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Angiogenic Squamous Dysplasia in Bronchi of Individuals at High Risk for Lung Cancer

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Abstract

Lung carcinogenesis is assumed to be a multistep process, but detailed understanding of the sequential morphological and molecular changes preceding invasive lung cancer remains elusive. To better understand early lung carcinogenesis, we initiated a program of fluorescence bronchoscopy in smokers at high risk for lung cancer. In the bronchial biopsies from these subjects, we observed a unique lesion consisting of capillary blood vessels closely juxtaposed to and projecting into metaplastic or dysplastic squamous bronchial epithelium, angiogenic squamous dysplasia (ASD). Serial sections of the capillary projections confirmed that they represent intramucosal capillary loops. Microvessel density in ASD was elevated in comparison to normal mucosa (P = 0.0003) but not in comparison to other forms of hyperplasia or dysplasia. ASD thus represents a qualitatively distinct form of angiogenesis in which there is architectural rearrangement of the capillary microvasculature. Genetic analysis of surface epithelium in a random subset of lesions revealed loss of heterozygosity at chromosome 3p in 53% of ASD lesions. No confirmed p53 mutations were identified. Compared with normal epithelium, proliferative activity was markedly elevated in ASD lesions. ASD occurred in 54 of 158 (34%) high-risk smokers without carcinoma and in 6 of 10 patients with squamous carcinoma who underwent fluorescence bronchoscopy. One early-stage invasive carcinoma was noteworthy for the occurrence of ASD juxtaposed to invasive tumor. Seventy-seven (59%) of the ASD lesions were detected by abnormal fluorescence alone. Twenty bronchial sites (11 patients) were rebiopsied 1 year after the initial diagnosis. At nine (45%) of these sites, the lesion was found to persist. The lesion was not present in biopsies from 16 normal nonsmoker control subjects. The presence of this lesion in high-risk smokers suggests that aberrant patterns of microvascularization may occur at an early stage of bronchial carcinogenesis.

Introduction

Carcinoma of the lung continues to be the leading cause of cancer death and years of life lost among both men and women (1). Although smoking prevalence is declining in the United States (2), former smokers, who make up half the American population, continue to be at risk for lung cancer many years after smoking cessation (3). Approximately half of all lung cancers are now diagnosed in former smokers (4, 5), with a large percentage harboring metastatic disease at the time of diagnosis. Significant reduction in morbidity and mortality from lung cancer will depend not only on aggressive efforts at smoking cessation, but also on earlier detection and treatment.

Strategies for early detection are changing as concepts of solid tumor carcinogenesis advance. The observation that many of the morphological and molecular abnormalities that exist in invasive tumors also occur to a lesser degree in preinvasive epithelium has led to the notion that many tumors, including lung carcinoma, are the result of a predictable progression of morphological and genetic changes in airway epithelium. This hypothesis has been especially well supported in the colon, where aberrant crypt foci have recently been recognized as the earliest of the precursor lesions that may eventuate in invasive carcinoma (6). Histological changes associated with lung carcinogenesis include reserve cell hyperplasia, squamous metaplasia, low- or high-grade dysplasia (atypia), CIS (3), and invasive carcinoma. However, stepwise progression through this series of morphological changes is rarely observed in single individuals because premalignant airway lesions are less easily recognized and characterized than lesions in organs such as the colon.

The most accessible stage of bronchial carcinogenesis is invasive carcinoma. Genetic alterations are a nearly universal feature of invasive lung cancers and include LOH (typically of regions on chromosomes 3, 5, and 9) and point mutations in tumor suppressor genes (p53 and Rb) or oncogenes (K-ras). These genetic alterations have also been assessed in preinvasive bronchial epithelium from smokers. The most consistently observed abnormality in preinvasive epithelium from this group is

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3 The abbreviations used are: CIS, carcinoma in situ; LOH, loss of heterozygosity; ASD, angiogenic squamous dysplasia; MVD, microvessel density; LIFE, laser-induced fluorescence emission; PBS, phosphate buffered saline.
allelic loss (LOH; Refs. 7–11). The high frequency of LOH in bronchial epithelium of smokers diminishes the potential utility of these specific alterations as predictive markers, and the overall prognostic value of histological and genetic alterations in respiratory epithelium remains to be established.

