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Analysis of K-*ras* and *p53* mutations in mesotheliomas from humans and rats exposed to asbestos

Zuyao Ni [a,b], Yu-Qing Liu [b], Nagalakshmi Keshava [a], Gu Zhou [a], Wen-zong Whong [a], Tong-man Ong [a,*]

[a] *Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, HELD, NIOSH, m / s 3014, 1095 Willowdale Road, Morgantown, WV 26505-2888, USA*
[b] *Department of Toxicology, West China University of Medical Sciences, Chengdu 610041, People's Republic of China*

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Abstract

Malignant mesothelioma is known to be associated with asbestos exposure. However, the mechanism of mesothelial carcinogenesis in relation to the activation of proto-oncogenes or inactivation of tumor suppressor genes remains unclear. In this study, the PCR-Primer Introduced Restriction Site (PCR-PIRS) assay was employed to examine mutations in the K-*ras* proto-oncogene in mesothelioma tissues from workers exposed to asbestos and from rats treated with asbestos. Mutations in exons 5–8 of the *p53* tumor suppressor gene were determined by direct DNA sequence analysis. Results of the PCR-PIRS analysis revealed no mutations in codons 12, 13 or 61 of the K-*ras* gene in any of the 17 human or 22 rat mesothelioma tissue samples. These results were confirmed by direct DNA sequence analysis. No mutations were found in exons 5–8 of the *p53* gene in any of the mesothelioma tissue samples analyzed. These results and the results reported by others indicate that the K-*ras* proto-oncogene and *p53* tumor suppressor gene may not play a critical role in the induction of mesothelioma by asbestos either in humans or in rats.

Keywords: Asbestos; Mesotheliomas; K-*ras* proto-oncogene; *p53* tumor suppressor gene

1. Introduction

Occupational exposure to asbestos may be one of the primary causes of mesotheliomas. Malignant mesothelioma is a rare neoplasm of mesodermal origin with an incidence of approximately 2000 cases annually in the United States [1]. Since the first report of the association between mesothelioma and asbestos exposure [2], efforts have been made to study the etiology of asbestos-induced mesotheliomas. Chromosomal aberrations, including aneuploidy, translocations and chromosomal breakage induced by asbestos, appear to be associated with mesothelioma [3,4]. Little is known, however, regarding the molecular mechanism of mesothelioma carcinogenesis.

Asbestos-induced mesothelioma is characterized by a long latency from the time of exposure [5]. The length of the latency period suggests that multiple genetic alterations may be required for tumorigenic

* Corresponding author. Tel.: +1-304-285-5817; fax: +1-304-285-6194.
E-mail address: too2@cdc.gov (T. Ong).

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conversion of mesothelial cells [6]. Mutations at codons 12, 13 or 61 of the *ras* gene have been detected in a variety of cancers such as cancer of pancreas [7], colon [8] and lung [9]. Also, over 50% of human cancer tissues carry mutations in *p53*, a tumor suppressor gene. Most mutations in this gene occur in exons 5–8, the evolutionarily conserved region of the gene that is essential for normal function [10]. Mutations in the K-*ras* and *p53* genes have been investigated in mesotheliomas induced by asbestos. Several studies have shown that there is not a good correlation between K-*ras* or *p53* alterations and the development of mesotheliomas [11–13]. However, in one study using human mesothelioma cell lines, 2/20 cell lines tested showed point mutations in the *p53* gene [14]. Studies using PCR-SSCP on mesotheliomas from a Chinese population have detected mutations in exons 5–8 of *p53* in 7/10 cases [15]. Additional studies are needed to resolve this inconsistency. The present study compares the K-*ras* and *p53* mutational patterns of the rat and human mesothelioma samples in order to further investigate whether mesotheliomas from workers and rats exposed to asbestos carry any mutations in the K-*ras* and *p53* genes.

2. Materials and methods

2.1. Human mesothelioma samples

Mesothelioma (autopsy) samples from 17 workers exposed to asbestos were collected from the hospital of West China University of Medical Sciences. Histological diagnosis was performed and the tissues were embedded in paraffin blocks.

