

Multiple Clonal Abnormalities in the Bronchial Epithelium of Patients With Lung Cancer

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Background: Several molecular changes, including loss of heterozygosity (i.e., deletion of one copy of allelic DNA sequences) and alterations in microsatellite DNA, have been detected early in the pathogenesis of lung cancer, even in histologically normal epithelium. In the bronchial epithelium of patients with lung cancer, we have determined the frequency, size, and patterns of molecularly abnormal clonal patches. **Methods:** We studied formalin-fixed, paraffin-embedded samples from 16 surgically resected lung carcinomas (five squamous cell carcinomas, four small-cell carcinomas, six adenocarcinomas, and one large-cell carcinoma). From each carcinoma, we microdissected foci (each containing about 200 cells) of tumor tissue and equivalent samples of histologically normal and abnormal epithelium. Furthermore, multiple discontinuous foci of bronchial epithelium were analyzed from methanol-fixed samples from three additional patients with lung cancer (two with squamous cell carcinoma and one with adenocarcinoma). We used two-step polymerase chain reaction-based assays involving 12 microsatellite markers at seven chromosomal regions frequently deleted in lung cancer. **Results:** Two hundred eighteen foci of nonmalignant bronchial epithelium (195 of histologically normal or slightly abnormal epithelium and 23 of dysplastic epithelium) were studied from the 19 surgically resected lobectomy specimens. Thirteen (68%) of the 19 specimens had at least one focus of bronchial epithelium with molecular changes. At least one molecular abnormality was detected in 32% of the 195 histologically normal or slightly abnormal foci and in 52% of the 23 dysplastic foci. Extrapolating from our two-dimensional analyses, we estimate that

most clonal patches contain approximately 90 000 cells. Although, in a given individual, tumors appeared homogeneous with respect to molecular changes, the clonally altered patches of mildly abnormal epithelium were heterogeneous. **Conclusions:** Our findings indicate that multiple small clonal or subclonal patches containing molecular abnormalities are present in normal or slightly abnormal bronchial epithelium of patients with lung cancer. [J Natl Cancer Inst 1999;91:1863-8]

As with other epithelial malignancies, lung cancers are believed to arise after a series of progressive histopathologic changes (preneoplastic lesions) in the bronchial epithelium. In centrally arising squamous cell carcinomas, the changes include (from the earliest to the most advanced) hyperplasia, metaplasia, dysplasia of increasing severity, carcinoma *in situ*, invasive cancer, and metastatic cancer (1-3). However, the sequence of events preceding small-cell lung carcinomas and peripherally arising adenocarcinomas are not fully understood (4,5). Preneoplastic changes frequently are extensive and multifocal, occurring throughout the respiratory tree, a phenomenon referred to as field cancerization (6). Tests for loss of heterozygosity (LOH) by use of polymorphic microsatellite DNA markers are frequently used to identify allelic losses at specific chromosomal loci. Allelic losses at the short arms of chromosomes 3, 9, and 17 (3p, 9p, and 17p, respectively) occur relatively early in the multistage development of invasive lung cancer (7-11). Microsatellite alterations (representing changes in the size of one or both alleles) are found in many human cancers, including lung cancer, and may serve as clonal markers for early cancer detection (12,13). Very high incidences of LOH and microsatellite alterations have been detected in the histologically normal and mildly abnormal bronchial epithelia (hyperplasia or squamous metaplasia) of most subjects with lung cancer and in current and former smokers (8,14-16).

The purpose of this study was to determine the frequency, size, and patterns of molecularly abnormal clonal patches in the bronchial epithelium of patients with lung cancer. A determination of the size of the clonal patches with such changes may be important for understanding tu-

morigenesis and for the evaluation of chemoprevention studies.