An improved understanding of premalignant bronchial epithelial cell biology is essential to identify reliable intermediate biomarkers for screening and chemoprevention. An increasingly better-established biological property of invasive lung carcinoma is angiogenesis, which is now understood to be a requirement for invasive tumor growth and metastasis (12) and has been identified as an independent prognostic variable in lung cancer (13, 14). Blood vessel formation in normal tissues is maintained at a static low level by a balance between angiogenesis activators and inhibitors. During tumorigenesis, alteration of this balance is thought to occur abruptly, and progenitor cells assume an “angiogenic phenotype,” stimulating the formation of new blood vessels (15, 16). This sudden change in tumor progenitor cells is referred to as an “angiogenic switch” (17). Although invasive tumors clearly may exhibit an angiogenic phenotype, the occurrence and timing of angiogenic switching in preinvasive lesions are not well delineated, particularly in the airways.

To better define morphological and genetic abnormalities of the airways during lung carcinogenesis, we performed both white light and fluorescence bronchoscopy on a population of current and ex-smokers previously shown to be at increased risk for dysplasia and lung cancer (18). Fluorescence bronchoscopy is an investigational tool based on the observation that atypical or neoplastic epithelium emits a different spectrum of light from that of normal respiratory mucosa, and this difference can be exploited to identify atypical bronchial mucosa for molecular analysis (19, 20). It is currently being evaluated in clinical trials to identify premalignant bronchial epithelium and early lung cancer. In the course of these studies, we noted a microscopic lesion consisting of capillary-sized vascular projections occurring in association with highly proliferative metaplastic or dysplastic bronchial epithelium, ASD. ASD was absent from the airways of normal volunteer subjects but was frequently present in individuals at high risk for lung cancer. We determined that the lesion is associated with increased stromal MVD and allelic loss on chromosome 3p. ASD reflects a unique form of neangiogenesis associated with dysplastic epithelium in the lower airways of individuals at high risk for lung cancer.

**Patients and Methods**

**Subjects and Specimens.** Specimens for this study were obtained during bronchoscopy of subjects at high risk for the development of lung cancer enrolled in clinical trials of the Colorado Specialized Program of Research Excellence in Lung Cancer. The Institutional Review Board approved all trial protocols. Each subject provided written informed consent before the investigation. Subjects entering these trials met one of the following sets of criteria: (a) 30 pack-year smoking history, evidence of airflow limitation on spirometry with an FEV₁/FVC ratio of less than 0.70 and FEV₁ criteria of Saccomanno moderate or worse atypia on sputum cytology [according to the criteria of Saccomanno et al. (21)], and no mass on chest roentgenogram; (b) sputum cytology with at least moderate atypia and prior history of invasive lung carcinoma; (c) current or past smoking history, moderate atypia (or worse) on sputum cytology, and abnormal chest roentgenogram; or (d) asymptomatic volunteer with normal sputum cytology.

Persons fulfilling at least one set of these sets of criteria underwent both white light bronchoscopy using a fiberoptic bronchoscope (Olympus BF20D; Olympus America Inc., Melville, NY) and fluorescence light bronchoscopy using a LIFE device (Xillix LIFE-Lung Fluorescence Endoscopy System; Xillix Technology Corp., Richmond, British Columbia, Canada). The same set of bronchoscopists performed all examinations. Biopsies were obtained from suspicious areas identified during either white light or fluorescent examinations as well as from two normal-appearing sites per patient.

