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**Syntenic relationships between genomic profiles of fiber-induced murine and human malignant mesothelioma**

Didier Jean <sup>1,2</sup>, Emilie Thomas <sup>3</sup>, Elodie Manié <sup>4,5</sup>, Annie Renier <sup>1,2</sup>, Aurélien de Reynies <sup>3</sup>, Céline Lecomte <sup>6</sup>, Pascal Andujar <sup>7,8,9</sup>, Jocelyne Fleury-Feith <sup>10,11</sup>, Françoise Galateau-Sallé <sup>12,13</sup>, Marco Giovannini <sup>14</sup>, Jessica Zucman-Rossi <sup>1,2</sup>, Marc-Henri Stern <sup>4,5</sup>, Marie-Claude Jaurand <sup>\* 1,2</sup>

<sup>1</sup>INSERM, U674, Paris, F-75010, France.

<sup>2</sup>Université Paris Descartes, UMR-S674, Paris, F-75010, France.

<sup>3</sup>Ligue Nationale Contre le Cancer, Paris, F-75013, France.

<sup>4</sup>INSERM, U830, Paris, F-75248, France.

<sup>5</sup>Institut Curie, Centre de Recherche, Paris, F-75248, France.

<sup>6</sup>Université du Havre, UFR de Sciences et Techniques, UPRES-EA3222, Laboratoire d'Ecotoxicologie, Le Havre, F-76000, France.

<sup>7</sup>Hôpital Intercommunal de Créteil, Service de Pneumologie et de Pathologie Professionnelle, Créteil, F-94010, France.

<sup>8</sup>INSERM, U955, Equipe 4, IMRB, Créteil, F-94010, France.

<sup>9</sup>Université Paris-Est, Faculté de Médecine, Créteil, F-94010, France.

<sup>10</sup>AP-HP, GHU Est, Hôpital Tenon, Service d'Histologie et de Biologie Tumorale, Paris, F-75020, France.

<sup>11</sup>Université Pierre et Marie Curie, Paris, F-75012, France.

<sup>12</sup>Service d'Anatomie Pathologique, Hôpital de la Côte de Nacre, Caen, F-14033, France.

<sup>13</sup>INSERM, ERI3, Caen, F-14032, France.

<sup>14</sup>House Ear Institute, Center for Neural Tumor Research, Los Angeles, CA 90057, USA.

\*To whom correspondence should be addressed.

Phone: +33 1 53 72 51 88; Fax: +33 1 53 72 51 92; Email: marie-claude.jaurand@inserm.fr

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**Corresponding author:**

Marie-Claude Jaurand

INSERM, U674 - 27, rue Juliette Dodu - Paris, F-75010, France.

Phone: +33 1 53 72 51 88; Fax: +33 1 53 72 51 92; Email: marie-claude.jaurand@inserm.fr.

**Reprint requests:**

Marie-Claude Jaurand

INSERM, U674 - 27, rue Juliette Dodu - Paris, F-75010, France.

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**Abbreviations:**

aCGH: array-comparative genomic hybridization; chr: chromosome; CNT: carbon nanotube; Hs: *Homo sapiens*; Mm: *Mus musculus*; MM: malignant mesothelioma; *Nf2*<sup>+/-</sup>: heterozygous *Nf2*<sup>+/-</sup> mice; RCF: refractory ceramic fibers; SNP: single nucleotide polymorphism; WT: wild type

## **Abstract**

**Background:** Malignant mesothelioma is an aggressive tumor with a poor prognosis mainly linked to past asbestos exposure. Murine models of MM based on fiber exposure have been developed in order to understand the mechanism of mesothelioma formation. Genomic alterations in murine MM have now been partially characterized.

**Methods:** To gain insight into the pathophysiology of mesothelioma, 16 murine and 35 human mesotheliomas were characterized by array-comparative genomic hybridization and were screened for common genomic alterations.

**Findings:** Alteration of the 9p21 human region, often by biallelic deletion, was the most frequent alteration in both species, in agreement with the *CDKN2A/CDKN2B* locus deletion in human disease and mouse models. Other shared aberrations were losses of 1p36.3-p35 and 13q14-q33, and gains of 5p15.3-p13 regions. However, some differences were noted, such as absence of recurrent alterations in mouse regions corresponding to human chromosome 22. Comparison between altered recurrent regions in asbestos-exposed and non-exposed patients showed a significant difference in the 14q11.2-q21 region, which was also lost in fiber-induced murine mesothelioma. A correlation was also demonstrated between genomic instability and tumorigenicity of human mesothelioma xenografts in nude mice.

**Conclusions:** Overall, these data show similarities between murine and human disease and contribute to the understanding of the influence of fibers in the pathogenesis of mesothelioma and validation of the murine model for preclinical testing.

