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Immunoreactivity for p53 and mdm2 and the detection of p53 mutations in human malignant mesothelioma

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Abstract Previous immunohistochemical studies on malignant mesothelioma with antibodies recognizing both the wild and the mutant types of the p53 protein have shown immunoreactivity in 25–70% of cases. This study was designed to determine whether there is immunoreactivity for p53 and mdm2 protein in malignant mesothelioma and to correlate p53 expression with the detection of mutations in *p53* at DNA level. In 10 of 15 cases there was immunoreactivity for p53. In 6 of these cases immunoreactivity for mdm2 was also detected. In one p53-immunonegative case, a mutation of the *p53* gene resulting in a stop codon was found. These results suggest that mdm2 might be involved in the inhibition of p53 in malignant mesothelioma. Also, these data suggest the existence of other proteins than mdm2 that may associate with p53.

Key words Malignant mesothelioma · p53 · mdm2 · Automated sequencing

Introduction

Malignant mesothelioma is a relatively rare, highly malignant tumour associated with asbestos exposure [23] and characterized by a long interval between the exposure and the appearance of the tumour. Survival rates after diagnosis are short [10].

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Suppressor genes are currently of great interest in cancer research, and the best investigated is the *p53* gene located on chromosome 17p13.1 [17]. Genetic changes in the *p53* gene are described in several neoplasms, including colon cancer [32], non-small-cell lung cancer [21], breast cancer [5], hepatocellular carcinoma [24], oral squamous-cell carcinoma [31], epithelial skin cancer [22] and thyroid carcinomas [11].

However, loss of the tumour suppressor function by point mutations in the *p53* gene is not the only mechanism by which the p53 protein function can be altered [6]. Binding to proteins of DNA tumour viruses such as the SV40 large T antigen, the adenovirus E1B protein and the papillomavirus E6 protein and to cellular proteins such as the mdm2 protein may disrupt the p53 pathway [33]. The human *mdm2* gene is mapped to chromosome 12q13-q14 [26] and is amplified in about a third of sarcomas of bone and soft tissue. Overexpression of the mdm2 protein can inhibit the binding of p53 to target genes [27]. Mutation of the *p53* gene and amplification of the *mdm2* gene operate by similar mechanisms to interfere with the regular growth control pathway mediated by p53, suggesting that one genetic alteration, but not both, is required to obtain a similar effect [16, 26]. The levels of the mdm2 protein in normal cells are low, giving only a weak signal or none at all on immunohistochemistry [8, 16].

The scope of the present study was to investigate by immunohistochemical testing whether there is expression of p53 and mdm2 protein and to correlate p53 expression with the detection of mutations in *p53* at DNA level.

Materials and methods

Tissue

Fifteen primary malignant mesothelioma tissues (8 epithelial, 3 sarcomatous and 4 mixed mesotheliomas) were included in this study. These malignant mesotheliomas were classified according to the guidelines of Henderson et al.[13]. The mean age of the me-

sothelioma patients was 65.8 (range 41–86) years; there were 10 men and 5 women. None of the patients had received any previous treatment. The tumour specimens were snap-frozen in liquid nitrogen and stored at –80°C. Representative haematoxylin-eosin stained sections and paraffin-embedded blocks of these specimens were used to reach the diagnosis of malignant mesothelioma.

Cell lines

Two cell lines served as a positive control for the detection of *p53* mutations and were obtained from the American Type Culture Collection. The osteosarcoma cell line HOS harbours a mutation at codon 156 [29] in exon 5 of the *p53* gene, while the colon cancer cell line COLO 320 DM shows a mutation at codon 248 [25] in exon 7.

DNA extraction

Genomic DNA was extracted from all 15 malignant mesotheliomas, from the two cell lines and from peripheral blood lymphocytes of a normal control individual using the proteinase K-phenol-chloroform extraction method [3].

