Genetic Alterations of the p53 Gene Are a Feature of Malignant Mesotheliomas

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ABSTRACT

A putative tumor suppressor gene, p53, has been shown to be altered in a variety of human tumor types. The primary mechanism of p53 inactivation is believed to be mutation of one allele followed by loss of the second allele. Malignant mesothelioma is a tumor that has been highly associated with exposure to asbestos fibers, which are known to cause chromosomal abnormalities in mesothelial cells. We have examined four mesothelioma cell lines for genetic abnormalities in p53. Cytogenetic analysis revealed that two of the four tumors had abnormalities (numerical and/or structural) of chromosome 17 (the locus of the p53 gene). Restriction fragment length polymorphism analysis using a chromosome 17p-specific probe (pYNZ22) revealed that two tumors had loss of heterozygosity in the region of 17p13. The relative level of p53 mRNA expression was examined by Northern analysis, with one tumor showing negligible expression of p53 mRNA. The complementary DNA of p53 was generated from the three tumors showing detectable mRNA expression, and the region between codons 70 and 319 was amplified by the polymerase chain reaction and sequenced. DNA single-base substitutions were detected in two of the tumor cell lines, each resulting in amino acid substitutions. One tumor had an arginine to histidine substitution at position 175, and one tumor had a glycine to aspartic acid substitution at position 245. The observed mutations took place in regions of high cross-species sequence homology, indicating that these regions may be functionally important. The correlation of chromosomal loss in 17p on the cytogenetic and molecular level along with p53 mRNA expression and DNA sequence data indicate that genetic alterations in p53 could be a feature of malignant mesotheliomas and may reveal an important role of asbestos fibers in tumor suppressor gene inactivation.

INTRODUCTION

Chromosomal abnormalities have been described for a wide variety of human tumors (1, 2). The best characterized examples occur in the hematopoietic malignancies, such as Burkitt's lymphoma and chronic myelogenous leukemia, in which specific reciprocal translocations involving protooncogenes have been described (1, 2). These translocations are thought to activate the affected protooncogenes, resulting in disregulation of cell growth and promotion of tumorigenesis. While chromosomal abnormalities have been described in solid tumors, these changes are generally much more complex as compared with hematological malignancies.

In addition to promotion of tumorigenesis by activation of protooncogenes, there has been a long-term interest in normal cellular genes which may play a role in controlling the proliferation processes that could lead to tumorigenesis, the so-called tumor suppressor genes (3–6). The first of these genes to be identified was the retinoblastoma (RB) gene (3, 7–9). The mechanism of tumor suppressor gene inactivation is postulated to be mutation of one allele with loss of the second allele. There has been a great deal of activity in examining the karyotype of human tumors for specific deletions, because it is postulated that these areas may contain tumor suppressor genes (3–6).

Recently, attention has been focused on chromosome 17. Specific deletions involving chromosome 17, and more particularly 17p, have been described in a variety of solid tumors, including colon, lung, brain, bone, and bladder cancers (10–14). Furthermore, it has been demonstrated that the gene coding for the nuclear protein p53 resides in 17p (15, 16). This protein, once thought to be a dominant oncogene product, is now believed to be the product of a tumor suppressor gene (17, 18). In this regard, it has now been demonstrated that a variety of human tumors show mutations in the p53 gene, often accompanied by deletion of the second allele (11, 19–23).

A large number of chromosome abnormalities have been described in malignant mesotheliomas, including deletions in chromosome 17 (24–27). We describe here that genetic abnormalities involving the region coding for p53, including loss of heterozygosity, loss of p53 mRNA expression, and point mutations in p53, can occur in malignant mesotheliomas and may be involved in the pathogenesis of this tumor.

