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Deletions of 17p and *p53* Mutations in Preneoplastic Lesions of the Lung[1]

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ABSTRACT

Cytogenetic and *p53* mutation analysis in two cases of severe dysplasia of the bronchial epithelium in lung cancer patients and *p53* immunostaining in a third one are reported. The finding of both chromosomal deletions of 17p and *p53* mutation indicates that these changes may take place early in the process of lung carcinogenesis.

INTRODUCTION

Multiple genetic lesions either activating dominant oncogenes or inactivating tumor suppressor genes have been described in human lung tumors. Dominant oncogene activation includes K-*ras* mutation in adenocarcinomas, deregulation of all the *myc* gene family members mainly in small cell lung cancer, and of growth factor receptor genes in non-small cell lung cancer (1–3). The involvement of known or presumed tumor suppressor genes has been suggested by the presence of recurrent chromosomal deletions or losses and confirmed by restriction fragment length polymorphism analyses showing loss of heterozygosity for specific loci on chromosomes 3p, 9p, 11p, 13q (RB gene) and *17p* (*TP53* gene) (1–3).

In lung cancer, as in many other human cancers, inactivation of the *p53* gene has been shown to occur by loss of one copy of the gene and mutations in the remaining allele (4, 5). A crucial role of this change is underscored by the finding that *p53* mutation is one of the most common genetic defect in lung cancer, being present in about 50% of non-small cell lung cancer and 75% of small cell lung cancer (4–6), and wild-type *p53* suppresses the growth of human lung cancer cell lines bearing other multiple genetic lesions (7).

Little is known about the timing of genetic changes during lung carcinogenesis, since preneoplastic lesions of the bronchial epithelium have rarely been analyzed (8). Nevertheless, since the bronchial epithelial cells are widely exposed to environmental carcinogens, early genetic lesions, eventually resulting in an altered cellular morphology, are likely to occur in the bronchial mucosa.

In a previous study we have shown that recurrent cytogenetic abnormalities and overexpression of growth factor receptors occur in both lung tumor cells and in histologically normal bronchial epithelium (9). These findings indicated that early genetic lesions may be present in normal appearing bronchial mucosa and that the progression toward full tumorigenicity would then be acquired through accumulation of further genetic defects.

In an attempt to define the type and temporal sequence of somatic genetic changes that precede the onset of the invasive phase of lung cancer, we have performed a cytogenetic study and *p53* mutation analysis in two cases of severe dysplasia of bronchial mucosa adjacent to lung cancer. In addition, a case of carcinoma *in situ* was analyzed by *p53* immunostaining.

MATERIALS AND METHODS

Lung tumor samples were obtained at surgery from three previously untreated patients. Bronchial dysplastic mucosa specimens were taken at resection margins in two cases and adjacent to the tumor sample in a third one. One-half of the samples were immediately frozen and the histopathological diagnosis was done on hematoxylin-eosin stained frozen sections. The remaining part of the sample was processed for the establishment of cell culture. Small bronchial explants were obtained by accurate mechanical digestion of the specimens containing representative areas of dysplasia, and were grown in Petri dishes with the use of a modified culture RPMI 1640 medium supplemented with 5% fetal calf serum (Flow Laboratories, Irvine, Scotland), penicillin (200 units/ml), streptomycin (200 μg/ml), transferrin (10 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (5 ng/ml) (Sigma Chemical Co., St. Louis, MO), and insulin (5 μg/ml) (Sigma).

Harvest of the primary cultures was performed after 8–10 days of culture according to the method of Gibas *et al.* (10). G-banding was achieved by Wright's stain after trypsin denaturation. Chromosome abnormalities were described, following the International System for Human Cytogenetic Nomenclature (11).

Immunocytochemical staining of the p53 protein on frozen sections was carried out by avidin-biotin complex technique with silver-diaminobenzidine enhancement according to the method of Frigo *et al.* (12), using the monoclonal antibody PAb240, raised against mutant forms of *p53* (13) at the dilution of 1:1000. On paraffin-embedded sections immunocytochemistry was performed by using the polyclonal antiserum CM-1 against wild-type human p53 (Novocastra Laboratory, Newcastle, England), using a modification (14) of the method described by Shi *et al.* (15).