**Histopathology.** All biopsies were fixed in 10% buffered formalin and embedded in paraffin wax. Paraffin blocks were sectioned according to a standard protocol at 5 μm, and serial sections were mounted in consecutive order on silane-coated glass slides (Superfrost-plus; Fisher Scientific). Twelve slides containing eight sections/slide were prepared from each paraffin block. Slides 4 and 9 were stained with H&E and examined for histological diagnosis by light microscopy. The remaining slides underwent immunohistochemical analysis or microdissection for DNA extraction.

Epithelial abnormalities were classified according to recent WHO criteria as normal, reserve cell hyperplasia, squamous metaplasia, or dysplasia (low or high grade). The presence or absence of ASD as defined below was noted in each biopsy. Two examiners evaluated the specimens, and joint review and consultation resolved diagnostic disagreements.

**Endothelial Cell Identification and MVD.** Endothelial cells were highlighted in tissue sections by immunohistochemical staining using the endothelial marker CD-31. This marker was used to confirm the presence of capillary cores in the ASD lesions and to identify capillary vessels for MVD determinations. To demonstrate this antigen, paraffin sections were stained with mouse anti-CD-31 antibody (DAKO, Carpinteria, CA) using protease for antigen retrieval and avidin-biotin/3,3’-diaminobenzidine (Ventana Medical Systems, Tucson, AZ) for detection on the NEXUS automated immunohistochemical staining (Ventana Medical Systems).

Sections stained with anti-CD-31 were examined for MVD in the mucosa beneath normal epithelium, ASD, and other abnormal epithelium using the Image-Pro Plus image processing program (Media Cybernetics, Silver Spring, MD). Images of bronchial mucosa, usually to the level of smooth muscle or cartilage, were digitally captured using a ×20 microscope objective. Cross-sectional mucosal areas were determined by manually tracing the region of interest on the digital image. Capillary-sized blood vessels within the area of interest were then counted, and MVD was expressed as microvessels/mm².

**Epithelial Cell Proliferation Rate.** Epithelial cell proliferation rates were estimated by immunohistochemical staining for Ki-67, a nuclear antigen that is expressed in G₁, S phase, G₂, and M phase of the cell cycle but is absent in the G₀ phase (22, 23). Ki-67 immunostaining correlates well with thymidine labeling index (24) and has proven to be comparable to mitotic...
count and flow cytometric analysis in defining tumor growth fraction.

MIB-1, an anti-Ki-67 monoclonal antibody (Ref. 25; Immunotech Inc., Westbrook, ME), was used in an antigen retrieval procedure (Antigen Retrieval Citra; BioGenex, San Ramon, CA) to demonstrate Ki-67 in paraffin sections. A light hematoxylin counterstain was applied before viewing. Ki-67 growth fractions were calculated by counting the proportion of 400 epithelial cell nuclei that stained brown, regardless of intensity of staining, and multiplying by 100. Counts were performed in areas exhibiting ASD and in regions of histologically normal mucosa (when available).

Molecular Analysis. For molecular analysis, slides containing ASD lesions were deparaffinized and stained with H&E or methyl-green pyronine. We found that although morphological detail was superior in H&E-stained sections, amplification or methyl-green pyronine. We found that although morphological detail was superior in H&E-stained sections, amplification sometimes failed in DNA isolated from sections stained in this manner. In such cases, sections were stained with methyl-green pyronine, with successful results. Five μm deparaffinized specimens were immersed in 100% ethanol for 5 min and subsequently covered with a glycerol solution [95% glycerol from Fisher Biotech (Fair Lawn, NJ)/5% PBS]. Epithelial cells were then microdissected along with a representative stromal sample (predominantly tissue leukocytes) from the same anatomic site using a glass micropipette and a dissecting microscope. DNA was extracted from microdissected cells by overnight proteinase K digestion at 55°C and was isolated using the QIAmp Tissue Kit according to the manufacturer’s instructions (Qiagen Inc., Valencia, CA). Subjects enrolled in the study also submitted blood samples, and DNA was isolated using a 341 GenePure Nucleic Acid Purification System (Applied Biosystems, Foster City, CA).