MATERIALS AND METHODS

Tumor Specimens

Specimens from 19 surgically resected small-cell lung carcinomas and non-small-cell lung carcinomas were studied. These specimens were from 13 male patients and six female patients who ranged from 37 to 77 years of age (mean, 58 years). All specimens were from curative intent lung cancer resections (lobectomies). Patients were staged by use of the International System for Staging Lung Cancer, and all had stage I and II disease (17). Specimens were obtained as part of an Institutional Review Board-approved research study. The 15 patients for whom data were available were current or past heavy smokers (>20 pack-years of smoking exposure [1 pack-year = one pack smoked per day for 1 year]). Archival microslides were obtained from 16 carcinomas (five squamous cell carcinomas, four small-cell carcinomas, six adenocarcinomas, and one large-cell carcinoma). From each carcinoma, a single cross-section of one lobar, segmental, or subsegmental bronchus with partially or completely intact histologically normal or abnormal bronchial epithelium was selected. Whenever possible, the cross-section was selected as close to the proximal resection margin, provided that the tumor was not identified in the same microsection. Serial 5- μ m sections were cut from formalin-fixed, paraffin-embedded tissues. All slides were stained with hematoxylin-eosin, and one of the slides was coverslipped. The coverslipped slide was used as a guide to localize regions of interest for microdissection of the other slides. Contiguous foci of approximately 200 cells along the entire epithelial surface were carefully counted and microdissected. We selected this cell number because it was the minimum number of cells required for multiplex analysis of the multiple markers studied and because it was the minimum number of epithelial cells present in bronchial biopsy specimens with an intact layer of his-

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tologically normal cells (Gazdar AF: unpublished observations).

In addition, methanol-fixed samples prepared by the epithelial aggregate separation and isolation method (EASI preparations) (19) were available from three more patients (two with squamous cell carcinomas and one with adenocarcinoma). For EASI preparations, the surfaces of large bronchi near the proximal resection margin were gently scraped with the edge of a plain uncharged microscope slide. The cellular materials so obtained were evenly spread onto the surface of one or more uncharged slides. The slides were immediately fixed in 95% methanol, stained with hematoxylin-eosin, and not covered with a coverslip. Tumors and preneoplastic lesions were identified by three pathologists (S. Milchgrub, A. Maitra, and A. F. Gazdar) who used standard published criteria (18).

We used laser-captured microdissection (20) or a manual micromanipulator, as previously described (7), for the serial microdissection of foci, each containing approximately 200 cells (Figs. 1 and 2). Stromal cells or lymphocytes from the same slides were used as a source of constitutional (normal) DNA from each specimen. Multiple foci of a similar size were also microdissected from the EASI preparations. To investigate the heterogeneity of tumor tissue, we microdissected several areas of 200–1000 cells. After DNA extraction, 5 μ L of the proteinase K-digested samples was used for each multiplex polymerase chain reaction (PCR).

Polymorphic DNA Markers and PCR-Based LOH Analysis

To evaluate LOH and microsatellite alterations, we used primers flanking 12 dinucleotide and mul-

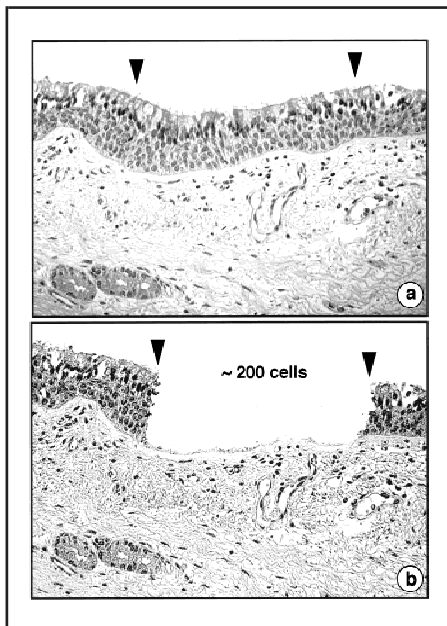


Fig. 1. Hyperplastic bronchial epithelium before (a) and after (b) microdissection of a focus of approximately 200 cells. The basement membrane is intact in the microdissected specimen, confirming lack of contamination by nonepithelial cells. *Note:* Not all nuclei visualized under microscopic examination are discernible in the photomicrograph. The distance between the two arrowheads is approximately 300 μ m.

tinucleotide microsatellite repeat polymorphisms located at the following seven gene or chromosomal locations: 3p12 (D3S1274), 3p14–21 (D3S1766), 3p21 (D3S1029), 3p22–24.2 (D3S2432, D3S1351, and D3S1537), 3p24.3 (D3S1244 and D3S1293), 9p21 (IFNA and D9S1748), and 17q13.1 (the dinucleotide and the pentanucleotide repeats of the TP53 [p53] gene). These genes or locations were selected because they are sites of frequent allelic losses in lung cancers (21). Primer sequences can be obtained from the Genome Database, with the exceptions of two pentanucleotide and dinucleotide repeats in the p53 gene (22). Nested PCR (8) or two-round PCR (15) methods were used. Multiplex PCR was performed during the first amplification, followed by uniplex PCR for individual markers. LOH was scored visually as more than a 50% reduction of the autoradiographic signal corresponding to one of the two alleles in informative specimens (in most cases, loss was complete). Microsatellite alterations were detected by a shift in the mobility of one or both alleles with respect to the parental alleles.