PCR-SSCP analysis

The PCR (polymerase chain reaction) primers used to amplify exons 5–9 of the *p53* gene have been published previously [31]. The PCR reactions were performed in a volume of 25 µl containing 100 ng genomic DNA, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 50 µM of each dNTPs, 25 pM of each primer, 1 U of Taq DNA polymerase (Perkin Elmer, Branchburg, N.J., USA) and 0.2 µl of [-32P]dCTP (3000 Ci/mmol, Amersham, Bucks., UK). An initial denaturation step at 94°C during 2 min was carried out in a Perkin-Elmer 480 DNA Thermal Cycler, followed by 35 cycles at 94°C for 1 min, at 68°C for 1.5 min, and at 72°C for 2 min. The reactions were terminated with a 7-min final extension step at 72°C, after which 5 µl of the PCR product was added to 3 µl of a loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were denaturated at 95°C for 5 min and chilled on ice for 5 min. Subsequently, 4 µl was loaded on a Hydrolink MDE gel (AT Biochem, Malvern, Pa., USA) with 10% glycerol and without glycerol. Electrophoresis was performed at 15 W for 18–20 h at room temperature. The gel was dried at 70°C for 45 min under vacuum and exposed to an X-ray film at –80°C for 16–24 h.

Sequencing of PCR products

Sequencing reactions were performed directly on 5 µg of PCR product by fluorescent dye terminator sequencing using PRISM T7 Terminator Sequencing Kit (Applied Biosystems, Foster City, Calif., USA). Afterwards the sequencing products were ethanol precipitated and washed with 70% ethanol. The dried pellet was then resuspended in 83% formamide, 8 mM EDTA and loaded on a 6% acrylamide gel containing 8 M urea, prepared in accordance with the Applied Biosystems sequencing manual instructions. All reactions were separated and analysed on an 373 A automated DNA sequencer (Applied Biosystems, USA).

Immunohistochemistry

Frozen sections 5 µm thick were cut and fixed in acetone at 4°C for 10 min. Slides were immersed in methanol supplemented with 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase. After rehydration through graded ethanol and distilled H_2O, sections were incubated with normal rabbit serum (dilution 1/20, Dako, Glostrup, Denmark) to block the nonspecific Fc receptor activity. The sections were then incubated with the primary anti-p53 antibody PAb 1801 (dilution 10 µg/ml, Oncogene Science, Uniondale, N.Y., USA) and with the anti-mdm2 antibody IF-2 (dilution 5 µg/ml, Oncogene Science, USA). The anti-p53 antibody PAb 1801 recognizes an epitope located between amino acids 32 to 79 of both wild-type and mutant human p53 proteins [8]. The anti-mdm2 antibody IF2 is directed against a protein containing aminoacids 27–168 of mdm2 [16].

The sections were then overlaid with biotinylated rabbit anti-mouse polyclonal antibody (Dako, Denmark) diluted 1/350 as the secondary antibody. Binding was detected by applying the avidin biotin-peroxidase complex (ABC; Dako, Denmark). Peroxidase was revealed by incubating the sections with a solution containing 3,3'diaminobenzidinetetrahydro-chloride in 20 ml TRIS buffer pH 7.6 containing 0.03% hydrogenperoxide.

The specificity of the immunohistochemical reactions was controlled as follows: (1) omitting the first antibody; (2) substituting the primary antibodies for an unrelated monoclonal antibody of the same isotype IgG_1 in the same concentration but directed against an unrelated antibody (monoclonal mouse anti-human CD 68 antibody, isotype IgG_1, Dako, Denmark).

Frozen sections of a colon adenocarcinoma and of a malignant fibrous histiocytoma were used as positive control for p53 and mdm2 respectively. For counterstaining, Mayer's haematoxylin was applied for 1 min.

Statistical analysis

Survival data were available in all patients but 1, and survival time from the date of diagnosis until death or last follow-up was determined for each. Survival curves were constructed using the Kaplan-Meier method, with statistical significance determined by the log-rank test. The relative magnitude of the difference in prognosis was estimated by a hazard ratio using a Cox proportional hazards model.