3p Analysis. Sites chosen for PCR amplification are highlighted in the ideogram of the short arm of chromosome 3 (left side of Fig. 9). These regions have previously been shown to be homozygously deleted in lung tumors or cell lines or are consistently deleted in premalignant lesions and may contain putative oncogenes or tumor suppressor genes (10, 26). Purified DNA samples underwent PCR to amplify the following microsatellite markers: (a) 3p21.33, D3S1611, D3S1298, and D3S1260; (b) 3p21.31, D3S2968, and D3S1573; (c) 3p14.2, D3S1300 and D3S1481; (d) 3p13, AFM320, D3S1598, and I284; and (e) 3p12.1, D3S1577, D3S1604, D3S1776, and D3S1274 [primer sequences were published previously in Ref. (27)].

The initial PCR consisted of either 10 or 15 rounds involving nonbiotinylated forward and reverse primers synthesized by Research Genetics (Huntsville, AL) performed on blood, epithelial, and stromal samples using a GeneAmp PCR System 9700 from Perkin-Elmer (Norwalk, CT). For the tested individuals, only sites that proved to be heterozygous were examined. PCR conditions were optimized so that primers with similar amplification requirements were paired for a first multiplex round. An aliquot of this initial PCR product was then subjected to a second round of PCR (touchdown), varying from 20 to 35 rounds, making use of biotinylated forward primers from Macromolecular Resources (Fort Collins, CO) and the same reverse primers. Conditions were adjusted to produce distinct bands at a control template concentration of 200 pg (25 diploid genomes) without detectable signal in a control water blank. The final product was run in a 7.0% acrylamide/7M urea/32% formamide gel, followed by the bands of interest being transferred to a magna nylon transfer membrane from Micron Separations Inc. (Westboro, MA). GelMarker 1, a 1-kb ladder, was supplied by Research Genetics and run on all gels to aid in DNA fragment size determination.

Chemiluminescence was used to detect regions of interest using the Phototope—Star Detection kit (New England Biolabs, Beverly, MA). In keeping with previously published work, LOH was determined by the complete absence or a decrease in visual intensity of at least 50% in one allele (10). The amount of DNA in the microdissected samples was estimated by comparison with a known quantity of DNA from peripheral blood lymphocytes of the individual. Microsatellite instability was detected by a shift in the mobility of one or both alleles (8). All determinations of loss were based on direct visual comparison with blood and stromal samples from the same patient. In situations where shadow bands existed above and/or below the principal allelic band, a common occurrence in microsatellite studies, the most intense band was considered the allele.

p53 Analysis. DNA was analyzed for p53 mutation by single-strand conformation polymorphism with direct sequencing, as described previously (28). DNA was amplified for single-strand conformation polymorphism after a proteinase K digestion, using six nested primer sets encompassing exons 5A, 5B, 7, and 8 (28). These exons were chosen because of their association with the large majority of p53 mutations. DNA from suspected mutation sites was isolated and sequenced after amplification with nonlabeled internal primers and gel purification. For sequencing, the fmol DNA sequencing kit (Promega Corp., Madison, WI) was used with autoradiography. DNA from cell lines with known p53 mutations and water blanks were run as controls for all sequencing reactions.
Results

A wide range of bronchial lesions, most of which have been well described (29), was observed in this patient cohort. However, one lesion was observed that has not been recognized in standard classifications, a lesion we refer to as ASD.

