p53 Protein Accumulates Frequently in Early Bronchial Neoplasia


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ABSTRACT

p53 mutations are common in human lung cancer and frequently generate levels of p53 protein that are detectable by immunohistochemistry. For this reason, p53 protein accumulation is a candidate biomarker, but little is known about its timing or frequency in multistage bronchial carcinogenesis. We studied human lung tissues containing preinvasive squamous neoplasms from 34 donors with and without lung cancer. Nuclear p53 protein was present in 6% of normal mucosas, 6.7% of squamous metaplasias, 29.5% of mild dysplasias, 26.9% of moderate dysplasias, 59.7% of severe dysplasias, 58.5% of carcinomas in situ, 67.5% of microinvasive carcinomas, and 79.5% of invasive tumors. These data indicate that (a) p53 protein accumulates in about 30% of the earliest recognized neoplastic lesions (i.e., mild dysplasia), (b) there is an increasing frequency of p53 protein accumulation starting with mild dysplasia, and (c) p53 protein accumulates infrequently in normal or metaplastic mucosa. In a subset of six patients whose most advanced lesion was carcinoma in situ without evidence of invasive cancer, p53 protein was detected in 0% of normal mucosas, 8.3% of squamous metaplasias, 37.5% of mild dysplasias, 12.5% of moderate dysplasias, 93.8% of severe dysplasias, and 55% of carcinoma in situ lesions. These data show clearly that p53 alterations can occur before invasion and suggest that the frequency is similar to that observed in the full series. Since two-thirds or more of lung cancers have p53 alterations, the timing and frequency of p53 protein accumulation make the p53 tumor suppressor gene an attractive marker for early diagnosis and evaluation of chemoprevention agents.

INTRODUCTION

Squamous lung cancer is thought to develop through multiple stages that are recognized histologically. The earliest changes include squamous metaplasia, followed by three grades of dysplasia, carcinoma in situ, and microinvasive and invasive cancer (1–6). Preinvasive morphological changes have been indirectly linked to invasive lung cancer by the dose-response relationship between the number of cigarettes smoked per day and the frequency of dysplastic lesions in bronchial epithelium (7).

The sequence of morphological changes is consistent with a multistage model of carcinogenesis, and it is thought that the genetic changes found in advanced lung cancer occur stepwise with the morphological changes. We have investigated the p53 tumor suppressor gene in preinvasive lung lesions for the following reasons: (a) p53 mutations are the most common genetic alteration in human cancer, including lung cancer (8); (b) p53 mutations and allelic deletions have been detected in dysplastic bronchial lesions (9–12); (c) missense mutations frequently stabilize the protein, which accumulates to levels detectable by immunohistochemistry (13); and (d) p53 is both a transcription factor involved in a G1 arrest checkpoint response to certain types of DNA damage and one component controlling gene amplification (14–20).

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Although previous studies by us and others (9–12) document some of the genetic changes to p53, there is little information regarding the usual frequency and timing of p53 alterations, especially in metaplastic and early dysplastic lesions. To address these questions, we examined progenitor lesions from 34 individuals with a polycyonal rabbit antiserum, CM-1, that binds both mutant and wild-type p53 protein. Although wild-type p53 protein degrades rapidly and is generally undetected by immunohistochemistry, mutant p53 protein usually has a longer half-life and accumulates to detectable levels. Based on our findings, we suggest that (a) analysis of p53 protein accumulation and/or mutation in biopsies and sputum samples may contribute to early diagnosis and treatment and (b) p53 protein and/or mutation may be useful in the evaluation of chemoprevention agents.

MATERIALS AND METHODS

Case Selection. Formalin-fixed bronchial resections containing one or more preinvasive lesions with or without invasive squamous cancer were collected at the Mayo Clinic (Rochester, MN) (19 cases); the University of Maryland (Baltimore) (5 cases); the Armed Forces Institute of Pathology (Washington, DC) (one case); the University of New Mexico (Albuquerque, NM) (3 cases); the Shanghai Cancer Institute (Shanghai, People’s Republic of China) (2 cases); and the University of Oulu (Oulu, Finland) (4 cases). The Mayo Clinic cases were collected during the Mayo Lung Project, a screening program for the detection of early lung cancer (21–23). These cases had extensive histological analysis; in 5 cases, the most advanced lesions were carcinoma in situ, and in 12 cases microinvasive carcinoma was the most advanced lesion. In one case from the University of Oulu, carcinoma in situ was the most advanced grade.

