

Persistence of Bronchial Dysplasia Is Associated with Development of Invasive Squamous Cell Carcinoma

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Abstract

Bronchial dysplasia (BD), a presumed precursor of pulmonary squamous cell carcinoma (SCC), rarely progresses to invasive cancer. A high-risk cohort at the University of Colorado provided an opportunity to directly sample airway epithelium at mapped sites on successive bronchoscopies. We have hypothesized that persistent dysplastic lesions showing a similar or higher level of dysplasia on follow-up biopsy, are associated with increased risk for the development of SCC. Endoscopic biopsies from 188 high-risk subjects were histologically classified according to the current WHO classification for BD using a numeric histology score ranging from 1 to 8 representing normal bronchial mucosa through invasive lung cancer. Differences in follow-up histology scores were compared between sites classified by clinical, histologic, and immunohistochemical variables. Subjects with a higher frequency of

sites that persist or progress to high-grade dysplasia ($\geq 37.5\%$ persist/progress, $N = 35$ versus $< 37.5\%$ persist/progress, $N = 114$) show a significant association with development of incident invasive SCC (adjusted HR, 7.84; 95% confidence interval, 1.56–39.39), and those with incident lung SCC have adjusted mean follow-up histology scores 1.55 U higher than in subjects without lung cancer. Current smoking, elevated Ki67 growth fraction, histologic features of angiogenic squamous dysplasia (ASD) and higher histology score in baseline biopsies are significantly associated with increased follow-up histology scores. These results show that persistent BD is associated with the development of invasive SCC. Furthermore, increased expression of Ki67, the presence of angiogenic change and degree of baseline atypia are associated with persistence of BD. *Cancer Prev Res*; 9(1); 96–104. ©2015 AACR.

Introduction

Bronchial dysplasia (BD; ref. 1) is a presumed precursor lesion of squamous cell carcinoma (SCC) of the lung, and elimination of these lesions has been proposed as a way to prevent invasive SCC of the lung (2). A scoring system for BD that stratifies these lesions by severity of squamous atypia has provided a framework for using histology scores to assess the effect of chemoprevention agents on bronchial mucosa (3–6). Understanding the behavior

of BD could provide means to assess risk for the development of lung cancer and elucidate mechanisms underlying progression.

Molecular alterations that parallel those seen in invasive cancer for site-specific loss of heterozygosity (LOH), and mRNA, miRNA, and immunohistochemical marker expression levels that become more prominent in higher grades of BD have provided data to support a relationship between BD and invasive SCC of the lung (7–10). In addition, chromosomal aneusomy, gene copy-number gains, increased PI3K pathway activation, and alterations in telomere length have been shown to be more common in BD from patients with known lung cancer as compared with those without (11–14). A relationship between increased expression of multiple tumor-related markers or specific LOH in BD and subsequent development of carcinoma-*in-situ* (CIS) or SCC has also been shown (15, 16). In a few selected cases, an increase in SOX2 amplification has been described as sites of BD progressed in atypia and ultimately developed invasive SCC (17). Progression of atypia or development of cancer has also been related to baseline histologic atypia and other clinical features, but has frequently produced contradictory findings. Although some studies have correlated baseline histology with smoking status, more frequent progression of atypia or development of cancer (18–23), others have failed to detect significant relationships with these parameters (15, 18, 24). Important issues compromising interpretation of these data include use of CIS as a malignant outcome, small numbers of cases per individual report, frequent pre-selection of cases with some cohorts being composed entirely of patients with previous lung or head and

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neck cancer, confounding of outcome by use of therapeutic intervention, and variable, often short periods of time to follow-up assessment of lesions. The limitations associated with these factors were also noted in a recent meta-analysis, in which findings suggested that higher degrees of atypia in BD are associated with more frequent progression or persistence than are lesions of lower grade (25). These findings were not related to development of lung cancer.

Prediction of outcome in BD is of paramount importance to the establishment of reliable, informative screening programs, and effective prevention measures. Surveillance techniques such as autofluorescence bronchoscopy have improved sensitivity for detection of BD (26–28), thus providing an opportunity to accurately follow these lesions over time. We have examined the relationship between differences in follow-up histology scores and a variety of parameters and found that more atypical outcomes are associated with subsequent incidence of SCC, increased baseline histologic atypia, smoking status, features of angiogenic squamous dysplasia (ASD), and Ki67 expression levels. We have identified a pattern of persistent BD that defines a subset of subjects with aggressive airway disease and increased risk for development of invasive SCC that may benefit from close follow-up and potential preventive measures.

Materials and Methods

Patients at high risk for the development of lung cancer were recruited to bronchoscopy protocols established through the Colorado SPORE in Lung Cancer program. High-risk subjects included those with tobacco smoking histories of greater than 20 pack years and those with a personal history of lung cancer or prior cancer of the upper aerodigestive tract. In subjects without a history of cancer, sputum screening was performed and bronchoscopy was offered to individuals with moderate or worse cytologic atypia. Informed consent for enrollment in the protocols and collection of associated clinical data were approved by the Colorado Multiple Institutional Review Board (CoMIRB). Histologic, clinical, and other data from subjects for which there was any bronchial site that was sampled on at least two occasions

were assembled for this study. Specimens that were collected less than 3 months after the original baseline biopsy at a specific site were excluded. During the timeframe of specimen collection (1993–2010), two Colorado Lung SPORE sponsored chemoprevention trials were conducted in which all-trans retinoic acid or the prostacyclin analog, Iloprost, were used as potential chemopreventive agents (3, 4). Iloprost, but not all-trans retinoic acid, was shown to be associated with a significant reduction in histology score on follow-up biopsy among former smokers as compared with former smokers on the placebo arm of the study. Therefore, all biopsies from treatment arm subjects collected after the Iloprost trial enrollment bronchoscopy were excluded.