Histopathology of ASD

Collections of capillary-sized blood vessels closely juxtaposed to dysplastic epithelial cells were observed in biopsies obtained during fluorescence bronchoscopy. Capillary loops frequently projected upward into the bronchial mucosa, imparting a papillary microscopic configuration to lesions (Fig. 1). These lesions were easily recognizable in histological sections stained with H&E. The constituent epithelium of the lesion was dysplastic to a variable degree as indicated by: (a) overall increase in the cellularity of the mucosa; (b) expansion of the basilar zone; (c) variable progression of cell maturation from the basal to luminal surfaces, frequently with flattening of epithelial cells in upper layers and loss of superficial mucociliary cells; (d) nuclei vertically oriented in the lower two-thirds of mucosa; (e) increased nuclear:cytoplasmic ratio; (f) nuclear angulations, grooves, lobulations,
and asymmetry; (g) frequent presence of nucleoli; and (h) occasional mitotic figures, often above base of the mucosal epithelium. Thickened basement membranes were a variable feature of ASD, with some lesions exhibiting a marked increase in basement membrane thickness. ASD lesions could be single or multiple.

In some specimens, ASD projections had a superficial resemblance to folds in the bronchial mucosa. To confirm that these structures represented more than mucosal folds, we constructed three-dimensional images of these lesions as described above. A typical reconstruction is illustrated in Fig. 2, along with two representative cross-sectional planes. The three-dimensional image confirms that ASD lesions represent projections of capillary loops into the bronchial mucosa.

Endothelial Marking and MVD

Immunohistochemical stains for CD-31 (Fig. 3) confirmed the presence of endothelial cells lining the capillaries in the cores of the papillary projections. This immunostain also revealed that MVD immediately beneath ASD was markedly increased in comparison to MVD beneath normal epithelium (Fig. 4). The mean MVD for ASD was 370/mm² compared with 229/mm² beneath normal epithelium ($P = 0.0003$; two-tailed Student’s $t$ test). Other epithelial lesions without ASD were also associated with an increase in MVD (415/mm²) in comparison to normal epithelium ($P = 0.00002$; two-tailed Student’s $t$ test) but lacked intraepithelial capillary loops, suggesting that ASD represents a qualitatively distinct, histologically recognizable form of angiogenesis in airway mucosa.

Proliferation Index of ASD

ASD lesions exhibited intense proliferative activity, as illustrated in Fig. 5. Immunohistochemistry for Ki-67 revealed a significantly elevated growth fraction ($n = 11$, median = 35.1%, range = 7.7-68.9%). Histologically normal biopsies from this cohort were also stained and had a significantly lower proliferative fraction ($n = 7$, median = 1.5%, range = 0.7-3.5%). When compared with normal epithelium, ASD lesions...
had a significantly higher proliferation index ($P = 0.00005$; one-tailed Student’s $t$ test).

**Bronchoscopic Appearances of ASD**

During fluorescence bronchoscopy, autofluorescence of the bronchial lining decreases in regions of dysplasia and CIS. In this study, areas judged to exhibit significant attenuation of autofluorescent hue and intensity were classified as “abnormal”, and biopsies were obtained from these bronchial sites. The fluorescence and white light findings were recorded at 130 sites that proved to have an ASD lesion. As indicated in Fig. 6, a large majority (101 of 130; 78%) of ASD lesions were found at biopsy sites judged to be abnormal by fluorescence bronchoscopy, and 59% of the lesions were found in areas that were thought to be abnormal by fluorescence but not by white light bronchoscopy.

**Prevalence and Persistence**

To date, 34% (54 of 154) of the high-risk subjects enrolled in fluorescence bronchoscopy trials have had at least one area of ASD (Table 1). Twelve percent (161 of 925) of the biopsies from these smokers contained ASD. Multiple biopsies from the same patient often displayed ASD. ASD was present in more that one biopsy in 70% (38 of 54) of subjects with the lesions, and in 25 of these multifocal cases (66%); ASD lesions were bilateral. In 11 subjects, a total of 20 sites containing ASD were rebiopsied 1 year after the initial diagnosis. ASD was found to persist at 9 of these 20 sites (45%). No particular bronchial site was found to be preferentially involved. ASD was not identified in 39 biopsies from 16 never smokers and 2 volunteers with minimal (0.5 and 3 pack-years) smoking histories ($P = 0.001$; $\chi^2$ test). The lesion occurred in 37% of males and 26% of females (Table 2) evaluated; however, by $\chi^2$ analysis, this difference was not statistically different ($P > 0.20$). When clinical parameters in high-risk subjects with ASD were compared with those in high-risk subjects without ASD (Table 3), no differences in age or tobacco exposure were found.

