Tumorigenesis and Neoplastic Progression

Syntenic Relationships between Genomic Profiles of Fiber-Induced Murine and Human Malignant Mesothelioma

Didier Jean,^{*,†} Emilie Thomas,[‡] Elodie Manié,^{§¶}
 Annie Renier,^{*,†} Aurélien de Reynies,[‡]
 Céline Lecomte,^{||} Pascal Andujar,^{**,††‡‡}
 Jocelyne Fleury-Feith,^{§§¶¶}
 Françoise Galateau-Sallé,^{||||***}
 Marco Giovannini,^{†††} Jessica Zucman-Rossi,^{*,†}
 Marc-Henri Stern,^{§¶} and Marie-Claude Jaurand^{*,†}

From INSERM, U674,* Paris, France; the Université Paris Descartes,[†] UMR-S674, Paris, France; the Ligue Nationale Contre le Cancer,[‡] Paris, France; INSERM, U830,[§] Paris, France; Institut Curie,[¶] Centre de Recherche, Paris, France; the Laboratoire d'Ecotoxicologie,^{||} Université du Havre, UFR de Sciences et Techniques, UPRES-EA3222, Le Havre, France; the Service de Pneumologie et de Pathologie Professionnelle,^{**} Hôpital Intercommunal de Créteil, Créteil, France; INSERM, U955,^{††} Equipe 4, IMRB, Créteil, France; the Faculté de Médecine,^{‡‡} Université Paris-Est, Créteil, France; the Service d'Histologie et de Biologie Tumorale,^{§§} AP-HP, GHU Est, Hôpital Tenon, Paris, France; the Université Pierre et Marie Curie,^{¶¶} Paris, France; Service d'Anatomie Pathologique,^{||||} Hôpital de la Côte de Nacre, Caen, France; INSERM, ERI3,^{***} Caen, France; and the House Ear Institute,^{†††} Center for Neural Tumor Research, Los Angeles, California

Malignant mesothelioma (MM) 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 to elucidate the mechanism of mesothelioma formation. Genomic alterations in murine MM have now been partially characterized. 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. 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 murine models. Other shared alterations 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 altera-

tions in mouse regions corresponding to human chromosome 22. Comparison between altered recurrent regions in asbestos-exposed and non-asbestos-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. 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. (Am J Pathol 2011; 178:881–894; DOI: 10.1016/j.ajpath.2010.10.039)

Malignant mesothelioma (MM) is a severe primary neoplastic disease. Its frequency has dramatically increased in recent decades because of occupational exposure to asbestos fibers and the long latency period between first exposure and diagnosis, ranging from 20 to more than 40 years.^{1,2} 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.^{3–6} This raises the question of the role of very low levels of asbestos, as described in the context of environmental exposure or

Supported by the Ligue Nationale Contre le Cancer: Carte d'Identité des Tumeurs (CIT) CIT1 and CIT2 programs; Ministère de l'Emploi, de la Solidarité n°1D004C; de l'Environnement n°AC008B; Ligue Nationale Contre le Cancer (comité de l'Oise, France); Agence Nationale de la Recherche 05 9 31/ANR 05; and Agence Française de Sécurité Sanitaire de l'Environnement et du Travail RD-2004-015.

Accepted for publication October 21, 2010.

Supplemental material for this article can be found at <http://ajp.amjpathol.org> or at doi:10.1016/j.ajpath.2010.10.039.