MATERIALS AND METHODS

Cell Lines. The mesothelial cell lines were established from surgically explanted primary epithelial mesotheliomas seen at Memorial Sloan-Kettering Cancer Center. The methods for establishing these cell lines have been described (28). The Memorial Sloan-Kettering Cancer Center designations (in parentheses) of the cell lines used in this study are tumor A (Mes.Cl.-1), tumor B (Mes.Cl.-2), tumor C (Mes.Cl.-3), and tumor D (Mes.Cl.-4).

Cytogenetic Analysis. The cell lines were harvested and chromosome preparations were prepared as described previously (28). Giemsa (G) and quinacrine (Q) banding methods were used as needed. Approximately 20–30 metaphases from each cell line which showed good spreading and banding were selected and photographed. The cells were analyzed for chromosome abnormalities from enlarged prints, and at least three karyotypes were prepared to establish clonal rearrangements. The chromosomal abnormalities were identified and designated according to the standard chromosome nomenclature (29).

Southern Analysis to Determine Loss of Heterozygosity and Germ Line Configuration of the p53 Gene. For this analysis the tumor cell line and normal DNAs were isolated, digested with TaqI, electrophoresed, and transferred to nylon filters according to protocols recommended by the manufacturer (Oncor, Gaithersburg, MD). The filters were then hybridized with the chromosome 17(p13)-specific probe pYN22 (American Type Culture Collection catalogue 57574) (30). In addition, Southern blotting analysis of the EcoR1-digested cell line DNA was performed to detect gross structural abnormalities of the p53 gene. A p53 cDNA1 plasmid (American Type Culture Collection catalogue 57255) (31) was digested with BamHI. The 2.2-kilobase insert (15 ng) was radioactively labeled with 50 mCi [α-32P]dCTP (NEN) by the random primer method (32) using the randomly primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The labeled probe was separated from unincorporated nucleotide using a G50 spin column (Worthington, Freehold, NJ) and hybridized with the digested tumor DNA (33).

1 The abbreviations used are: cDNA, complementary DNA; GAPDH, glyceraldehyde phosphate dehydrogenase; RFLP, restriction-fragment length polymorphism.
Northern Analysis of p53 mRNA Expression. RNA was prepared from the cell lines using a single-step acid guanidinium thiocyanate-phenol chloroform extraction (34). The integrity of the RNA was determined by gel electrophoresis (1% agarose) for the presence and absence of rRNAs. The filter was washed in 0.25% sodium citrate-0.1% sodium dodecyl sulfate at 60°C for 15 min. In all cases there was no significant degradation of the RNA, as determined by spectral absorption at 260 nm using the random primer-labeled pS3 cDNA probe. Amplification of Tumor p53 cDNA. Reverse transcription of p53 mRNA was performed using 1 µg of total cellular RNA as template (31) by standard methods (33). The filter was then rehybridized with a probe to GAPDH (35). The nitrocellulose filter probed with the p53 cDNA was stripped by addition of boiling water for 30 min. This was repeated, and the filter was exposed overnight to verify that no radioactivity was present. The filter was then rehybridized with a probe to GAPDH (35).

Amplification of Tumor p53 cDNA. Reverse transcription of p53 mRNA was performed using 1 µg of total cellular RNA as template and Moloney murine leukemia virus reverse transcriptase as polymerizing enzyme. The primer for the reverse transcription reaction was a 3′ oligonucleotide complementary to codons 320 (third base) to 326 of the wild-type p53 cDNA clone (31) showed no obvious structural abnormalities in the p53 gene, because all tumors showed normal copies of chromosome 17. The original tumors from which these cell lines were derived have also been characterized cytogenetically. Such an analysis has shown that the karyotypes seen in the short-term cultures of tumors are identical with the karyotypes from the respective cell lines (Fig. 1). In addition to recurrent abnormalities of chromosomes 1, 3, and 22, the tumor cell lines also exhibited abnormalities (numerical and/or structural) of chromosome 17 (Fig. 1). Tumors A and B showed abnormality (deletion or rearrangement) of the short arm of chromosome 17; tumors C and D, on the other hand, had normal copies of chromosome 17.