## Introduction

Malignant mesothelioma (MM) is a severe primary neoplastic disease. Its frequency has dramatically increased in recent decades due to occupational exposure to asbestos fibers and the long latency period between first exposure and diagnosis, ranging from 20 to more than 40 years<sup>1,2</sup>. The growing frequency of mesothelioma was also aggravated by the delayed recognition of asbestos diseases and implementation of asbestos regulations. Despite recent epidemiological data suggesting that the peak of MM incidence may occur sooner than expected, this malignancy remains a major concern in view of the poor results of treatment and reports of mesotheliomas with no known exposure to asbestos in 20% to 40% of cases<sup>3-6</sup>. This raises the question of the role of very low levels of asbestos, as described in the context of environmental exposure, or other as yet unidentified risk factors<sup>7-11</sup>. New manufactured fiber-shaped products may also be of concern, as recent studies have demonstrated that carbon nanotubes (CNT) can reach subpleural tissue in mice<sup>12,13</sup> and induce inflammation after injection in the peritoneal cavity of mice<sup>14</sup>. Mesotheliomas also occur in genetically-modified cancer-sensitive mice and in conventional Fisher 344 rats exposed to CNT by intraperitoneal and intrascrotal administration, respectively<sup>15,16</sup>.

To improve the outcome of MM, a better knowledge of the somatic alterations in neoplastic cells is necessary in order to improve diagnosis, treatment and prognosis. Several techniques are available to identify MM genomic alterations, such as cytogenetics, comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) array, or gene mutations by DNA sequencing. Data from the literature show that most human MM cases exhibit complex patterns of cytogenetic changes with chromosomal losses being more frequent than gains and numerous partial or total chromosome losses have been shown to be recurrent<sup>17-26</sup>. Somatic molecular abnormalities affecting tumor suppressor genes (TSGs) have also been described in human MM. They mainly consist of codeletion of *p16/CDKN2A*,

*p14/ARF* and *p15/CDKN2B* genes, and mutations of the *NF2* gene in approximately one half of cases. In contrast, the *TP53* TSG is less frequently inactivated<sup>27-30</sup>.

Murine models of mesothelioma have been recently developed in wild-type (WT) and hemizygous *Nf2*<sup>+/-</sup> mice after asbestos exposure by intraperitoneal inoculation, offering a unique opportunity to more clearly characterize the genomic changes caused by asbestos on mesothelial cells<sup>31-33</sup>. These animals developed mesotheliomas 9 to 24 months after exposure, with frequent occurrence of ascitic fluid. *Nf2* hemizygosity resulted in a higher rate of mesothelioma when compared to WT mice, with no significant difference in terms of time to tumor occurrence<sup>31</sup>. Mesothelioma cells isolated from tumor ascites obtained from these mice displayed similar gene mutations as human MM, characterized by frequent LOH at the *Nf2* locus in *Nf2*<sup>+/-</sup> mice and, in all genetic backgrounds, frequent inactivation of *p16/Cdkn2a*, *p19/Arf*, *p15/Cdkn2b* TSGs and infrequent inactivation of *Trp53*<sup>32,34,35</sup>. Only limited data on global analysis of genomic alterations have been reported to date in rodents. Only recurrent alterations in chromosome 4 were described in murine mesothelioma cells<sup>36</sup>. A CGH study was also performed in rats after intraperitoneal administration of iron saccharate<sup>37</sup>.

The present study was designed to investigate genomic alterations in MM by comparing the genomic profile of human MM cases to that of fiber-induced murine MM assessed by array-comparative genomic hybridization (aCGH). Genomic profiles showed numerous alterations in both species with fifteen similar regions of chromosome imbalance. In human MM cells, a link was found between genomic instability and the ability of MM xenografts to develop subcutaneous tumors in immunocompromized mice. Our data also suggest that loss of human 14q11.2-q21 region is related to asbestos exposure.

These results suggest that genes in altered regions may be damaged or deregulated by asbestos exposure, and may subsequently sustain carcinogenesis. Research is currently

underway to identify the genes, gene families and pathways involved in mesothelial carcinogenesis.

## **Materials and Methods**

### *Mesothelioma cells*

Murine mesothelioma cells were obtained from previous experiments in which mineral fibers, asbestos and carcinogenic refractory ceramic fibers (RCF) were inoculated into the peritoneal cavity of WT and *NF2*<sup>+/-</sup> mice. This strategy allowed the generation of mesotheliomas mimicking the morphology and histopathology of the corresponding human cancer<sup>31,35</sup>. Sixteen cell cultures were established from tumor ascites obtained from 12 *Nf2*<sup>+/-</sup> mice and 4 WT mice, as reported elsewhere<sup>31</sup>. The mesothelial origin was assessed by immunocytochemistry using cytokeratin and vimentin antibodies according to a previously described method<sup>31</sup>. Histological subtypes were epithelioid (15.4%), sarcomatoid (30.8%) and mixed (53.8%) MMs. Histopathological features of murine mesotheliomas were independent of the fiber type, asbestos or RCF. The genetic background did not influence tumor morphology or genetic alterations, apart from more frequent LOH at the *NF2* locus in *Nf2*<sup>+/-</sup> mice<sup>34</sup>.