Address reprint requests to Marie-Claude Jaurand, Ph.D., INSERM, U674, 27, rue Juliette Dodu, Paris, F-75010, France. E-mail: marie-claude.jaurand@inserm.fr.

other, as yet unidentified, risk factors.^{7–11} New manufactured fiber-shaped products may also be of concern, as recent studies have demonstrated that carbon nanotubes can reach subpleural tissue in mice^{12,13} and induce inflammation after injection in the peritoneal cavity of mice.¹⁴ Mesotheliomas also occur in genetically modified cancer-sensitive mice and in conventional Fisher 344 rats exposed to carbon nanotubes by intraperitoneal and intrascrotal administration, respectively.^{15,16}

To improve the outcome of MM, a better knowledge of the somatic alterations in neoplastic cells is necessary 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 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.^{17–26} 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.^{27–30}

Murine models of mesothelioma have been recently developed in wild-type (WT) and hemizygous *Nf2*^{+/-} mice after asbestos exposure by intraperitoneal inoculation, offering an unique opportunity to more clearly characterize the genomic changes caused by asbestos on mesothelial cells.^{31–33} 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.³¹ 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*^{+/-} mice and, in all genetic backgrounds, frequent inactivation of *p16/Cdkn2a*, *p19/Arf*, *p15/Cdkn2b* TSGs and infrequent inactivation of *Trp53*.^{32,34,35} 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.³⁶ A CGH study was also performed in rats after intraperitoneal administration of iron saccharate.³⁷

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 15 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 immunocompromised 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 were inoculated into the peritoneal cavity of WT and *NF2*^{+/-} mice. This strategy allowed the generation of mesotheliomas mimicking the morphology and histopathology of the corresponding human cancer.^{31,35} Sixteen cell cultures were established from tumor ascites obtained from 12 *Nf2*^{+/-} mice and four WT mice, as reported elsewhere.³¹ The mesothelial origin was assessed by immunocytochemistry using cytokeratin and vimentin antibodies according to a previously described method.³¹ 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 refractory ceramic fibers. The genetic background did not influence tumor morphology or genetic alterations, apart from more frequent LOH at the *NF2* locus in *Nf2*^{+/-} mice.³⁴

A total of 35 human cell cultures were obtained from confirmed malignant mesothelioma case patients: 29 male (82.8%) and six female (17.2%), with a mean ($\pm$ SD) age of 63 $\pm$ 11 years and 69 $\pm$ 5 years, respectively. 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.³⁸ In this series, asbestos exposure was ascertained in 24 MM cases, in two cases that involved possible exposure, and no exposure was found in eight cases. In one case, no data were available on asbestos history.

DNA Extraction and Qualification

Cells were grown in RPMI 1640 medium with Glutamax and 25 mmol/L 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,³⁴ quantified by absorption measurement, and qualified by deposition of 100 ng on a 0.8% agarose gel. DNA ladder was λ HindIII (Invitrogen).

Human and Murine aCGH

The human genome-wide CIT-CGH array (V6) contained 5822 BAC clones, with a higher coverage in genomic re-

gions known to contain genes involved in cancer. This array was developed by partnership among the Ligue Nationale Contre le Cancer, the Genoscope, the Curie Institute, and Integragen. 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.^{39,40} 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,⁴¹ and data were visualized on VAMP interface.⁴² 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 University of California, Santa Cruz, Genome Bioinformatics Group to each murine BAC of the array. Gains and losses were defined by the GLAD algorithm.⁴³ Amplifications and biallelic 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 Alteration Analysis

Gene mutations and deletions were analyzed from genomic DNA extracted from all murine and human MM subjects. 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.^{34,35} 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 μ L PBS and injected subcutaneously into the scapular region of athymic nude mice. At least four 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 (range, 4–18) altered autosomes per tumor. Interestingly, these alterations were mostly gains or

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**.

Table 1. Recurrent Regions of Chromosomal Alterations (Frequency Greater than 30%) in Murine Mesothelioma Cells

Alteration	Chromosomal region	Maximal frequency (%)	Start	End	Size (Mb)
Gain	1qA1–qC1.3	31	0	65343589	65
	5qG1.3–qG3	38	124245465	152000000	28
	Chr6*	56			
	Chr8*	50			
	10qC2–qD3	38	92606277	130000000	37
	Chr15*	81			
	16qA1–qB3	31	0	37335257	37
	Chr17*	38			
	Chr19*	75			
	1qH2.1–qH5	38	158533294	188288765	30
Loss	2qE1–qE3	31	88372743	113343671	25
	Chr4*	81			
	Chr7*	63			
	12qA1.1–qE	44	0	105426793	105
	Chr14*	69			

*Several gained or lost regions encompassed whole chromosomes in some murine MMs (see Supplemental Figure S1, A–C, for details at <http://ajp.amjpathol.org>).