The tumor DNA was extracted from a frozen surgical specimen using conventional methods (16). Previously identified dysplastic areas on stained sections were microdissected from parallel 5-μm frozen sections and collected in microfuge tubes, at which time conventional DNA extraction was performed. Oligonucleotide sequence and single strand conformational polymorphism procedures are described by Gaidano *et al.* (17). Gel-purified polymerase chain reaction products were sequenced directly by using 5′-^{32}P-labeled primers and AmpliTaq polymerase as described by Murray (18).

RESULTS

The histological diagnosis of the tumor samples of all three cases was of invasive squamous cell carcinoma of the lung, whereas the analysis of the adjacent bronchial mucosa revealed areas of moderate and severe dysplasia in two cases (Figs. 1 and 2) and of carcinoma *in situ* in the third one.

The primary cultures from the bronchial dysplasia samples of Patients 1 and 2 showed typical epithelial growth without evident fibroblast contamination.

The cytogenetic analysis of Case 1 showed a pseudodiploid karyotype with del(17)(p13) as a single chromosomal abnormality in eight cells analyzed (Fig. 3). In Case 2 the 10 cells analyzed showed del(17)(p13) (Fig. 3) and an extranumerary

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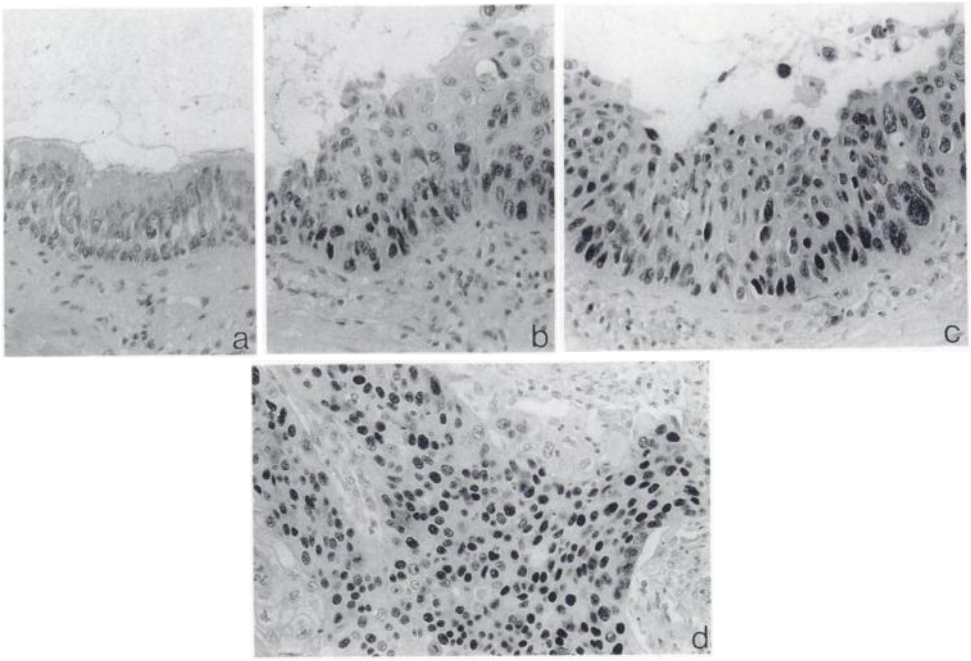

Fig. 1. Immunocytochemistry of *p53* in Case 1. *a*, normal bronchial mucosa; *b*, area of moderate, and *c*, severe dysplasia showing a progressively higher proportion of nuclei immunoreactive with the antibody CM-1; *d*, the invasive component of the tumor shows numerous nuclei immunostained with the same antibody.

