mutations in the p53 gene in lung cancer are associated with cigarette smoking and asbestos exposure

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Abstract

It has been proposed that the patterns of mutations in the p53 tumor suppressor gene will provide clues to the mechanisms of cancer occurrence. Cigarette smoking is known to be the greatest risk factor for lung cancer. Epidemiological evidence has also implicated radon and asbestos as exposures that significantly increase this disease risk; asbestos exposure synergistically enhances the lung cancer risk of smokers. Previous studies of the mutational spectra of the p53 gene in lung cancer have shown cigarette smoke and radon exposure to be associated with the induction of particular lesions or classes of lesions. We have investigated the p53 gene in surgically resectable lung cancers in 85 patients from the Massachusetts General Hospital. We found 25 (29%) patients to have somatic p53 mutations in their tumors. The patients with p53 mutations who were current smokers were significantly older (75.1 versus 59.8 years; P < 0.01) and had smoked for significantly more years (56.8 versus 41.2 years; P < 0.01) than those without p53 changes. Consistent with other reports, we observed a large number (40%) of G:C to T:A transversion mutations, noting that their occurrence increased with increasing cumulative exposure to cigarette smoke. Interestingly, we also found that p53 mutations occurred significantly more frequently in patients with a history of occupational exposure to asbestos [3 of 60 (5%) for patients without p53 mutations versus 5 of 25 (20%) of those with p53 mutations; P < 0.05]. Additionally, 4 of the 5 patients with asbestos exposure and p53 alterations had G:C to T:A transversion mutations, and 3 of 3 double mutations that were seen in the p53 gene occurred in patients who smoked and had a history of asbestos exposure. This suggests that asbestos exposure may increase the frequency of G:C to T:A transversion mutations in the p53 gene. Because these lesions can be induced by polycyclic aromatic compounds found in cigarette smoke, our data also suggest that one possible important role of asbestos is to increase delivery of these substances to the respiratory epithelium. Asbestos might also act to alter clonal selection through other mechanisms, including apoptosis, oxyradical generation, or altered proliferation.

Introduction

Carcinogenesis is a multistep process in which the activation of oncogenes and inactivation of tumor suppressor genes participate in the process of neoplastic transformation. Clonal expansion of mutant cells under selection is thought to eventually result in the formation of clinical tumors (1, 2). p53 is a tumor suppressor gene that is believed to act as either a transcriptional activator of multiple genes or a factor in the assembly of the initiation-replication complex (3). Mutation of the p53 gene is a frequent event in carcinogenesis. It has been found to have frequent structural alterations in more than 30 different types of tumors, and more than 50% of human tumors have p53 mutations (4–6). A broad distribution of mutations have been observed in tumors, and the precise nature of the mutation in a particular tumor may, in some instances, reflect the biological activity of the causative mutagen. These characteristics of the p53 mutational spectrum may allow molecular epidemiologists to identify certain carcinogens as being involved in the production of an individual tumor because some agents appear to induce characteristic mutations. In addition, examination of the mutational spectrum of lesions in genes that are associated with the genesis of a tumor may help to elucidate possible mechanisms involved in the genesis of cancer.

The incidence of lung cancer has increased, and lung cancer has become the leading cause of death in the United States. Cigarette smoking is the greatest risk factor for lung cancer (7–10). Slightly more than 600 lung cancer cases have been studied for p53 mutations (11–20). Some of the studies characterizing the nature of p53 mutations also investigated the relationship of the mutational spectra to patient smoking history (11–14). Although the effect of smoking on the p53 mutational spectrum is not completely clear, it has been reported that, unlike the tumors from other tissues, G:C to T:A transversions are the most prevalent nucleotide changes distributed throughout the gene. Because benzo[a]pyrene can also induce G:C to T:A transversions in p53 in murine skin tumors (21), it has been further suggested that these G:C to T:A transversions in lung cancer are related to cigarette smoke exposure (6, 22).