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 greater than 30% (Table 1). Frequent recurrent gains (more than 50% of cases) included chr15 (75% to 81%), 19qC3–qD2 (75%), 19qA–qC1 (63% to 75%), 6qA1–qB1 (50% to 56%), and 8qC2–qC5 (50%), and frequent losses included regions in 4qC4–qD1 (50% to 81%), 14qD3–qE2.1 (50% to 69%), 4qD3–qE2.1 (50% to 63%), chr7 (56% to 63%), and 14qB (50%) (see Supplemental Figure S1, A–C, 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 to identify potential tumor suppressor genes and the miRNAs lost in murine mesothelioma cells (Table 2 and Supplemental Figure S1, A–C 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 of 16 MM cells, containing the *Diap3*, *Pcdh9*, and *Pcdh20* candidate genes. Deletion of the *Nf2* region in 11qA1 was infrequent (3/16) and found only in a *Nf2*^{+/–} background. Three high-level gained regions containing the proto-oncogene *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

Table 2. Minimal Regions of Chromosomal Imbalance Based on Alteration Frequency and Occurrence of Interstitial Deletions, Amplifications, and Biallelic Deletions in 16 Murine Mesothelioma Cells

Alteration	Chromosomal region	Number of MMs (%)	Start	End	Size (Mb)	Gene number	Gene	miRNA
Minimal regions of deletion	1qH2.2–qH2.3	6 (38)	162074549	171028275	9	>10	<i>Gas5</i>, <i>Rxrg</i>, <i>Pbx1</i>	Mir214, Mir2134-1, Mir689-1
	4qC4	13 (81)	84800547	89483516	5	>10	<i>Cdkn2a</i>, <i>Cdkn2b</i>	Mir491, Mir31
	4qC7	9 (56)	102961540	114389001	11	>10	<i>Cdkn2c</i>, <i>Faf1</i>	Mir761
	12qC1–qC2	7 (44)	60575140	71056044	10	>10	<i>Sav1</i>	Mir681
	14qB	8 (50)	39021984	43790944	5	>10	—	—
	14qE2.1	11 (69)	85113991	96312501	11	5	<i>Diap3</i>, <i>Tdrd3</i>, <i>Pcdh20</i>, <i>Pcdh9</i>, <i>Klhl1</i>	—
							<i>Elavl1</i>, <i>Gas6</i>	
Amplification	8qA1.1	1 (6)	0	23875369	24	>10	<i>Mmp2</i>, <i>Mmp15</i>, <i>Cdh11</i>, <i>Cdh5</i>, <i>Cdh8</i>	Mir1968, Mir2144
	8qC5–qD1	1 (6)	91443012	107427526	16	>10	<i>Mmp2</i>, <i>Mmp15</i>, <i>Cdh11</i>, <i>Cdh5</i>, <i>Cdh8</i>	Mir138-2
Biallelic deletion	15qD1	1 (6)	58895730	66093803	7	>10	<i>Myc</i>	—
	1qA1	1 (6)	0	10359697	10	>10	—	—
	4qC4	6 (38)	84800547	89483516	5	>10	<i>Cdkn2a</i>, <i>Cdkn2b</i>	Mir31
	12qC1–qC2	1 (6)	60575140	71056044	10	>10	<i>Sav1</i>	Mir681
	14qB	1 (6)	39021984	43790944	5	>10	—	—

Genes clearly or potentially involved in oncogenesis are indicated in bold.

