Multiple High-Grade Bronchial Dysplasia and Squamous Cell Carcinoma: Concordant and Discordant Mutations

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ABSTRACT

Loss of heterozygosity (LOH) involving chromosomes 3p, 5q, 9p, or 17p and aberrant expression or mutation of p53 are reported previously in selected bronchial dysplasias and squamous cell cancers (SCCs). Yet, comprehensive analyses of LOH patterns at these chromosomal sites and of p53 alterations are not reported for histologically normal bronchial epithelium, high-grade bronchial dysplasia, and SCC present in the same pulmonary resections. Whether concordant or discordant genetic changes are detected in these bronchial tissues, especially when multiple high-grade dysplastic bronchial lesions are present, was studied. Genomic DNA was microdissected from eight pulmonary SCCs and high-grade dysplastic lesions that were associated with SCC. In four cases, two independent high-grade dysplastic bronchial lesions were identified. When available, histologically normal bronchial epithelium was microdissected. Germ-line genomic DNA was isolated from normal lymph nodes. LOH was assessed for 15 microsatellite markers on chromosomes 3p, 5q, 9p, or 17p, sites frequently deleted in lung cancers. Immunohistochemical p53 expression was studied and correlated with p53 DNA sequence analyses. Progressive LOH for these markers was found when SCCs were compared with high-grade dysplasia and histologically normal bronchial epithelium present in the same resections. Histologically normal bronchial specimens had LOH in up to 27% of informative markers. High-grade dysplastic lesions exhibited LOH for 18–45% and SCC had LOH for 18–73% of the markers. Common regions of LOH were found in some dysplasias compared with SCCs. In other dysplasias, discordance was found relative to SCCs, especially for p53 mutations. In cases with a single or second high-grade dysplasia associated with SCC, heterogeneity in LOH markers was detected. These concordant and discordant changes were consistent with convergent and divergent clonal selection pathways in pulmonary squamous cell carcinogenesis. Some histologically normal bronchial epithelial tissues had genetic changes more similar to those in the SCCs than in dysplastic lesions. DNA loss or mutations accumulate in SCC, but discordant genetic changes can exist in the same carcinogen-exposed bronchial tissues. These findings have implications for lung cancer prevention trials.

INTRODUCTION

Lung cancer is a leading cause of cancer mortality for men and women in the United States (1). Although curative therapy for disseminated NSCLC does not yet exist, effective chemoprevention of lung tumors offers a therapeutic strategy for reducing lung cancer mortality. For this reason, it is important to identify genetic changes in epithelial lesions that precede development of invasive NSCLC. Once found, these could determine individuals at high risk for NSCLC. These individuals are potential candidates for lung cancer prevention trials.

Clinical and preclinical evidence support the view that pulmonary SCCs derive from progressive changes in the bronchial epithelium beginning with cellular metaplasia or atypia and progressing through varying degrees of dysplasia on to carcinoma in situ and then to invasive carcinomas (2–5). Those genetic changes that cause bronchial dysplastic lesions to progress to SCC are poorly understood. LOH involving chromosomes 3p, 5q, 9p, or 17p are reported frequently in selected preneoplastic or malignant lung lesions, and aberrant p53 expression or p53 mutations are also detected in these lesions (6–19). Of these genetic changes, LOH of chromosomes 3p or 9p are among the most common during lung carcinogenesis (6, 7, 8).

Prenecoplastic bronchial epithelial lesions often exhibit LOH with increasing incidence of LOH for chromosomal 3p and 9p microsatellite markers occurring during lung carcinogenesis (7, 8). Analysis of preneoplastic and malignant lung lesions derived from the same patient reveal that LOH involving chro-
MATERIALS AND METHODS

Pulmonary Tissues. Eight SCC cases containing high-grade bronchial dysplasia were identified retrospectively from the surgical pathology files at Memorial Sloan-Kettering Cancer Center. The relevant Institutional Review Board approved use of these found tissue specimens. The diagnosis of high-grade dysplasia was made in accordance with the WHO criteria (22). Two pathologists (F. L. and D. K.) confirmed independently the histopathology and that the high-grade dysplastic lesions were anatomic from the associated SCC. In three cases, histologically normal bronchial epithelium was available. Germ-line DNA was isolated from benign lymph nodes present in each pulmonary resection.

