8	ADC	E	4574	2	60	17	30	2307+1G>A	-	-	310C>T, Q104X	G	A
5091	Male	65.9	SCC	U	0	1	100	15	50	-	-	-	581T>G, L194R	G	A
5099	Male	64.7	ADC	E	1097	1	47	16	48	-	-	-	-	G	A
5103	Male	68	ADC	U	33	2	40	20	20	-	34G>T, G12C	-	-	G	A
5117	Male	70.3	ADC	U	58	2	23	37	31	-	34G>T, G12C	-	465G>T, V157F ; 796G>T,	G	A
5119	Male	76.7	SCC	U	0	1	24	27	53	-	-	-	833C>G, P278R	G/A	A/C
5121	Male	49.3	ADC	U	0	1	34	15	34	-	-	-	-	G	A
5125	Male	63	SCC	E	2985	2	41	19	42	-	-	-	-	G/A	A/C

NSCLC cases	Gender	Age (year)	Histology	Asbestos exposure	Asbestos bodies per g dry lung tissue	Smoking status	P-Y	Smoking age at onset (year)	Cumulative duration smoking (year)	EGFR mutations (NM_005228.3)	KRAS mutations (NM_033360.2)	NF2 mutations (NM_000268.3)	TP53 mutations (NM_000546.4)	TP53 SNP rs12947788	TP53 SNP rs12951053
5127	Male	70.9	SCC	U	0	2	59	18	49	-	-	-	-	G	A
5129	Male	70.5	SCC	E	1812	1	45	15	55	-	34G>T, G12C	-	375G>T, T125T	G	A
5130	Male	65.6	ADC	E	1933	1	48	15	50	-	-	-	-	G/A	A
5131	Female	62	ADC	E	1803	0	0	-	-	-	-	-	473G>T,R158L	G	A/C
5132	Male	57.9	ADC	U	0	1	42	15	42	-	-	-	-	G	A
5134	Male	53.1	ADC	E	2399	1	35	17	35	-	-	-	892G>T, E298X	G	A
5135	Male	64.6	SCC	U	16	2	20	22	40	-	-	-	-	G	A
5136	Male	56.1	SCC	E*	204	2	45	20	30	-	-	-	730G>T,G244C	G	A
5138	Male	49.5	ADC	E*	760	1	28	10	38	-	-	-	-	G	A
5139	Male	49.6	ADC	E	1948	1	53	17	53	-	-	-	729G>C, M243I ; 730G>T,	G	A
5141	Male	61.7	SCC	U	82	2	40	20	40	-	-	-	-	G	A
5142	Male	63.9	SCC	U	98	1	48	20	44	-	-	-	-	G	A
5145	Male	58.3	ADC	U	84	1	37	21	37	-	34G>T, G12C	-	769C>G,L257V	G	A
5147	Male	66.8	ADC	E	21200	2	23	15	47	-	-	-	573-5_573-1 del (TACAG)	G	A
5152	Male	55.2	ADC	E	4996	1	35	20	31	-	-	-	818G>A, R273H	G/A	A/C
5153	Male	52.2	ADC	U	43	0	0	-	-	-	-	-	-	G	A
5154	Male	50.9	ADC	U	161	1	37	20	31	-	-	-	473G>T,R158L ; 917G>C,	G	A
5170	Male	64.3	SCC	E	2603	1	54	19	45	-	-	-	-	G/A	A/C
5172	Male	82.1	ADC	E	1394	2	80	21	41	-	-	-	-	G	A
5179	Male	63	ADC	U	95	1	39	20	35	-	-	-	517 del (G), V173FsX173	G	A
5181	Male	50.3	ADC	E	1101	2	35	15	25	-	34G>T, G12C	-	-	G	A
5184	Male	67.5	SCC	E	1875	1	96	20	48	-	-	-	734G>T,G245V	G	A
5185	Male	73	SCC	E	1857	1	38	18	54	-	-	-	471-493 del	G	A
5193	Male	71.2	SCC	U	104	2	36	18	48	2508C>T, R836R	-	-	1105-12_1107 del	G	A
5194	Male	57.1	ADC	E	1492	2	16	14	34	-	34G>T, G12C	-	-	G	A
5200	Female	72.5	ADC	U	0	0	0	-	-	2237-2251del	-	-	-	G	A
5202	Male	70	ADC	U	0	2	40	18	20	-	35G>A, G12D	-	734G>A,G245D	G	A
5204	Male	63.5	SCC	U	0	1	2	20	43	-	-	-	1024C>T,R342X	G	A
5205	Male	75.4	SCC	U	0	2	47	20	47	-	-	-	375G>T, T125T ; 743G>C,	G	A
5211	Male	60.5	ADC	U	16	1	51	18	27	-	-	-	842A>G, D281G	G	A
5212	Male	50.2	ADC	U	0	1	45	20	30	-	-	-	-	G	A
5223	Male	71.1	SCC	U	0	1	51	20	51	-	-	-	711G>T,M237I	G	A