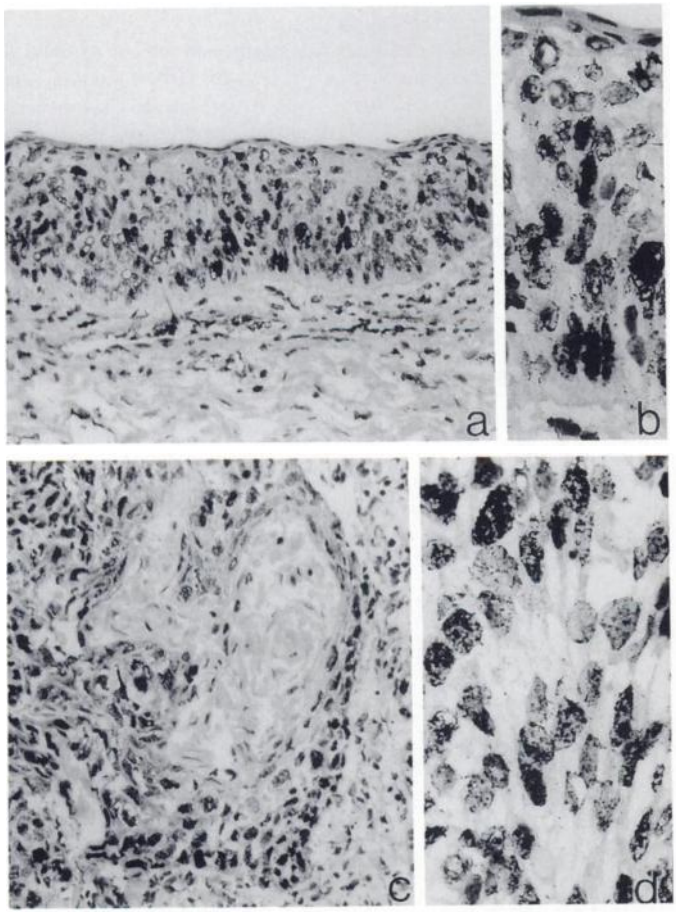

Fig. 2. Immunocytochemistry of *p53* in Case 2. *a*, *p53* immunostain of a section of the bronchial epithelium showing severe dysplasia which reveals nuclear staining with the antibody PAb240; *b*, a high power field of a selected area; *c*, invasive component of the tumor in which the majority of the nuclei are immunoreactive for *p53* with the same antibody; *d*, a high power field showing that the immunoreactivity is confined to the nuclei.

marker chromosome of unidentified origin. In both cases a clone with a normal 46,XY karyotype was present. No constitutional chromosomal abnormalities were observed in the peripheral blood lymphocytes of the two patients.

p53 immunostaining of sections from the tumor specimens of the three patients displayed positivity in the majority of nuclei, using the antibody PAb240, which specifically detects the conformationally altered mutant *p53* protein, and the polyclonal antiserum CM-1, directed at wild-type forms of *p53* (Fig. 1*d* and Fig. 2, *c* and *d*).

With the same antibodies, sections of dysplasia and of carcinoma *in situ* showed a high number of immunoreactive nuclei. As shown in Figs. 1 and 2, immunoreactive nuclei were more numerous in the areas of severe dysplasia then in those with moderate dysplasia, whereas the normal bronchial mucosa did not show any staining.

In addition, DNA of the tumor and the dysplastic areas from Case 2 was analyzed for the presence of *p53* mutations in exons 5, 6, 7, 8, and 9. The single strand conformational polymorphism analysis revealed the presence of a mutated exon 5 in both tumor and dysplasia as indicated by the presence of abnormal bands with shifted electrophoretic mobility (Fig. 4*a*). In peripheral blood lymphocytes of the same patient, only the wild-type configuration of the same exon was detected (data not shown). The DNA sequencing reaction of the PCR amplified exon 5 showed the same point mutation at codon 161 in both tumor and dysplasia (Fig. 4*b*).

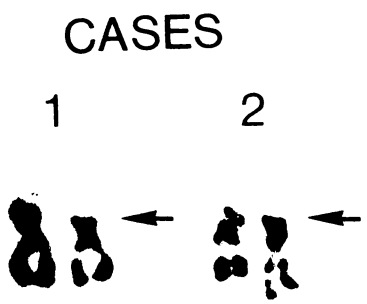

Fig. 3. Pairs of chromosomes 17 from Case 1 (*left*) and Case 2 (*right*). *Arrows* indicate the del(17)(p13).