It is of importance that occupational asbestos exposure also interacts with smoking to synergistically enhance lung cancer risk (23). Exposure to both cigarette smoke and asbestos can confer risks up to 50–90-fold higher than that of compa-

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rable, unexposed individuals. Many workers have studied this new well established multiplicative interaction but the precise mechanism of action remains obscure. One hypothesis that has received considerable attention involves an asbestos-mediated enhanced delivery of mutagenic polyaromatic compounds found in tobacco smoke to the respiratory epithelium (24–26). This hypothesis would predict that the nature of the genetic lesions in the lung that lead to cancer associated with smoking alone would reflect the action polyaromatic hydrocarbons in a fashion that was similar to that involved in cancer associated with exposure to the combination of smoking and asbestos. Additionally, because asbestos is known to produce chromosomal mutations and aneuploidy (27–29), one also might expect an increase in the occurrence of p53 mutations in asbestos-associated disease because of the induction of loss of heterozygoty. However, to date, very little work has focused on the possible effect of asbestos exposure on the p53 mutation spectra in lung cancers. Here, we report our analysis of alterations in p53 in primary tumor tissue from 85 patients with lung cancer, studying the relationship of these changes with smoking and asbestos exposures.

Materials and Methods

Tissue Samples. Fresh frozen tumor tissue was obtained from 85 consecutive lung cancer patients who underwent surgical excision of their cancers at Massachusetts General Hospital, after voluntary consent was obtained. The protocol used was approved by the Committees on the Use of Human Subjects in Research at the Massachusetts General Hospital and the Harvard School of Public Health. When tumors were large enough for macroscopic dissection, specimens were obtained at the time of surgery from pathologically sectioned material. Care was taken to ensure that all material was examined by a pathologist; this was done to minimize contamination of the samples by normal tissue. DNA was extracted with the use of a modified method from Miller et al. (17).

Patient Medical and Lifestyle History. Each patient was given an extensive interviewer-administered questionnaire (the Harvard food frequency questionnaire), which included questions on demographic characteristics, smoking, and other lifestyle exposure history, occupational history, environmental exposure to potential carcinogens, family history of cancer, and diet (30). Questions about the previous medical history and medication use of the patient were also included in the questionnaire.

Asbestos Exposure Characterization. We used a historical exposure index, as defined below, to assess cumulative asbestos exposure. This index, modified from the version of Sprince et al. (31), was based on the knowledge of asbestos usage in commercial construction, shipbuilding, and homes in the New England area. The heaviest occupational exposures occurred before 1965, when fiberglass insulation was introduced and began to be widely used. Exposure continued to occur until 1965 when the use of asbestos for newly installed insulation and fireproofing decreased and was eventually discontinued. The promulgation of a permissible exposure limit for asbestos by Occupational Safety and Health Administration in 1972 also helped to reduce workplace asbestos exposure.

The index used represents a weighted average of asbestos exposure calculated for each subject based on their duration of work during three periods: (a) before 1965; (b) 1965–1972; and (c) after 1972. A weight of 4 was given to each year worked before 1965, a weight of 2 was given to each year worked from 1965–1972, and a weight of 1 for each year worked after 1972. Jobs that were considered asbestos exposed included auto or truck repair, boilermaker, brake mechanic, building maintenance, carpentry, building demolition, drywall hanging, foundry work, insulation installation, iron/steel manufacture, pipe fitting, pipe insulation/covering, shipbuilding/repair, smelting, tunnel construction, and welding. We also rated environmental exposure to asbestos, including weights of 0, 1, or 2 to represent nonoccupational exposures. For example, removing and replacing asbestos insulation in one’s home was weighted 2, and living in an apartment with known friable asbestos was rated 1. All other (“background”) exposures were rated 0 or given no additional weighting to any occupational exposure index calculation.

PCR-Single-Strand Conformational Polymorphism Tumor Tissue Analysis. Oligonucleotide primers for PCR amplification of exons 5–9 were those of Toguchida et al. (32). PCR products were generated in a 50–μl reaction mixture including 50 ng DNA, 20 μM dNTP, 10 μM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 0.1% Triton X-100, 25 pmol of each primer, 0.25 unit Taq polymerase (Perkin Elmer Cetus, Norwalk, CT), and 3.3 μCi [32P]dCTP (DuPont New England Nuclear, Boston, MA). The PCR reaction was carried out with the use of 35 cycles (94°C for 1 min, 55–63°C for 1 min, and 72°C for 2 min) on a thermal cycler from Ericomp (San Diego, CA). Ten μl of PCR product were diluted with 90 μl of 0.1% SDS-10 mM EDTA mixture. The diluted sample was then mixed 1:1 with stop solution from United States Biochemical Corp. (Cleveland, OH) and heated at 94°C for 4 min. The sample was put on ice and loaded immediately on 6% nondenatured PAGE with 10% glycerol. The gel was running at 4°C for 16 h at 15 W and was exposed for 16 h for autoradiographic detection of bands.