Thirty five human cell cultures were obtained from confirmed malignant mesothelioma cases: 29 males (82.8%) and 6 females (17.2%) with a mean age of 63 ± 11 years and 69 ± 5 years, respectively (mean ± standard deviation). The study was approved by the local Ethics Committee and human cells were obtained with the informed consent of the patients. Detailed information about the tumors was obtained from pathology reports. Most cases were epithelioid subtypes (80%). Sarcomatoid and mixed subtypes of MM were observed in 6.7% and 13.3% of cases, respectively. Patients' asbestos exposure was estimated by interviewer-administered questionnaire. This questionnaire comprised complete job

history, including past occupational, domestic, and environmental exposures to asbestos. It was completed by face-to-face interview<sup>38</sup>. In this series, asbestos exposure was ascertained in 24 MM cases, 2 cases were possibly exposed and no exposure was found in 8 cases. In one case, no data was available on asbestos history.

#### *DNA extraction and qualification*

Cells were grown in RPMI 1640 medium with Glutamax and 25 mM HEPES, supplemented with 8% fetal calf serum, 50 IU/ml penicillin and 50 mg/ml streptomycin (Invitrogen, Cergy Pontoise, France). All genetic analyses were carried out with cultures of less than 10 passages. Genomic DNA was extracted as previously described<sup>34</sup>, quantified by absorption measurement, and qualified by deposition of 100 ng on a 0.8% agarose gel. DNA ladder was  $\lambda$ HindIII (Invitrogen).

#### *Human and murine aCGH.*

The human genome-wide CIT-CGH array (V6) contained 5,822 BAC clones, with a higher coverage in genomic regions known to contain genes involved in cancer. This array was developed by partnership between the Ligue nationale contre le cancer, the Genoscope, the Curie Institute and Integragen (<http://cit.ligue-cancer.net>). The genome-wide CIT M3 Mus musculus 1K BAC CGH array containing 958 unique BAC clones was manufactured by the Curie Institute (GEO record: GPL3972). Hybridizations and analyses were performed as previously reported<sup>39,40</sup>. Fluorescent signals were detected with GenePix 4000B scanner (Molecular Devices, Union City, CA) and analyzed with GenePix Pro 5.1 software. Normalization was performed by the MANOR routine<sup>41</sup> and data were visualized on VAMP interface<sup>42</sup>. A syntenic conversion tool was developed to visualize the “humanized” profile of murine tumors and to facilitate comparison with human profiles. This tool attributes human



syntenic coordinates obtained from the UCSC website [<http://genome.ucsc.edu/>] to each murine BAC of the array. Gains and losses were defined by the GLAD algorithm<sup>43</sup>. Amplifications and bi-allelic deletions were defined by a normalized fluorescence ratio greater than 3 and less than 0.5, respectively. Recurrent regions of chromosomal alteration were defined as regions that encompassed at least two adjacent probes that were both gained or lost.

#### *Gene alterations analysis.*

Gene mutations and deletions were analyzed from genomic DNA extracted from all murine and human MM cases. PCR amplifications and sequencing were carried out on a GeneAmp 9700 apparatus (Perkin Elmer, Courtaboeuf, France) and ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Courtaboeuf, France), respectively, according to previously published methods<sup>34,35</sup>. Analyses concerned human genes *TP53*, *CDKN2A*, *CDKN2B* and *NF2*, and the murine orthologs *Trp53*, *Cdkn2a*, *Cdkn2b* and *Nf2*.

#### *Human MM cell xenografts.*

Female athymic BALB/c nude mice (6-8 weeks old) were purchased from Charles River laboratories (Les Oncins, Saint Germain sur l'Arbresle, France) and maintained under pathogen-free conditions. Animal care and experimentation were conducted in compliance with institutional guidelines in France. Human MM cells were harvested using a 0.25% trypsin, 0.2% EDTA solution (w/v) (Invitrogen). Three million cells with more than 90% viability were suspended in a volume of 200  $\mu$ l PBS and injected subcutaneously into the scapular region of athymic nude mice. At least 4 mice were inoculated for each MM, and subcutaneous tumor growth was monitored twice a week.

*Statistical analyses.*

Statistical analyses were performed using Prism software, version 4.0c (GraphPad Software, San Diego, CA). A t-test was used to analyze the association between tumorigenic phenotype and number of chromosomal breakpoints. Statistical differences in the frequency of gene mutations between human and murine MMs and comparison between the occurrence of recurrent alterations in chromosomal region and tumorigenic phenotype or asbestos exposure were assessed by Fisher's exact test.