Results

Immunohistochemistry

The 15 malignant mesotheliomas included 10 that displayed a fine granular nuclear immunoreactivity with the anti-p53 antibody (Table 1). In 2 there was diffuse im-

Table 1 Immunoreactivity (*IR*) detected for the p53 and mdm2 proteins (+ focal IR in less than 10% of cells, ++ focal IR with over 50% positivity in the clusters, +++ diffuse IR in more than 50% of cells, *E* epithelial, *S* sarcomatous, *M* mixed)

Case no.	Type	p53 IR	mdm2 IR
1	E	+	+
2	E	+	+
3	S	+	+
4	E	++	–
5	E	–	–
6	E	–	–
7	E	–	–
8	E	+++	–
9	S	+++	–
10	M	+	+
11	E	–	–
12	M	+	+
13	M	++	–
14	M	+	+
15	S	–	–

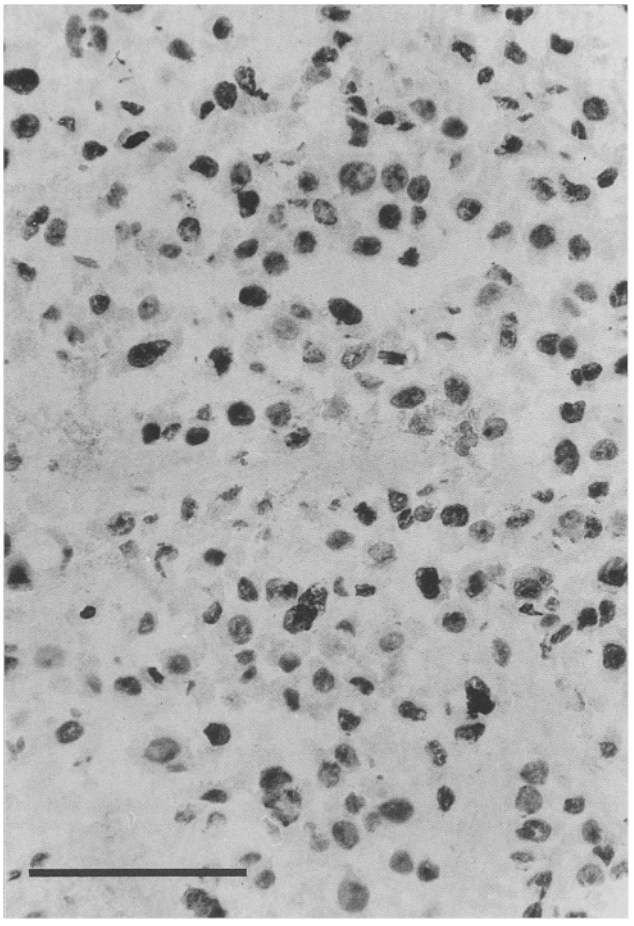

Fig. 1 Neoplastic cells of an epithelial mesothelioma displaying strong nuclear immunoreactivity for p53 with the PAb 1801 antibody. ×290; *scale bar*=100 μm