RESULTS

Microdissection of Foci of Bronchial Epithelium

From the 19 surgical resection specimens, a total of 218 foci of bronchial epithelium (each containing approximately 200 cells) were dissected (175 from archival paraffin-embedded slides and 43 from methanol-fixed EASI preparations). The molecular findings are summarized in Table 1. For the archival materials, the foci were dissected from the entire bronchial circumference selected for study and were consecutively numbered from a preselected starting point (Fig. 2), although, in some cases, the mucosa was not intact throughout the cross-section. The number of foci per specimen varied from five to 25 (mean = 11.4 foci per specimen). The foci from EASI preparations represented isolated epithelial cell clumps, and their spatial relationship to each other could not be determined. Foci of squamous metaplasia were present in five of the 19 specimens, and dysplasia (mild or moderate) was present in four. Of the 218 individual foci microdissected from both types of preparation, 23 were dysplastic and the remainder were histologically normal or had mild changes (i.e., metaplasia or hyperplasia). At least one focus was dissected from all of the corresponding tumors, and multiple foci were dissected from four tumors.

Identification of Clonal (or Subclonal) Patches of Bronchial Epithelium

Molecular changes were detected in at least one focus of nonmalignant epithelium from 13 (68%) of the 19 specimens

studied. Of the 16 paraffin-embedded preparations, 60 (34%) foci from 10 specimens had at least one molecular change (either LOH or microsatellite alteration; Fig. 2). When adjacent foci contained identical changes, the contiguous foci were regarded as a single patch of altered cells (Fig. 2). A total of 54 patches were identified that consisted of 49 single foci, and five larger patches were identified that each had two or three foci. Thus, by two-dimensional analysis, the vast majority (91%) of the patches contained a single focus of about 200 cells, and the remainder contained 400–600 cells. Of three EASI preparations, 15 (35%) of 43 foci from all preparations had one or more molecular changes. Because the spatial relationship between the foci could not be determined, each altered focus was regarded as a separate clonal patch.

