p53 and Kirsten-ras Mutations in Human Mesothelioma Cell Lines


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Abstract

Twenty cell lines from 17 individuals with malignant mesothelioma have been examined for p53 alterations by direct sequencing of genomic DNA, by evaluation of mRNA expression levels, and by immunocytochemical analysis of p53 protein expression in comparison with normal human pleural mesothelial cells. The results of this study show p53 abnormalities in cell lines from 3 individuals. These include 2 point mutations and one null cell line. Interestingly, while both cell lines with point mutations exhibit high levels of p53 protein, normal mesothelial cells as well as 12 of the mesotheliomas evaluated express low but significant levels. In addition, sequencing of K-ras at codons 12, 13, and 61 reveals wild-type sequence in all 20 mesothelioma cell lines. The capacity to induce tumors in athymic nude mice did not correlate with the presence of a p53 mutation or elevated p53 protein levels. These data suggest that neither p53 alteration nor K-ras activation constitutes a critical step in the development of human mesothelioma.

Introduction

Malignant mesothelioma is a rare cancer the development of which is strongly associated with asbestos exposure (1, 2). This disease is characterized by a long latency from onset of exposure and a short survival after diagnosis (3, 4). The length of the latency period suggests that multiple genetic alterations may be required for tumorigenic conversion of mesothelial cells (5). A search for molecular changes which have relevance for the development of mesothelioma benefits from studies of other tumor types which specify specific oncogenes and tumor suppressor genes. One such gene is the Kirsten-ras protooncogene; examples of Ki-ras genes activated by mutations in codons 12, 13, and 61 have been found with substantial frequencies in adenocarcinomas of the exocrine pancreas, colon, and lung (6–10). If activated ras were involved in the genesis of mesothelioma, mutations in the p53 tumor suppressor gene might be expected to act synergistically for tumor development. It has been shown in murine systems that normal embryonic cells are converted to tumorigenicity by the combination of an activated ras oncogene and overexpression of a mutated p53 gene (11–13). Many recent studies indicate that loss of function of the p53 suppressor gene through mutation or allelic loss is an event that is frequently associated with pathogenesis of human tumors including lung carcinomas (14–20). Evidence suggesting alteration of the p53 suppressor gene in mesothelioma includes reports of deletions of chromosome 17p which contains the p53 locus (21, 22) in some malignant mesotheliomas (23–26).

This study examines the hypothesis that activating mutations of Ki-ras and/or mutation or loss of p53 may be common events in the carcinogenic process leading to malignant mesothelioma. We report here the analysis of 20 cell lines from 17 individuals for abnormalities in c-Ki-ras by direct sequencing of PCR-amplified genomic DNA at codons 12, 13, and 61 and for p53 alterations by immunocytochemical analysis of protein expression, Northern hybridization analysis for mRNA expression level, and genomic DNA sequencing after PCR amplification. In contrast to most other tumor types and a previous report on mesothelioma cell lines (27), the frequency of mutation in p53 is relatively low.

Materials and Methods

Cell Lines and Culture Conditions. Human mesothelioma cell lines were cultured as described in LHC MM growth medium (28). Twenty cell lines derived from 17 patients were analyzed in this study: JMN and DND (24); VAMT 1 (29); HUT 28, HUT 226, and HUT 290 (30); MT 3 (31); M9K, M10K, M14K, M14P, M14M, M19, and M20 (32); and M15, M24K, M25K, M28K, M32K, and M33K. All mesothelioma cell lines were established from pleural fluid or tumor samples from patients whose diagnosis of malignant mesothelioma was pathologically confirmed. All cell lines with the M prefix in their designation were specimens from patients in whom the diagnosis of mesothelioma was reviewed by the Finnish National Mesothelioma Panel and the European Organization for Research and Treatment of Cancer Mesothelioma Panel. All tumor material was reviewed and grouped into epithelial, mixed, or fibromatous subtypes. Cell lines M14M, M14P, M14K, and M20 are derived from the same individual. M14P and M20 were established from pleural effusions with M14P being established before and M20 after mitoxantrone chemotherapy (total dose, 50 mg = 27 mg/m²) (32). In addition, M9K, M10K, and M15 were established from patients who had received previous mitoxantrone chemotherapy. M15 and M10K were the only cell lines from patients who had received prior radiotherapy. All M-prefix cell lines with the exception of M19 and M25K were from patients who had documented asbestos exposure. All cell lines were unique by karyotypic analysis with the exception of M14M which was a tetraploid subclone of M14P.