RESULTS

Cytogenetic Analysis. A detailed cytogenetic analysis of mesothelioma cell lines included in this investigation will be presented elsewhere; only the description of predominant abnormal clones in each of the cell lines relevant to the present study is presented here (Table 1). In addition to recurrent abnormalities of chromosomes 1, 3, and 22, the tumor cell lines also exhibited abnormalities (numerical and/or structural) of chromosome 17 (Fig. 1). Tumors A and B showed abnormality (deletion or rearrangement) of the short arm of chromosome 17; tumors C and D, on the other hand, had normal copies of chromosome 17.

The original tumors from which these cell lines were derived have also been characterized cytogenetically. Such an analysis has shown that the karyotypes seen in the short-term cultures of tumors are identical with the karyotypes from the respective cell lines (Fig. 2).4

RFLP and p53 DNA Analysis. Southern blot analysis of EcoRI-digested DNA from the mesothelioma cell lines using the wild-type p53 cDNA clone (31) showed no obvious structural abnormalities in the p53 gene, because all tumors showed normal sized (16 kilobases) EcoRI fragments (Fig. 3).

Table 1 Correlation of cytogenetics, RFLP (17p), DNA, mRNA expression and point mutations for p53 in mesotheliomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Predominant abnormal clone</th>
<th>Loss of heterozygosity for 17p allele</th>
<th>p53 gene (Southern)</th>
<th>p53 mRNA (expression)</th>
<th>p53 mutation (codon)</th>
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<td>A</td>
<td>69-77,X,-Y,Y,X(3n±),-1,-1,-4, -10,-14,-15,-16,-17,-18,-18, -18,-19,-21,-22,+2,+3,+7,+11, +2X(1q11)+4(5p1,der(6;57)q27;7), +2Xder(15)(1;15)(p11;p13), +2Xder(16)(16;17)(q24;q11), +2X(18q), +2Xder(19)(9;13;7)+mar1</td>
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<td>Not informative</td>
<td>Normal</td>
<td>+</td>
<td>245</td>
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4 S. C. Jhanwar, manuscript in preparation.
RFLP analysis of tumor DNA using a probe for chromosome 17p, pYNZ22 (30) showed loss of heterozygosity in tumors A and B (Fig. 4). Tumor C showed no detectable loss of alleles for this marker (Fig. 4). While tumor D appears to be heterozygous at the locus identified by pYNZ22, the bands are extremely close, making interpretation difficult (Fig. 4).

p53 mRNA Expression. The relative level of p53 mRNA expression was examined by Northern blot analysis using a wild-type human p53 cDNA clone (31) and the results are shown in Fig. 5A. Because no inference could be made about the “normal” level of p53 mRNA expression in mesothelial cells, the relative levels of expression were compared for the four tumors. Tumors A, C, and D (Fig. 5A, lanes 1, 3, and 4, respectively) showed expression at significant levels, while tumor B showed negligible expression of p53 mRNA (Fig. 5A, lane 2). In order to control for loading artifacts, the filter was stripped and rehybridized with a GAPDH probe (35). As shown in Fig. 5B, all tumors expressed significant levels of GAPDH mRNA, indicating that tumor B expresses very little or no steady state levels of p53 mRNA.

Amplification and DNA Sequence of p53 cDNA. In order to determine the DNA sequence of the coding region of the p53 gene, p53 mRNA was reverse transcribed to cDNA. The cDNA was then amplified by the polymerase chain reaction and sequenced by the dideoxynucleotide method. Previous analyses indicated that there are regions of high homology across species lines in the wild-type p53 sequence (40), located between the codons for amino acids 117 and 286. It is within this region that the majority of mutations in the human p53 gene have been described (11, 20–23). We amplified a portion of the cDNA which included the region between the codons for amino acids 70 and 319, producing an amplified product approximately 800 bases in length (including the 5’- and 3’-oligonucleotide primers). The sequence of the entire amplified length of the p53 cDNA was obtained using a series of internal oligonucleotide primers described in “Materials and Methods” (20). Typically, 150 nucleotides could be read from each sequencing reaction. In this way complete sequence information in the area of interest was obtained from tumors A, C, and D. Tumor B could not be sequenced because it did not express adequate levels of p53 mRNA for reverse transcription and amplification as shown above.