Histological Criteria and Classification of Lesions. The histological criteria used to classify progenitor lesions have been described by Saccomanno (4), Auerbach (3), and the WHO (24). Based on these descriptions, we recognized eight categories: normal ciliated columnar epithelium; squamous metaplasia and basal cell hyperplasia; mild dysplasia; moderate dysplasia; severe dysplasia; carcinoma in situ; microinvasive carcinoma; and invasive carcinoma. All lesions were independently classified by two pathologists (W. D. T. and T. V. C.), and the interpretations were recorded on tissue diagrams. The results were compared, and differing interpretations were resolved by review of the specific lesions by both pathologists at a multiheded microscope. Diagnostic differences of one grade (i.e., mild versus moderate dysplasia) were considered irreproducible; in these instances, a third pathologist (W. P. B.) made the final classification.

Immunohistochemistry for p53 Protein. Immunohistochemical analysis was performed using an avidin-biotin peroxidase detection system as previously described and outlined below (25). After dewaxing, inactivating endogenous peroxidase activity, and blocking cross-reactions with normal goat serum, 3-aminopropyltriethoxysilane was used to coat the sections. For positive controls, a subcutaneous tumor of human cells was explanted from an athymic nude mouse, fixed in 100% ethanol, embedded in paraffin, and serially sectioned. The tumor nuclei from these human cells...
Table 1  Frequency of p53 protein accumulation at multiple stages of bronchial carcinogenesis

<table>
<thead>
<tr>
<th>Source</th>
<th>Result</th>
<th>Normal mucosa</th>
<th>Squamous metaplasia*</th>
<th>Dysplasia</th>
<th>Carcinoma</th>
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<td>6.7</td>
<td>29.5</td>
<td>58.5</td>
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<td>1/15</td>
<td>3.25/11</td>
<td>14.6/25</td>
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<td>26.6/29.4</td>
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<tr>
<td></td>
<td>No. positive/no. total</td>
<td>0/23</td>
<td>1/15</td>
<td>4.25/16</td>
<td>15.2/28</td>
</tr>
</tbody>
</table>

* Squamous metaplasia includes basal cell hyperplasia.

The abbreviations used are: CIS, carcinoma in situ; Microinv., microinvasive; No., number.

RESULTS

Bronchial resections from 34 patients contained one or more pre-invasive squamous lesions. Sixteen of the 34 patients had invasive squamous carcinoma, 12 had microinvasive squamous cancer, and 6 had carcinoma in situ as the most advanced lesion. Overall, p53 immunohistochemistry showed protein accumulation in bronchial lesions in 24 of 34 cases (71%). Table 1 and Fig. 4 show the immunohistochemistry results according to histological category. Also shown in Table 1 are results from the current literature. Examples of morphology and immunohistochemistry are shown in Figs. 1–3.

The immunohistochemistry slides were reviewed independently by four pathologists who graded each lesion as positive, negative, or equivocal. To average and summarize the results, numeric values were assigned: positive was scored as 1.0, and negative and equivocal were scored as 0. Most of the equivocal scores occurred in the normal, metaplastic, and severely dysplastic mucosas. Positive staining was seen in 0% of normal mucosas (0 of 22 foci), 6.7% of squamous metaplasias (one of 15), 29.5% of mild dysplasias (3.25 of 11), 26.9% of moderate dysplasias (4.58 of 17), 59.7% of severe dysplasias (15/25), 79.5% of microinvasive carcinomas (10/13), and 75.1% of invasive tumors (11/27) (see Table 1). These data indicate that (a) p53 protein accumulates in approxi-
See bottom of p. 4820 for legend to Fig. 2.
p53 IN EARLY BRONCHIAL CARCINOGENESIS

Fig. 3. Case 4129 had mild dysplasia (A) with moderately enlarged nuclei and occasionally prominent nucleoli (arrow). p53 immunohistochemistry of a serial section (B) showed a few darkly stained nuclei (arrows) and probably artifactual staining of the basement membrane (arrowheads). Case 2-988 had moderately dysplastic mucosa (C) with enlarged, darkly stained nuclei in the lower and middle mucosal layers. p53 immunohistochemistry of a serial section showed dark, nuclear staining (arrows) of the enlarged nuclei within the lower levels of mucosa (D). A and C, H&E stains; B and D, CM-1 immunohistochemistry. Original magnifications: A and B, ×400; C and D, ×200.