Immunohistochemistry. Immunohistochemical staining for p53 was performed on these lung tissues. Histologically normal bronchial epithelium, high-grade bronchial dysplasia, and SCC were each evaluated for p53 expression using established immunohistochemical methods (10, 21), and formalin-fixed and paraffin-embedded tissue sections were evaluated using the pAb1801 monoclonal antibody (Oncogene Sciences, Uniondale, NY).

Microdissection. Five-μm sections were obtained from paraffin-embedded tissue blocks and were mounted onto glass slides, using standard techniques (10, 21). Histologically normal bronchial epithelium, high-grade bronchial dysplasia, and SCC were microdissected individually using a standard microdissection microscope and routine techniques. Histologically benign lymph nodes were isolated from each case and were the source of germ-line genomic DNA that was used to determine LOH for each informative marker.

H&E staining of representative sections confirmed the precision of the microdissection. Unstained specimens were placed in xylene (Sigma, St. Louis, MO) solutions to dissolve the paraffin. Subsequently, 250 μl of 70% ethanol were added, and specimens were pelleted for 5 min using a microfuge. The xylene was removed, and the pellet was air-dried for 1–2 h. Tissue pellets were digested in 1% SDS with 5 mg/ml proteinase K added to the solution in a 50°C bath for 72 h or until the pellet was completely digested. Fresh proteinase K solution was added every 12 h. This solution was extracted three times with equal volumes of a phenol/chloroform solution saturated with Tris-EDTA buffer (pH 7.9). Genomic DNA was isolated from the aqueous phase by precipitation with ammonium acetate and cold 100% ethanol using glycogen (Boehringer Mannheim, Indianapolis, IN) as a carrier. Genomic DNA was precipitated at −20°C with a microfuge for 20 min. Pellets were washed twice with cold 70% ethanol and air-dried before suspension in Tris-EDTA buffer before storage at 4°C.

LOH Markers. LOH was evaluated by PCR assays using informative microsatellite markers and previously established techniques (7, 8, 13–15). The microsatellite markers were chosen to reflect those DNA regions frequently lost in preneoplastic bronchial lesions or SCC. These LOH markers were located on chromosomes 3p, 5q, 9p, or 17p. These regions encompass the p16 locus on chromosome 9p, frequently deleted segments of chromosome 3p, the commonly deleted region involving the MCC/APC loci on chromosome 5q, and an affected region of chromosome 17p.

The primer sequences for these markers were available from a microsatellite genomic database (23), and conditions for the PCR assays were optimized for each LOH marker. PCR assays were performed typically for 25–35 cycles at an annealing temperature of 55–62°C. Prior to annealing, reactions were incubated at 95°C before proceeding with the optimal cycle number for each LOH marker. The final extension reactions were for 5 min at 72°C. AmpliTaq gold (Perkin-Elmer, Norwalk, CT) was used as the polymerase. Reactions were performed with 32P- or 33P-cytosine nucleotides (Amersham, Piscataway, NJ). The reaction products were size-fractionated on a 6% polyacrylamide gel. Gels were dried and autoradiographed was performed using standard techniques. Autoradiographs were analyzed for evidence of LOH and were scored as showing
retention of heterozygosity, LOH, or noninformative loci or markers that were not evaluable for examination for technical reasons.