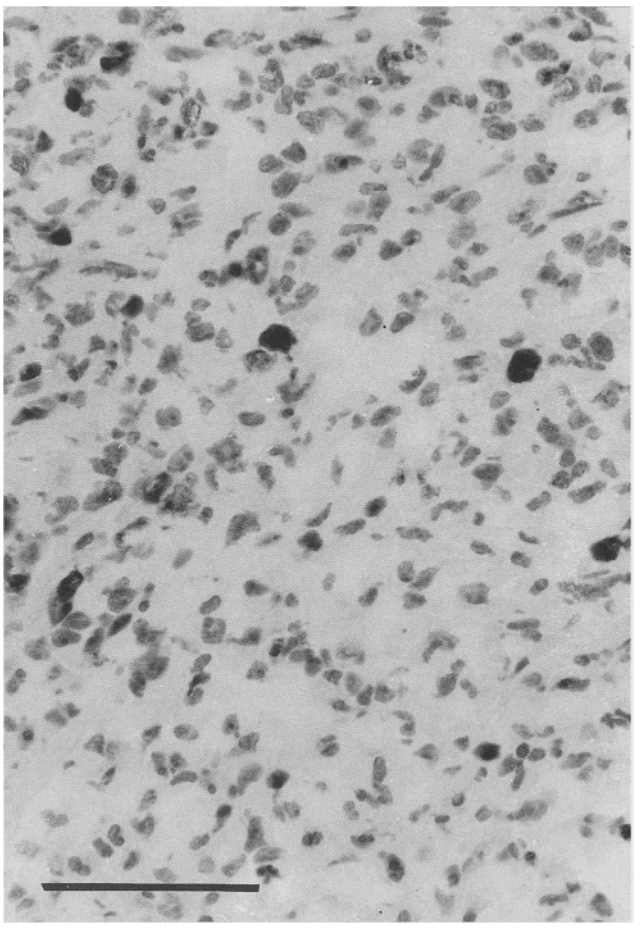

Fig. 2 A sarcomatous mesothelioma showing scattered nuclear immunoreactivity for mdm2 with the IF2 antibody. ×290; *scale bar*=100 μm

munoreactivity in more than 50% of the cells (Fig. 1). In another 2 there was focal immunoreactivity with clusters containing more than 50% positive cells. In 6 cases focal immunoreactivity was seen in less than 10% of the cells. These 6 mesotheliomas also displayed focal nuclear immunoreactivity with the anti-mdm2 antibody in less than 10% of the cells (Fig. 2, 3a, b), while the other 9 mesotheliomas were negative.

Immunoreactivity was not seen in stromal cells. The colon adenocarcinoma and the malignant fibrous histiocytoma, used as a positive control, were positive for p53 and mdm2 respectively.

PCR-SSCP analysis

The mutations of the *p53* gene for the HOS cell line and for the COLO 320 DM cell line at codon 156 and 248 are localized in exon 5 and 7 of the *p53* gene respectively. SSCP (single-strand conformation polymorphism) analysis revealed an abnormal mobility shift in exons 5 and 7 for the control cell lines (Fig. 4a, b). In one malignant mesothelioma (case 5) an abnormal mobility shift was detected in exon 8 (Fig. 4c). The case with the abnormal mobility shift on the PCR-SSCP analysis (case 5) did not show immunostaining. None of the other 14 malignant mesotheliomas or the peripheral blood lymphocytes of the control individual showed a shift in the migration pattern for the exons 5–9 screened.

Sequencing analysis

To see whether the mobility shift in exon 8 in the 1 mesothelioma was caused by a mutation, direct PCR DNA sequencing was performed on genomic DNA of the malignant mesothelioma as well as on that of peripheral blood lymphocytes. The malignant mesothelioma showed a G-to-T transition at the first base (nucleotide) of the codon 266. It is predicted this base change will result in replacement of glycine (GGA) by a stop codon (TGA; Fig. 5). The DNA sequence of exon 8 of the normal lymphocytes was identical to the published sequence (Fig. 5) [15].