Biopsy site classification

Biopsies were assigned a numeric histology score (see Fig. 1) ranging from 1 to 8 for normal (score = 1), basal cell hyperplasia (score = 2), squamous metaplasia without atypia (score = 3), mild dysplasia (score = 4), moderate dysplasia (score = 5), severe dysplasia (score = 6), CIS (score = 7), and invasive carcinoma (score = 8) with each group being defined by the histologic features described in the WHO classification (1). Baseline histologic score was defined as the diagnosis for the first biopsy at a given site. Follow-up histologic scores were classified into three groups: those with biopsies collected between 3 months to 2 years, 2 to 4 years and >4 years after baseline biopsies. If more than one biopsy had been collected during the timeframe of one of these groups, the biopsy with the highest diagnosis was used. Most analyses used grouping of histology scores into nondysplastic (scores 1–2), low-grade dysplasia (LGD, scores 3–4), and high-grade dysplasia (HGD, scores 5–7) histologic groups. In analyses comparing groups defined by a pre-assigned persistent/progressive or regressive classification, persistent/progressive dysplasia (referred to as "persistent" BD throughout manuscript) was defined as any baseline LGD that showed LGD or higher histologic score on follow-up and any baseline HGD that showed HGD or higher histologic score on follow-up unless otherwise noted. Biopsies of histology score 3 or greater were also characterized as ASDs if they showed projections of vascular structures into

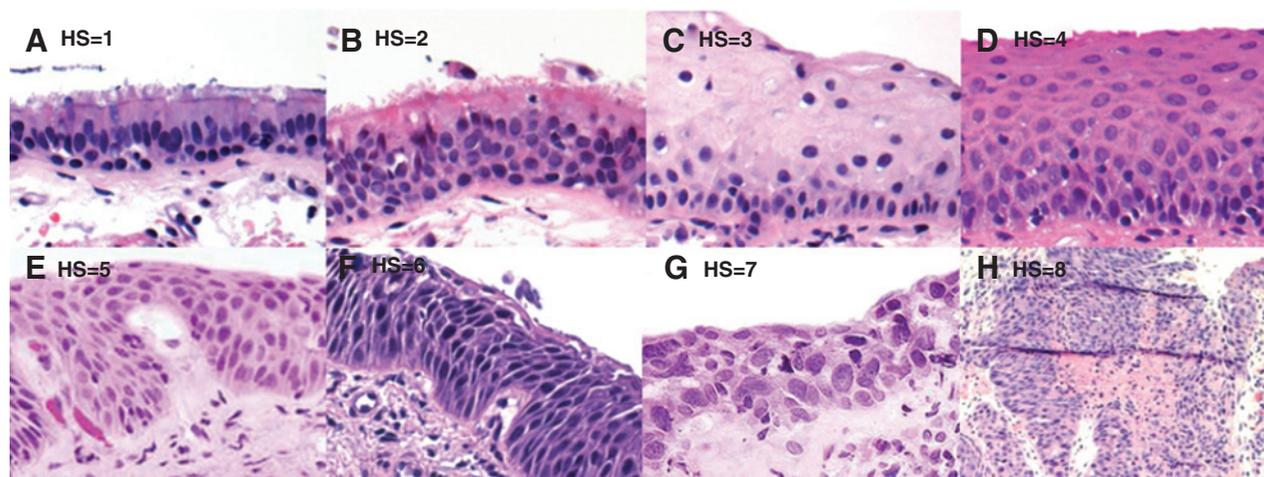


Figure 1.

Photomicrographs demonstrating histologic features and histology scores (HS) of bronchial epithelium from sites of nondysplasia (A, normal; B, reserve cell hyperplasia), low-grade dysplasia (C, squamous metaplasia without atypia; D, mild dysplasia), high-grade dysplasia (E, moderate dysplasia; F, severe dysplasia; G, CIS) and invasive SCC (H). Note, vascular papillary projections of ASD in E and F; Magnification, A–G, $\times 400$; H, $\times 100$.

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overlying epithelium as described in previous publications (29) and shown in Fig. 1E and F.

Cancer status was based on subject-level tissue diagnoses with incident carcinoma defined as those cases in which a diagnosis of invasive carcinoma was made 6 months or longer after the date of the baseline bronchoscopy. All other subjects with known lung cancer diagnoses were considered prevalent (or prior) cancer cases. Twenty three of the 41 cases with associated lung carcinomas demonstrated incident carcinomas (in one subject, two synchronous, incident carcinomas occurred in contralateral lung lobes). Fourteen of these were SCC, 4 adenocarcinoma, and 6 not otherwise specified (NOS). Tumor diagnoses and sites were established by bronchoscopic biopsy in 8 cases (all SCC), cytologic sampling in 3 (2 SCC and 1 adenocarcinoma) or resection specimens in 7 (4 SCCs, 2 adenocarcinomas, and 1 NOS), whereas in 6 cases the method of diagnosis/site were unknown (1 adenocarcinoma and 5 NOS). Among the incident cases, 6 represented second or higher primaries (4 SCC, 1 adenocarcinoma, and 1 NOS). Among the 18 cases associated only with prevalent lung carcinomas, 12 were SCC, 5 adenocarcinomas, and 1 NOS. Throughout the article, analyses of cancer-associated cases indicate whether this group includes prevalent and incident cancers or incident or prevalent cancers alone. Each baseline biopsy was associated with clinical data that were indexed at the time of the baseline biopsy, including subject age, gender, smoking status (current, former, and never), and pack-year smoking history. A subject was considered a former smoker if they had quit at least 12 months before the baseline biopsy was collected.