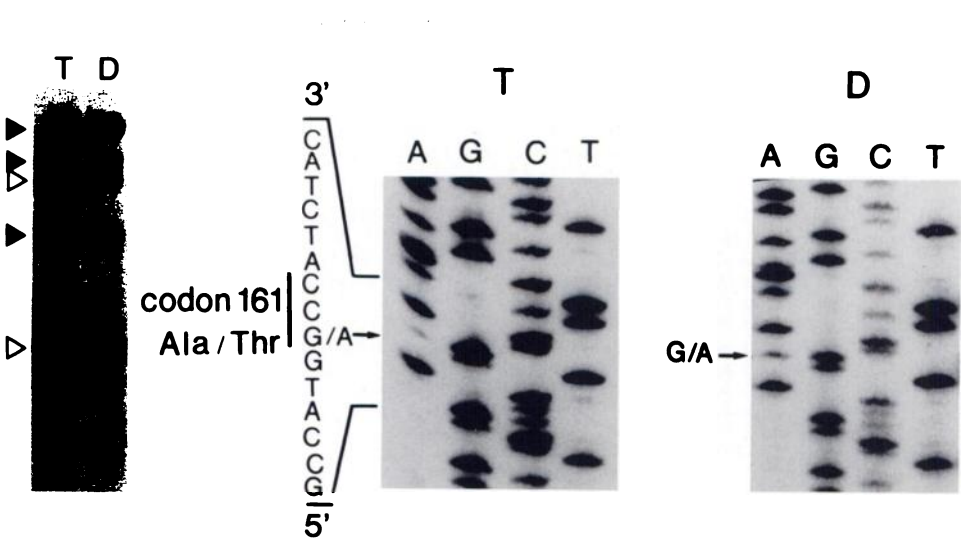

Fig. 4. *a*, single strand conformational polymorphism analysis of *p53* exon 5 from tumor (*T*) and dysplasia (*D*) DNA of Case 1. ▶, abnormal bands with shifted mobility, ▷, wild-type bands; *b*, DNA sequencing reactions by polymerase chain reaction-amplified exon 5 fragments from tumor (*T*) and dysplasia (*D*) DNA of Case 1. The codon at which the mutation occurs is indicated. *Arrows* show the bands corresponding to the mutated base pair.

DISCUSSION

Dysplasia of the bronchial epithelium is considered a premalignant change that may evolve into lung cancer. The recognition of the genetic changes associated with dysplasia would be helpful in clarifying the pathway of neoplastic development of bronchial tumors.

We report here that in contrast to the cytogenetic and molecular complexity of the invasive tumors of the lung, bronchial dysplasia showed limited but nevertheless clonal and specific changes at both levels. In fact, in two cases we found near-diploid karyotypes with a recurrent deletion of 17p involving the band where the *p53* gene is localized, whereas immunocytochemistry showed an increased amount of *p53* protein in all three cases. In addition, in one of the two cases with dysplasia the same *p53* missense point mutation was also found in the adjacent invasive carcinoma. This constitutes the first report of a combined cytogenetic, immunocytochemical, and molecular evidence of *p53* loss and mutation in premalignant lesions of the bronchial epithelium.

To our knowledge, only two other studies have investigated this issue. In a survey of archival material of radon-associated lung cancer patients, Vähäkangas *et al.* (8) reported immunocytochemical and molecular evidence of *p53* mutation in preinvasive, microinvasive, and invasive lesions of one patient. Rabbitts *et al.* (19) recently found allelic losses at various loci on chromosome 3p and loss of one allele of the *p53* gene, as well as *p53* mutations in preinvasive lesions of the bronchus.

While there is evidence that in several human tumors such as colon (20), ovarian (21) and thyroid cancer (22),[3] the inactivation of *p53* gene is a late event associated with tumor progression, the present findings strongly suggest that this change may take place early in the dysplastic lesions of the bronchial mucosa.

Nevertheless, our previous (9) and unpublished findings of several clonal rearrangements in the normal bronchial mucosa, particularly those affecting chromosomes such as 3p, 7, and 11p, suggest that the genetic damage might affect other known or putative tumor suppressor genes. In any event, a selective alteration of the *p53* gene on chromosome 17 is likely to lead to a clonal expansion and, through accumulation of further genetic hits, to transition to the malignant phenotype. The recent observation (7) that the reintroduction of the wild-type *p53* gene suppressed the growth of human lung cancer cell lines bearing other multiple genetic lesions seems to indicate that in spite of the numerous genetic changes observed in lung cancer, *p53* inactivation represents a crucial step in lung carcinogenesis.

Altogether, these data provide convincing evidence that somatic genetic alterations may occur in early stages of lung tumorigenesis, raising the possibility that cytogenetic and molecular analyses will be useful in the early diagnosis of precancerous lesions of the bronchial mucosa.

[3] R. Donghi, A. Longoni, S. Pilotti, P. Michieli, G. Della Porta, and M. A. Pierotti. *p53* mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland, submitted for publication.

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