**DNA Sequence Analysis of p53.** Exons 5 through 9 of p53 were sequenced using established techniques (24) and genomic DNA was derived from histologically normal bronchial epithelium, high-grade bronchial dysplastic lesions, or SCC present in the same pulmonary resections. The desired DNA regions were amplified using PCR-based purification of two segments, a 500-bp segment spanning exons 5 and 6 and a 750-bp segment spanning exons 7–9. The desired PCR fragments were size-fractionated using agarose gel electrophoresis. These DNA fragments were purified using a Qiaex II gel extraction kit, (Qiagen Inc., Hilden, Germany). Sequence analysis was performed using a thermostequean kit (Amersham, Piscataway, NJ) and 33P- or 32P-labeled dideoxynucleotide to terminate the PCR reactions. The reaction products were then electrophoresed on a 6% acrylamide sequencing gel that was subsequently dried. Autoradiography was performed after exposure to XAR film (Eastman Kodak, Rochester, NY) at ~70°C for an appropriate length of time. Because of the limited genomic DNA isolated from the high-grade dysplastic lesions, the exon sequenced was that identified as mutant in the associated SCC. Mutations were confirmed independently by repeat sequence analysis after reamplification of the region of DNA containing the p53 exon of interest.

**RESULTS**

Genomic DNA was isolated independently from paraffin-embedded tissue sections obtained from eight cases with SCC in which at least one high-grade dysplasia was evident in the same pulmonary resection. Four of these primary SCC cases were associated with a single high-grade dysplastic lesion, whereas the remaining four cases had a second high-grade bronchial dysplasia. Histologically normal bronchial mucosa was available in three cases. Germ-line genomic DNA, isolated from a histologically benign lymph node from each case, was used to determine whether there was LOH for these microsatellite markers.

Results of the LOH analyses for each microsatellite marker appear in Fig. 1A. Informativity for each of the 15 LOH markers was greater than 50% except for the D9S162 marker for which only 2 of 8 examined cases were informative. Ninety-one loci were informative and 12 loci were invaluable because of insufficient genomic DNA isolated from the high-grade bronchial dysplasias. Fig. 1A maps comprehensively LOH for the depicted microsatellite markers in the histologically normal bronchial epithelium, high-grade dysplastic bronchial epithelial tissues, and associated SCC. Representative autoradiographs using radiolabeled PCR products and independent microsatellite markers for chromosomes 3p, 5q, 9p, or 17p are displayed in Fig. 1B. A representative result for an individual chromosome 3p marker examined in the histologically normal bronchial epithelium adjacent to two independent high-grade dysplastic lesions and an associated SCC from the same case is shown in Fig. 1C. A photomicrograph for p53 immunohistochemical expression in one representative case is displayed in Fig. 1D.

Progressive LOH was detected in the examined high-grade dysplastic lesions. LOH for informative markers is summarized in Fig. 2. Histologically normal bronchial epithelial specimens exhibited LOH in up to 27% of informative markers. High-grade dysplastic bronchial lesions exhibited LOH in 18–45% of cases using these markers. An even higher percentage (18–73%) of LOH was detected in the SCC cases. As lesions advanced from high-grade dysplasia to SCC, there is increasingly frequent genetic alterations. Notably, when two high-grade dysplastic lesions were examined for common or discordant mutations, each lesion was found to harbor LOH at chromosomes 9p and 3p, although regions that were lost occasionally differed. This discordance was most evident for p53 mutations. For example, in cases 1 and 3, discordant p53 mutations were found (see Fig. 1A and Table 1).

These cases exhibited common DNA regions lost in the SCC as compared with the associated high-grade dysplastic lesions. For instance, in case 1, two dysplastic lesions and the SCC each had LOH for the D9S1752 marker. Also, the high-grade dysplastic lesions and SCC derived from case 2 had common LOH for IFN-α on chromosome 9p and the D3S1110 marker on chromosome 3p. All of the preneoplastic and malignant lesions examined in case 3 had LOH for the D9S171 marker. All of the preneoplastic and malignant lesions examined in case 4 exhibited LOH for the D9S1747 marker. When two independent high-grade dysplastic lesions were analyzed (as in cases 1 through 4), these lesions displayed LOH for common loci, with as many as three markers having LOH. In cases 5 through 8, in which only one high-grade dysplastic lesion was associated with a SCC, these lesions had a similar spectrum of LOH. For cases 6 and 8, the preneoplastic and malignant lesions studied had an identical pattern of LOH involving chromosome 3p. These findings strongly implicate a clonal relationship between the cells present in these dysplastic lesions and those in the associated SCC.