DNA Sequencing. DNA sequencing was performed with the use of the pT7 Blue clone (the clones isolated were pooled for analysis) and direct PCR product-sequencing methods. For clone sequencing, PCR product was ligated into pT7 Blue vector and transformed into Escherichia coli bacteria (kit from Novagen, Madison, WI). Minipreps were carried out with the use of the alkaline lysis method of Maniatis et al. (33). Sequencing reactions were carried out with the use of Sequenase Ver. 2.0 kit (United States Biochemical Corp.) with the same primer that was used for the PCR reaction. In the majority of cases, a second sequence analysis was done to confirm initial findings. For direct sequencing, PCR products were digested with alkaline phosphatase and exonuclease and sequenced as above (PCR Direct Sequence kit; United States Biochemical Corp.). Six % denatured PAGE was run at room temperature for 4 h and exposed for 16 h for autoradiography. As above, in the majority of cases, the product was reamplified and resequenced to confirm the initial findings.

Statistical Analysis. Statistical comparisons of the characteristics of the study population were made with the use of Student’s t test or Fisher’s exact test, as indicated.

Results

Tumor DNA from 85 lung cancer patients was screened for mutations with the use of PCR-single-strand conformational polymorphism, examining exons 5–9 of the p53 gene. Sequence analysis was carried out in all cases where band shifts were observed on single-strand conformational polymorphism. When tumor DNA was found to contain a sequence alteration, we also examined the germline DNA for the presence of a similar mutation. Twenty-eight p53 mutations from 25 patients were found. Three patients had double mutations. Overall, 29.4% of the tumors had p53 mutations.
### Table 1: Characteristics of the p53 mutations in lung cancer: demographics and sequence alterations

| ID* | Age | Sex | Smoking (Pack-Years) | Years quit* | Asbestos exposure index* | Family history | Histology† | Codon | Mutation | AA* change | Conserved domain |
|-----|-----|-----|----------------------|-------------|--------------------------|----------------|------------|--------|----------|------------|--------------|------------------|
| 1   | 77  | F   | 48.9                 | 0.0         | 0                        | ?              | SQ         | 149    | TCC-ACC  | Ser-Thr    | N             |
| 13  | 81  | M   | 70.0                 | 2.1         | 0                        | Y              | LC         | 273    | CGT-CTT  | Arg-Leu    | Y             |
| 9   | 78  | M   | 184.7                | 0.0         | 0                        | Y              | SQ         | 135    | One T del | Y          | Y             |
| 17  | 68  | M   | 100.0                | 1.8         | 0                        | Y              | AD         | 204-210 Del | N          | N           |
| 18  | 47  | M   | 62.0                 | 0.1         | 0                        | N              | AD         | 248    | CGG-CTG  | Arg-Leu    | Y             |
| 19  | 70  | M   | 44.1                 | 3.5         | 0                        | N              | AD         | 127    | TCC-TAC  | Ser-Tyr    | Y             |
| 20  | 76  | M   | 59.0                 | 0.5         | 12                       | Y              | SQ         | 249    | AGG-ATG  | Arg-Met    | Y             |
| 30  | 65  | M   | 87.0                 | 24.6        | 0                        | N              | AD         | 289    | CTC-CCC  | Leu-Pro    | N             |
| 34  | 80  | F   | 51.4                 | 0.0         | 0                        | N              | AD         | 245    | GGC-GCC  | Gly-Ala    | Y             |
| 36  | 80  | F   | 43.0                 | 6.5         | 76                       | Y              | SQ         | 152    | CCG-CTG  | Pro-Leu    | N             |
| 38  | 65  | F   | 80.0                 | 6.8         | 0                        | Y              | SQ         | 249    | AGG-AGT  | Arg-Ser    | Y             |
| 41  | 69  | M   | 51.0                 | 0.1         | 0                        | Y              | SQ         | 274    | One G del | Y          | Y             |
| 42  | 43  | F   | 12.5                 | 0.5         | 0                        | Y              | AD         | 220    | TAT-TGT  | Cys-Tyr    | N             |
| 44  | 72  | M   | 22.4                 | 0.0         | 0                        | N              | AD         | 261    | AGT-GGT  | Ser-Gly    | Y             |
| 46  | 74  | F   | 26.5                 | 4.2         | 0                        | Y              | AD         | 240    | AGT-GGT  | Ser-Gly    | Y             |
| 54  | 65  | F   | 20.5                 | 4.5         | 0                        | N              | AD         | 273    | CTG-CTT  | Arg-Leu    | Y             |
| 57  | 69  | M   | 171.0                | 0.0         | 0                        | Y              | SQ         | 156    | CCG-CAC  | Cys-Tyr    | N             |
| 62  | 53  | M   | 13.0                 | 22.1        | 8                        | Y              | AD         | 269    | ACG-GGC  | Ser-Gly    | Y             |
| 72  | 62  | M   | 129.0                | 2.8         | 366                      | Y              | SQ         | 282    | CGG-TGG  | Arg-Trp    | Y             |
| 73  | 74  | F   | 23.6                 | 21.8        | 0                        | Y              | LC         | 132    | AAG-AAC  | Lys-Asn    | Y             |
| 82  | 78  | M   | 47.0                 | 10.1        | 0                        | N              | SQ         | 176    | TGC-TAC  | Cys-Tyr    | Y             |
| 86  | 51  | M   | 59.5                 | 0.5         | 0                        | Y              | LC         | 151    | One C del | Y          | Y             |
| 87  | 72  | M   | 67.5                 | 24.0        | 73                       | Y              | SC         | 153    | CCC-GCT  | Pro-Pro    | N             |
| 96  | 75  | M   | 49.3                 | 0.1         | 0                        | N              | AD         | 251    | ATC-TTC  | Ile-Phe    | Y             |
| 100 | 70  | F   | 27.5                 | 0.1         | 0                        | N              | LC         | 245    | cGGG-cTGC | Gly-Trp    | Y             |