DNA single-base substitutions were detected in tumors A and D. In tumor A there was a G to A transition in the second base of codon 175 (Fig. 6A), resulting in an arginine to histidine substitution. In tumor D there was a G to A transition in the second base of codon 245 (Fig. 6B), resulting in a glycine to aspartic acid substitution. In the case of tumors A and D, both the coding and complementary strands of the cDNA in the region of the putative mutations were sequenced. The presence of the point mutations at codons 175 (tumor A) and 245 (tumor D) was confirmed in each case (data not shown). No mutation was observed in the p53 transcript from tumor C. DNA was extracted from normal tissues from patients A and D and sequenced in the regions where the mutations were identified. The wild-type sequence was observed, indicating that the mut-
The base which would correspond to the wild-type sequence (G specific to this tumor are presented. Chromosomes which show recurrent abnormality in short-term culture (A) and permanent cell line (B). Note similarity in the cytogenetics, DNA analysis (including RFLP analysis of chromosomes 1, 3, 17, and 22) and only structural abnormalities specific to this tumor are presented.

The analysis described here was for the most part performed on material derived from mesothelioma cell lines. The use of culture-derived material has the risk of identifying genetic events which occur after the establishment of the cell line. However, we have shown that the cytogenetic abnormalities seen in the tumor cell lines studied here are identical with those seen in the primary tumor explants, including abnormalities in chromosome 17p, clearly demonstrating that the cytogenetic abnormalities (and presumably other genetic abnormalities) described for the cell lines are features shared by the original tumor as well. This has also been demonstrated in colon and renal cell carcinomas and in sarcomas. Furthermore, it has recently been shown that p53 abnormalities in colon tumors were found at similar frequencies in primary tumor samples and in cell lines derived from the tumors (23).

Asbestos fibers are known to cause proliferation of human mesothelial cell organ cultures (43). In addition, mesotheliomas can be induced in experimental animals by injection of asbestos (44, 45). Asbestos has been shown to bind nucleic acids and come into contact with chromosomes and the mitotic apparatus of mammalian cells grown in vitro (46, 47). When human mesothelial cells are exposed to asbestos in vitro, chromosomal abnormalities have been induced, including loss of chromosomal material (43). This mechanism is particularly interesting with regard to inactivation of tumor suppressor genes, in which loss of chromosomal material is a key event (3). Asbestos has also been shown to elicit an intense inflammatory response in the pleura of experimental animals, composed primarily of activated macrophages (48). These macrophages have been shown to release reactive oxygen metabolites (e.g., hydroxyl radicals) which may induce chromosomal damage, including point mutations. While loss of chromosomal material by asbestos may be random, loss or mutations in regions coding for tumor suppressor genes might be expected to confer a growth advantage to that cell, resulting in selection of those cells at increased risk for progression to malignancy.

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Northern blot analysis of p53 mRNA expression revealed that three of the mesotheliomas had normal sized transcripts of 2.8 kilobases. However, one of the tumors (B) showed virtual loss of expression of p53 mRNA. This tumor had a normal p53

DISCUSSION

We have shown that genetic abnormalities involving the short arm of chromosome 17 can occur in malignant mesotheliomas. These include karyotypic abnormalities involving deletions in 17p and loss of heterozygosity, which demonstrates loss of chromosomal material at the molecular level. We have further shown that the p53 gene can show abnormalities in malignant mesothelial cell lines, where loss of mRNA expression and point mutations in the coding region have been demonstrated.