Immunohistochemistry

All immunohistochemical (IHC) stains had been performed previously in studies assessing the relationship between bronchial histology and marker expression levels (3, 4, 9). None of the markers, except for a subset of Ki67, had been previously correlated with follow-up histologic scores of biopsy sites. The Ki67 scores represent an expanded set of data, and this analysis of the relationship between expression of this parameter and lesion outcome has not been previously published. IHC analyses used marker levels from the first biopsy at a site stained with the marker. Follow-up histology scores were based on biopsies collected subsequent to the biopsy on which the IHC was performed. IHC scoring has been previously described (3, 4, 9). Briefly, EGFR and HER2 immunostains were classified as normal if the staining was confined to the basal layer of the bronchial epithelium and overexpressed if the staining was seen to extend into the upper half of the epithelium. Ki67, MCM2, and p53 were scored as the percentage of positive epithelial cells with a goal of counting 400 cells per biopsy from the area that established the diagnosis for that site.

Statistical analysis

Descriptive statistics were used to summarize demographic and clinical characteristics of the data. Mean, SD, median, and range were used for continuous variables and frequency and percentage were used for categorical variables. The follow-up time was grouped into three periods: 3 months-2 years, 2-4 years, and greater than 4 years. The dysplasia status was categorized into persistent/progressive or regressive as described in the earlier section. The χ^2 test was used to assess the association between grouped baseline histologic score distribution (nondysplastic,

LGD, and HGD) and demographic or clinical characteristics. Multivariable linear mixed effects models were used to evaluate the association between the follow-up histology score, primarily at the window of 3 months to 2 years, and predictor variables including dysplasia status, cancer status, tobacco use, smoking status, and biomarker expression, while adjusting for baseline histology score and gender, etc. Correlation among biopsies at different sites from the same patient was accounted for by including a random patient effect. Kaplan–Meier survival curves were obtained for developing SCC based on categorized persistent dysplasia. Cox proportional hazards regression models were used to estimate HRs for developing cancer for persistent dysplasia while adjusting for worst baseline diagnosis score and smoking status only due to a limited number of available events. Persistent dysplasia was modeled as a continuous variable and also as a categorical variable based on quartile cutoff values of its distribution initially, and then combining categories if similar survival functions were observed based on the $\log[-\log(\text{survival curves})]$. The more appropriate functional form between the continuous and categorical was then selected on the basis of the smaller AIC (Akaike information criterion). A bootstrap sampling approach was used to obtain robust HR estimates and their associated 95% confidence limits. Overall predictive accuracy of the Cox model was assessed using ROC curves following the approach of Heagerty and Zheng (Heagerty and Zheng 2005 survival model predictive accuracy and ROC curves). SAS 9.2 (SAS Inc. Cary) and R (3.12) were used for analysis. A *P* value of ≤ 0.05 was considered statistically significant throughout the article.

Results

Study subject and biopsy site characteristics

Three thousand and forty-two biopsies representing 1,170 biopsy sites from 188 subjects were included in the analysis (see Supplementary Table S1). Four hundred and two sites were sampled on three or more occasions. Subjects were more frequently male than female (72.3% male), and ages ranged from 39 to 83 (mean and median 61) years. There were fewer current (79) than former (104) smokers and five subjects were never smokers. One hundred and forty-eight biopsy sites were from 41 subjects with diagnoses of lung cancer, and 75 of these sites were from 23 subjects with incident carcinomas. Two hundred and two biopsies with histologic features of ASD were associated with follow-up biopsies. Table 1 describes the relationship between baseline histology score and a variety of clinical characteristics. Strong, significant correlation between higher baseline histology score and carcinoma status, cancer subtype, tobacco use, tobacco pack years, gender and age were noted. As described below, several of these variables were also associated with differences in follow-up histology scores although age and gender were never or only infrequently associated with differences in follow-up histology scores, respectively. This led to the inclusion of all of these parameters except age in multivariable analyses of differences in follow-up histology score presented below. Male gender was adjusted for in those analyses when sample size or number of events was adequate to allow for this.