2.2. Rat mesothelioma samples

Wistar rats (30 males and 30 females) from the Laboratory Center at West China University of Medical Sciences in China were used for the induction of mesothelioma. Crocidolite fibers (20 mg/ml rat) were injected (two times with 1 month apart) with less than 1 μm in diameter and varying in lengths from 5 to 10 μm (51%; longer than 10 μm, 22%; shorter than 5 μm, 27%). Rats were kept under controlled conditions until tumors developed. Tumor tissues were removed from tumor-bearing animals and fixed with 10% buffered formalin, then embedded in paraffin blocks.

2.3. DNA extraction

DNA was extracted from paraffin-embedded tissue sections using 200–500 μl of digestion buffer (50 mM Tris at pH 8.0, 1 mM EDTA, 0.5% Tween 20 and 500 μg/ml of proteinase K) over 5 days at 37°C. The proteinase K was inactivated by boiling the sample at 94°C for 8 min. DNA extracted from leucocytes of healthy persons and from fresh rat liver tissues was used as normal controls for human and rat samples, respectively.

2.4. Nested PCR

A larger DNA fragment was amplified first using outer primers, then 5 μl of the amplified PCR product underwent a second round of PCR using inner primers. The primers used for exons 1 and 2 of the K-*ras* and exons 5–8 of the *p53* are listed in Table 1. After heating for 10 min at 94°C, PCR reactions were performed for 35 cycles at 94°C for 1 min, 40°C for 1 min, and 74°C for 1 min. 10 μl of the second PCR products were electrophoresed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

2.5. PCR-Primer Introduced Restriction Site (PCR-PIRS)

K-*ras* gene mutations in codons 12, 13 and 61 were determined by the PCR-PIRS method previously described by Jacobson and Mills [16]. Briefly, the first PCR was performed in a reaction mixture containing 10 μM of each nucleotide, 1.2 mM $MgCl_2$, and fully matched primers flanking the desired DNA fragments. An aliquot of the first PCR product was used as the template for the second PCR. The second PCR reaction mixture contained 4 μM of each nucleotide, 0.6 μM $MgCl_2$ and 100 pmol of 5′ mismatched primer (BstNI: 5′AAA CTT GTG GTA GTT GGA *C*CT 3′; Bg1 I: 5′ CTT GTG GTA GTT GG*C C*CT GGT 3′; Bcl I: 5′ G GAT ATT CTC GAC ACA GC*T* G*A*T 3′ for codons 12, 13 and 61, respectively), to introduce a new enzyme restriction site into PCR products derived from alle-