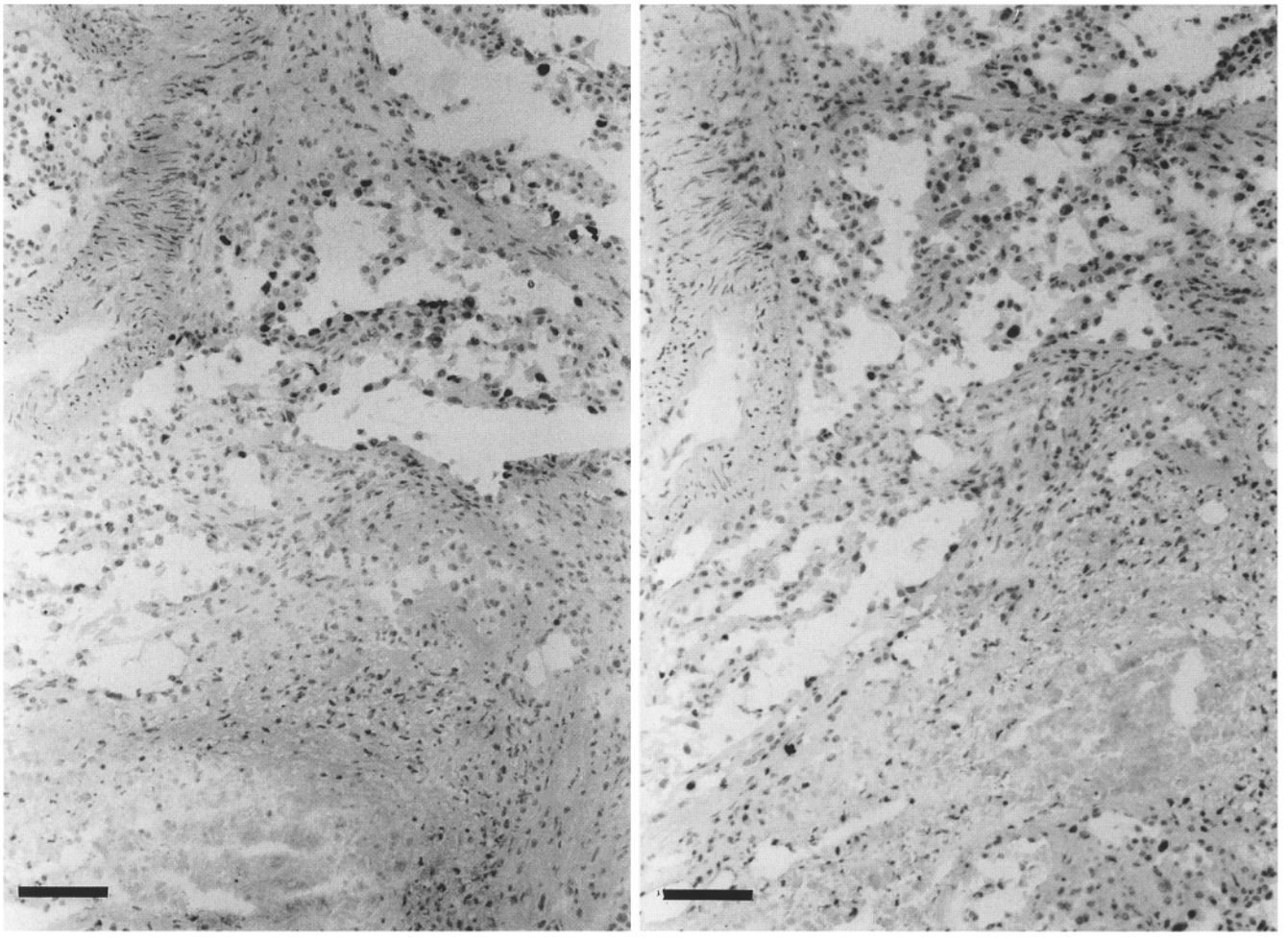

Fig. 3 Immunoreactivity for **a** mdm2 and **b** p53 in the same tumour area of an epithelial malignant mesothelioma. ×116; *scale bar*=100 μm

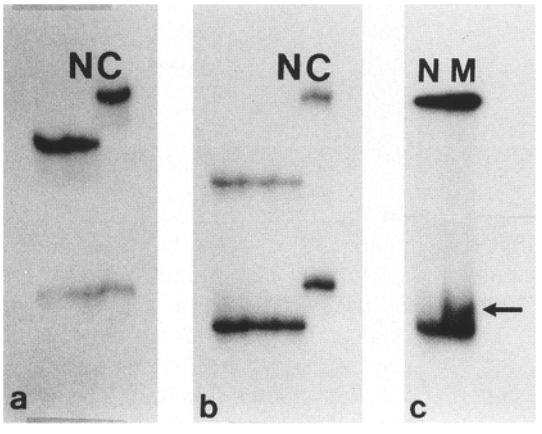

Fig 4 a–c PCR-SSCP analysis of various exons in various malignant mesotheliomas. **a** Exon 5 (*left lane* case 14; *N* normal lymphocytes, *C* HOS cell line). **b** Exon 7 (*left lanes* cases 2 and 10, respectively, *N* normal lymphocytes, *C* COLO 320 DM cell line). **c** Exon 8 of the *p53* gene, showing an abnormal mobility shift (*arrow*) in case 5 (*N* normal lymphocytes, *M* case 5)