## Results

### *Genomic characterization of murine mesotheliomas.*

The molecular profile of murine mesotheliomas was investigated in a series of 16 murine mesotheliomas. An overview of chromosome alteration patterns is presented in Figure 1. This analysis revealed numerous genomic alterations, with an average of 10.7 altered autosomes per tumor (range: 4-18). Interestingly, these alterations were mostly gains or losses of entire chromosomes. Recurrent regions of chromosomal alterations were then defined as regions with at least two adjacent probes gained or lost at a frequency higher than 30% (Table 1). Frequent recurrent gained regions (more than 50% of cases) included: chr15 (75-81%), 19qC3-qD2 (75%), 19qA-qC1 (63-75%), 6qA1-qB1 (50%-56%), 8qC2-qC5 (50%) and frequent losses included regions in 4qC4-qD1 (50-81%), 14qD3-qE2.1 (50-69%), 4qD3-qE2.1 (50-63%), chr7 (56-63%) and 14qb (50%) (supplementary Figure 1s.A-C, see Figure 1s at <http://ajp.amjpathol.org>). Minimal regions of chromosomal loss were then delineated on the basis of the alteration frequency and the presence of an interstitial deletion in at least two MMs in order to identify potential tumor suppressor genes and the miRNAs lost in murine mesothelioma cells (Table 2 and supplementary Figure 1s.A-C, see Figure 1s at <http://ajp.amjpathol.org>). The most remarkable characteristic was the high frequency of interstitial deletion in chromosome 4 (81%), which was biallelic in six tumors (Table 2). The 5 Mb minimal region of this recurrent deletion located in 4qC4 contained the *Cdkn2a/Cdkn2b* gene locus. Another recurrent interstitial deletion was observed on chromosome 14 (14qE2.1) in 11/16 MM cells, containing the *Diap3*, *Pcdh9* and *Pcdh20* candidate genes. Deletion of the *Nf2* region in 11qA1 was infrequent (3/16) and only found in a *Nf2*<sup>+/-</sup> background. Three high-level gained regions containing the protooncogene *Myc* and several cadherins were identified.

Four regions of homozygous deletions were observed including *Sav1*, the putative TSG involved in the Hippo signaling pathway as candidate target gene (Table 2).

#### *Genomic characterization of human mesotheliomas*

The genomic characterization of a series of 35 human MMs also demonstrated a high rate of rearrangements (Figure 2). Two tumors displayed up to 21 altered autosomes (mean of 13.8 altered autosomes per tumor; range: 1-21). However, most alterations did not involve entire chromosome, and monoallelic gains were less frequent than in murine MMs. Nine recurrent regions of loss with a frequency higher than 50% were observed: 9p22-p13 (51%-91%), 22q (57%-80%), 3p22-p14 (51%-63%), 13q11-q21 (51%-60%), 6q21-q22 (51%-57%), 4q21-q24 (51%-54%), 12p13 (54%), 1p36.3 (51%), 6q24-q25 (51%) and only one region of gain was observed: 5p15.3 (51%) (supplementary Figure 2s.A-Q, see Figure 2s at <http://ajp.amjpathol.org>). Recurrent regions of chromosomal alterations with a frequency higher than 30% are shown in Table 3. Ten high-level gained regions and twelve regions containing homozygous deletions were detected (Table 4). Homozygous deletions in 9p21 (22/35), 22q11.2 (3/35), 1p21 (2/35) and 9p23 (2/35) were identified in several human MMs, but none of the amplicons were recurrent.

#### *Humanized murine aCGH*

Syntenic analysis of the murine genomic profiles revealed similarities between their humanized profiles and the genomic profiles obtained from the human series of MM. In particular, twelve regions recurrently altered (frequency higher than 30%) in both the human disease and in the mouse model were identified (Table 5). Human and murine MM cells shared the same lost regions with the highest frequency: Hs (*Homo sapiens*) 9p24-p13 (91%) and Mm (*Mus musculus*) 4qC3-C5 (81%). Other altered regions, such as Hs 1p36.3-p35 (Mm

4qD2.3-4qE2) and Hs 13q14-q33 (Mm 14qD1-qE2.3), showed a frequency of loss higher than 50%. Gain in Hs 5p15.3-p13 (Mm 15qA1-qB3.1) and losses in Hs 3p21-p14 (Mn 14qA1-14qA3) and Hs 13q12 (Mn 14qC3) were also characterized as recurrent in both species. However, it must be noted that some loci within these human recurrent regions (Hs 3p21, Hs 13q13) were not altered in mice (see supplementary Figure 2s.D,L, see Figure 2s at <http://ajp.amjpathol.org>). Furthermore, several regions of chromosomal imbalance in human MM were not recurrently altered in the murine genomic profile. The most striking difference was the absence of recurrent alteration in mouse genomic regions corresponding to human chromosome 22. Others human regions characterized by a frequency of loss higher than 50%, such as Hs 4q21-q24, Hs 6q21-q22, Hs 6q24-q25, and Hs 12p13, were also not recurrently lost in the murine syntenic regions. Furthermore, regions of high-level gain differed between human and murine MMs and only the frequent homozygous deletion in the Hs 9p21 region was detected in murine MM (Mn 4qC4).