There was heterogeneity in LOH detected in cases with two high-grade dysplastic lesions, as in cases 1 through 4. In each case in which two high-grade dysplastic lesions were present, there was at least one LOH microsatellite marker that did not show LOH concordance when these different dysplastic lesions were compared. Discordance was evident for some chromosomal 3p and 9p markers. Further discordance was noted when the LOH patterns were compared for the dysplastic lesions and the associated SCC.

LOH was identified in the histologically normal bronchial epithelium for chromosome 9p markers in cases 2 and 3. In case 2, the D9S171 marker exhibited LOH in the histologically normal bronchial epithelium and in the associated SCC, but not in either of the examined high-grade dysplastic bronchial lesions. In case 3, the D9S171 marker was lost in the histologically normal bronchial epithelium, in two dysplastic lesions, and in the SCC present in this resection. D9S1747 and D9S1751 markers were lost from chromosome 9p in the histologically normal bronchial epithelium in this case. These findings indicate how genetic alterations can be detected in the bronchus even when there is no histological evidence of preneoplastic changes in the bronchial epithelium. For example, in case 3, the normal bronchial epithelium had more frequent genetic alterations involving chromosome 9p markers than either high-grade dysplastic bronchial lesion present in this pulmonary resection. This is...
Fig. 1 A, the LOH for chromosome 3p, 5q, 9p, or 17p and presence (+) or absence (−) of p53 mutations (p53 mut.) in histologically normal or preneoplastic bronchial tissues associated with SCC. This study demonstrates the frequent LOH at chromosomes 3p and 9p in bronchial preneoplasia and in malignant (CA) bronchial epithelium. These analyses represent a comprehensive evaluation for these microsatellite markers in histologically normal bronchial epithelium (NBE), high-grade dysplasias (DysA and DysB), and SCC present in the same pulmonary resections. Concordant and discordant mutations are detected. B, LOH (arrows) for microsatellite markers are depicted for chromosomes 3p (D3S1007), 9p (D9S1747), 5q (MCC), and 17p (17p2) markers. This LOH study depicts representative results using microsatellite markers for these chromosomal sites assayed using genomic DNA from normal lymph node (N) and malignant lung tissue (T) derived from the same surgical resections. These representative autoradiographs were obtained using 33P PCR products after PAGE and size-fractionation. These and other LOH markers were used to generate the data displayed in A. C, LOH (arrow) for a representative microsatellite marker for chromosome 3p (D3S1110) analyzed in normal bronchial epithelium (N) adjacent to two independent high-grade dysplastic lesions (DysA and DysB) and to an associated SCC (T). Similar analyses were performed using normal bronchial epithelium, high-grade bronchial dysplasias and SCC to obtain the findings shown in A. D, a representative photomicrograph showing positive staining for p53 in a nest of invasive SCC. In contrast, the surrounding stromal cells did not stain for p53. Immunohistochemistry for p53 was performed as described in “Materials and Methods,” and findings are summarized in Table 1.
Fig. 2  Progressive accumulation of LOH markers in histologically normal versus preneoplastic and malignant bronchial epithelium. This study reveals the frequent LOH evident in the SCC (CA), and less frequent LOH in high-grade dysplasias (DysA and DysB) present in the same pulmonary resections. LOH was examined in the histologically normal bronchial epithelium (NBE) in cases 1 through 3.

Table 1  Analysis of p53 immunostaining and p53 mutations in the histologically normal bronchial epithelium, high-grade bronchial dysplasias (designated DysA and DysB), and associated SCC (CA).

<table>
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<tr>
<th>Case no.</th>
<th>Histology</th>
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<th>Affected codon</th>
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<td>+</td>
<td>163</td>
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<td>Tyr→stop</td>
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<tr>
<td></td>
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<td>163</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>198</td>
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</tr>
<tr>
<td></td>
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<td>−</td>
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<td>NE</td>
<td>NE</td>
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a  NBE, histologically normal bronchial epithelium; WT, wild type; NE, not evaluated; FS, frame shift.

demonstrated in the LOH for D9S171, D9S1747, and D9S1751 chromosome 9p markers.