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 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 chromosomes, and monoallelic gains were less frequent than in murine MMs. Nine recurrent regions of loss with a frequency greater than 50% were observed: 9p22–p13 (51% to 91%), 22q (57% to 80%), 3p22–p14 (51% to 63%), 13q11–q21 (51% to 60%), 6q21–q22 (51% to 57%), 4q21–q24 (51% to 54%), 12p13 (54%), 1p36.3 (51%), and 6q24–q25 (51%), and only one region of gain was observed: 5p15.3 (51%) (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Recurrent regions of chromosomal alterations with a frequency greater than 30% are shown in Table 3. Ten high-level gained regions and 12 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, 12 regions recurrently altered (frequency greater than 30%) in both the human disease and the murine 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

Table 3. Recurrent Regions of Chromosomal Alterations (Frequency Greater than 30%) in Human Mesothelioma Cells

Alteration	Chromosomal region	Maximal frequency (%)	Start	End	Size (Mb)
Gain	5p15.3–p11	51	0	49464271	49
	7p22–p11.2	37	0	57346208	57
	20q11.2–q13.1	34	34168802	43536662	9
Loss	1p36.3–p35	51	0	30383282	30
	1p31–p12	40	75229478	119360570	43
	3p23–p14	63	34981835	64352460	29
	Chr4	54			
	6q14–q27	57	80213243	170629779	90
	8p23–p12	31	1790686	31056165	29
	9p24–q21	91	0	70025358	70
	10p15–p12	37	0	21068361	21
	10q23–q26	37	86173438	135071951	49
	12p13	54	10844968	12614264	2
	13q	60			
	14q11.2–q21	40	0	42832378	43
	14q24–q32	40	76307162	106230236	30
	15q13–q21	40	27018560	44046064	17
	17p13–p11.2	34	5782075	17473056	12
	18q12–q23	46	23824248	75939456	52
	19p13.1–p12	31	18161120	24246700	6
	19q13.2–q13.4	31	47672188	59385609	12
	22q	80			

Table 4. Amplification and Biallelic Deletions in Human Mesothelioma Cells

Alteration	Chromosomal region	Number of MMs (N _T = 35)	Start	End	Size (Mb)	Gene number	Gene	miRNA
Amplification	1p34.2–p34.1	1	40464163	43936177	3	>10	CDC20	MIR30E, MIR30C1
	2p12	1	75046439	76197958	1	5	POLE4, TACR1, FAM176A, MIRPL19, C2orf3	—
	4q31.2	1	147916169	150226685	2	>10	—	MIR548G
	5p15.3	1	0	2692035	3	>10	TERT	—
	7p21–p15	1	9198514	29922960	21	>10	TWIST1, GPNMB, IL6, NPY, HNRNPA2B1, HOXA9, HOXA13	MIR1302-6, MIR1183, MIR148A, MIR196B
	7p14	1	39469174	43369192	4	>10	—	—
	9p24	1	442668	8228482	8	>10	RLN2, KDM4C	MIR101-2
	10q26	1	123070217	126566539	3	>10	FGFR2	—
	19q12–q13.1	1	33184604	38838451	6	>10	—	—
	20q13.3	1	54336834	57746514	3	>10	AURKA	MIR296, MIR298
Biallelic deletion	1p21	2	101859311	105068099	3	8	OLFM3, COL11A1, RNPC3, AMY2B, AMY2A, AMY1A, AMY1B, AMY1C	—
	4p15.3	1	12344454	13298102	1	3	RAB28, NKX3-2, BOD1L	—
	5q34	1	166908572	170038731	3	>10	—	MIR103-1, MIR103-1AS, MIR218-2, MIR585
	9p23	2	8228482	13419660	5	4	PTPRD, TYRP1, C9orf150, MPDZ	—
	9p21	22	20172510	22317110	2	>10	CDKN2A, CDKN2B	MIR31, MIR491
	10q26	1	126566539	131109233	5	>10	—	—
	11q22–q23	1	106448945	114320480	8	>10	ATM, SDHD, ZBTB16, PPP2R1B	MIR34B, MIR34C
	13q21	1	65415360	70465908	5	3	PCDH9, KLHL1, DACH1	—
	16p13.3	1	5147035	8712134	4	4	A2BP1, TMEM114, C16orf68, ABAT	—
	18p11.3	1	5196459	7203642	2	6	ZFP161, EPB41L3, TTMA, L3MBTL4, ARHGAP28, LAMA1	—
	22q11.2	3	19570585	20084258	1	9	SNAP29, CRKL, AIFM3, LZTR1, THAP7, P2RX6, SLC7A4, GGT2, RIMBP3B	MIR649
	22q12	1	28155132	28374240	<1	1	RFPL1, NEFH, THOC5, NIPSNAP1, NF2	—