Malignant mesotheliomas are rare neoplasms which arise from mesothelium-lined tissue of the pleura, peritoneum, and pericardium. They have an extremely poor prognosis. These tumors have been highly associated with exposure to asbestos fibers (41). In fact, in the majority of mesotheliomas, exposure to asbestos can be demonstrated (42). Of the four tumors that are the focus of this study, three were derived from patients with histories of asbestos exposure.

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ALTERATIONS IN MALIGNANT MESOTHELIOMA

Fig. 4. Allelic deletions on chromosome 17 analyzed by TaqI-digested DNA from mesothelioma cell lines (lane CL) compared with normal lymphocyte DNA (lane N) or tumor (lane T) from the corresponding patient. Filters were hybridized with a probe for chromosome 17 (p13), pYNZ22. Tumors A and B show loss of heterozygosity (arrows). Tumor C shows no allelic deletion, because both bands are present in the cell line as well as normal DNA. Tumor D appears to be heterozygous at the locus identified by pYNZ22. However, the bands are extremely close together, making interpretation difficult. Note that the tumor DNA from patient A was used to assess heterozygosity because the normal DNA was unavailable from this patient. The discrepancy between the intensity of two bands seen in tumor tissue is due to contamination of tumor cells with normal cells so often seen in tumor tissues.

**EcoRI** digest by Southern blot. Thus, loss of p53 mRNA expression in this tumor may have been due to a more subtle structural abnormality not detectable by the Southern analysis (e.g., a mutation altering the stability of the message). Alternatively, loss of expression may have been due to a regulatory mutation decreasing the rate of transcription.

The mutations in the coding region of p53 associated with tumorigenesis have been shown to take place in a region of the gene showing the greatest cross-species homology, between the codons for amino acids 117–286 (40). Sequencing human tumor p53 cDNAs has shown that, when mutations are found, they reside in the region between codons 132 and 309 (11, 21–23). The sequence for the p53 cDNA from the three mesotheliomas showing p53 mRNA expression was done between the codons for amino acids 70 and 319. In one tumor, no mutations were observed. However, in two of the mesotheliomas, point mutations were detected; both of these resulted in amino acid substitutions. In tumor A, there was a G to A transition in the second base of codon 175 (CGC to CAC) resulting in an arginine to histidine substitution at that site. In tumor D, there was a G to A transition in the second base of codon 245 (GGC to GAC), resulting in a glycine to aspartic acid substitution.

Examination of the sequencing gels for the tumors which show point mutations reveals that only one p53 allele (the mutant one) is present, as demonstrated by the appearance of only the mutated base at that position.

Point mutations in the coding region of p53 have now been described in several human tumor types, including colon, lung, and breast carcinomas and glioblastoma multiforme (11, 21, 22). As mentioned, these mutations have all been found between codons 132 and 309. However, the mutations are apparently not randomly distributed and appear to cluster in four “hot spots” (codons 132–143, 174–179, 236–248, 272–281) (11). Of 50 point mutations described (11, 21–23) (49 missense and one frame shift), 35 (70%) occur in these hot spots. The four hot spots are within the four most highly conserved regions of the p53 gene (40), suggesting that the mutations seen in tumors may take place in the most functionally important areas.

The two mutations described here also occur in the hot spot areas. One of these mutations (tumor A) is the single most common mutation described. The G to A transition in the second base codon 175 has now been described in a total of 10 different tumors: one mesothelioma (the present study), one brain tumor, and eight colon cancers (11, 23). The amino acid substitution caused by this mutation (arginine to histidine) is interesting in that both are positively charged and the substitution might, therefore, not be expected to result in a major conformational change in that region of the protein. The second