#### *Human candidate genes associated with minimal regions of chromosomal imbalance*

To determine genes or miRNAs potentially involved in human mesothelial carcinogenesis, minimal altered regions were defined on the basis of several criteria. First, CGH array data were compared between murine and human MMs and four minimal regions of loss were delineated in both species: 1p36.3, 1p36.1, 9p21 and 13q21 (Table 6 and supplementary Figure 2s.A,B,I,M, see Figure 2s at <http://ajp.amjpathol.org>). The 9p21 region, which encompassed the *CDKN2A* and *CDKN2B* genes, showed the highest alteration frequency in both species. The presence of homozygous deletions in this region was also detected in 63% and 40% of human and murine MMs, respectively (Table 5). Three other regions also contained putative TSG, such as *CHD5* (1p36.3), *NBL1* (1p36.1), *PCDH9* and *DACHI* (13q21). Interestingly, the 13q21 region, in which one of the human MMs showed a

homozygous deletion (Table 4), contained only three coding genes (Table 6). Second, thirteen new minimal regions of chromosomal loss in human MM were defined on the basis of the alteration frequency and the presence of an interstitial deletion in at least three MMs (Table 6 and supplementary Figure 2s.A-Q, see Figure 2s at <http://ajp.amjpathol.org>). Seven of these regions (3p21, 4q22-23, 6q24-q25, 10q23-q24, 12p13, 13q13 and 15q14-q21) contained genes that have been previously described as being involved in cancer progression, as shown in Table 6. The other six regions (1p22-p21, 4p16-p15.3, 4q13, 6q22, 9p22, 9p11.1 and 15q14-q21) did not contain any potential TSG, but encompassed several miRNAs loci. Third, despite the entire loss of 22q in 57% of human MMs, the smaller q12-q13 region, characterized by the highest alteration frequency (80%), was delineated. This region contains two potential genes previously described to be involved in tumor progression (*EP300*, *BIK*) and four miRNAs (Table 6). The *NF2* gene locus located in 22q12 showed an alteration frequency of 71% (supplementary Figure 2s.Q, see Figure 2s at <http://ajp.amjpathol.org>). Fourth, genes and miRNAs localized in high-level gained regions and in regions with homozygous deletions were identified (Table 4). The most remarkable finding was the presence of the *NF2* locus in one of the regions bearing biallelic deletion (22q12). Furthermore, several human genes possibly involved in tumor progression of MM such as *CDC20* (1p34.2-p34.1), *TERT* (5p15.3), *FGFR2* (10q26) and *AURKA* (20q13.3) were localized in high-level gained regions (Table 4).

#### *Gene mutations in human and murine MM*

The rate of mutations in human *TP53*, *CDKN2A/CDKN2B*, and *NF2* genes, and in the orthologous murine genes are reported in Table 7, confirming previously published data, but based on a larger series<sup>34,35,44</sup>. No significant difference was observed for the percentage of human and murine MMs showing gene alterations. Mutations in *TP53* TSG were found in a

fairly low percentage in both human and murine MMs (20% and 25%, respectively). In contrast, *CDKN2A* and *CDKN2B* genes were altered by high frequency deletions, especially in human MM. Comparison between *NF2* alterations in mice and humans is more difficult, as 75% (12/16) of murine MM cultures were derived from mesotheliomas developed in *Nf2*<sup>+/-</sup> mice. Nevertheless, deletions were the most frequent type of mutation in both human and murine MMs (65% and 92%, respectively).

#### *Association between genomic alterations and histological subtype of MM in mice and human*

Comparison of recurrent regions of chromosomal alterations with histological MM subtypes underlined different frequencies among the altered regions. As biphasic subtype is a mixed population, comparisons were only made between epithelioid and sarcomatoid subtypes. Results should be considered with caution regarding the low percentage of sarcomatoid human MM (6.7%) and murine epithelioid MM (15.4%). Gain in Hs 5p15.3-p11 and losses in Hs 3p23-p14, Hs 6q14-q27 and Hs 10p15-p12 were observed in more than 50% and 0% of epithelioid and sarcomatoid subtypes, respectively. In mice, loss of Mm 2qE1-qE3 showed the same distribution. At the opposite, Mm 1qH2.1-qH5 and Mm 16qA1-qB3 alterations showed a frequency higher than 50% in sarcomatoid subtypes and 0% in epithelioid subtypes. However, there were no similarities between human and mouse MM in terms of type and location of recurrent alterations according to histological subtype.

#### *Association between genomic alterations and xenograft growth of human MM cells*

Tumorigenicity of 32 human MM cells was determined in immunocompromised mice. *In vivo* tumor growth was observed with 23 MMs. For each case, the tumorigenic potency was compared to that of genomic alterations. Several recurrent genomic alterations were more frequent in tumorigenic (T) than in non-tumorigenic (NT) human MM, corresponding to

losses in 3p23-p14 (T: 74%; NT: 33%), 1p31-p12 (T: 52%; NT: 22%), 10p15-p12 (T: 43%; NT: 22%) and 17p13-p11.2 (T: 43%; NT: 22%), and gain in 5p15.3-p11 (61%; NT: 33%). Statistical comparison between the occurrence of minimal regions of chromosomal alterations (Table 6) and this biological feature revealed that losses of 1p22-p21 ( $p<0.05$ ), 3p21 ( $p<0.05$ ), and 9p22 ( $p<0.02$ ) regions were more frequent in tumorigenic than in non-tumorigenic MM. Interestingly, the 3p21 region contained several genes previously described as being involved in tumor progression (*SETD2*, *MST1R*, *PLXNB1*, *RASSF1*, *RBM5*, *SEMA3B*, *SEMA3F*, *HYAL1*, *HYAL2*).