Because this study only surveyed the prevalence of this lesion and not its long-term prognostic significance, no information is yet available on the prognostic importance of the lesion. However, we did note that the lesion was present in the airways 7 of 19 of the patients with primary carcinoma elsewhere in the lung. Ten of these tumors were squamous carcinoma, and six of the seven individuals with coexisting ASD and carcinoma had squamous carcinomas. In one patient with a central stage I carcinoma, ASD was contiguous with the invasive carcinoma (Fig. 7).

**Genetic Analysis**

**Chromosome 3p.** To assess the mutational status in the ASD epithelial component, a subset of biopsies from 20 consecutive subjects entered in the LIFE bronchoscopy protocol...
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shift (Fig. 8), was observed in one specimen. Microsatellite instability, presenting as a loss occur in ASD as compared to airway epithelium without specific histological abnormalities, nor did more extensive (66.7%). Thus, whereas a high incidence of LOH at 3p was analyzed separately. Loss was observed in at least one region in subjects, exhibited ASD, and these were microdissected and analyzed in this manner. Nineteen of these biopsies, from 12 different subjects, were analyzed for LOH in five regions on chromosome 3p. Fig. 8 contains a representative gel illustrating LOH in an epithelial sample as compared to the heterozygous stromal sample dissected from the same biopsy specimen.

A total of 53 biopsies from 20 individuals were evaluated in this manner. Nineteen of these biopsies, from 12 different subjects, exhibited ASD, and these were microdissected and analyzed separately. Loss was observed in at least one region in 8 of these 12 individuals. Fig. 9 contains a summary of the genetic analysis. The extent of loss varied, with some subjects exhibiting LOH only in one 3p region, whereas others had multiple areas of loss, as illustrated by patient 11. Ten of 19 biopsies with ASD (53%) harbored regions of 3p allelic loss. Subjects in our cohort without ASD had a similar rate of LOH (66.7%). Thus, whereas a high incidence of LOH at 3p was observed at sites of ASD, LOH was not preferentially associated with specific histological abnormalities, nor did more extensive loss occur in ASD as compared to airway epithelium without ASD.

As indicated in Fig. 9, a small number of cases were homozygous for all primers in a particular 3p region and therefore uninformative. Microsatellite instability, presenting as a shift (Fig. 8), was observed in one specimen.

**p53 Analysis.** No p53 mutations (codons 5, 7, and 8) were confirmed by direct sequencing of the ASD biopsies. As we reported previously (28), dysplastic bronchial epithelium from one patient did have a distinct p53 mutation at several endobronchial sites, including an ASD lesion, consisting of a G:C to T:A transversion in exon 7, codon 245 (GGC to TGC). To date, this p53 mutation is the only p53 abnormality detected in our cohort with ASD.

**Discussion**

The close juxtaposition of capillary tufts to dysplastic epithelium in the distinctive microscopic endobronchial lesion we refer to as ASD suggests that an angiogenic stimulus may be associated with epithelial dysplasia in the lower airways before the occurrence of lung carcinoma. These angiogenic changes were observed in many high-risk smokers and all patients with squamous cell lung cancer and may represent an important intermediate pathological biomarker preceding lung cancer development.

The histopathology of a similar lesion was described as early as 1949 in an autopsy series from Finland (30). No association with smoking was suspected at this time, which was before the landmark work of Wynder and Graham (31) linking tobacco smoking with lung cancer. In another autopsy study in 1963, a lesion described as “micropapillomatosis” was reported as an accompaniment of invasive lung carcinoma (32). Dysplasia of bronchial epithelium in “micropapillomatosis” as well as a possible link between angiogenesis and preinvasive bronchial epithelial dysplasia was recognized by Muller, who also described the ultrastructure of these lesions, including documentation of their capillary cores (33). However, these early studies have not been widely appreciated.