Baseline histology is directly correlated with persistence on follow-up biopsy

Each site was evaluated for follow-up histology score at three, 2-year interval follow-up time periods. A direct comparison of the

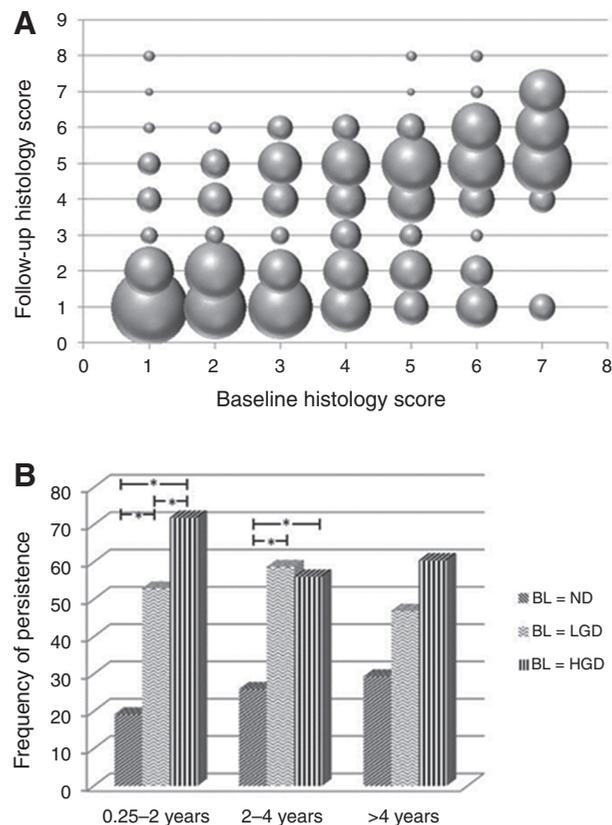
Table 1. Histologic and clinical characteristics of baseline biopsies

	Non-dys (%)	LGD (%)	HGD (%)	P
Lung cancer status^a				
Cancer negative	598 (59.0)	179 (17.7)	237 (23.4)	<0.001
Prevalent lung cancer	34 (46.6)	8 (11.0)	31 (42.5)	
Incident lung cancer	33 (42.9)	14 (18.2)	30 (40.0)	
Lung cancer subtype^b				
Cancer negative	598 (58.7)	179 (17.8)	237 (23.5)	<0.001
SCC positive	34 (37.6)	19 (16.8)	53 (45.5)	
Adenocarcinoma positive	16 (85.0)	1 (5.0)	2 (10.0)	
Tobacco status				
Current smoker	231 (42.8)	138 (25.6)	171 (31.7)	<0.001
Former smoker	423 (68.9)	66 (10.8)	125 (20.4)	
Never smoker	13 (81.3)	0 (0.0)	3 (18.8)	
Tobacco—pack years				
Never smoker	13 (81.3)	0 (0.0)	3 (18.8)	<0.001
0–40 pack years	273 (64.8)	73 (17.3)	75 (17.8)	
40–72 pack years	238 (48.7)	107 (21.9)	144 (29.4)	
>72 pack years	143 (58.6)	24 (9.8)	77 (31.6)	
Gender				
Male	478 (54.9)	162 (18.6)	231 (26.5)	0.0369
Female	189 (63.2)	42 (14.1)	68 (22.7)	
Age				
<55 y.o.	189 (59.1)	66 (20.6)	65 (20.3)	0.0079
55–65 y.o.	259 (52.6)	88 (17.9)	145 (29.5)	
>65 y.o.	219 (61.2)	50 (14.0)	89 (24.8)	

NOTE: Correlation between baseline histology and clinical variables.

^aCancer status unknown for 6 sites.^bCancer subtype unknown for 31 sites.

frequency of follow-up histology scores at each unique baseline histologic diagnosis indicates two distinct groups of lesions at 3 months to 2 years of follow-up: Those that show dysplasia (histology score ≥ 3) and those that are nondysplastic (histology score < 3) on repeat biopsy (Fig. 2A). These follow-up data show that the proportion of follow-up biopsies with dysplastic morphology increases with increasing baseline histology score. To investigate this relationship, sites with LGD or HGD were compared with those with nondysplastic histology at baseline and found to show significantly higher frequencies of persistence (follow-up histology score of 3 or greater) in the 3 months to 2 years follow-up period with crude risk ratios (RR) of 2.68 [95% confidence interval (CI), 1.66–4.34] and 5.22 (95% CI, 3.39–8.04), respectively (Fig. 2B). Persistence was more frequent in the HGD versus the LGD baseline groups in this time interval (crude RR 1.95, 95% CI, 1.21–3.14). Significantly higher frequencies of persistence for baseline LGD and HGD compared with nondysplastic sites were also seen at 2 to 4 years of follow-up (LGD: crude RR, 2.19; 95% CI, 1.15–4.18; HGD: crude RR, 2.13; 95% CI, 1.14–3.95). Although a similar trend was seen in the analysis of sites with follow-up biopsies collected greater than 4 years post-baseline biopsy (Fig. 2B), these differences in frequency were not statistically significant possibly due to much smaller number of sites followed for this length of time (see Supplementary Table S1). Multivariable linear mixed effects model analyses showed that adjusted follow-up histology scores in the 3 months to 2 year post-baseline group were on average 0.68 (95% CI) units higher in HGD compared with LGD baseline sites and 0.78 (95% CI) units higher in LGD compared with nondysplastic baseline sites following adjustment for tobacco status (current/former/never), pack-year tobacco exposure and cancer status (Supplementary Table S2). Similar, statistically significant, but progressively smaller differences were seen in the 2 to 4 year and >4 year follow-up groups with the exception that the mean follow-up histology