Genes clearly or potentially involved in oncogenesis are indicated in bold.

(Mm 4qD2.3–4qE2) and Hs 13q14–q33 (Mm 14qD1–qE2.3), showed a frequency of loss greater 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 Supplemental

Table 5. Comparison of Chromosomal Alterations (Frequency Greater than 30%) between Human and Murine Mesothelioma Cells

Alteration	Human					Murine	
	Chromosomal region	Maximal frequency (%)	Start	End	Size (Mb)	Chromosomal region	Maximal frequency (%)
Gain	5p15.3–p13	49	7387737	39283396	32	15qA1–qB3.1	75
	7p22–p21	37	188237	12020141	12	5qG2/6qA1	38/58
	7p15–p14	37	24340863	30917523	7	6qB2.3–qB3	44
Loss	1p36.3–p35	51	2058046	29574368	28	4qD2.3–4qE2	63
	3p21–p14	60	52571782	63683864	11	14qA1/14qA3	38/38
	6q15–q16	40	89148060	100061960	11	4qA3–qA5	38
	8p21	31	22048391	26316410	4	14qD1	44
	9p24–p13	91	8228482	36895443	29	4qA5–B1/4qC3–C5	44/81
	10q23	34	86679954	87489928	1	14qA3–qB	38
	10q26	37	121395903	127901902	7	7qF2–qF3	56
	13q12	57	19505755	19703538	0	14qC3	44
	13q14–q33	57	42908596	101265081	58	14qD1–qE2.3	69
	14q11.2–q21	40	19150554	42832378	24	12qB3–qC1/14qC1–qC2	44/44
	14q24–q32	37	77705005	94277131	17	12qD3–qE	38
	19q13.2–q13.4	31	50052470	54919450	5	7qA2/7qB3	56/56
	9p21	63	20172510	22317110	2	4qC4	40
Biallelic deletion							

Table 6. Minimal Regions of Chromosomal Alterations in Human Mesothelioma Cells

Alteration (loss)	Chromosomal region	Maximal frequency (%) (human/mouse)	Start	End	Size (Mb)	Gene number	Gene	miRNA
Human/mouse comparison	1p36.3	51/63	4356069	6292626	2	>10	CHD5	—
	1p36.1	46/50	19083345	20986850	2	>10	NBL1	MIR1290
	9p21	91/81	20172510	22317110	2	>10	CDKN2A, CDKN2B	MIR31, MIR491
Interstitial deletions in humans	13q21	49/69 (human)	62637997	70465908	8	3	PCDH9, KLHL1, DACH1	—
	1p22–p21	40	92918111	101318454	8	>10	—	MIR760, MIR137, MIR553
	3p21	60	45214909	50409942	5	>10	SETD2, MST1R, PLXNB1, RASSF1, RBM5, SEMA3B, SEMA3F, HYAL1, HYAL2	MIR1226, MIR2115, MIR711, MIR425, MIR191, MIR566
	4p16–p15.3	46	9614707	12344454	3	5	SLC2A9, WDR1, ZNF518B, CLNK, HS3ST1	MIR572
	4q13	49	63518558	65274228	2	1	TECRL	—
	4q22–23	54	92471635	101247956	9	>10	BMPR1B, UNC5C	—
	6q22	57	115807782	120525326	5	>10	—	MIR548B
	6q24–q25	51	144840012	155794638	11	>10	LATS1, AKAP12	—
	9p22	49	14010653	14732051	1	4	NFIB, ZDHHC21, CER1, FREM1	—
	9p11.1	43	42843412	66195312	23	4	ANKRD20A3, FAM75A6, CNTNAP3B, FAM75A7	—
	10q23–q24	37	87489928	108067973	21	>10	BMPR1A, SNCG, PTEN, FAS, LGI1, NFKB2, SUFU	MIR346, MIR107, MIR607, MIR1287, MIR608, MIR146B, MIR1307, MIR936, MIR609
	12p13	54	11584100	12614264	1	6	ETV6, BCL2L14, LRP6, MANSC1, LOH12CR1, DUSP16	MIR1244
	13q13	60	31067328	33059957	2	9	RXFP2, FRY, ZAR1L, BRCA2, N4BP2L1, N4BP2L2, PDS5B, KL, STARD13	—
	15q14–q21	40	30654132	44046064	13	>10	THBS1	MIR1233, MIR626, MIR627
	22q12–q13	80	32678146	42309461	10	>10	EP300, BIK	MIR658, MIR659, MIR1281, MIR33a