Table 1
The primers used for exons 1 and 2 of K-*ras* and exons 5–8 of *p53*

Gene			Primer sequence	Base pair
Rat and human K-ras				
Exon 1	Outer	Forward	5′CTT GAT AAT CTT GTG TGG AAC-3′	226
		Reverse	5′CCA CCC TTT ACA AAT TGT AC3′	
	Inner	Forward	5′TTT ATT ATA AGG CCT GCT G3′	141
		Reverse	5′GCA TTT ACC TCT ATC GTA GG3′	
Exon 2	Outer	Forward	5′AGG TGC ACT GTA ATA ATC C3′	264
		Reverse	5′AAT TAC TCC TTA ATG TCA GC3′	
	Inner	Forward	5′AGA CTG TGT TTC TCC CTT C3′	212
		Reverse	5′TTA AAC CCA CCT ATA ATG G3′	
Human p53				
Exon 5	Outer	Forward	5′TTG CTT TAT CTG TTC ACT TGT G3′	359
		Reverse	5′TCC TTC CAC TCG GAT AAG3′	
	Inner	Forward	5′GTC TCC TTC CTC TTC CTA C3′	218
		Reverse	5′TGC TCA CCA TCG CTA TC3′	
Exon 6	Outer	Forward	5′TGA TTC CTC ACT GAT TGC TC3′	300
		Reverse	5′AAC TGT GCA ATA GTT AAA CCC3′	
	Inner	Forward	5′TGA TTG CTC TTA GGT C3′	237
		Reverse	5′GAG GTC AAA TAA GCA G3′	
Exon 7	Outer	Forward	5′CCC CAA GGC GCA CTG3′	264
		Reverse	5′TAG TAG TAT GGA AGA AAT CGG3′	
	Inner	Forward	5′CTT GGG CCT GTG TAA3′	156
		Reverse	5′TGT GCA GGG TGG CAA3′	
Exon 8	Outer	Forward	5′CTA TCC TGA GTA GTG GTA ATC3′	172
		Reverse	5′TGT CCT GCT TGC TTA CCT C3′	
	Inner	Forward	5′TAA TCT ACT GGG ACG GAA C3′	143
		Reverse	5′TAC CTC GCT TAG TGC TC3′	
Rat p53				
Exon 5	Outer	Forward	5′TGA CCT TTG ATT CTT TC 3′	291
		Reverse	5′GCA AGA ATA AGT CAG AGG C 3′	
	Inner	Forward	5′TCT TTC TCC TCT CCT AC3′	260
		Reverse	5′GAC AAC CAG TTC TAA C 3′	
Exon 6	Outer	Forward	5′GGT TGT CCA GGG TCT C 3′	286
		Reverse	5′CAC AGC TTC CTA CCT G 3′	
	Inner	Forward	5′TCT GAC TTA TTC TTG C 3′	254
		Reverse	5′TAC CTG GAG TCT TCC 3′	
Exon 7	Outer	Forward	5′GGT AGT GGG AAT CTT C3′	220
		Reverse	5′AAG GAG AGA GCA AGG 3′	
	Inner	Forward	5′TAG TGG GAA TCT TCT G 3′	173
		Reverse	5′TCC ACC TTC TTT GTC3′	
Exon 8	Outer	Forward	5′GCA AAG AGA GGT GAG 3′	165
		Reverse	5′AAG GGT GAA ATA TTC TC 3′	
	Inner	Forward	5′AAG GGT GAA ATA TTC TC3′	142
		Reverse	5′ACA AAG AAG GTG GAG3′	

les containing the normal sequence at relevant positions. After heating for 10 min at 94°C, the first and second PCR reactions were performed for 35 cycles at 94°C for 1 min, 40°C for 1 min, and 74°C for 1 min. The second PCR products were digested with a specific enzyme (Table 2; New England BioLabs, Beverly, MA) and separated by electrophoresis on 4% agarose gel with 0.5 μg/ml ethidium bromide. The result of a digestion-resistant band indicates the presence of a K-*ras* gene mutation located at codon

Table 2
The enzymes and restriction sites to detect mutations in K-*ras* gene

Enzyme	Codon	Enzyme recognition site	Temperature (°C)	Time (h)
BstN I	12	5′...CCA[a](T)[a]GG...3′ 3′...GGT (A)[a]CC...5′	60	4
Bgl I	13	5′...GCCN NNN[a]NGGC...3′ 3′...CGGN[a]NNN NCCG...5′	37	2
Bcl I	61	5′...T[a]GATC A...3′ 3′...A CTAG[a]T...5′	50	2

[a] Digestion site of the enzyme.

12, 13 or 61 using enzymes BstN1, Bgl I or Bcl I, respectively. DNA fragments amplified by PCR using the primer-introduced point mutation were employed as mutant positive controls.

2.6. DNA sequencing

The sequencing of PCR-amplified DNA fragments was conducted by the fluorescent-labeled dideoxynucleotide triphosphate terminator method with the Tag Dideoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) using an automated sequencer (373A Sequencer, PE Applied Biosystems).

3. Results and discussion

Of the 60 rats exposed to crocidolite fibers, 22 developed tumors with the latency ranging from 1 to 1.5 years. The tumors were malignant mesotheliomas by histological examination. There were three different types of histological appearance: fibroblastic, demographic and a mixture of these two types.