**Figure 2.**

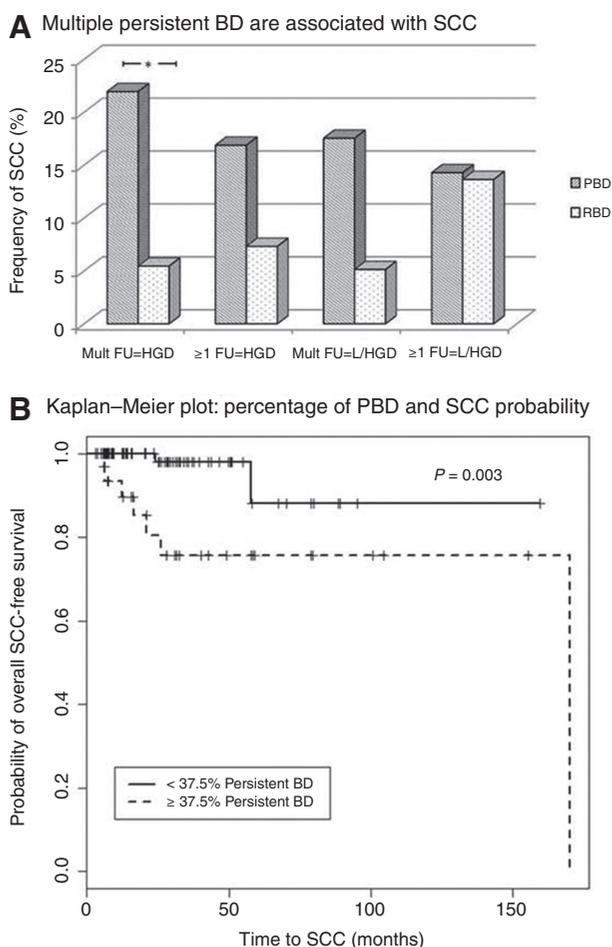
A, the plot of the frequency of specific follow-up histology scores (3 months–2 years post-baseline biopsy) associated with a given baseline histology score. The area of each sphere is proportional to the percentage of biopsies with the baseline histology score shown on the x-axis that correspond to the follow-up histology score represented on the y-axis. B, the frequency of persistence (follow-up histology score greater than or equal to 3) in nondysplastic (ND), low-grade (LGD), and high-grade (HGD) dysplasia baseline (BL) groups at three follow-up time intervals. *, $P < 0.05$ after adjustment for age, tobacco status (current/former/never), pack-year exposure, and cancer status.

scores were nearly identical in the baseline LGD and HGD groups for the 2 to 4 year follow-up period (Supplementary Table S2).

Relationship between development of lung cancer and persistence of BD

Per subject classifications of airway disease were undertaken to determine whether persistence of BD could act as an indicator of risk for lung cancer in patients undergoing bronchoscopic evaluation. Two subgroups of cases with baseline BD (LGD or higher) were assessed. These subgroups were composed of cases with one or more BDs at baseline or cases with multiple (2 or more) BDs at baseline. For each of these subgroups two subject level definitions of persistent BD were used. In one set, persistent BD was defined as presence of LGD or HGD in follow-up biopsies of sites with BD at baseline, and in the second, presence of HGD only on follow-up biopsy of dysplastic baseline sites was considered to represent persistent BD. χ^2 analyses revealed a statistically significant correlation with SCC for the persistent group defined by multiple dysplastic sites persisting as or progressing to HGD but not for the three other alternatively defined persistent BD groups (Fig. 3A).

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**Figure 3.**

A, univariable analysis comparing frequency of SCC using four different definitions of persistent BD (PBD; RBD = regressive BD) for subjects with dysplasia at baseline (BL HS > 3). The presence of two or more dysplastic sites that persist or progress to HGD (Mult FU = HGD, $n = 96$) shows a statistically significant correlation with presence or development of SCC ($P = 0.02$). When PBD is defined as one or more dysplastic sites with HGD on follow-up (>1 FU = HGD, $n = 120$; $P = 0.14$), two or more with low- or high-grade dysplasia on FU (Mult FU = L/HGD, $n = 96$; $P = 0.07$) or one or more with L/HGD on FU (>1 FU = HGD, $n = 120$; $P = 0.63$), PBD is not correlated with SCC. B, the Kaplan–Meier plot demonstrating significantly higher probability of developing SCC in patients ($n = 35$) with 37.5% or more of all sites showing persistence as or progression to HGD compared with those for patients ($n = 114$) with less than 37.5% of their dysplastic sites showing persistence as or progression to HGD ($P = 0.003$ by log-rank test).

The same relationship was found when considering persistence or progression to dysplasia for sites of any baseline histology (Supplementary Fig. S1). Cox proportional hazards regression analysis was used to assess the association between the time to diagnosis of incident SCC and the percentage of sites with HGD on follow-up biopsy. The model fit criteria indicated that the data fit either a continuous or dichotomized model equally well. When considered as a continuous variable with correction for tobacco status and baseline diagnosis, the percentage of persistent sites showed an HR of 1.34 (bootstrap 95% CI, 1.03–1.97; $P = 0.017$; incidence of SCC, $n = 9$) for every 10% increase in the percentage of persistent sites. Dichotomization was based on a cutoff ($\geq 37.5\%$ of dysplastic sites showing persistence vs. less) value

selected using AIC as described in the statistical methods section that corresponded to the 75th percentile of the percentage of persistent dysplastic sites. An HR of 7.84 (95% CI, 1.56–39.39; $P = 0.003$) was obtained for the group in which 37.5% or more sites showed persistence as or progression to HGD compared with the group with less frequent persistence (Fig. 3B). However, this relationship was not significant and showed a much wider confidence interval using a bootstrap sampling approach (HR, 6.63; 95% CI, 0.44 – $>10^7$), indicating that larger numbers of case events are needed to define a reliable cutoff for risk and a more precise estimate of the HR. The mean baseline histologic score, but not highest baseline histology score, also showed a correlation with SCC in univariable analyses (Supplementary Fig. S2). When mean baseline histology score was treated as a continuous variable, a 2.4-fold (95% CI, 1.2–4.6) increase in the hazard for developing SCC with each unit increase in histology score was seen. However, a statistically significant cutoff value incorporating time to diagnosis for risk of invasive SCC based on mean baseline histology score could not be identified.