Summary statistics

Patients without p53 mutations (n = 60)

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<td>67.3</td>
<td>F = 28</td>
<td>58.6 ± 40.5</td>
<td>8.4 ± 1.7</td>
<td>n = 3</td>
<td>33</td>
<td>SQ23</td>
<td>25</td>
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Patients with p53 mutations (n = 25)

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<tr>
<td>68.6</td>
<td>F = 9</td>
<td>62.0 ± 44.6</td>
<td>5.8 ± 8.3</td>
<td>n = 5</td>
<td>16</td>
<td>SQ9.SC1</td>
<td>8</td>
<td>M = 16</td>
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*ID, patient identification number; M, male; F, female; Y, yes; N, no; del, deletion.
†Exposure intensity multiplied by duration of exposure, weighted by decade of exposure (pre-1965, post-1965 but pre-1972, or post-1972).
‡SQ, squamous cell; LC, large cell; AD, adenocarcinoma; SC, small cell; mixed, more than one histological subtype.
§AA, amino acid.
Mean ± SD.
*Number shown represents those with occupational exposure to asbestos; index value > 1.

In the 28 mutations, 23 (82%) were missense mutations, 4 (14%) were deletions, and 1 (4%) was a nonsense mutation. There were 15 transitions and 9 transitions. G:C to T:A transitions were most prevalent, occurring in 10 cases (only 1 of which occurred on the nontranscribed strand ID number 19); A:T to G:C transitions were seen in 4 cases. Three G:C to T:A mutations at CpG sites were also detected.

The demographics of the study group are shown in Table 1. There were no differences in age, gender, family history of cancer, or lung tumor histology between the group of patients whose tumors had p53 mutation and those without mutations. There were seven patients who had tumors with p53 mutations who also had a medical history of a prior nonpulmonary malignancy; this frequency of previous malignancies did not differ from that in the group of patients without p53 mutations, in which 15 patients had a similar history of a prior cancer at a different site.

The cigarette smoking habits of all of the patients studied were investigated with the use of never- (n = 2), ex- (n = 68), and current- (n = 15) smoking status as the indicator variable and pack-year history as a measure of long-term exposure to cigarette smoke. Neither smoking status nor pack-year history was associated with the presence of a mutation of the p53 gene. The 2 patients who were never smokers did not have p53 mutations. The mean age of smoking initiation was not different between the p53-positive (mutated) and p53-negative (no mutation in p53) patients (Table 2).

The group of patients who did not have p53 mutations quit smoking, on average, 8.4 ± 11.7 years before surgery, whereas the patients who had mutations quit a mean of 5.8 ± 8.3 years before surgery. In the p53-positive group, 12 of the 25 (48%) individuals either were current smokers or had quit within the last year. In the p53-negative group, 23 of 60 (38%) had smoked within the year before thoracic surgery. The patients...
with p53 mutations had smoked an average of 44 ± 13.0 years, whereas the p53-negative patients had smoked an average of 38.6 ± 14.8 years (P > 0.3).