DNA from 17 human and 22 rat mesothelioma tissues isolated from sections of paraffin-embedded blocks were analyzed for mutations in K-*ras* and *p53* genes. Each of the desired DNA fragments containing exons 1 and 2 of K-*ras* and exons 5–8 of *p53* was amplified by nested PCR with outer and inner primers, respectively. To determine point mutations in codons 12, 13, and 61 of the K-*ras* gene, PCR-PIRS, a highly specific technique, was used for the analysis of mutations in these codons. As shown in Fig. 1, no mutations in these codons were found in any of the 17 human or the 22 rat mesothelioma tissues as seen by the complete digestion of the PCR products by the related restriction enzyme. The absence of mutations in exons 1 and 2 of K-*ras* in human and rat mesothelioma tissues was confirmed by direct DNA sequence analysis. These results appear to be in agreement with results previously reported in the literature. No K-*ras* mutations were found by Metcaff et al. [14] in 20 mesothelioma cell lines. Southern blot analysis by Tiainen et al. [17] revealed no rearrangements and no amplifications of the *ras* gene in 23 mesothelioma tissues. An absence of K-*ras* mutations has also been reported in primary mesotheliomas related to asbestos exposure [12]. It is possible that mesothelioma could develop by other mechanisms.

Direct DNA sequencing was also used for the analysis of the *p53* gene in mesothelioma tissues. No mutations were detected in exons 5, 6, 7, and 8 in any of the 17 human or 22 rat mesothelioma samples. These results are in agreement with several

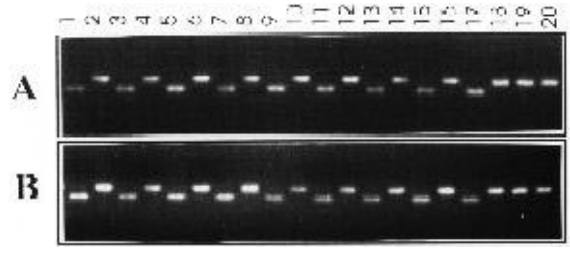

Fig. 1. Representative banding pattern of the PCR products digested by Bcl I for codon 61 of K-*ras* gene in human and rat mesothelioma samples. Panel A: human K-*ras* gene; odd numbered lanes are digested PCR products; even numbered lanes are undigested PCR products; lanes 19 and 20 are positive controls. Panel B: rat K-*ras* gene; odd numbered lanes are digested PCR products; even numbered lanes are undigested PCR products; lanes 19 and 20 are positive controls.

other studies reported in the literature. Using PCR-SSCP and PCR-Denaturing Gradient-gel Electrophoresis, Mor et al. [13] found no *p53* mutations in 13 primary mesothelioma tissues. Similar results were reported, recently by Kitamura et al. [12] in seven primary mesotheliomas by the PCR-SSCP analysis. All of these results seem to indicate that gene mutations in K-*ras* and *p53* may not play a crucial role in mesothelioma carcinogenesis in humans or in rats.

It needs to be noted that immunohistochemical analysis has been used for the detection of the *p53* gene product, the *p53* protein. Results of one study showed that none of the 20 reactive mesothelial proliferation samples had any *p53* immunoreactivity while 14 of 20 malignant mesothelioma samples had *p53* staining [18]. In another study, 10 of 15 malignant mesothelioma samples were found to show immunoreactivity for *p53* [19]. Immunoreactivity, however, was not found in 36 and 8 malignant mesothelioma cases by Ramael et al. [20] and Kishimoto [21], respectively. Further studies are needed to determine whether accumulation and/or overexpression of the *p53* protein is related to mesothelioma carcinogenesis.

Sixty percent of human mesotheliomas are found to contain and express SV40 sequences. It is thought that the SV40 large T-antigen (*Tag*) interferes with the normal expression of tumor suppressor gene, *p53* in human mesotheliomas. Carbone et al. [22] found that SV40 *Tag* binds and inactivates *p53*. Abnormal mitosis and apoptosis was observed in asbestos-exposed rat pleural mesothelial cells expressing SV40 *Tag* [23]. Therefore, it is possible that SV40 may also contribute to the development of human mesotheliomas.

Studies related to the genotoxicity of asbestos seem to suggest that asbestos fibers may (1) physically interfere the formation of mitotic spindles and the segregation of chromosomes [24,25] and (2) generate reactive oxygen species which could react with DNA [26–28]. Extensive chromosomal aberrations have been found in mesothelioma tissues by cytogenetic analysis [4,17]. Frequent and multiple chromosomal abnormalities have also been found in tissue cultures treated with asbestos [29–31]. Chromosome breaks have also been found in cultured human primary mesothelial cells following treatment with asbestos [32]. It has also been reported that asbestos is capable of impairing the cell cycle checkpoint. It seems, therefore, that chromosomal aberrations and impairment of cell cycle checkpoint may be related to, at least in part, the initiation and development of mesotheliomas induced by asbestos.