Fig. 4. Data from the National Cancer Institute series (see Table 1) show the frequency of p53 protein accumulation at multiple stages of bronchial carcinogenesis. The ratios shown at the top of each bar are the number of positive/total, which is the number of immunostain positive lesions divided by the total number of lesions of a specific histological grade.

mately 30% of the earliest recognized neoplastic lesions (i.e., mild dysplasia), (b) there is an increasing frequency of p53 protein accumulation starting with mild dysplasia, and (c) unequivocal p53 protein accumulation is uncommon in normal or metaplastic mucosa.

p53 Immunohistochemistry in the Absence of Invasive Cancer.
To address the question of whether p53 alterations precede the onset of invasion, the immunohistochemistry data were tabulated from six patients whose resected tissues had CIS as the most advanced lesion (five patients from the Mayo Clinic and one patient from the University of Oulu). The results of this group show conclusively that p53 protein accumulation may precede invasion. There was positive staining in 0% of normal mucosas, 8.3% of squamous metaplasias (0.25 of 3), 37.5% of mild dysplasias (0.75 of 2), 12.5% of moderate dysplasias (0.25 of 2), 93.8% of severe dysplasias (3.75 of 4), and 55% of CIS lesions (2.75 of 5).

p53 Immunohistochemistry of Normal and Metaplastic Mucosas. None of the four observers found definite, unequivocal nuclear staining in normal mucosa, but three found equivocal staining in four different cases. Three observers found definite nuclear staining in squamous metaplasias from three different cases, and three found equivocal staining in six cases.

DISCUSSION
We and others (9–12) have reported that p53 mutations and protein accumulation can precede invasion. However, these studies concentrated on advanced lesions (i.e., severe dysplasia and carcinoma in situ) and did not identify sufficient early lesions to estimate the frequency and timing of p53 protein accumulation. Therefore, we undertook an immunohistochemical study to examine a larger series of cases. To minimize the subjective aspects of interpreting histological sections and immunohistochemical stains, six different pathologists reviewed all the slides, and their interpretations were averaged. The histological classification was made independently by two different pathologists; a third pathologist decided only the minor discrepancies (i.e., mild versus moderate dysplasia). Four pathologists
with experience in interpreting p53 immunohistochemistry independently reviewed the immunostains. They used a simple grading scheme (positive, negative, or equivocal), and their findings were averaged. Only one of the six observers contributed to both the histological and immunohistochemical interpretations.

**p53 Protein Frequently Accumulates in Preinvasive Lesions.** Analysis of 34 cases indicates that p53 protein accumulation occurs in approximately 30% of mild and moderate dysplasias and two-thirds of severe dysplasias, CIS, and invasive cancers. The results support a multistage model for squamous bronchogenic carcinoma in which genetic damage to the p53 tumor suppressor gene occurs in 30% of the earliest recognized neoplastic lesions (illustrated in Fig. 3), and the majority of p53 mutations occur before the onset of invasion. Since two-thirds or more of lung cancers have p53 alterations (reviewed in Ref. 8), these features make the p53 tumor suppressor gene an attractive marker for early diagnosis and evaluation of chemoprevention agents.

**p53 Alterations Precede the Onset of Invasive Cancer.** The most common lesions in this series are severe dysplasias and CIS, and most occur next to invasive cancers. There are three main reasons for this: (a) advanced dysplasias occur frequently near invasive cancers; (b) surgical resections are designed to remove cancers with the minimum of normal tissue; and (c) dysplastic lesions are generally undetectable macroscopically and are usually found by chance. Because most of the observed dysplasias are near or continuous with invasive tumors, there is an unresolved debate over whether (a) dysplastic lesions and invasive tumors are separate, independent neoplasms, (b) malignant cells from the invasive tumors have migrate through the adjacent mucosa to mimic preinvasive, dysplastic lesions, or (c) invasive tumors develop in preexisting dysplastic mucosas. Eventually, the question will be settled using a panel of genetic markers, but for now the microscopic size of the lesions is a barrier to genetic analysis. Fortunately, the issue of whether p53 mutations precede invasion can also be answered by examining cases lacking invasive cancers. The data from this series as well as three cases examined by Sundaresan and co-workers (10) conclusively demonstrate that p53 protein can accumulate before invasion. Furthermore, these data support the conclusions drawn from the full data set, which is composed mostly of progenitor lesions associated with invasive tumors.