Currently, quantification of angiogenesis in human specimens relies on the counting of foci of vascular proliferation (“hot spots”) in sections stained with antisera to vascular markers such as coagulation factor VIII and CD-31 or CD-34 antigens (15). Such a method captures a snapshot of angiogenesis at a particular point in time but does not reflect the chronology of the process in the same tissue (34). The frequent finding of thickened basement membranes in ASD suggests variable remodeling of the vascular extracellular matrix over an extended period of time.

Until recently, no means of locating such a microscopic lesion in the lower airways has been available except for random biopsy. Our experience with this lesion suggests that it may be recognized in situ by fluorescence bronchoscopy. As summarized in Fig. 6, 59% of ASDs were abnormal under fluorescent light bronchoscopic examination but normal under white light. Detection of preinvasive lesions by fluorescence bronchoscopy is based on a reduction or quenching of the fluorescent signal in suspicious lesions. The biochemical basis for this phenomenon is unknown, but similar alterations in patterns of fluorescence have been observed in early diagnosis of cervical cancer (35). We believe that the increased or aberrant mucosal microvascularity associated with ASD results in increased blood content in the mucosa. This may result in quenching of the autofluorescent signal and contribute to the recognition of these lesions in the lower airway.

That the epithelium of ASD is abnormal is suggested not only by histological changes (dysplasia) but also by the increased proliferative rate of the epithelium and the presence of allelic loss in microdissected epithelial cells. The proliferative rate of normal respiratory epithelium, as measured by Ki-67 labeling index, is low (36–38). In this series, in histologically normal epithelium, the mean Ki-67 labeling rate was 1.8%, whereas that of ASD epithelium was 38% (P < 0.00005). The significantly elevated proliferative rate in ASD may be a reaction to airway injury or may be due to endogenous proliferative stimuli resulting from mutation.

Genetic alteration of ASD epithelium is indicated by allelic losses at chromosome 3p. For the present study, alleles in five 3p regions were chosen for study. These regions were previously shown to have undergone frequent heterozygous or homozygous loss in tumors and, in some cases, in preneoplastic epithelium as well. Sites of possible tumor suppressor genes are 3p14.2 and 3p21.31. The former region encompasses the most
common inducible fragile site in the human genome, FRA3b, as well as the fragile histidine triad (FHIT) gene. The latter region, 3p21.31, is gene rich and includes the H. SemaIV gene (39). LOH at 3p21.31 has been demonstrated in invasive lung tumors and preinvasive lesions (11). Homozygous losses have also been found here in a limited number of small cell lung cancer cell lines (27, 40). Other 3p regions in which homozygous losses have been found in lung cancer cell lines include 3p21.33, 3p13, and 3p12.3. Primers for all five of these 3p regions were used to amplify the DNA from microdissected epithelial cells from ASD in the current study.

Our findings document a high frequency of allelic loss by ASD epithelium at one or more of the tested loci on 3p, with two-thirds of subjects having lost at least one allele at one or more bronchial sites. No locus was preferentially affected, and losses appeared to be randomly distributed among the loci tested. Expansion of a single mutant epithelial cell may have occurred in some cases, as suggested by the finding that 17% of subjects exhibited loss of the same allele at more than one site. These findings indicate that the epithelium of ASD, in addition to being histologically abnormal, is often genetically altered as well. The allelic loss, however, cannot be interpreted as specific for ASD because 3p LOH has been reported in smokers regardless of histological appearance of the mucosa (9, 10).