The number of breakpoints across the 22 autosomes was quantified in order to estimate genomic instability in human MM. Breakpoints were defined as the junction between a non-altered chromosomal region and a lost region. The breakpoint index, which corresponds to the sum of all breakpoints observed, ranged from 2 to 35 with a median of 22.5 (Figure 3). Interestingly, a statistically significant correlation was demonstrated between a high breakpoint index and the ability of MMs to develop a tumor in *nude* mice ( $p=0.031$ ).

#### *Comparative genomic alterations related to fiber exposure*

Comparison of recurrent regions of chromosomal alterations between asbestos-exposed (24 cases) and non-exposed (8 cases) patients showed a difference in the 14q11.2-q21 region (Figure 4). The maximum frequency of chromosomal alterations was 46% and 0% in asbestos-exposed and non-exposed cases, respectively ( $p<0.03$ ). In our murine model of fiber-induced MM, the syntenic regions 12qB3-qC1 and 14qC1-qC2 were also altered at a frequency of 44% (Table 5).



## Discussion

This study was designed to compare genomic alterations in human MMs and in fiber-related murine MMs as models of human MM. To our knowledge, an aCGH study of mineral fiber-induced murine MM was recently reported by Altomare *et al.* (2009)<sup>36</sup>. This investigation concerned 4 MMs developed in *Arf*<sup>+/-</sup> mice and primarily identified losses or gains of whole chromosomes, but the only recurrent genomic imbalance was a focal loss in chromosome 4C6 containing the *Cdkn2a* locus and the *Faf1* gene<sup>36</sup>. The present study based on 16 murine MMs demonstrated numerous genomic alterations involving several chromosomes. Similarly, 2 minimal regions of chromosomal loss were identified, containing *Cdkn2a/Cdkn2b*, and *Cdkn2c* and *Faf1*, respectively. In addition to recurrent deletions in mouse chromosome 4, frequent losses included regions in chromosomes 7 and 14, and frequent gains were detected in chromosomes 6, 8, 15 and 19, likely due to the greater number of MMs investigated in this series. Another aCGH study was carried out in 11 cases of iron saccharate-induced MM (6 epithelioid and 5 sarcomatoid) in rats<sup>37</sup>. Epithelioid MMs showed minimal alterations, while a few chromosomal amplifications and deletions were found in sarcomatoid MM including homozygous deletion of the *Cdkn2a/Cdkn2b* locus. Further studies would be necessary to determine whether these differences are related to the type of MM-inducing agent and/or to species specificities. However, *Cdkn2a/Cdkn2b* locus deletion seems to be an independent and specific feature of MM.

Human MMs have been previously studied by various cytogenetic methods, classical CGH, CGH array, SNP array and representational oligonucleotide microarray analysis (ROMA). Human MM is characterized by frequent losses of chromosomes 1p, 3p, 4, 6, 9p, 13q, 14q and 22q, and gains mostly involving chromosomes 5p, 7p, 8q and 17q<sup>21-26,45,46</sup>. In agreement with data in the literature, aCGH in our series detected chromosomal losses in similar regions, confirming the recurrence of these alterations in human MM. Other regions of

loss identified in 8p, 10p, 14q, 15q, 17p, 18q, 19p and 19q have also been reported in some studies<sup>47,48</sup>. In the 12p region, a translocation breakpoint with X chromosome was described in one human MM<sup>49</sup>. In the present study recurrent regions of gain were only identified in 5p, 7p and 20q. Data in the literature data tend to indicate that MM tumors show more marked diversity of chromosomal gains.

Genomic data obtained with cultured MM cells are in good agreement with those found in MM primary tumors, i.e. all recurrent regions of chromosomal alterations identified in cultures of human MM, except for losses in 8p23-p12 and 12p13, have been previously described in primary tumors using CGH, CGH array or ROMA (supplementary Table 1s, see Table 1s at <http://ajp.amjpathol.org>). Nevertheless, a higher frequency of alterations was observed in MM cultured cells probably due to contamination of tumor specimens by normal tissue.

Similarities and differences were clearly demonstrated after “humanizing” murine MM genomic profiles. The most striking similarity between human disease and its mouse model is the deletion of *CDKN2A/CDKN2B* locus, with a frequency as high as 91% and 81% in humans and mice, respectively, and this deletion is frequently bi-allelic in both species (Table 5). These results are in agreement with published data reporting that *CDKN2A/CDKN2B* deletions are a feature of MM<sup>28,29,50,51</sup>. The present results suggest codeletion of *CDKN2A* and *MTAP* (methylthioadenosine phosphorylase), a gene under study as a potential therapeutic target<sup>51,52</sup>. Interestingly, the orthologous *Mtap* gene is located in the syntenic region (Mm chr4C4), and could be accordingly co-deleted with *Cdkn2a* in the murine genome.