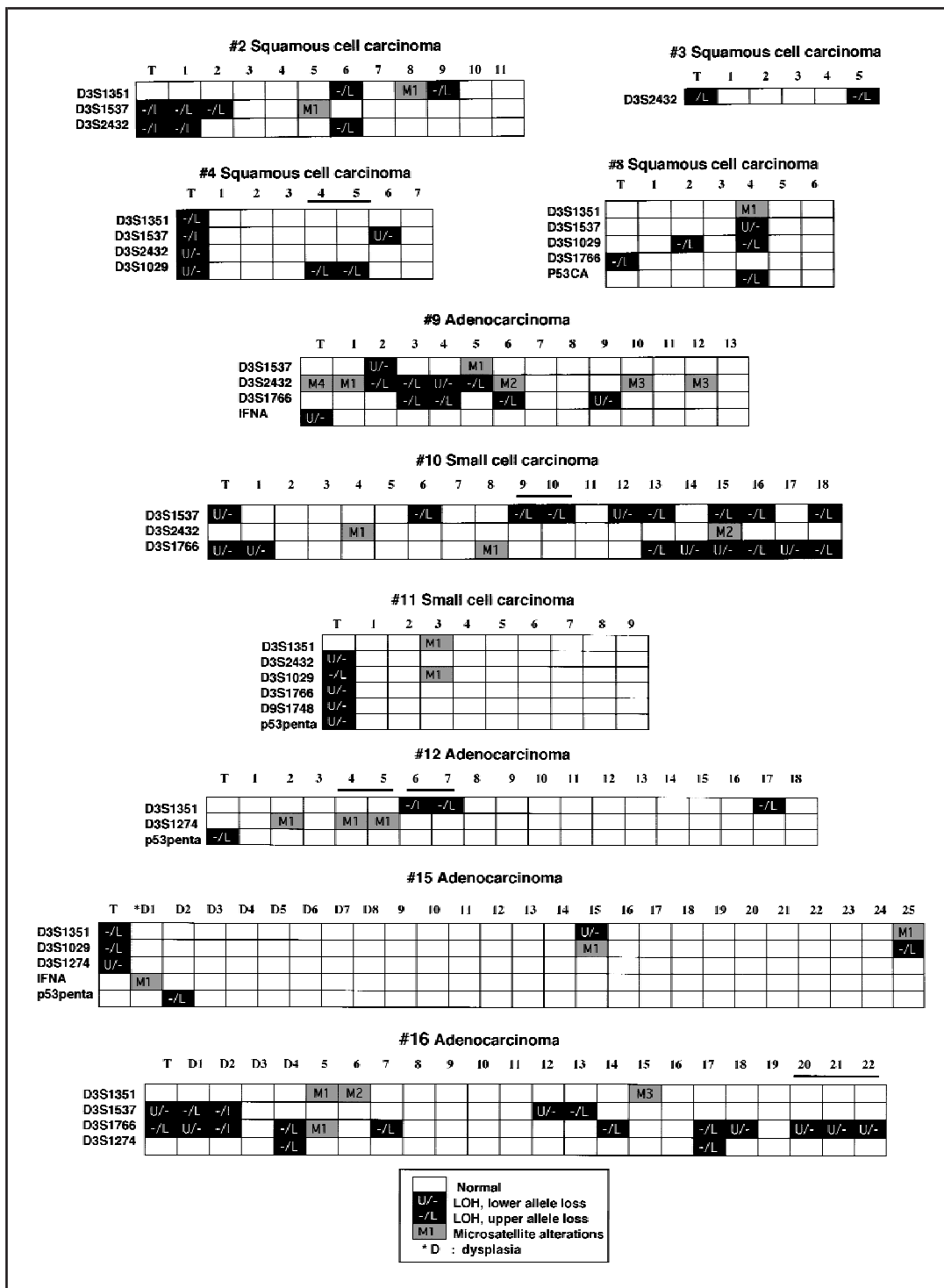
Molecular Changes in Clonal Patches

At least one molecular change was identified in 12 (52%) of the 23 total dysplastic foci and in 63 (32%) of the remaining 195 foci with normal or mildly abnormal histology. As discussed previously, these 75 foci with molecular changes were believed to represent 69 clonal or subclonal patches. The most frequently detected abnormality was LOH at one or more regions of 3p (49 [71%] of the molecularly altered patches). Microsatellite alterations were detected in 22 patches (32%). LOH at p53 gene was detected in seven patches (10%). Although we failed to detect LOH of 9p in any patch, only five of the 16 samples were informative for either of the two 9p markers used.

Relationship Between Changes in Clonal Patches and in Corresponding Tumors

Molecular changes were present in all 13 tumor specimens, with one or more altered clones present in the accompanying nonmalignant epithelium (Figs. 2 and 3). We compared the pattern of changes in the clonal patches with those present in their corresponding tumors. If the changes in the clonal patches were identical with those present in the corresponding tumor (even if the tumor had more extensive changes), we regarded the clonal patches as possibly representing direct precursor lesions. We regarded the changes in the clonal patches as different from the corresponding tumor if 1) both lesions had LOH with a particular marker but the losses involved different parental alleles

Fig. 2. Representative examples of molecular analyses of bronchial cross-sections from 10 resected non-small-cell lung carcinoma specimens with clonal patches of molecular changes. Each column represents a single focus of approximately 200 cells microdissected from paraffin-embedded microslides. Each focus is identified by a number at the top of the column. The identification number of dysplastic foci is preceded by the letter "D." All other foci had normal or slightly abnormal histology (i.e., hyperplasia or metaplasia). Data from the corresponding tumors are presented in columns T. Polymorphic markers that showed loss of heterozygosity (LOH) or microsatellite alterations are indicated. LOH is indicated as **solid boxes**, and microsatellite alterations are indicated by numbers preceded by the letter "M." If different foci had multiple forms of microsatellite alterations, each form is identified by an "M" followed by a number (e.g., M1, M2, etc.). In the majority of cases, only single foci demonstrate a specific pattern of changes. When contiguous foci demonstrated identical changes, they were regarded as clonal patches as indicated by **bars under the identification numbers** (e.g., foci 4 and 5 in sample 10).



or if 2) LOHs or microsatellite alterations present in the patches were not present in the corresponding tumor. If we apply these criteria to the 69 clonal patches, nine (13%) would represent possible precursor lesions and 60 (87%) would represent clones independent of the corresponding tumor.