Genes clearly or potentially involved in oncogenesis are indicated in bold.

Figure S2, D and L, 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. Other human regions characterized by a frequency of loss greater 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 re-

gions 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 Supplemental Figure S2, A, B, I, and M 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 **DACH1** (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, 13 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 Supplemental Figure S2

Table 7. Percentage of Cases Showing Gene Alterations in Human and Murine Mesothelioma Cells

Human/murine gene	Alteration frequency in humans (%)	Alteration frequency in mice (%)	P value*
<i>TP53/Trp53</i>	20	25	0.72
<i>NF2/Nf2</i>	46	75	0.07
<i>CDKN2A/Cdkn2a</i>	82	56	0.09
<i>CDKN2B/Cdkn2b</i>	76	60	0.43

*Fisher's exact test.

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 miRNA 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% (see Supplemental Figure S2, Q, 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 rates of mutation 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.^{34,35,44} 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*^{+/-} 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 Humans

Comparison of recurrent regions of chromosomal alterations with histological MM subtypes underlined differ-

ent frequencies among the altered regions. As biphasic subtype is a mixed population, comparisons were made only 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 greater 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 fre-

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 nontumorigenic (NT) and tumorigenic (T) MM cells. Medians are indicated by horizontal bars.

Human 14q11.2-q21

Figure 4. Schematic diagram of chromosomal alterations in the Hs 14q11.2-q21 region in asbestos-exposed and non-asbestos-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 clone 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 gray shaded area, region of loss; light gray shaded area, region of gain. Corresponding mouse chromosomal regions are shown when alteration frequency of BAC clones is greater than 30%. Alteration frequency of each BAC clone is also specified for each group.

quent in tumorigenic (T) than in nontumorigenic (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 nontumorigenic MM. Interestingly, the 3p21 region contained several genes previously described as being involved in tumor progression (*SETD2*, *MST1R*, *PLXNB1*, *RASSF1*, *RBM5*, *SEMA3B*, *SEMA3F*, *HYAL1*, and *HYAL2*).