Statistical analysis

The log-rank test performed on the actuarial survival curves constructed using the Kaplan-Meier method revealed no statistical significance for p53 (P=0.42) or mdm2 (P=0.74), as seen in Fig. 6; there was no association between immunoreactivity for p53 and mdm2 and prognosis.

Discussion

Previous immunohistochemical studies conducted on malignant mesothelioma using antibodies recognizing both the wild type and the mutant type of the p53 protein showed immunoreactivity in 25–70% [4, 14, 18, 28]. In the present study 66% of the cases displayed immunoreactivity for p53. Recently, the assumptions that all positive immunostaining indicates p53 mutations and/or that all p53 mutations cause positive immunoreactivity have been questioned [2, 12]. An important mechanism that stabilizes the wild-type p53 is its interaction with oncoproteins of tumour viruses or with cellular gene products such as mdm2 [6]. In our study, 6 of the cases that were positive for p53 were also positive for mdm2, suggesting that the p53 detected here could be of the wild-type

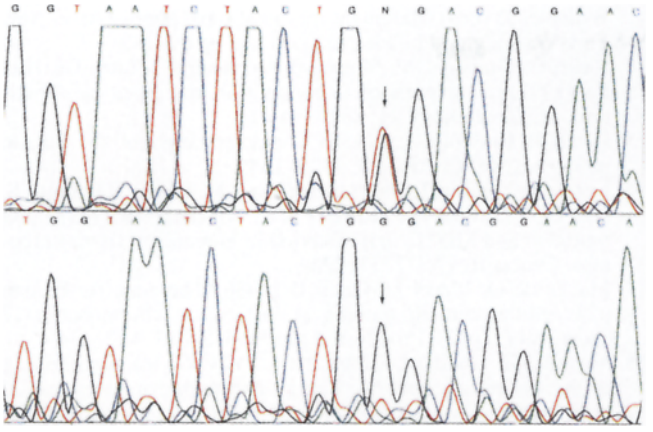

Fig. 5 Printout from an automated Applied Biosystems DNA sequencer. *Top* DNA sequence analysis showing a G-to-T transition at the first letter of codon 266 (*arrow*), resulting in a stop codon. *Bottom* the sequence analysis of the normal lymphocytes is shown for comparison

form, not excluding the mutated form, because the antibody used against p53 cannot discriminate between the two forms. These results correlate well with those of the PCR-SSCP analyses, which showed no mobility shift in these 6 cases. Whether the mdm2 immunoreactivity in malignant mesothelioma is due to DNA gene amplification, to increased transcription or to other mechanisms that could increase the level of the mdm2 protein has to be investigated further. Cordon-Cardo et al. [8] found in sarcomas that there was not always a correlation between the overexpression of the mdm2 protein and amplification of the *mdm2* gene and stated that because of multiple forms of mdm2 proteins, this antibody might fail to detect the mdm2 protein in some cells. If there is binding of p53 and mdm2 in malignant mesothelioma cells this has to be confirmed by binding studies.

The 4 other mesotheliomas contained more cells that were positive for p53, and heavy staining in the majority of the cells was frequently associated with the mutated form [12]. However, none of these 4 mesotheliomas showed a shift in the migration pattern on the PCR-SSCP analysis. Since only exons 5–9 (encompassing 95% of p53 mutations [19]) were analysed for the detection of genetic changes, it is possible that mutations in other exons of the p53 gene give rise to the mutated form. Other mechanisms that would explain immunopositivity for p53 are an alteration in an enzymatic pathway responsible for p53 degradation [9], overexpression of the c-myc protein [30], alterations outside the coding region [1] or other cellular proteins than mdm2 that can stabilize p53 [33].