Clonal Changes in Tumors

To determine whether heterogeneity was present in tumor tissue, we compared

the pattern of molecular changes present in multiple areas. From four tumors (one squamous cell, one small-cell lung carcinoma, and two adenocarcinomas), we microdissected six to 11 foci per tumor, each containing 200–1000 cells. Foci were selected from several individual tumor blocks and as far away from each other as possible on individual microslides. In all cases, all foci from individual tumors had identical patterns of molecular changes (data not shown).

Validation of Results of Molecular Analyses

PCR-based assays by use of small quantities of DNA from formalin-fixed, paraffin-embedded materials may result in preferential amplification of the smaller of a pair of alleles and spurious band shifts, resulting in the artifactual appearance of apparent allelic losses and microsatellite alterations. For these reasons, we did further analyses to confirm the valid-

Table 1. Summary of molecular analyses*

Sample type	No. with molecular changes/ No. tested (%)
Tumor specimens	13/13 (100)†
Foci	
All	75/218 (34)
Normal or mildly abnormal histology	63/195 (32)
Dysplastic histology	12/23 (52)
Foci from paraffin-embedded specimens	60/175 (34)
Foci from EASI* preparations	15/43 (35)
Size of clonal patches, cell No. in two-dimensional analysis	No. of clonal patches (n = 54)
200 cells	49
400 cells	4
600 cells	1

*Foci consisted of approximately 200 cells microdissected from paraffin-embedded sections or preparations made by the epithelial aggregate separation and isolation method (EASI). If adjacent foci (microdissected from paraffin sections) had identical molecular changes, they were regarded as a single clonal patch. The spatial relationships of foci microdissected from these preparations could not be ascertained, and the size of the clonal patches in these preparations could not be estimated.

†These 13 tumors represent a subset of the original 19 tumors tested. This subset represents the tumors in which one or more molecular changes were detected in the corresponding nonmalignant epithelial foci.

ity of our results. As mentioned above, molecular analyses of 32 independently dissected areas of four tumors were 100% reproducible. Allelic losses or alterations in foci of bronchial epithelium were confirmed by redissection and reanalysis of the same foci from replicate slides (24 of 24 foci). Electronic capture of images of the bronchial cross-sections analyzed aided precise redissection of the foci. Of all specimens with allelic losses, the larger allele was lost in 57% and the lower allele was lost in 43%.

DISCUSSION

We determined the size of clonal patches of bronchial epithelium (both normal and abnormal) by studying molecular changes (allelic losses and microsatellite alterations) at several locations that are frequently involved in lung cancer pathogenesis. We used a two-dimensional model and carefully microdissected foci of epithelium from the circumference of cross-sections of large bronchi, usually

near the proximal surgical resection margin.

We dissected epithelial foci, each containing approximately 200 cells, because, in our experience, this is the minimum number of epithelial cells present in a single 5- μ m section of a histologically normal biopsy specimen obtained by fluorescence bronchoscopy. The major finding of our study is that multiple small clonal patches of molecular abnormalities are present in the histologically normal, as well as abnormal, bronchial epithelium of patients with lung cancer. At least one molecular abnormality was detected in 32% of histologically normal or slightly abnormal foci and in 52% of dysplastic foci. Thus, about one third of the epithelial surface of large bronchi of patients with lung cancer may have molecular

damage. We had previously demonstrated at least one molecular abnormality in 48% of histologically normal bronchial biopsy specimens from smokers without cancer (16). However, areas of abnormal fluorescence were preferentially selected. In this study, the somewhat lower percentage (32%) of changes in histologically normal or slightly abnormal epithelium from lung cancer patients may reflect the nonselected circumferential analysis of a single bronchus. As described in the "Results" section, our data were confirmed and validated by redissection and reanalysis of epithelial foci. This study and previous studies (14,16) support the concept that numerous small clonally altered foci are present in the bronchial epithelium of the majority of smokers and patients with lung cancer.