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Fig. 5. Northern blot of mesothelioma RNA. RNA (15 μg) from each tumor was loaded onto gel. Lane 1, tumor A; lane 2, tumor B; lane 3, tumor C; lane 4, tumor D. A, filter hybridized with wild-type p53 cDNA probe. RNA from tumors A, C, and D show normal sized (2.8 kilobases) p53 mRNA. However, there is no detectable expression of p53 mRNA in tumor B (lane 2). B, the same filter seen in A, stripped and rehybridized with a GAPDH probe. All tumors expressed significant levels of GAPDH mRNA, indicating that the RNA preparations from each tumor contained approximately equal levels of intact RNA.
mutation described here [tumor D, codon 245, GGC to GAC, glycine (uncharged) to aspartic acid (negatively charged)] takes place in a hot spot but has not been previously described. However, we have recently sequenced the p53 cDNA from a Ewing's sarcoma and have found the identical mutation. It is interesting to speculate that these amino acid substitutions (at codons 175 and 245) may be at sites of significant functional importance.

It has been found that codon 175 is the location of a 5-methylcytosine (CpG) site (49). These sites are postulated to be sites of significant functional importance. There was notable correlation between the findings of the cytogenetic, RFLP, Northern, and DNA sequence analyses for the tumors in this study. Two tumors (A and B) showed deletions in chromosome 17p and loss of heterozygosity in the region of the p53 locus. Two tumors (A and D) demonstrated DNA base substitutions resulting in amino acid substitutions, and another tumor (B) showed virtual loss of p53 mRNA expression. Tumor C had cytogenetically normal copies of chromosome 17, and no loss of heterozygosity was seen at the 17p locus using the probe pYN22. No mutations in the coding region of p53 were detected in tumor C. It is particularly interesting that the tumors from patients with histories of asbestos exposure (A, B, and D) all showed abnormalities in p53, while tumor C, with no detectable abnormality in chromosome 17 or 17p13 and no mutation in p53, was from a patient with no history of asbestos exposure. This suggests an important role of asbestos fibers in tumor suppressor gene inactivation.

A variety of cytogenetic abnormalities have been described in human mesotheliomas. Deletions in 17p, including loss of 17, appear to be fairly common (24–26). However, chromosome 3p deletions are more common that 17p deletions in mesotheliomas (24–26). The major sites of deletion in chromosome 3 are p14-21. The breakpoint at p14 is the location of an inducible constitutive fragile site (50, 51) and may, therefore, be particularly prone to damage by asbestos fibers. It has been postulated that 3(p14-21) may be the site of an as yet unidentified tumor suppressor gene, an idea which is supported by the observation of 3(p14-21) deletions in a wide variety of human tumors including renal, lung, ovarian, and bladder carcinomas and hematological malignancies as well as mesotheliomas (12, 52–55). Furthermore, specific deletions in chromosomes 1 and 22 are also frequently observed in mesotheliomas (27). It is, therefore, possible that while p53 abnormalities can occur in malignant mesotheliomas, other tumor suppressor gene abnormalities may also be important in the pathogenesis of this disease. If this is the case, it will be interesting in the future to study the interactions of these factors and their roles in mesothelial carcinogenesis.

We have also shown that genetic abnormalities in the p53 gene, a putative tumor suppressor gene, are found in mesotheliomas. There are four possible mechanisms for loss of tumor suppressor gene function: (a) mutation in one allele with loss of the second allele, (b) mutation in both alleles, (c) loss of expression of the gene, and (d) loss of both genes (10, 11, 13, 14, 19–21). The majority of cases reported in the literature involve p53 mutations in one allele with loss of the second allele. This was observed for two of the four mesotheliomas in this study. In addition, one of the mesotheliomas in the current study demonstrated loss of expression of p53 mRNA, even though the gene was apparently present and at least grossly intact. Furthermore, we have recently observed complete loss of both p53 alleles in a sarcoma, and it is conceivable that this can occur in mesotheliomas as well. It will now be interesting to study the pathogenesis of p53 mutations in mesotheliomas, including the possible role of asbestos in inducing the type of p53 abnormalities seen here.

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