Legends of supplementary Table 2s :

ADC : Adenocarcinoma

E : Exposed; E* : Exposed (definite occupational exposure to asbestos for more than 10 years)

NSCLC : Non-small cell lung cancer

P-Y : Pack-years

SCC : Squamous cell carcinoma

Smoking status : 0 = never smoker; 1 = current smoker; 2 = former smoker

U : Unexposed

Supplementary Table IIIs : Mutations in malignant pleural mesothelioma

MPM cases	Gender	Age (year)	Histology	Asbestos exposure (CEI)	EGFR mutations (NM_005228.3)	KRAS mutations (NM_033360.2)	NF2 mutations (NM_000268.3)	TP53 mutations (NM_000546.4)	TP53 SNP rs12947788	TP53 SNP rs12951053
MESO_MJ_HS_031103	Male	72.2	MME	High	-	-	-	-	G/A	A/C
MESO_MJ_HS_120602	Male	71.3	MME	High	-	-	-	-	A	C
MESO_MJ_HS_110592	Male	51.6	MMB	High	-	-	558-?_890+? del	-	G/A	A/C
MESO_MJ_HS_070591	Female	76.6	MME	U	-	-	-	743G>A, R248Q	G	A
MESO_MJ_HS_141189	Male	56.4	MME	U	-	-	-	-	G	A
MESO_MJ_HS_220103	Male	63.4	MMS	Moderate	-	-	1151T>A, L384X ; 1890-?_2017+? del ; 2181-?_2231+? del	-	G	A
MESO_MJ_HS_191290	Male	69.0	MME	High	-	-	-	-	G	A
MESO_MJ_HS_290391	Male	77.7	MME	Moderate	-	-	1043-?_1118+? del	-	G	A
MESO_MJ_HS_200594	Male	80.4	MME	Moderate	-	-	294 del (C), A98FsX122	-	G	A
MESO_MJ_HS_181089	Male	78.2	MME	High	-	-	-	-	G	A
MESO_MJ_HS_240589	Male	46.9	MME	U	-	-	-	-	G	A
MESO_MJ_HS_070790	Male	59.8	MME	Moderate	-	-	576C>G, Y192X	-	G	A
MESO_MJ_HS_270492	Male	58.5	MME	Low	-	-	-	-	G	A
MESO_MJ_HS_061190	Male	67.5	MME	Low	-	-	-	-	G	A
MESO_MJ_HS_311091	Female	64.0	MME	High	-	-	1-?_1118+? del ; 1254-?_1783+? del	-	G/A	A/C
MESO_MJ_HS_140602	Male	76.6	MME	High	-	-	1-?_557+? del ; 949 G>T, E317X	-	G	A
MESO_MJ_HS_080390	Male	65.0	MME	Low	-	-	-	-	G	A
MESO_MJ_HS_180393	Male	69.2	MME	High	-	-	-	-	G	A
MESO_MJ_HS_151003	Male	52.6	MMB	High	-	-	-	-	G/A	A
MESO_MJ_HS_140795	Male	47.0	MME	High	-	-	-	742C>T, R248W	A	C
MESO_MJ_HS_100593	Male	46.1	MME	Moderate	-	-	1357C>T, Q453X	-	G	A
MESO_MJ_HS_160994	Male	53.7	MMD	High	-	-	-	-	G	A
MESO_MJ_HS_180791	Male	37.5	MMB	Low	-	-	-	747G>T, R249S	G	A
MESO_MJ_HS_040203	Male	69.7	MME	High	-	-	-	-	G	A
MESO_MJ_HS_200103	Female	69.3	MMB	Low	-	-	558-?_2017+? del	-	G	A
MESO_MJ_HS_010391	Male	71.1	MME	Moderate	-	-	558-?_890+? del	-	G	A
MESO_MJ_HS_280291	Male	64.2	MME	High	-	-	1890-?_2017+? del	-	G	A
MESO_MJ_HS_190790	Female	64.3	MME	U	-	-	-	-	G	A
MESO_MJ_HS_140891	Male	48.2	MME	U	-	-	-	-	G	A
MESO_MJ_HS_110789	Male	58.6	MME	High	-	-	-	-	G	A
MESO_MJ_HS_010290	Male	64.9	MME	High	-	-	1258G>T, E420X	517G>A, V173M	G	A
MESO_MJ_HS_020592	Male	50.3	MME	High	-	-	-	-	G/A	A
MESO_MJ_HS_240295	Male	66.6	MME	High	-	-	-	-	A	A
MESO_MJ_HS_260402	Male	43.1	MMS	High	-	-	1784-?_1889+? del	-	G	A

Legends of supplementary Table 3s :

CEI : Cumulative exposition index

MMB : Malignant mesothelioma biphasic

MME : Malignant mesothelioma epithelioid

MMS : Malignant mesothelioma sarcomatoid

MMD : Malignant mesothelioma desmoplastic

MPM : Malignant pleural mesothelioma

U : Unexposed