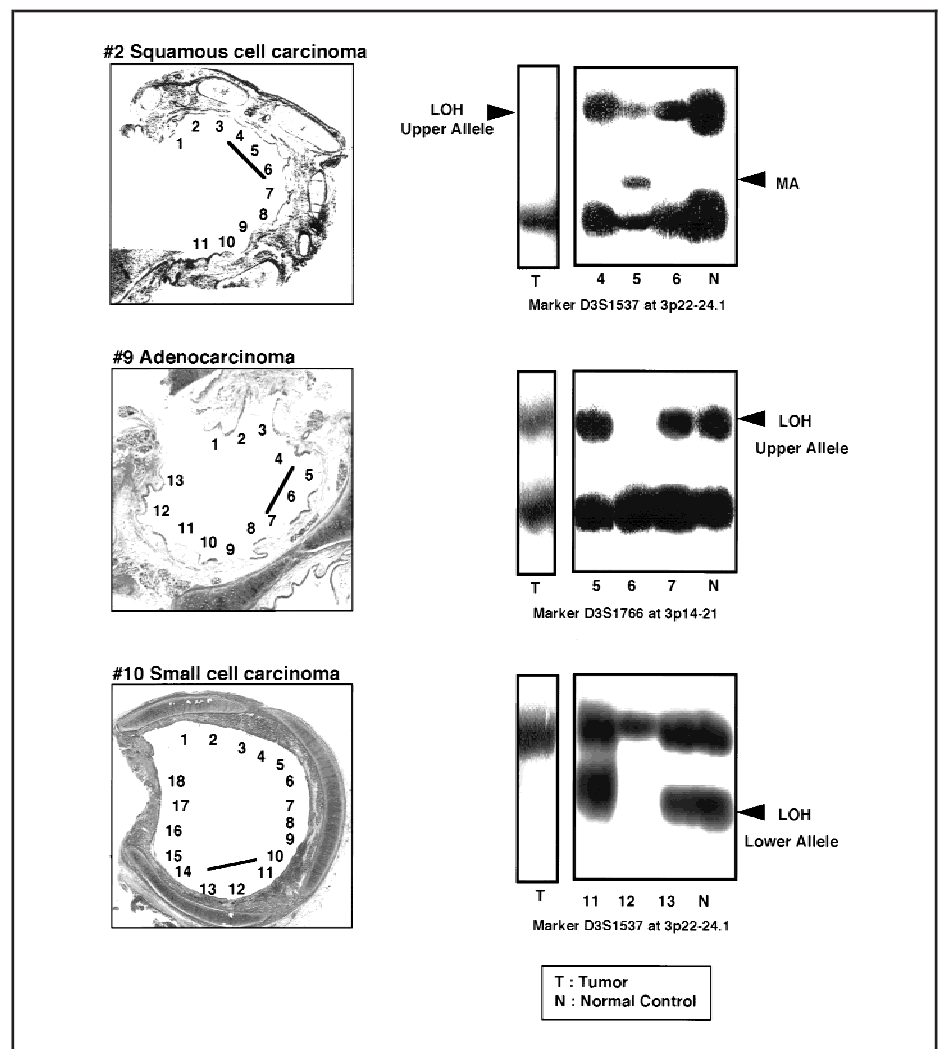


Fig. 3. Examples of clonal changes present in bronchial epithelium. In #2 squamous cell carcinoma, focus 5 has a microsatellite alteration with marker D3S1537 that is not present in the corresponding tumor (T) or adjacent foci. In the #9 adenocarcinoma, focus 6 has loss of heterozygosity (LOH) of the upper allele with marker D3S1766, whereas the corresponding tumor retains heterozygosity. In the #10 small-cell carcinoma, focus 12 and the corresponding tumor have LOH of the lower allele of marker D3S1537. N = normal control stromal cells.

Previous studies (14–16) have demonstrated multiple molecular abnormalities, especially allelic losses at 3p and 9p, in bronchial biopsy specimens from smokers and patients with squamous cell lung cancer. Losses at chromosome 3 were the most common molecular change observed in this study. Because relatively few of our specimens were informative for the two 9p loci studied, we could not evaluate the role of 9p losses fully. The clonal patches, as analyzed in our two-dimensional examination, indicated that the patches were relatively small, usually between 200 and 400 cells. The largest clonal patch identified was 600 cells. If we assume that the surface dimensions of the patches are approximately equal in size and accept 300 cells as the average number of cells in a patch as measured in two dimensions, then the approximate number of cells per clonal patch would be 90 000. This number is less than the average number of epithelial cells present in hyperplastic or dysplastic biopsy specimens (Gazdar AF, MacAulay C, Smith A, Lam S: unpublished results).