**Mechanisms of p53 Protein Accumulation.** Accumulation of p53 protein within the nucleus of a cancer cell usually signals a missense mutation (13), although exceptions have been reported (28-34). Non-mutational mechanisms for p53 protein accumulation include (a) inactivation of an enzymatic pathway responsible for p53 protein degradation (35), (b) stabilization of normal p53 protein through complex formation with a cellular oncoprotein such as mdm2 (36, 37) or a DNA tumor virus protein (38-41), (c) DNA damage-induced post-translational modification conferring extended protein half-life (42), (d) altered expression of the p53 gene by cellular transcriptional regulators (43), and (e) physiological accumulation during late G1 and S phases of the cell cycle (44). Regardless of mechanism, the excess protein provides a distinct marker of both preinvasive lesions and invasive tumors. It should be noted that not all mutations lead to protein accumulation. For example, chain terminating mutations, deletions, and some splice site mutations block protein synthesis and/or accumulation (28).

**p53 Immunohistochemistry of Normal and Metaplastic Mucosa.** Until mutant-specific p53 antibodies can detect all mutant p53 proteins, we must rely on the detection of total protein, both wild type and mutant. Although there is considerable evidence that accumulation of p53 protein within neoplastic lung tissues correlates with missense mutation (9, 33, 45), there is no such evidence for p53 mutation within normal or metaplastic bronchial mucosas. Therefore, equivocal staining in some normal and metaplastic tissues may represent either experimental artifact or transient accumulation of wild-type protein during late G1 and S phases of the cell cycle (44). This suggests that false positives will occur when diagnostic cytology preparations are screened for malignancy using p53 immunohistochemistry.

**Genetic Analysis of p53 in Bronchial Progenitor Lesions.** Genetic and cytogenetic analyses have been made of a few preinvasive lesions. We reported one missense mutation in three different segments of immunopositive mucosas that were severely dysplastic, microinvasive, and fully invasive (9). Sundaresan and co-workers (10) studied six patients with severe dysplasia and invasive cancer; three of the six were informative at the p53 locus, and one of the three dysplasias showed deletion of one p53 allele, and one of the samples containing both alleles had a missence mutation in exon 5. They also studied three patients who had severe dysplasia, but no invasive cancer, and they found allelic deletion of p53 in one of two informative cases (10). Sozzi and colleagues (11) found the same exon 5 missense mutation in a segment of severe dysplasia and in the nearby invasive tumor. The same case plus a second showed deletion of the short arm of chromosome 17 by karyotypic analysis of metaphase spreads from severely dysplastic lesions. Advances in microanalytic techniques will provide additional genetic data on bronchial progenitor lesions.

**p53 Is a Candidate Biomarker for Squamous Cancer of the Lung.** An intermediate biomarker should appear before tumor invasion, to make early diagnosis possible. The timing of p53 mutation and protein accumulation has been studied best in colorectal adenocarcinoma. This cancer develops in multiple well-defined stages, and p53 mutation most frequently occurs between the stages of late adenoma and invasive carcinoma (46, 47). In addition to the colorectal model, recent studies indicate that damage to the p53 gene frequently occurs in preinvasive lesions of the breast, esophagus, skin, and testis (25, 48-53). However, p53 damage may occur later in other tumors. For example, in bladder cancer, the rate of p53 mutation is greater in high-grade and invasive tumors than in superficial and low-grade tumors (56). Similar observations have been made in low- and high-grade tumors of the brain and thyroid (54, 55). These findings suggest that, in some tissues, p53 mutation may contribute more to tumor progression than to the conversion to malignancy. These data indicate that the timing for p53 alteration may vary with tumor type and underscore the need for analysis of progenitor lesions in all tissues.

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**REFERENCES**

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