Analyses of LOH for microsatellite markers associated with the MCC locus on chromosome 5q reveal that four of the SCC cases exhibited LOH at this site. Two high-grade dysplastic lesions had LOH at chromosome 5q. Both were associated with SCCs that harbored this mutation. LOH for the p53 microsatellite marker was detected in three of six informative SCC cases. Two of these cases contained p53 mutations, and one contained wild-type p53 sequences within exons 5–9 (Table 1). Three high-grade dysplastic bronchial lesions exhibited LOH for the chromosome 17p marker and were associated with SCC that exhibited this LOH. In case 3, the SCC and one high-grade dysplastic bronchial lesion (designated DysA) exhibited LOH for the p53 microsatellite marker and the same p53 mutation was identified in these lesions. This mutation was absent in the
second dysplastic bronchial lesion (designated Dysb) that exhibited loss of the chromosome 17p LOH marker (Table 1).

DISCUSSION

Squamous cell carcinogenesis is a multistep process. For SCC, precursor lesions are often evident. These include hyperplasia, metaplasia, low grade or high-grade dysplasia, and in situ carcinoma. Focal cytological atypia is also observed. Bronchial lesions diagnosed as carcinoma in situ are typically viewed as preceding development of invasive and subsequent metastatic SCC. This study was undertaken to examine comprehensively for LOH at chromosomes 3p, 5q, 9p, and 17p in high-grade dysplastic bronchial lesions associated with a resected SCC. Findings indicate that common regions of chromosomal loss were detected in different high-grade dysplasias associated with a SCC.

Clonal divergence was found in some dysplasias as compared with the associated SCC. This divergence was evident when p53 mutations were searched for in different preneoplastic and malignant lesions. Although concordant genetic alterations were identified in different high-grade dysplasias, discordance was also observed. Notably, histologically normal bronchial epithelium can exhibit genetic alterations similar to those present in an associated SCC. This indicates how some individuals having histologically normal bronchial epithelium may still be at risk for lung cancer development. This study confirmed that pulmonary SCCs exhibit LOH at chromosomes 3p, 5q, 9p, or 17p. Aberrant p53 expression or p53 mutations are evident in some of these carcinomas. LOH for chromosomes 3p, 5q, 9p, or 17p and p53 alterations are reported in selected preneoplastic and malignant bronchial lesions (6–19). This study advances prior work by conducting a comprehensive analysis investigating the frequency and nature of these genetic changes in synchronous preneoplastic lesions and SCC present in the same pulmonary resections. LOH and mutational results were compared with findings obtained using germ-line DNA isolated from histologically benign lymph nodes present in each pulmonary resection.

Findings indicate that progressive LOH occurs for chromosomal markers on 3p, 5q, 9p, or 17p when high-grade dysplastic bronchial lesions are compared with SCC present in the same pulmonary resection. Histologically normal bronchial epithelium exhibited LOH for some markers. More frequent LOH was detected in associated bronchial preneoplasia. SCC exhibited the highest incidence of LOH for examined markers, as displayed in Fig. 2. When a second high-grade dysplasia was present, discordant LOH or DNA mutations were often detected. This was apparent when mutations for p53 were examined, as shown in Table 1. Some histologically normal bronchial epithelial specimens exhibited similar LOH patterns as in associated SCC, whereas LOH patterns for other high-grade dysplasias differed from those in SCC. This is shown in analyses of cases 1, 3, 5, and 6 in Table 1. This demonstrates how discordant p53 mutations are frequent in synchronous preneoplastic and malignant pulmonary lesions. These findings are consistent with discordant p53 immunostaining and mutational results, as previously reported in NSCLC (25).