Our previous studies (15,16,23) have indicated a possible clonal relationship between the molecularly altered foci of bronchial epithelium present in smokers and patients with lung cancer. For allelic losses, the same parental allele was lost in biopsy specimens obtained from widely dispersed parts of the bronchial epithelium, a phenomenon that we have referred to as allele-specific loss (7). Allele-specific loss may indicate the widely dispersed presence of individual molecularly altered clones of cells (24). A recent report (23) described a single identical point mutation in multiple areas of histologically abnormal bronchial epithelium in both lungs of a smoker without lung cancer and provided further evidence that individual clones may disperse progeny cells throughout the bronchial epithelium. In this study, we failed to find a clonal relationship (or allele-specific losses) between the molecularly altered patches or between the patches and the corresponding tumors. Thus, our current findings appear to contradict our previous studies. However, in this study, we blindly selected small contiguous patches of epithelium, whereas, in the previous studies, we selected, in general, patches that had abnormal histology or fluorescence patterns. This study is consistent with the findings of Hittelman (25,26) who found evidence for the presence of numerous small mono-

somic or trisomic clonal and subclonal patches in smoking-damaged upper aerodigestive tract epithelium, as determined by fluorescence *in situ* hybridization analyses. One possible explanation for these apparently contradictory findings is that numerous small individual clonal patches of histologically or molecularly altered epithelium are present in the lungs of smokers. Subsequently, a small number of more advanced clones acquire the ability to become widely dispersed, and these clones are the direct precursors of invasive tumors. A possible problem with interpretation of the studies cited above is that evidence directly linking the changes to smoking exposure is limited. Equivalent specimens from aged-matched nonsmoking controls are exceedingly difficult to obtain. Examination of a modest number of bronchial biopsy specimens from lifetime nonsmokers identified few (14) or no (16) molecular changes. However, further studies on specimens from nonsmokers are required for confirmation.

It is of interest to compare our findings with observations from other cancers arising in widely damaged epithelia that show a field effect. In Barrett's disease, a precursor lesion of esophageal adenocarcinoma, large patches of epithelium have metaplastic and dysplastic changes. Specimens of Barrett's epithelium from separate sites may have identical p53 mutations, suggesting a clonal origin (27). Thus, the clonally altered patches with molecular abnormalities are relatively large. These findings are in contrast to observations from sun-damaged skin, which predisposes to nonmelanotic skin cancer. Sun-exposed human skin contains clonal patches of p53-mutated keratinocytes, arising from the dermal-epidermal junction and from hair follicles (28). These clones of 60–3000 cells were present at frequencies exceeding 40 clones/cm² and together may involve as much as 4% of the epidermis. Clones in sun-exposed skin are both more frequent and larger than clones in sun-shielded skin. The authors concluded that, in addition to being a tumorigenic mutagen, sunlight acts as a tumor promoter by favoring the clonal expansion of p53-mutated cells (28). These combined actions of sunlight result in normal individuals carrying a substantial burden of keratinocytes predisposed to cancer. The findings in smoke-damaged epithelium may be more comparable to those present in sun-

damaged skin than to Barrett's esophagus and suggest that exposure to carcinogens present in tobacco smoke results in multiple molecularly altered cells that have a growth advantage for clonal expansion. Larger patches of molecularly altered epithelium, as identified by fluorescence bronchoscopy, may be more likely to be clonally related to each other and to any subsequently arising invasive tumor. As expected, all foci microdissected from tumor cells appeared to be clonally related, consistent with the theory that most if not all tumors are clonally derived.

In conclusion, our findings indicate that multiple small clonal or subclonal patches of molecular abnormalities (usually smaller in size than the average bronchial biopsy specimen obtained by fluorescence bronchoscopy) can be detected in normal or slightly abnormal bronchial epithelium of patients with lung cancer. The clonal patches of bronchial epithelium with molecular changes were usually small. Although the tumors were homogeneous for molecular changes, the clonally altered patches of mildly abnormal bronchial epithelium were usually heterogeneous. The presence of numerous small clonal patches of molecularly altered cells indicates that either arbitrarily selected (14) or fluorescence-guided (16) biopsy specimens of smoking-damaged bronchial epithelium will frequently contain the presence of such clones. Our findings may help in the design of chemoprevention studies by use of sequential bronchial biopsy specimens for the monitoring of intermediate biomarkers as end points.

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