Loss of Hs 1p36.3-p35 (Mm 4qD2.3-4qE2) is another notable similarity. Deletions in the short arm of chromosome 1 are a well known feature of human MM<sup>53,54</sup>, and were associated with high asbestos exposure level in one study<sup>55</sup>. In our series, losses of 1p36.3

were found in 51% and 63% of cases in human and murine MM, respectively (Table 5). One gene, *CHD5*, may be of interest in this region. *CHD5* is involved in chromatin remodelling, and has been defined as a tumor suppressor gene<sup>56</sup>. Monoallelic loss of this gene was observed in several human MMs in the present series. Other studies reported that *CHD5* inactivation was associated with silencing of the second allele by an epigenetic mechanism, as in neuroblastoma and several carcinomas<sup>57,58</sup>.

The minimal region of common deletion between Hs 13q14-q33 and Mm 14qD1-qE2.3 was found at a frequency of 58% and 69%, respectively. The minimal region of deletion, 13q21, contains only three genes, *PCDH9*, *KLHL1* and *DACHI*. Interestingly, a biallelic deletion was found in this region in one human MM. *PCDH9* is a member of the protocadherin family, involved in oncogenesis<sup>59</sup>. *PCDH9* was found to be mutated in one pancreatic primary tumor<sup>60</sup> and *DACHI* is a nuclear factor playing a role in breast, prostate and ovary cancers<sup>61</sup>.

The 5p15.3-p13 gains reported in human MM were found in different syntenic regions of the mouse genome (Table 5). Many genes of interest are located in these regions, including several cadherins, *PRMD9*, *SKP2* and *RAD1*. *TERT* is located in 5p15.3, a recurrent region of gain in human MM, but not in the syntenic mouse region. 5p15.3 was also amplified in one human MM. Telomerase activity has been reported in a high proportion of human pleural MM, and telomere maintenance was recently reported to be due to both telomerase activity and alternative lengthening of telomeres in peritoneal MM<sup>62,63</sup>. *Tert* appears to be constitutively active in most murine cells<sup>64</sup>, and the Mm 13qC1 region in which it is located is not deleted in murine MM, in accordance with a potential role of this enzyme in both species.

In contrast, several regions of genomic alterations differed between human and murine MMs. In the present series, a high rate of deletions was detected in Hs 22q mainly consisting of loss of the entire chromosome, and *NF2* inactivation is a specific alteration in MM<sup>18,27,53,55</sup>.

Monoallelic deletions at *NF2* were found in 71% of cases, and a biallelic deletion was observed in one MM. Lower percentages were observed in murine MM genomic profiles. However, genetic analyses confirmed loss of *Nf2* in murine MM, in agreement with a role of this gene in mesothelial carcinogenesis (Table 7). The *Nf2* gene has been shown to enhance the incidence of MM in mice without shortening the latency, in comparison with WT mice, supporting a role as a susceptibility factor, a hypothesis previously formulated in human MM<sup>65</sup>. Furthermore, MM occurred at a higher incidence when mice knockout for *Nf2* were co-inactivated for either *Ink4a/Arf* or *Trp53*<sup>66</sup>. These observations indicate that inactivation of other genes potentiates *Nf2* gene loss and substantially contributes to development of MM. Other genes located in Hs 22q could also play a role in mesothelial carcinogenesis, such as *EP300* and *BIK* located in the region lost at the highest frequency. Further studies should be developed to identify other genes relevant to mesothelial oncogenesis.

Deletions of 3p regions are among the most frequent alterations in human cancers, including human MM. Frequent loss of the Hs 3p21 region, in which several candidate genes are located, was observed, but not in mice. A cluster of tumor suppressor genes has been reported in the 3p21.3 region, including Ras-associated factor 1 (*RASSF1*), which appears to be inactivated by methylation in MM<sup>67-69</sup>. Alterations in this 3p21 region were significantly more frequent in human MM exhibiting tumorigenicity in nude mice than in non-tumorigenic MM, suggesting an important role of the associated genes in tumor formation. However, as for chromosome 22, the syntenic region of human 3p21 was not altered accordingly in mice. These differences could be explained by technical issues, including differences in resolution of human and murine arrays used for genomic profiling, as well as species specificities.

Other regions of chromosome imbalance found exclusively in human MM contain well known TSG such as *BRCA2* (13q13, 60% frequency of loss) or *PTEN* (10q23-24, 37% frequency of loss). Downregulation of *PTEN* has been previously described in human MM<sup>70</sup>.

In contrast, some recurrent regions of chromosomal alterations did not contain any potential TSG or oncogene, but encompassed loci of several miRNAs. Differential expression of miRNAs has been previously observed between mesothelioma and mesothelial cells<sup>71,72</sup>. Copy number alteration is one of the mechanisms affecting miRNAs gene expression. MiRNAs are thought to act as TSGs and oncogenes due to their ability to modulate the transcriptional regulation of their target genes. Indeed, MIR31, located in the 9p21 recurrently lost region (Table 6), was recently demonstrated to inhibit cell proliferation and invasion of mesothelioma cells<sup>73</sup>. Further investigations of other miRNAs, such as MIR760, MIR137 and MIR553, would be of interest. These miRNAs are located in 1p22-21, a region devoid of coding genes, but altered at a higher frequency in tumorigenic MM than in non-tumorigenic human MM. The present study identified several miRNAs which could be deregulated in both human and murine MMs.