Primary mesothelial cell cultures were cultivated as described in LHC MM growth medium (28). The three cultures evaluated were derived from pleural fluid samples from three human subjects with noncancerous conditions.

Tumorigenicity. In order to evaluate the tumorigenic potential of mesothelioma cell lines, 5 × 10⁶ cells were inoculated s.c. into athymic nude mice. Mice were exposed to 350 rads 24 h before inoculation. Mice were maintained for 52 weeks and observed weekly for presence and size of tumors. Tumors were scored as positive when the cross-sectional area was 5 mm or greater and regression did not occur.

Immunocytochemical Analysis. Cells were seeded onto glass multiwell chamber slides (LAB-TEK No. 177402; Nunc, Naperville, IL) at an initial concentration of 10,000 cells/cm². After incubating overnight at 37°C the cells were fixed in acetone at −20°C for 10 min and stored at −20°C. Endogenous peroxidase activity was quenched for 20 min at room temperature with a 0.3% H₂O₂ solution in phosphate-buffered saline. After copious washing in phosphate-buffered saline, antigenic...
cross-reactivity was blocked with a 1:50 dilution of normal horse serum for 30 min at room temperature. Saturating concentrations of murine monoclonal primary antibodies were incubated overnight at 4°C and with two monoclonal antibodies in at least two separate experiments.

An epitope near the amino terminus was recognized by Pab 1801 (AB-CA). The chromogen was diaminobenzidine (final concentration, 0.05 mg/ml) osmicated with nickel chloride (final concentration, 0.03%).

p53 protein expression was demonstrated by concordant staining with two monoclonal antibodies in at least two separate experiments. An epitope near the amino terminus was recognized by Pab 1801 (AB-CA) and there was no counterstain. A proliferation marker, Ki-67, demonstrated the viability of the cells under analysis (M722; Dakopatts, Glostrup, Denmark). A monoclonal antibody to SV40 large T-antigen served as an isotype-matched negative control (Pab 416, AB-2; Oncogene Science, Inc., Manhasset, NY).

The results of immunocytochemical analysis of cultured cells for p53 protein expression are presented in Table 1 and representative photomicrographs are shown in Fig. 2. The JMN cell

### Table 1 Analysis of mesothelioma cell lines for p53 status and tumorigenicity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exons sequenced</th>
<th>Codon, mutation amino acid</th>
<th>Immunocytochemistry*</th>
<th>mRNA (p53/GAPDH)*</th>
<th>Tumorigenicity</th>
<th>Latency (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DND</td>
<td>2-11</td>
<td>Wild type</td>
<td>2+</td>
<td>1.1</td>
<td>5/5</td>
<td>14</td>
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<td>1.1</td>
<td>0/10</td>
<td></td>
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<tr>
<td>HUT 226</td>
<td>2-11</td>
<td>Wild type</td>
<td>ND</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUT 290</td>
<td>4-11</td>
<td>Wild type</td>
<td>ND</td>
<td>1.9</td>
<td>3/5</td>
<td>20</td>
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<tr>
<td>JMN</td>
<td>4-11</td>
<td>245 GGC-AGC Gly-Ser</td>
<td>4+</td>
<td>4.9</td>
<td>5/5</td>
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<td>MT 3</td>
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<td>1+</td>
<td>0.8</td>
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<td>1.9</td>
<td>3/5</td>
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<td>1+</td>
<td>2.3</td>
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<td>20</td>
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<td>1.2</td>
<td>2/5</td>
<td>28</td>
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<td>Wild type</td>
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<td>ND</td>
<td>2/7</td>
<td>45</td>
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<td>Wild type</td>
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<td>2.4</td>
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<td>4-11</td>
<td>278 CCT-TCT Pro-Ser</td>
<td>3+</td>
<td>1.3</td>
<td>3/5</td>
<td>54</td>
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<td>45</td>
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<td>VAMT 1</td>
<td>2-11</td>
<td>Wild type</td>
<td>Neg</td>
<td>0</td>
<td>5/5</td>
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</table>