When the duration and intensity of smoking was compared by mutation status within groups of current and exsmokers, however, the current smokers with p53 alterations were significantly older and had smoked significantly longer than the patients without mutations (Table 2; P < 0.01). As expected, age and duration of smoking were correlated (r² = 0.42; P < 0.01). Smoking intensity, measured by pack-years of exposure, was higher in the current smoking patients with p53 mutations but did not differ significantly from the patients without changes in p53 (Table 2, P > 0.3). Age, smoking duration, and smoking intensity were also compared by mutation status in exsmokers alone, and no significant differences were observed (Table 2).

When the data from the subjects with G:C to T:A transversions were analyzed separately, the mean number of pack-years smoked did not differ from either the other patients with p53 mutations or those without p53 mutations. When the data were plotted in a fashion after Takeshima et al. (16), however, there was an apparent linear trend in the induction of these mutations when their occurrence was compared to tobacco exposure, measured by pack-year smoking history (Fig. 1). The frequency distribution of smoking history in the cases with p53 mutations that were not G:C to T:A transversions showed an apparent reversal of this trend. When the frequency distribution of pack-year history of smoking in the cases without discernible p53 gene alteration was considered, it showed a pattern very similar to that for the tumors with these specific (G to T or C to A) transversions (Fig. 2).

When the cumulative asbestos exposure index was examined by p53 mutation status, we found that 5 of the 25 individuals with p53 mutations in their tumors had a history of significant (i.e., occupational in our index) exposure to asbestos, whereas only 3 of the 60 people without p53 mutations had nonzero asbestos exposure scores (P < 0.05; Fisher’s exact test). All of the asbestos-exposed patients had a history of cigarette smoking. The remaining 77 patients did not have a history of occupational exposure to asbestos. It is interesting that the sequence alterations in the p53 gene from the asbestos-exposed individuals showed a predominance of G:C to T:A transversions. Four of the 5 patients with both asbestos exposure histories and p53 mutations had G:C to T:A mutations. In addition, 3 of 3 double mutations that were seen occurred in patients with significant asbestos exposure histories. In each of these 3 cases where the patients were exposed to asbestos, 1 of the mutations was a G:C to T:A transversion.

One patient had a p53 lesion reported previously to be in lung tumors of smoking, radon-exposed miners. Review of the occupational history of this patient revealed no clear source of radon exposure. He was a police officer and had worked in an automotive garage, repairing brakes, for approximately 35 years. He had also worked for 2 years in a sand and gravel pit involved in concrete production. It is conceivable, however, that he was exposed to radon in his home.

Discussion

We have studied 85 patients with newly diagnosed lung cancer and found that 25 (29.4%) of these individuals had somatic alterations in exons 5–9 of the p53 gene. In our case series we also found 82% of the mutations to be missense; G:T to T:A transversions were the most common type of alteration within this class. These observations are consistent with those reported by many investigators studying lung cancer (12–21). Some workers have reported the frequency of alterations in the p53 in
Our study further found that asbestos exposure most frequently occurred in those who had G:C to T:A transversion mutations, as well as double mutations in the gene. Again, this is consistent with the assertion that asbestos exposure facilitates the delivery of polyaromatic compounds to the respiratory epithelium because it is known that G:C to T:A lesions can be induced by these compounds (22). In addition, the 3 double mutations observed occurred only in asbestos-exposed individuals. Although asbestos is not mutagenic in many mammalian systems, recent work has shown that it induces large scale mutagenic damage in human-hamster hybrid cells (38). Asbestos is also well known to induce chromosomal damage, including deletions and rearrangements (27–29). Additionally, p53 mutations have been observed previously in mesotheliomas, a malignancy almost always attributable to asbestos exposure (39). Finally, asbestos has also been associated with G:C to T:A transversions in the ras oncogene in patients with lung cancer (40).

Hence, our data suggest that asbestos exposure acts to augment the action of cigarette smoke in producing somatic lesions in critical target genes in the lung epithelium. It may accomplish this either directly by enhancing the formation of adducts on the DNA at the site of damage or through the process of clonal selection, where the asbestos would provide a growth advantage to clones that either have or are more likely to receive cigarette-induced lesions. Regardless of the mechanism, our data suggest that asbestos plays an important role in the induction of p53 mutations in lung cancer. Additional study is required before its precise role in the induction of lung cancer can be determined, but occupational and environmental histories detailing asbestos and other carcinogen exposures are critical to the evaluation of p53 spectra in lung cancer.

Acknowledgments

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References


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