The case with the nonsense mutation in exon 8 did not show immunoreactivity for p53 and mdm2. This was most probably because the base substitution resulted in a nonsense mutation. This observation is also described in soft tissue sarcoma [8]. This illustrates that not all mutations in the p53 gene can be detected immunohistochemically. Other false-negative results may be caused by total deletion of the gene, by an unstable mutated protein form or by inappropriate fixation methods [2]. The sequencing results suggest that the mutation detected here is heterozygous in nature, affecting only one allele, because both the wild-type nucleotide and the mutant-type nucleotide are present. Another possibility is that the cell population is not homogeneous and consists of cells without genetic alterations in this part of the gene (normal stromal cells or tumour cells with a different clonal origin) and of cells with a totally or partially deleted allele and another allele with a point mutation, the latter being the way in which in most tumours both p53 alleles are inactivated [6].

Previous studies on p53 mutations performed on 24 mesothelioma cell lines from 21 individuals [9, 20] have

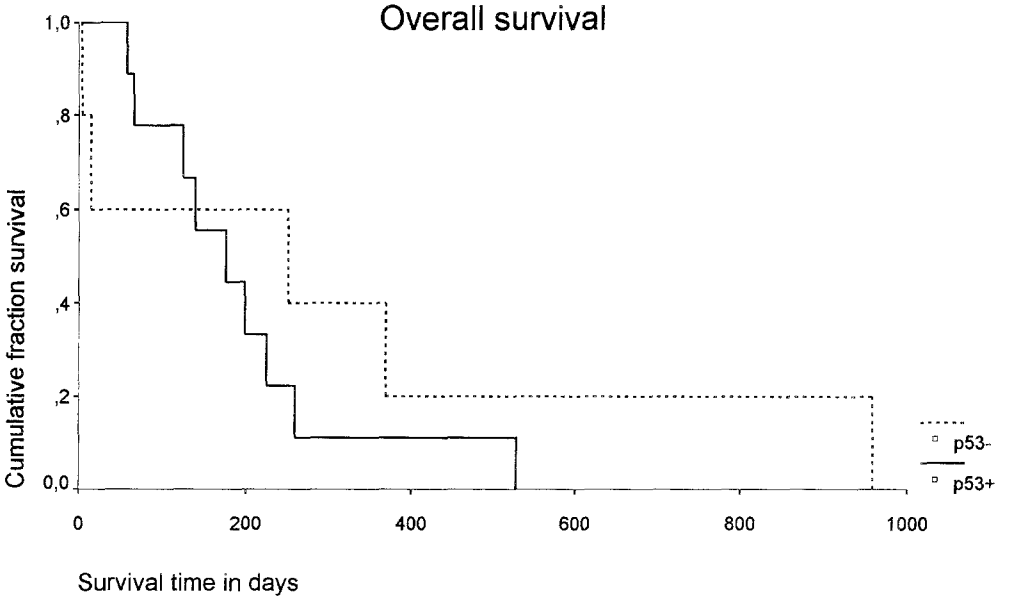

Fig. 6 Actuarial survival curve comparing mesothelioma cases with immunoreactivity for p53 and without immunoreactivity for p53. The curve obtained for mdm2 had a similar shape

documented four missense mutations located at codons 175, 245 and 278 situated at the mutational 'hot spots' of the p53 gene (codon 129–146, 171–179, 234–260, 270–287 [33]). In two of these cell lines no p53 mRNA was detected . These findings and our report demonstrate that mutations are an uncommon finding in malignant mesothelioma cell lines as in biopsies of malignant mesotheliomas.

This study reports immunoreactivity in malignant mesotheliomas for p53 and mdm2 in more than half of the cases with p53 positive immunostaining. Since the occurrence of mutations of the p53 gene in exon 5–9 is not a common finding in biopsies of malignant mesotheliomas, alternative mechanisms, such as p53 binding by cellular proteins other than mdm2 [33], may play a part in the escape from p53-growth control in this malignancy.

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