The number of breakpoints across the 22 autosomes was quantified to estimate genomic instability in human MM. Breakpoints were defined as the junction between a nonaltered 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-asbestos-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-asbestos-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. An aCGH study of mineral fiber-induced murine MM was recently reported by Altomare et al (2009).³⁶ This investigation concerned four MMs developed in *Arf*^{+/-} 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.³⁶ The present study, based on 16 murine MMs, demonstrated numerous genomic alterations involving several chromosomes. Similarly, two 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 because of the greater number of MMs investigated in this series. Another aCGH study was carried out in 11 cases of iron saccharate-induced MM (six epithelioid and five sarcomatoid) in rats.³⁷ Epithelioid MMs showed minimal alterations, whereas 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, single nucleotide polymorphism array, and representational oligonucleotide microarray analysis. Human MM is characterized by frequent losses of chromosomes 1p, 3p, 4, 6, 9p, 13q, 14q, and 22q and by gains mostly involving chromosomes 5p, 7p, 8q, and 17q.^{21–26,45,46} 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.^{47,48} In the 12p region, a translocation breakpoint with X chromosome was described in one human MM.⁴⁹ In the present study, recurrent regions of gain were identified only in 5p, 7p, and 20q. Data in the literature 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; ie, 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 representational oligonucleotide microarray analysis (see Supplementary Table S1 at <http://ajp.amjpathol.org>). Nevertheless, a higher frequency of alterations was observed in MM cultured cells, probably because of 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 murine 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 biallelic in both species (Table 5). These results are in agreement with published data reporting that *CDKN2A/CDKN2B* deletions are a feature of MM.^{28,29,50,51} The present results suggest codeletion of *CDKN2A* and *MTAP* (methylthioadenosine phosphorylase), a gene under study as a po-

tential therapeutic target.^{51,52} Interestingly, the orthologous *Mtap* gene is located in the syntenic region (Mm chr4C4), and could be accordingly codeleted 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,^{53,54} and were associated with high asbestos exposure level in one study.⁵⁵ 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 remodeling, and has been defined as a tumor suppressor gene.⁵⁶ Monoallelic loss of this gene was observed in several human MMs in the present series. Other studies reported that *CDH5* inactivation was associated with silencing of the second allele by an epigenetic mechanism, as in neuroblastoma and several carcinomas.^{57,58}

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 *DACH1*. Interestingly, a biallelic deletion was found in this region in one human MM. *PCDH9* is a member of the protocadherin family, involved in oncogenesis.⁵⁹ *PCDH9* was found to be mutated in one pancreatic primary tumor⁶⁰ and *DACH1* is a nuclear factor playing a role in breast, prostate, and ovary cancers.⁶¹

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. In one human MM, 5p15.3 was also amplified. 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.^{62,63} *Tert* appears to be constitutively active in most murine cells,⁶⁴ 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.^{18,27,53,55} 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.⁶⁵ Furthermore, MM occurred at a higher incidence when mice knockout for *Nf2* were co-inactivated for either *Ink4a/Arf*

or *Trp53*.⁶⁶ 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.^{67–69} Alterations in this 3p21 region were significantly more frequent in human MM exhibiting tumorigenicity in nude mice than in nontumorigenic 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.⁷⁰ 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.^{71,72} Copy number alteration is one of the mechanisms affecting miRNAs gene expression. MiRNAs are thought to act as TSGs and oncogenes because of 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.⁷³ 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 nontumorigenic human MM. The present study identified several miRNAs that 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. Although the differences are not significant, at least partly because of 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 have emphasized that epithelioid MM was more frequent in

humans than in mice after asbestos exposure.^{31,32} More recently, Jongsma et al⁶⁶ 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 two or three 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 humans as opposed to mice 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.^{74,75} 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 that was not found in non-asbestos-exposed patients but that also was lost in the syntenic region in murine 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.^{21,76} However, to date, it has been related to asbestos exposure in only one study by Björkqvist et al,⁷⁶ who found a clear history of asbestos exposure in nine of 13 MMs with deletions at 14q, compared with only one of five MMs with no known exposure to asbestos. 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)⁷⁷ 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 these alterations 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 interest 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 murine 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.

Acknowledgments

Murine aCGH was performed at the CIT platform at Institut Curie by Camille Blanchard. Human aCGH was performed at the CIT platform at IGBMC. We thank A. Janin for her participation in histological examinations. We are grateful to clinicians and histologists for having provided tissue samples: Dr. Isame Abd-Alsamad and Prof. Jean-Claude Pairon (CHI, Créteil, France), Françoise Le Pimpec-Barthes and Claire Danel and Prof. Marc Riquet (HEGP, Paris, France), and Philippe Astoul and Christian Boutin (Hôpital la Conception and CHU, Marseille, France).

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