References

[1] T. Reynolds, Asbestos-linked cancer rates up less than predicted: news and commentary, J. Natl. Cancer Inst. 84 (1992) 560–562.

[2] J.C. Wagner, C.A. Sleggs, P. Marchand, Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province, Br. J. Ind. Med. 17 (1960) 260–271.

[3] B.L. Libbus, J.E. Craighead, Chromosomal translocations with specific breakpoints in asbestos-induced rat mesotheliomas, Cancer Res. 48 (1988) 6455–6461.

[4] N.C. Popescu, A.P. Chahinian, J.A. DiPaolo, Non-random chromosome alterations in human malignant mesothelioma, Cancer Res. 48 (1988) 142–147.

[5] I.J. Selikoff, E.C. Hammond, H. Seidman, Latency of asbestos disease among insulation workers in the United States and Canada, Cancer 46 (1980) 2736–2740.

[6] E.R. Fearon, B. Vogelstein, A genetic model for colorectal tumorigenesis, Cell 61 (1990) 759–767.

[7] C. Almoguera, D. Shibata, K. Forrester, J. Martin, N. Arnheim, M. Perucho, Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes, Cell 53 (1988) 549–554.

[8] J.L. Bos, E.R. Fearon, S.R. Hamilton, M. Verlaan-de Vries, J.H. van Boom, A.J. van der Eb, B. Vogelstein, Prevalence of *ras* gene mutations in human colorectal cancers, Nature 327 (1987) 293–297.

[9] R. Salgia, A.T. Skarin, Molecular abnormalities in lung cancer, J. Clin. Oncol. 16 (1998) 1207–1217.

[10] A.J. Levine, The *p53* tumor-suppressor gene, N. Engl. J. Med. 326 (1992) 1350–1352.

[11] R.J. Cote, S.C. Jhanwar, S. Novick, A. Pellicer, Genetic alterations of the *p53* gene are a feature of malignant mesotheliomas, Cancer Res. 51 (1991) 5410–5416.

[12] F. Kitamura, S. Araki, T. Tanigawa, H. Miura, H. Akabane, R. Iwasaki, Assessment of mutations of Ha- and Ki-*ras* oncogenes and the *p53* suppressor gene in seven malignant mesothelioma patients exposed to asbestos — PCR-SSCP and sequencing analyses of paraffin-embedded primary tumors, Ind. Health 36 (1998) 52–56.

[13] O. Mor, P. Yaron, M. Huszar, A. Yellin, O. Jakobovitz, F. Brok-Simoni, G. Rechavi, N. Reichert, Absence of *p53* mutations in malignant mesotheliomas, Am. J. Respir. Cell Mol. Biol. 16 (1997) 9–13.

[14] R.A. Metcalf, J.A. Welsh, W.P. Bennett, M.B. Seddon, T.A. Lehman, K. Pelin, K. Linnainmaa, L. Tammilehto, K. Mattson, B.I. Gerwin et al., *p53* and Kirsten-*ras* mutations in