Loss of genetic material on chromosomes 3p and 9p are early events in squamous cell carcinogenesis (7, 8). This chromosomal loss leads to inactivation of several known or potential tumor suppressors. Aberrant expression of G1 cyclins is reported in bronchial preneoplasia (21). These cyclins have been proposed as therapeutic targets for lung cancer prevention (26–28). These genetic changes can lead to cell cycle deregulation and uncontrolled proliferation that results in genomic instability and further abnormalities. Genomic instability is reflected in the findings displayed in Fig. 1. Clones of altered bronchial epithelial cells may acquire additional genetic changes that confer a growth advantage to these cells.

This study reports that clonal divergence is frequent because discordant p53 mutations are detected in preneoplastic versus malignant bronchial tissues (Table 1). Aberrant p53 expression was reported previously at late stages of bronchial preneoplasia (10, 21). These findings and that reported in this study indicate that p53 alterations are late events in squamous cell carcinogenesis. Aberrant expression of G1 cyclins (21) and the epidermal growth factor receptor is more frequent in bronchial preneoplasia than is altered p53 expression (10). How these changes in gene expression cooperate with chromosomal LOH is the subject of future work. Specific cassettes of dominant or recessive genetic events might be required to transform these preneoplastic lung lesions.

The pattern of genetic changes identified in these dysplastic lesions was often similar to those present in associated SCC. For example, LOH at chromosomes 5q or 17p present in dysplastic lesions was also detected in the associated SCC. Whereas discordant p53 mutations were evident in some SCC as compared with high-grade dysplasia, concordant mutations were detected in two cases. This reveals how a close genetic relationship can exist between SCC and associated preneoplastic lesions. Thus, multiple pathways may exist for malignant progression of preneoplastic lesions present in the same carcinoma-exposed bronchial epithelial field (29).

The findings reported in this study have implications for understanding squamous cell carcinogenesis and for developing lung cancer prevention strategies. Genetic deletions, especially involving chromosomes 3p and 9p, are not uncommon in histologically normal bronchial epithelium and dysplastic lesions of individuals exposed to tobacco-derived carcinogens. These deletions are often similar or identical to those in the associated SCC but can diverge from genetic changes in a second dysplasia. These and other findings are summarized in Fig. 3, which emphasizes how many carcinogenic steps precede and follow development of high-grade bronchial dysplasias. Solid arrows in Fig. 3 indicate how sequential alterations in preneoplastic bronchial lesions accumulate genetic changes in tissues before invasive SCC arise. A clinical implication of these findings relating to lung cancer prevention is depicted by dashed arrows in this figure.

It is known that clonal populations or fields (29) of bronchial epithelial cells acquire genetic mutations (13) even before high-grade dysplasia is evident, as shown in Fig. 1A. Distinct subsets of clonal bronchial epithelial cells can exist in the epithelium (13). The fate of these genetically altered cells is uncertain, as depicted by the dashed arrows in Fig. 3. It is not known whether these altered epithelial cells ultimately contribute to the development of a malignant lung tumor. Yet, evidence
for similar genetic changes in the carcinogen-exposed normal bronchial epithelium and associated precancerous or SCC indicate that these histologically normal bronchial epithelial cells may place the affected epithelium at risk for neoplastic transformation. On the basis of these findings and others reported in the bronchial epithelium of current or former smokers (30) and in the bronchial epithelium adjacent to small cell lung cancers (31) or NSCLC (13), future clinical trials might target genetically altered but histologically normal bronchial epithelium for treatment with chemopreventive agents to inhibit or block neoplastic progression.

In summary, this study demonstrates that concordant and discordant mutations exist in histologically normal bronchial epithelium and high-grade bronchial dysplasias associated with SCC. Progressive LOH is found for microsatellite markers of chromosomes 3p, 5q, 9p, or 17p. Aberrant expression or mutations of p53 are late events in bronchial neoplasia. Discordant mutations (especially involving p53) are evident in some preneoplastic lesions when compared with associated SCC. Because similar mutations can occur in histologically normal and preneoplastic or malignant bronchial epithelium, this should be considered in the design of lung cancer prevention trials.

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