* Immunostain criteria: 4+, more than 75% nuclei intensely stained; 3+, 50 to 75% nuclei stained; 2+, 25 to 50% nuclei stained; 1+, less than 25% nuclei stained; Neg, not stained.

p53/GAPDH ratios were determined as detailed in "Materials and Methods."

ND, not done.
Wild Type (M9K) Mutant (M15)

Fig. 1. Sequence analysis of two mesothelioma cell lines showing wild-type sequence or heterozygous mutation at codon 278. Sequencing gels of a wild-type mesothelioma cell line (M9K) or a heterozygous mutant mesothelioma cell line (M15). Sequences were obtained after PCR amplification as described in “Materials and Methods” and proceed from the 3’ direction. The figure displays sequence from codons 269 to 281 and shows the presence of two bands (G and A) at equal intensity in the first base pair of codon 278 in the M15 cell line.

Fig. 2. Immunocytochemical analysis of p53 protein expression in cells derived from human pleural mesothelium. A, intense nuclear staining in most of the JMN cells shown. There is variation in the intensity of nuclear staining, and two nuclei near the center of the field are unstained. B, characteristic extranucleolar, nuclear staining pattern as well as giant cell formation by some M15 cells. Although all the nuclei in this field are positive, there is variation in the staining intensity and unstained nuclei were observed in other fields. C, unstained nuclei in the null cell line VAMT 1. D, normal human pleural mesothelial cells including one darkly stained nucleus, several faintly stained nuclei, and scattered unstained nuclei. In all panels, the primary antibody was Pab 1801, and the original magnification was ×630.

line demonstrated high levels of p53 protein in 85% of the cell population; this staining (Fig. 2A) is explained by the presence of a missense mutation. M15 (Fig. 2B) also contained a missense mutation, and 62% of the nuclei were positively stained. Neither p53 mRNA nor protein were detected in the VAMT 1 cell line (Fig. 2C; Table 1). The remaining tumor cell lines lacked mutations within the coding regions examined and exhibited a range of levels of p53 protein expression: three had 3+ levels of protein expression; two had 2+ levels; and ten had 1+ levels. Three samples of normal human pleural mesothelial
cells exhibited a composite of 2+ level staining (i.e., 27.5% total positive nuclei) with individual positive percentages of 25, 37, and 20% (Fig. 2D). The proliferation (Ki-67) and negative (SV40 large T-antigen) controls showed that the cells were viable and that an isotype-matched negative control was negative as expected (see “Materials and Methods”).

Discussion

Utilizing direct genomic DNA sequencing after PCR amplification, this report presents an analysis of the frequency of single base alterations in codons 12, 13, and 61 of the K-ras gene and in major portions of the coding sequence (Table 1) of the p53 gene in 20 human mesothelioma cell lines from 17 individuals. Cytogenetic analyses of mesothelioma have shown numerous abnormalities involving almost every chromosome including 3p deletions and trisomy 7 (24, 25, 32), but no specific lesions which might serve as markers of this disease. The present study was designed to examine the possibility that activating mutations in K-ras, perhaps in combination with alterations of p53, might be associated with the genesis of mesothelioma. The involvement of ras was suggested by experiments showing that expression of mutant EJ-ras can provide growth factor independence to normal human mesothelial cells (39) and convert an SV40 large T-antigen-immortalized human mesothelial cell line (Met-5A) to tumorigenicity (40). All 20 cell lines were wild-type for K12-13 and K-61, suggesting that ras mutations are uncommon in mesothelioma.