human mesothelioma cell lines, Cancer Res. 52 (1992) 2610–2615.
[15] B.C. Liu, D.C. Fu, Q. Miao, H.H. Wang, B.R. You, *p53* gene mutations in asbestos associated cancers, Biomed. Environ. Sci. 11 (1998) 226–232.
[16] D.R. Jacobson, N.E. Mills, A highly sensitive assay for mutant *ras* genes and its application to the study of presentation and relapse genotypes in acute leukemia, Oncogene 9 (1994) 553–563.
[17] M. Tiainen, J. Kere, L. Tammilehto, K. Mattson, S. Knuutila, Abnormalities of chromosomes 7 and 22 in human malignant pleural mesothelioma: correlation between Southern blot and cytogenetic analyses, Genes, Chromosomes Cancer 4 (1992) 176–182.
[18] G. Kafiri, D.M. Thomas, N.A. Shepherd, T. Krausz, D.P. Lane, P.A. Hall, *p53* expression is common in malignant mesothelioma, Histopathology 21 (1992) 331–334.
[19] K. Segers, H. Backhovens, S.K. Singh, J. De Voecht, M. Ramael, C. Van Broeckhoven, E. Van Marck, Immunoreactivity for *p53* and mdm2 and the detection of *p53* mutations in human malignant mesothelioma, Virchows Arch. 427 (1995) 431–436.
[20] M. Ramael, I. Deblier, C. Eerdekens, G. Lemmens, W. Jacobs, E. VanMarck, Immunohistochemical staining of *ras* oncogene product in neoplastic and non-neoplastic mesothelial tissues: immunoreactivity for N-*ras* and lack of immunohistochemical staining for Ha-*ras* and K-*ras*, J. Pathol. 169 (1993) 421–424.
[21] T. Kishimoto, The distribution of various type of oncogenes products in the tumor tissue of malignant mesothelioma, Nihon Kyobu Shikkan Gakkai Zasshi 29 (1991) 1168–1173.
[22] M. Carbone, P. Rizzo, P.M. Grimley, A. Procopio, D.J. Mew, V. Shridhar, A. de Bartolomeis, V. Esposito, M.T. Giuliano, S.M. Steinberg, A.S. Levine, H.I. Giordano, Simian virus-40 large-T antigen binds *p53* in human mesotheliomas, Nat. Med. 3 (1997) 908–912.
[23] V. Levresse, S. Moritz, A. Renier, L. Kheuang, F. Galateau-Salle, J.P. Mege, P. Piedbois, B. Salmons, W. Guenzburg, M.C. Jaurand, Effect of simian virus large T antigen expression on cell cycle control and apoptosis in rat pleural mesothelial cells exposed to DNA damaging agents, Oncogene 16 (1998) 1041–1053.
[24] C.G. Jensen, L.C. Jensen, C.L. Rieder, R.W. Cole, J.G. Ault, Long crocidolite asbestos fibers cause polyploidy by sterically blocking cytokinesis, Carcinogenesis 17 (1996) 2013–2021.
[25] M. Yegles, L. Saint-Etienne, A. Renier, X. Janson, M.C. Jaurand, Induction of metaphase and anaphase/telophase abnormalities by asbestos fibers in rat pleural mesothelial cells in vitro, Am. J. Respir. Cell Mol. Biol. 9 (1993) 186–191.
[26] H. Dong, A. Buard, A. Renier, F. Levy, L. Saint-Etienne, M.C. Jaurand, Role of oxygen derivatives in the cytotoxicity and DNA damage produced by asbestos on rat pleural mesothelial cells in vitro, Carcinogenesis 15 (1994) 1251–1255.
[27] J.A. Hardy, A.E. Aust, Iron in asbestos chemistry and carcinogenicity, Chem. Rev. 95 (1995) 97–118.
[28] D.W. Kamp, P. Graceffa, W.A. Pryor, S.A. Weitzman, The role of free radicals in asbestos-induced diseases, Free Radical Biol. Med. 12 (1992) 293–315.
[29] J.F. Lechner, T. Tokiwa, M. LaVeck, W.F. Benedict, S. Banks-Schlegel, H. Yeager Jr., A. Banerjee, C.C. Harris, Asbestos-associated chromosomal changes in human mesothelial cells, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 3884–3888.
[30] K. Linnainmaa, B.I. Gerwin, K. Pelin, K. Jantuene, M.A. LaVeck, J.F. Lechner, C.C. Harris, Asbestos-induced mesothelioma and chromosomal abnormalities in human-mesothelial cell in in vitro, in: The Changing Nature of Work and Work Place, NIOSH, Cincinnati, OH, 1986, pp. 119–122.
[31] N.S. Wang, M.C. Jaurand, L. Magne, L. Kheuang, M.C. Pinchon, J. Bignon, The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture: a scanning and transmission electron microscopic study, Am. J. Pathol. 126 (1987) 343–349.
[32] K. Pelin, A. Hirvonen, M. Taavitsainen, K. Linnainmaa, Cytogenetic response to asbestos fibers in cultured human primary mesothelial cells from 10 different donors, Mutat Res. 334 (1995) 225–233.