

Analysis of c-ErbB1/Epidermal Growth Factor Receptor and c-ErbB2/HER-2 Expression in Bronchial Dysplasia: Evaluation of Potential Targets for Chemoprevention of Lung Cancer

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Abstract Purpose: Lung cancer is preceded by a premalignant phase during which intervention could decrease associated morbidity and mortality. Molecular characterization of factors involved in controlling progression of bronchial dysplasias will provide markers of premalignant change and identify targets for chemoprevention.

Experimental Design: Immunohistochemical analysis of epidermal growth factor receptor (EGFR; c-ErbB1/EGFR), HER-2/*neu* (c-ErbB2/HER-2), Ki-67, and minichromosome maintenance protein 2 (MCM2) expression in bronchial dysplasia was undertaken to characterize molecular alterations associated with the progression of these lesions in 268 bronchoscopically obtained biopsies from 134 subjects.

Results: Analysis of biopsies with the most severe diagnosis from each subject showed a linear relationship between increasing marker expression and severity of dysplastic change for EGFR ($P < 0.001$), Ki-67 ($P < 0.001$), and MCM2 ($P = 0.001$) but not HER-2 ($P = 0.102$). Increased expression of either EGFR or HER-2 was associated with increased levels of Ki-67 and MCM2 expression, and combined overexpression of these receptors was associated with the highest levels of proliferation, suggesting a synergistic effect. Finally, the lack of an associated trend toward increased EGFR expression when comparing the worst and best biopsies within each subject indicated a potential field effect in the induction of EGFR expression.

Conclusions: The results suggest a prominent role for EGFR overexpression in the development and progression of bronchial dysplasia and provide rationale for exploring inhibition of EGFR signaling in lung cancer chemoprevention.

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Identification and treatment of this disease in its preinvasive stage could greatly reduce the associated morbidity and mortality. Recent progress in bronchoscopic evaluation of the airways has allowed for the identification of premalignant airway lesions and has provided material for histologic and molecular characterization of bronchial dysplasia (2).

A histologic classification scheme has been generated (3), and its validity has been confirmed by early studies establishing a relationship between severity of dysplastic change and risk for subsequent progression to invasive carcinoma (4, 5). In addition, these premalignant lesions have been shown to be associated with many molecular alterations that also distinguish invasive lung cancers from normal bronchial tissue (6–8).

A distinctive feature of bronchial dysplasia is the presence of increased epithelial proliferation compared with normal bronchial mucosa. The ErbB receptor [epidermal growth factor receptor (EGFR)] family is thought to play a primary role in the control of epithelial cell proliferation. This family is composed of four semihomologous receptors that can interact with several ligands and generate intracellular signaling as homodimer or heterodimer pairs (9, 10). Ligand interaction and subsequent dimerization lead to activation of kinase activity associated with the intracellular domains of these transmembrane receptors. This leads to subsequent activation of downstream cytoplasmic and nuclear second messengers (11–13). Increased activity of c-ErbB1/EGFR is associated with the alteration of several important cellular processes, including induction of cellular proliferation, activation of angiogenesis, development of metastatic capacity, and reduced apoptosis (14–17). EGFR protein expression is normally limited to the basal layer of stratified squamous epithelium, whereas the expression of c-ErbB2/HER-2 (HER-2/*neu*) is less consistent, being found only

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in the basal layer of cervical squamous epithelium or the intermediate layers of oral mucosa (18, 19). The roles of the other members of this receptor family (c-ErbB3 and c-ErbB4) in the control of epithelial proliferation are less well characterized.