Comparison of recurrent regions of chromosomal alterations according to MM histology showed different frequencies between epithelioid and sarcomatoid subtypes. While the differences are not significant, at least partly due to the low power of the statistical analysis, they are likely species-dependent. This suggests that recurrent regions of chromosomal alterations are characteristic of a given subtype only in a given species. Differences in the proportion of histological subtypes between human and mouse complicated comparison between these two species. Several authors emphasized that epithelioid MM is more frequent in human than in mouse following asbestos exposure<sup>31-32</sup>. More recently, Jongma *et al.*<sup>66</sup> found the same result in spontaneous MM identified in mice carrying conditional TSG knockout alleles for *Nf2*, *Trp53* and *Ink4a/Arf*. In this study, eight different series of mice carrying co-inactivation of 2 or 3 TSGs were studied. The proportion of different subtypes was dependent on the genetic background with epithelioid MM being under represented in all but one series. These authors suggested that the predominant epithelioid

MM subtype seen in human opposed to mouse may occur as a result of species specific differences or relate to the route of MM induction. Our results would better suggest a species specific difference.

An interesting finding was the relationship between chromosomal instability, estimated by the number of breakpoints, and the ability of human MM xenografts to develop subcutaneous tumors in nude mice. To the best of our knowledge, this relationship with tumor aggressiveness has not been previously reported in mesothelioma. However, in other types of cancer, an increase in chromosomal aberrations is associated with higher grade and stage of tumors, which is consistent with increased genomic instability observed in tumor development and progression<sup>74,75</sup>. It would be of interest to determine whether the number of breakpoints could be a useful prognostic factor for MM progression.

The present study identified one recurrent region of chromosome loss, 14q11.2-q21, in asbestos-exposed patients not found in non exposed patients, but also lost in the syntenic region in mice mesotheliomas, suggesting that this region might be a target of action of mineral fibers. Frequent allelic loss has been detected in this region in human MM<sup>21,76</sup>. However, to date, it has been related to asbestos exposure in only one study by Björkqvist *et al.*, who found a clear history of asbestos exposure in 9 of 13 MMs with deletions at 14q, compared to only 1 of 5 MMs with no known exposure to asbestos<sup>76</sup>. Several genes involved in cell cycle regulation (*CCNB1IP1*), DNA repair (*PARP2*) and tumor promoting transcription factor (*NKX2-1*) are located in this region. Moreover Sugarbaker *et al.* (2008)<sup>77</sup> identified LOH in the 14q11 region involving *LRP10*, a member of low-density lipoprotein receptor-related proteins, in one patient with a history of asbestos exposure.

In conclusion, despite certain differences, our results show common genomic alterations between genomic profiles in murine and human MM and suggest that they can be of importance to account for asbestos-induced mesothelial cell neoplastic transformation.

Genes at the *CDKN2A/ CDKN2B* locus may play an important role in this process by committing cells to carcinogenic transformation. It would be of interested to determine whether other genes located in common regions of chromosomal imbalance in these two species could be involved in mesothelioma transformation. Genomic profiling of human and murine MM cells reported here validates the mouse model as a useful model to study the molecular mechanisms of this disease, and to test innovative therapeutic approaches. New generations of genomic profiling tools in combination with expression analysis will be useful to identify target genes involved in this dreadful disease.

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### **Legends to figures**

#### **Figure 1. Summary of genomic profiles in murine mesothelioma cells.**

Frequency plots of gains and losses of each chromosomal region in murine MM. Bars correspond to the percentage of samples with gain or loss in a given chromosomal region. Chromosome borders are indicated by solid vertical lines and centromere positions by dashed lines. Recurrent regions of chromosomal alteration are framed with a fine line.

#### **Figure 2. Summary of genomic profiles in human mesothelioma cells.**

Frequency plots of gains and losses of each chromosomal region in human MM. Bars correspond to the percentage of samples with gain or loss in a given chromosomal region. Chromosome borders are indicated by solid vertical lines and centromere positions by dashed lines. Recurrent regions of chromosomal alteration are framed with a fine line.

#### **Figure 3. Genomic instability and tumorigenic potency in nude mice xenografts of human mesothelioma cells.**

Breakpoint index was determined for each human MM and plotted separately for non-tumorigenic (NT) and tumorigenic (T) MM cells. Medians are indicated by horizontal bars.

#### **Figure 4. Schematic diagram of chromosomal alterations in the Hs 14q11.2-q21 region in asbestos-exposed and non-exposed cases, and in the syntenic mouse chromosomal regions.**

The start position in the human genome (NCBI Build 36), the cytogenetic location and the alteration frequency of each BAC clones are shown in the left columns. Each vertical column represents one individual MM: Open circle (○), no evidence of loss or gain; large black circle

(●), heterozygous loss; open circle with a central dot (⊙), gain; small black circle (•), not informative; dark grey shaded area, region of loss; light grey shaded area, region of gain. Corresponding mouse chromosomal regions are shown when alteration frequency of BAC clones are higher than 30%. Alteration frequency of each BAC clones are also specified for each group.

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