Immunocytochemical analysis as well as DNA sequencing were used to detect p53 mutations in mesothelioma cell lines. Two missense point mutations were identified in two cell lines expressing high levels of protein. Several lines of evidence indicate that overexpression of p53 protein within a tumor may signal the presence of missense mutation, but the correlation between p53 protein overexpression and mutation is imperfect (16, 20, 41, 42). In this series, the JMN cell line had the highest protein expression (i.e., 85% positive nuclei) and a mutation. The second mutation occurred in M15, which was one of four cell lines with 3+ expression of p53 protein (i.e., 50–75% positive nuclei). The remaining cell lines with 3+ staining (i.e., M9K, M14M, M14P) were derived from two patients and contained wild-type sequences. Mechanisms which may explain p53 protein overexpression in cells with wild-type genomic sequence include (a) inactivation of an enzymatic pathway responsible for p53 protein degradation (43), (b) stabilization of wild-type protein through complex formation with a DNA tumor virus protein (44) or a cellular oncogene; and (c) overexpression of the myc oncogene product (45). The last mechanism is relevant since myc amplification and/or overexpression is common in lung cancer (46, 47).

Lower levels of p53 protein (i.e., 1+ to 2+ staining) were detected in the remaining tumor cell lines as well as in primary mesothelial cell cultures. Wild-type p53 protein expression in normal human mesothelial cells is compatible with several observations in other nonneoplastic cell types. For example, the wild-type protein appears to play a role in normal proliferation of nontransformed cells such as human lymphocytes, normal mouse thymocytes, and NIH 3T3 fibroblasts (48–50). Wild-type p53 protein may be expressed at significant levels in cells in which it contributes to maturation and differentiation (51). The meaning of p53 protein expression in a small fraction of a tumor cell population is not clear. These data suggest that low levels of p53 protein may not signal p53 abnormality but may reflect physiological expression of a wild type protein. Alternatively, the previously cited mechanisms for p53 protein accumulation may apply. Clearly, many questions remain regarding the interpretation of p53 protein expression in normal and tumor-derived human mesothelial cells.

DNA sequence, RNA expression, and immunocytochemical analyses presented in Table 1 indicate that alteration of the p53 tumor suppressor gene occurs infrequently in the development of human mesothelioma. p53 protein accumulated in a majority of tumor nuclei in cell lines from only four individuals. In two cases, missense point mutations explain the protein overexpression; in the remaining pair, the examined sequences were wild-type, and some other alteration may exist. The remaining abnormality produced a p53 null cell (VAMT 1) as judged by analysis of p53 mRNA by Northern blot techniques and of p53 protein by immunocytochemistry. Genomic DNA sequencing after PCR amplification of exons 2–11 detected only a wild-type sequence in this cell line suggesting that the lack of p53 expression results from rearrangement, intronic mutation, or an alteration in downstream processes which regulate p53 gene expression. Since cell lines, as opposed to tumors, might be expected to develop mutations during extended passaging, the low frequency of mutations observed in the cell lines studied here strengthens the suggestion that loss of wild-type p53 expression is not a frequent occurrence in this tumor type. These results contrast with a recent report of three p53 alterations in a total of four mesothelioma cell lines (27). In agreement with the data presented here, two alterations were single base changes while a third resulted in a p53 null cell. The reported base changes in mesothelioma are all G:C to A:T transitions in codons 175 and 245 (27) and 245 and 278 (Table 1). Only the JMN transition mutation occurs at a CpG site which would be expected to have a high mutability because of modification of C in this pair to 5-methylcytosine (19, 52). In addition to these changes, two p53 null cells without documented mutations have been reported in these two studies. Therefore, combining the previous (27) and present data, four point mutations and two p53 null cells have been documented in 24 cell lines from 21 individuals. This frequency of approximately 29% is to be compared to frequencies of 45–79% reported for tumors of the lung (23 of 51) (15), stomach (19 of 24) (42, 53), bladder (9 of 16) (54), liver (13 of 26) (55, 56), and skin (14 of 28) (35). A low frequency of p53 mutations has been reported for medulloblastomas (0 of 12 tumors, 0 of 8 xenografts, and 1 of 3 cell lines) (57).