Point mutation constitutes a second mechanism for tumor suppressor gene inactivation in lung cancer. \( p53 \) is of particular interest in this regard because loss of wild-type \( p53 \) in Li-Fraumeni fibroblasts results in reduction of the angiogenesis inhibitor thrombospondin-1 and conversion to an angiogenic phenotype (41, 42). At least one report indicates that \( p53 \) overexpression may be associated with angiogenesis in lung cancer (14), although this report has been contradicted by other studies using different endothelial markers to assess angiogenesis (43). \( p53 \) mutation in preinvasive bronchial epithelium has only rarely been reported. One study documented an increase in the number of mutant \( p53 \) alleles in bronchial biopsies from the same site over a 9-month period (44). Another has described the presence of a single \( p53 \) mutant clone distributed through broad areas of the respiratory tract in a high-risk smoker without lung cancer (28). With these exceptions, there have been no reports of \( p53 \) mutation in preinvasive bronchial epithelium, and it is widely suspected that \( p53 \) mutation occurs mainly at a late stage of lung carcinogenesis. Our results support this suspicion because we found no \( p53 \) mutations in the epithelium of the ASD lesions we tested. This result also suggests that \( p53 \) mutation is not critical for conversion to an angiogenic phenotype, and the critical genetic alterations required for ASD formation remain to be determined.

ASD clearly indicates abnormal vascularization of the bronchial mucosa that is reflected by both increased microvas-

### LOH at Chromosome 3p in ASD

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<th>Microsatellite Markers</th>
<th>Cases with at least one site of LOH</th>
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<tr>
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<td>D3S1573/D3S2968 (H. SemaIV Gene)</td>
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<td>D3S1577/D3S1604/D3S1776/D3S1274 (L2029 Homozygous Deletion)</td>
<td>12.1</td>
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Fig. 9: Chromosome 3p ideogram. The short arm of chromosome 3 is depicted, with loci used for assessing LOH indicated on the left. Results of the molecular analyses are indicated on the right by specimen and case. Each circle represents an individual biopsy, with several subjects having multiple biopsies. - at least one allelic loss at the locus; gray circle, retention of polymorphic alleles; and circle enclosing an X, uninformative loci that could not be tested.
cular density and aberrant morphology of bronchial capillary bed. Although microvascular density may be elevated in the stroma beneath epithelium exhibiting a range of histological abnormalities, the constellation of microscopic features defining ASD indicates a mechanistically distinct form of angiogenesis in airway mucosa. Possible mechanisms of angiogenesis in ASD may involve small populations of dysplastic squamous cells harboring premalignant mutations transmitting angiogenic signals over very short distances.

Formation of new blood vessels (“neovascularization”) is crucial for tumor cell growth beyond a certain restricted size and rate. Vessel formation is mediated by a complex network of molecular signals including both inducers and inhibitors of proliferation and migration of endothelial cells (reviewed in Refs. 12 and 15–17). Isolation and characterization of proangiogenic endothelial growth factors [such as vascular endothelial growth factor, fibroblast growth factors, and certain CXC chemokines (45, 46)] and endogenous angiogenesis inhibitors (thrombospondin-1, IFN-α/β, platelet factor 4, angiostatin, and endostatin) and delineation of the role of the extracellular matrix in neovascularization have provided potential molecular mechanisms for blood vessel growth during tumorigenesis (47).

The precise timing of angiogenic switching during lung carcinogenesis is not yet clear, but in locations more accessible than the lung, such as the cervix, there is evidence that switching to angiogenic phenotype occurs at a preinvasive stage (48). Better definition of the morphological and molecular pathways involved in premalignant bronchial neoplasia may lead to means of effective intervention in lung carcinogenesis before irreversible progression to invasive lung cancer takes place. At the present time, longitudinal studies are in progress to determine the long-term prognostic significance of ASD. The frequent occurrence of this lesion in high-risk smokers and in association with invasive carcinoma suggests that it may represent an important marker of lung cancer risk and may be a useful intermediate end point biomarker for chemoprevention studies. The demonstration that ASD occurs frequently in high-risk individuals suggests that antiangiogenic strategies may be effective in this subset of smokers at risk for lung cancer.

References


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