Increased expression of EGFR and its association with induction of increased expression of proliferation markers, such as Ki-67 and proliferating cell nuclear antigen, has been shown previously in premalignant lesions of the skin and laryngeal mucosa (20, 21). Similarly, such changes are found in squamous metaplasia and premalignant dysplasias of uterine cervix (18, 22). Bronchial mucosa shows metaplastic change to a squamous mucosa in airway injury secondary to stimuli, such as tobacco smoke exposure. These metaplastic bronchial sites may or may not be associated with epithelial atypia (dysplasia). Increased expression of EGFR has been shown in the majority of non-small cell lung cancers with overexpression in up to 85% of squamous cell lung carcinomas (23–27). In some studies, EGFR overexpression has also been shown to have prognostic implications in lung cancer (28, 29), whereas, in others, including studies from our institution, no significant association with prognosis was found (26). Bronchial dysplasia is believed to be the precursor of squamous cell lung carcinoma; thus, EGFR might play a significant role in the progression of these lesions to invasive cancer. In addition, demonstration of a key role for the ErbB family of receptors in the development of premalignant lesions would have significance because several new therapeutic inhibitors of this pathway are now available. Promising results with EGFR inhibitors have been seen in patients with advanced non-small cell lung cancer where a survival benefit has been shown in cases in which prior treatment with conventional chemotherapy has failed (30). Furthermore, the toxicity associated with EGFR inhibitors seems to be less significant than that seen with conventional chemotherapeutics, making these agents potential candidates for chemoprevention purposes. Some previous immunohistochemical studies in bronchial dysplasia have shown EGFR expression and an association with increased cellular proliferation (31–35). However, an extensive evaluation of the relationship between levels of expression of the primary members of the ErbB receptor family and their association with the degree of bronchial dysplasia, as defined by WHO criteria, has not been done.

In this study, we analyze bronchial biopsies from 134 subjects. Expression of EGFR, HER-2, Ki-67, and minichromosome maintenance protein 2 (MCM2) is measured by immunohistochemistry to assess ErbB receptor pathway activity in bronchial dysplasia. EGFR and HER-2 expression in bronchial epithelium is characterized and the relationship between the levels of expression of these markers and degree of dysplasia and epithelial proliferation is evaluated to explore the potential role of EGFR blockage for chemoprevention of lung cancer.

Materials and Methods

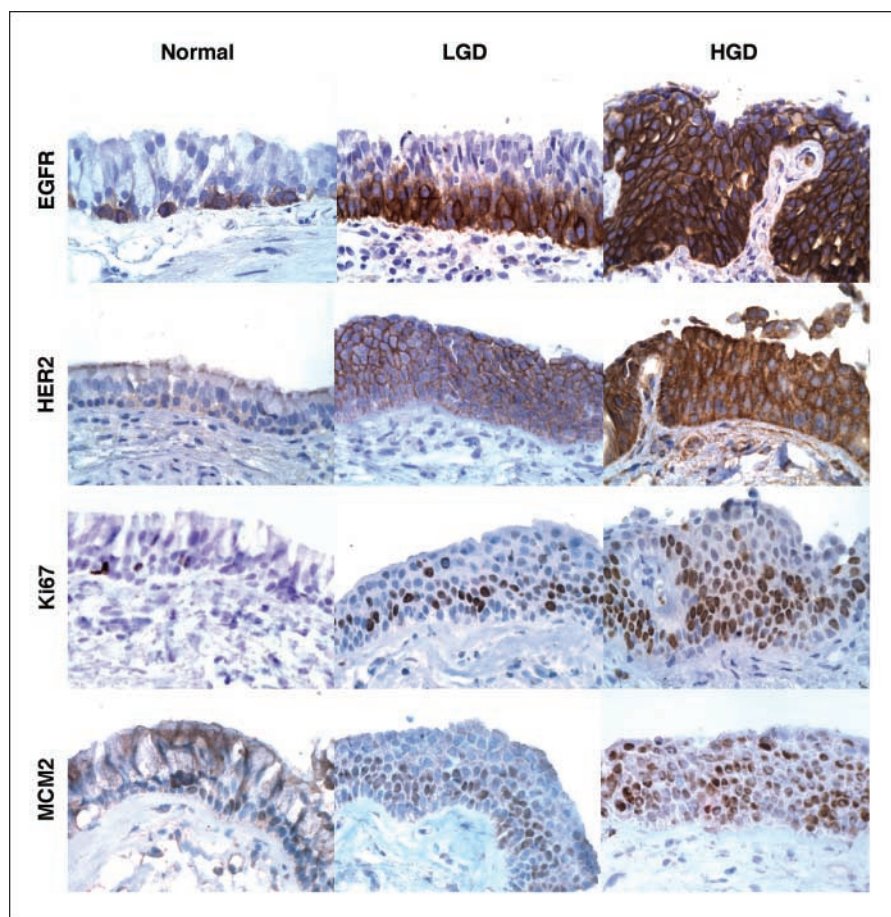
Subjects and tissue samples. Endobronchial biopsies were collected by fluorescence bronchoscopy from 134 consecutive subjects followed at the University of Colorado Health Sciences Center and associated hospitals. The majority (>75%) of subjects were recruited to bronchoscopy protocols with enrollment criteria consisting of prior moderate or worse atypia (sputum) or dysplasia (biopsy) and clinical history of (a)