Comparison of follow-up histology scores in SCC, adenocarcinoma, and non-cancer-associated cases

A determination of the degree of difference in follow-up histology score for sites from SCC-associated cases as compared with those from adenocarcinoma-associated cases or cases in which there had been no documented lung cancer was undertaken to characterize the features of persistence that are associated with increased risk for progression to invasive SCC. When considering follow-up histology scores from all prevalent and incident cancer cases together, multivariable linear mixed effects model analyses showed a significant mean increase of 0.82 U (95% CI, 0.32–1.32) in follow-up histology scores from SCC-associated sites compared with the non-cancer-associated sites (Table 2). Comparisons of sites from adenocarcinoma-associated cases with non-cancer- or SCC-associated sites did not show significant differences in follow-up histology scores. Compared with non-cancer-associated cases, sites from cases with prior SCC but not adenocarcinoma-associated cases again showed significantly increased follow-up histology score with a mean difference of 0.73 (95% CI, 0.17–1.29; Table 2). The highest increase in adjusted mean follow-up histology score was seen in the group of biopsies from cases with incident SCC. Incident SCC-associated sites showed adjusted mean follow-up histology scores that were 1.55 (95% CI, 0.80–2.30) higher than those from non-cancer sites, and the differences were statistically significant regardless of whether sites from cases in which the incident SCC represented a second primary were included or excluded (Table 2). In addition, incident and prevalent SCC-associated sites showed significantly higher frequencies of progression to or persistence as HGD in follow-up biopsies as compared with non-cancer-associated sites with the highest frequency seen in sites from patients with incident SCC (Supplementary Fig. S3).

Relationship between tobacco history and follow-up histology

Tobacco history showed a significant relationship with follow-up histology scores. In multivariable analyses, bronchial sites from current smokers showed a mean 0.37 (95% CI, 0.08–0.69) unit increase in follow-up histology score when compared with former smokers (Table 3). In addition, an interaction was noted between smoking status and lung cancer status. The effect of tobacco use was also evaluated by comparisons of subjects

Table 2. Cancer status and follow-up histology score

Follow-up histology score in subjects with incident or prevalent lung SCC or adenocarcinoma vs. subjects without associated lung cancer				
Comparison	N ^a	Difference in follow-up HS ^b	95% CI	P
SCC vs. No CA	90-1,022	0.82	0.32-1.32	0.001
Adenocarcinoma vs. No CA	19-1,022	0.10	-0.96-1.16	0.855
SCC vs. Adenocarcinoma	90-19	0.72	-0.44-1.88	0.221
Follow-up histology scores from subjects with prevalent lung SCC or adenocarcinoma vs. subjects without associated lung cancer				
Prevalent lung cancer vs. no cancer	N ^a	Difference in follow-up HS ^b	95% CI	P
Prevalent SCC vs. No CA	65-1022	0.73	0.17-1.29	0.011
Prevalent Adenocarcinoma vs. No CA	16-1022	0.01	-1.04-1.05	0.999
Follow-up histology scores from subjects with incident lung SCC vs. subjects without associated lung cancer				
SCC vs. no lung cancer	N ^a	Difference in follow-up HS ^b	95% CI	P
Incident SCC, All vs. No CA	61-1022	1.55	0.80-2.30	<0.001
Incident SCC, No prior CA vs. No CA	30-1022	0.99	0.08-1.90	0.034

NOTE: Differences in follow-up histology scores (HS) as compared with cases without known lung cancer (No CA) for all prevalent or incident lung cancer cases, prevalent lung cancer cases alone, or incident SCC cases alone.

^aNumber of biopsies (N) for each of the comparator groups, respectively.

^bData for all analyses are adjusted for baseline diagnosis and tobacco parameters in the multivariable linear mixed effects model.

divided into three groups characterized by increasing pack-year smoking histories (Table 3). Follow-up histology scores at biopsy sites from subjects with 40 to 72 pack-year smoking histories were significantly higher than those from subjects with less than 40 pack years. Interestingly, there was no difference in follow-up histology scores when comparing sites from subjects with more than 72 pack years to those with less than 40 or 40 to 72 pack-year histories.