Examination of the spectrum of p53 point mutations associated with a known carcinogenic agent has been utilized to produce a "molecular footprint" providing information concerning the nature of the molecular interactions which may be of importance in the carcinogenic process (19, 35, 58). Malignant mesothelioma is strongly associated with asbestos fiber exposure (1, 2). It was hoped that the sequencing analysis performed for this study might reveal a mutational spectrum found in mesothelioma containing patterns suggestive of molecular routes involved in the carcinogenic interaction of asbestos with the mesothelium. The relative infrequency of single base changes in mesothelioma supports the hypothesis that oxy radical-generated 8-hydroxydeoxyguanine adducts are not important in the genesis of this tumor. Earlier reports have shown that, while exposure to asbestos in vitro supplies a growth advantage to and induces chromosomal abnormalities in normal human mesothelial cells (59), it does not generate oxy radicals (60). However, it is known that oxy radicals are pro-
duced by the interaction of asbestos and macrophages or tracheal epithelial cells (61–63). Perhaps the mesothelial cell has sufficient DNA repair capability and/or can shift to repair mechanisms to render exposure from this source of minor consequence. Alternatively, it may be that, when the critical genetic targets for mesothelioma are identified, they may reveal a different mutational spectrum. The finding of frequent and multiple chromosomal abnormalities in human mesothelioma cells, coupled with the observations that asbestos associates with chromosomes (64) and induces chromosomal damage in tissue culture (59, 65), is consistent with the present report, suggesting that structural changes and chromosomal re-arrangements may be of great importance in the development of mesothelioma after fiber exposure. In support of this hypothesis, Cora and Kane (66) have recently reported that frequent deletions and two point mutations in the p53 gene were observed in tumorigenic cell lines isolated from tumors developed in C57BL/6 mice after weekly i.p. injections of 200 μg of UIICC crocidolite asbestos. Thus, the interaction of fibers and mesothelial cells appears to yield structural chromosomal changes with high frequency and, perhaps, with a greater probability than base pair mutations. Interestingly, a recent study of mutational spectra resulting from aerobic incubation of DNA with Fe2+ documented primarily single-base substitutions (67). In contrast to these results, a study which examined mutagenesis of a plasmid in H2O2-treated simian cells reported deletions in 45% of spontaneous or induced mutants and single or multiple base changes in 68 or 57% of induced or spontaneous mutants (68). The differences observed point out the importance of evaluating such spectra in carefully controlled studies. Thus, the factors involved in the lack of base substitutions in mesothelioma cell lines may be related to uncontrolled factors involved in asbestos carcinogenesis. It will be of interest in the future to examine mutational spectra resulting from interaction of asbestos and mesothelial cells or with mixed cultures of mesothelial cells and macrophages.

The findings of this report suggest that loss of wild-type p53 expression is less commonly associated with human mesothelioma than with many other tumor types. An additional study showing no abnormalities in retinoblastoma gene protein or mRNA expression in human mesothelioma cell lines3 makes it probable that these two tumor suppressor genes do not play critical roles in growth regulation in mesothelial cells. Thus, loss of function of the retinoblastoma gene has been associated with some but not all tumor types (69) while p53 alterations have been more commonly found (70). It is probable that specific cell types will differ with regard to which genes are most critical in the regulatory processes which define the "normal" cell. In mesothelial cells, it would appear that loss of function alterations in the p53 or the retinoblastoma gene are not rate limiting for tumor development.

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p53 STATUS IN HUMAN MESOTHELIOMA CELL LINES


p53 and Kirsten-\textit{ras} Mutations in Human Mesothelioma Cell Lines


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