Relationship between histologic features and follow-up histology

Immunohistochemical expression of several biomarkers was evaluated to determine whether altered expression was associated with an effect on follow-up histology. In univariable analyses,

HER2, p53, and MCM2 did not show associations with altered follow-up histology score, but EGFR and Ki67 overexpression were associated with increased follow-up histology scores. We had previously demonstrated that both of these parameters showed a direct correlation with baseline histology score, and therefore adjusted models were used (9). Ki67 expression retained a significant relationship showing a 0.11 (95% CI, 0.03-0.20) unit increase in follow-up histology score per 10% increase in baseline Ki67 positivity (Table 3). Analysis of the relationship between ASD and follow-up histology score was restricted to biopsies with a baseline diagnosis of squamous metaplasia without atypia or higher (histology score of 3-7) because the morphologic features of ASD are generally not seen in nondysplastic lesions. Of note, it was found that there was a significantly higher proportion of

Table 3. Relationship of tobacco use and histologic features to follow-up histology score

Tobacco status and follow-up histology score				
Current vs. former smokers	N (current, former)	Adjusted difference in follow-up histology score ^a	95% CI	P
All biopsies	954 (461-493)	0.37	0.08-0.69	0.0276
No lung cancer	873 (430-443)	0.46	0.15-0.76	0.005
SCC associated	81 (31-50)	0.41	-0.56-1.38	0.41
Tobacco exposure and follow-up histology score				
Pack year comparison	N	Adjusted difference in follow-up histology score ^b	95% CI	P
40-72 vs. 1-40 pack years	952 (514-438)	0.59	0.26-0.92	0.0005
>72 vs. 1-40 pack years	658 (220-438)	0.27	-0.16-0.70	0.22
>72 vs. 40-72 pack years	734 (220-514)	-0.32	-0.73-0.08	0.11
IHC expression and follow-up histologic score				
Biomarker	N	Adjusted difference in follow-up histology score ^c	95% CI	P
Ki67 ^d	468	0.11	0.03-0.20	0.0104
p53 ^e	79	-0.04	-0.20-0.09	0.5199
MCM2 ^e	78	0.06	-0.10-0.20	0.4650
EGFR ^e	79	0.71	-0.23-1.64	0.1192
HER2 ^e	79	0.11	-0.85-1.09	0.8207
ASD and follow-up histologic score				
	Raw mean follow-up histology score	Adjusted difference in follow-up histology score ^f	95% CI	P
ASD-positive sites	3.93	0.42	0.09-0.78	0.016
ASD-negative sites	3.40			

NOTE: Analysis of the relationship between follow-up histology score and tobacco use, immunohistochemical (IHC) marker expression, and presence of histologic features of ASD.

^{a,b}Outcome differences are adjusted for baseline diagnosis, carcinoma status and tobacco pack years or tobacco status.

^cChanges in histology score for Ki67, p53, and MCM2 are expressed as mean change per 10% increase in number of cells positive for the biomarker; EGFR and HER2 are expressed as mean change in overexpressing sites (staining extends into upper half of epithelium) versus nonoverexpressors.

^dKi67 data are adjusted for baseline diagnosis, age, gender, and tobacco status.

^ep53, MCM2, EGFR, and HER2 data are adjusted for baseline diagnosis only.

^fThe calculated difference in histology score is adjusted for baseline diagnosis, tobacco status, and pack year history in the multivariable linear mixed effects models.

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high-grade dysplasia at baseline in the ASD versus dysplasia without ASD groups (71.9% vs. 53.0%, respectively, $P < 0.001$). Nonetheless, after adjustment for a number of clinical parameters, including baseline diagnosis, the presence of histologic features of ASD was also associated with a significant increase in follow-up histology score as compared with sites without features of ASD (Table 3).

Discussion

The potential to use lesion-specific changes over time as an indication of risk for the development of invasive SCC of the lung was explored in this study. The findings demonstrated that an increased frequency of sites that show persistence as or progression to HGD was associated with a significant 7.84 (95% CI, 1.56–39.39) fold increase in risk for development of invasive SCC, and indicated that subjects with multiple dysplastic sites that persist or progress to HGD represent a subset of patients with aggressive airway disease. The demonstration that subjects with multiple sites of persistent disease show the strongest association with development of SCC emphasizes the importance of performing a thorough evaluation of the airway, and adds support to the role of field carcinogenesis in the development of invasive lung cancer. Increased risk for invasive SCC has previously been associated with multiple sites of abnormal appearing mucosa by autofluorescence bronchoscopy (AFB; ref. 30). In addition, the data demonstrate that higher histology scores in follow-up biopsies imply important differences in potential for progression. For instance, cases with dysplastic sites that persisted or progressed to HGD showed a significant increase in risk for development of SCC whereas those in which persistence as low- or high-grade BD did not, despite the fact that the latter definition of persistence allowed for inclusion of several more cases in the overall evaluation. This finding is similar to those of Alaa and colleagues (31), who demonstrated that the development of new severely dysplastic lesions, regardless of the baseline histology, was more common in subjects that developed invasive cancer or CIS. Our data showed similar findings, demonstrating a significant relationship between development of SCC and presence of multiple persistent HGDs when sites of any baseline histology score were included (see Supplementary Fig. S2). However, as discussed below, we and others have observed that CIS often regresses (20). Therefore, establishment of a relationship between persistence of BD and risk for invasive SCC is an important extension of these previous findings.

The demonstration of a relationship between HGD in follow-up biopsies and risk for invasive SCC may also have implications for the management of patients at risk for aggressive airway disease. The tumors that develop in association with persistent BD are more centrally located and are less likely to be associated with identifiable radiographic abnormalities in the early course of disease. Thus, screening for BD and identification of patients at high risk for progression to invasive SCC will likely require a different modality from high-resolution CT to be effective. Our findings suggest that multiply sampling the airways may be important and that the employment of a bronchoscopic technique that increases the sensitivity for detection of BD, such as AFB, may be advisable (27, 30). Furthermore, in patients with features of aggressive airway disease, close follow-up would likely be indicated and consideration of potential benefit from preventive therapy might be suggested. With respect to invasive cancer,

our study showed that subjects with persistent BD developed invasive cancer both at sites that were associated with and remote from those with baseline dysplastic change. Eight subjects developed incident SCC at sites that were previously biopsied and five at sites that had not been previously biopsied (including one patient that developed synchronous, incident SCCs in two different contralateral lung lobes). Six of the previously biopsied sites showed dysplasia in baseline biopsies, of which two had demonstrated persistence before development of SCC (SCC was diagnosed in the second biopsy for the other four). Thus, two SCCs developed at nondysplastic sites and six others developed at sites that had not been previously sampled, suggesting that they did not appear abnormal on AFB. This may indicate that chemopreventive rather than local therapy will be necessary to significantly reduce the incidence of SCC in this setting. Finally, the findings also support the use of reduced bronchial histology scores as an informative endpoint in trials evaluating efficacy of potential preventive agents. Although we have shown that the frequency of persistent BD is associated with subsequent development of SCC, a potential drawback associated with our analysis is the inclusion of some incident SCC cases in which this tumor represents a second lung primary. Information regarding therapies that patients with prior lung cancer may have received was not available. It is possible that such treatments could influence the course of BD in the group of patients with prior carcinoma. However, our finding that primary incident SCC is also associated with increased follow-up histology scores further supports a relationship between persistence or progression of BD and risk for the development of invasive SCC.

The association of higher grades of dysplasia at baseline with increased histologic scores on follow-up corroborates findings from the prospective study of Bota and colleagues (21) and the meta-analysis of follow-up data from four different chemoprevention trials performed in the British Columbia Lung Health Study that included more than 700 subjects (2). Although different classifications of outcome were used in the latter study, their finding of a 4- to 5-fold higher rate of progression in sites with baseline diagnoses of moderate or severe dysplasia as compared with those with lower diagnoses is consistent with the findings in our analysis. Strengths of our study that may more firmly establish some of these relationships include fewer numbers of sites coming from subjects with prior lung or head and neck cancers (8.8%), inclusion of sites with lengthy follow-up (48.2% with >2 years), and confinement of our study group to subjects from the non-treatment protocols or the placebo arm of prevention trials with positive findings. Previously, CIS has been reported to progress to invasive frequently with the majority progressing to cancer in some reports (19). In our cohort, 9 subjects had 23 sites that showed CIS and had follow-up biopsies. Although histologically normal at the baseline biopsy, one of these sites developed CIS and progressed to invasive SCC 7 months later. In addition, 2 other subjects developed incident SCCs, but not at their sites of CIS. Although 16 of 22 (72.7%) CIS sites persisted as HGD, including all of those in cases with associated SCC, six sites in 2 subjects regressed to nondysplastic histology, including five that were followed over a course of 35 months and were re-biopsied 1 to 3 times. Taking the biopsy with the highest diagnosis before development of SCC, one site with CIS at baseline (1/22, 4.54%), five sites with baseline moderate or severe dysplasia (5/282, 1.77%), one with baseline LGD (1/204, 0.49%) and four with nondysplastic baseline diagnoses progressed to SCC (4/667,

0.60%). Although the overall number of CIS lesions is small in this cohort, the findings support the aggressive nature that other publications have found to be associated with these lesions, but also suggests that the rate of progression is not high and document regression of CIS.

Our data show that tobacco use has an impact on the course of BD with current tobacco users having higher follow-up histology scores than former tobacco users. These findings compliment the findings of Clément-Duchêne and colleagues (32) in which duration of smoking history was found to correlate with increased incidence of BD. This information could be useful clinically for physicians counseling their patients to quit tobacco use as a measure to prevent the development of lung cancer.

ASDs were also shown to be associated with an increased level of atypia on follow-up biopsy. Angiogenesis is well established as a prognostic factor in invasive carcinoma, and we have previously shown that expression of VEGF increases with higher grades of BD (33). Furthermore, our recent analysis of vandetanib, the VEGFR2 inhibitor with multitarget inhibitory capacity, showed preventive activity of this agent in a mouse model of lung carcinogenesis (34). These findings also correlate with previous work that has demonstrated more frequent ASD in subjects with lung cancer than in those without (35). Angiogenic changes could support an increased level of epithelial cell proliferation that may be important in promoting BD persistence and progression. Given that poor vascular integrity has been associated with VEGF dominant neoangiogenesis (36), it is also possible that ASD lesions are associated with an altered microenvironment that promotes progression. In addition, our IHC analyses suggest that higher levels of expression of Ki67 could also serve as biomarkers of increased risk in BD.

The results of this study suggest that an important subset of aggressive airway disease is represented by cases that show the presence of multiple dysplastic lesions that persist or progress to HGD, and demonstrate that in patients with this presentation there is increased risk for invasive SCC. Further characterization of

these persistent lesions should allow for the development of more precise predictive markers. Furthermore, obtaining an understanding of the biologic characteristics that drive these BD with a high risk for progression to invasive lung cancer will help identify effective targets for prevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Persistence of Bronchial Dysplasia Is Associated with Development of Invasive Squamous Cell Carcinoma

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