>20 pack-year smoking history and/or (b) clinical airflow obstruction defined as $FEV_1 < 75\%$ predicted. Five subjects were enrolled in protocols for which they qualified simply by virtue of atypia/dysplasia on a prior sputum or biopsy. Three of these subjects were never smokers, and smoking histories were not available for the other two subjects. Additionally, 9 never-smoker, volunteer control subjects, 10 subjects recruited to protocols as healthy smoker controls (>30 pack-years but normal FEV_1 and sputum), and 8 subjects with clinical indications for bronchoscopy were included. Bronchoscopies were done per protocol and consent was obtained from all patients. Protocols and consent forms were approved by the Colorado Multiple Institutional Review Board. Multiple endobronchial biopsies were collected from six predetermined sites and any additional suspicious areas as described in the bronchoscopy study protocols. The biopsies were formalin fixed, paraffin embedded, and stained with H&E for subsequent morphologic evaluation and classification. Bronchial tissue was classified into one of eight histologic categories as defined by the recent WHO classification (3). These categories are as follows: 1, normal; 2, basal cell hyperplasia; 3, squamous metaplasia without dysplasia; 4, mild dysplasia; 5, moderate dysplasia; 6, severe dysplasia; 7, carcinoma *in situ*; and 8, invasive carcinoma. Differences in these numeric assignments were used to calculate the difference in histologic grade when doing comparisons between worst and best biopsies from a single subject. Grouping of these diagnostic categories into low-grade dysplasia (mild) and high-grade dysplasia (moderate, severe, and carcinoma *in situ*) categories are used for data presentation in Figs. 1 and 2. Additionally, within the squamous metaplasia without dysplasia through carcinoma *in situ* categories, biopsies were subclassified as angiogenic squamous dysplasias (ASD) when they showed characteristic vascular papillary structures projecting into overlying epithelium (36). Features of ASD were noted in 3 squamous metaplasia without dysplasia, 9 low-grade dysplasia, and 16 high-grade dysplasia biopsies. The biopsies showing the lowest and highest degree of dysplasia were selected from each subject and used for immunohistochemical staining with a panel of antibodies to EGFR, HER-2, Ki-67, and MCM2. Five endobronchial specimens (one squamous metaplasia, one mild dysplasia, and three carcinoma *in situ*) came from five subjects in whom a separately processed transbronchial biopsy was later found to have shown an invasive carcinoma. However, all of these cancers were in different lobes than the biopsies included in this study.

Immunohistochemistry. Immunostaining was done according to previously described protocols (26). Briefly, the procedure generally consisted of (a) 20-minute antigen retrieval using DAKO (Carpinteria, CA) target retrieval solution, (b) 10-minute peroxide block in water, (c) 1-hour primary antibody incubation, (d) 30-minute secondary antibody incubation with EnVision+ anti-mouse peroxidase (3,3'-diaminobenzidine)-conjugated antibody (DAKO), (e) 5- to 7-minute substrate incubation with 3,3'-diaminobenzidine + 20 μ L chromogen (DAKO), and (f) 25-second counterstain using Hematoxylin Gill II. Antibody-specific alterations included (a) 10-minute antigen retrieval step using proteinase K solution (DAKO) for EGFR, (b) peroxide block in methanol for EGFR and Ki-67, (c) an additional binding block step between the peroxide block and primary antibody incubation using Biogenex (San Ramon, CA) power block for EGFR and Ki-67, (d) use of a biotinylated link anti-mouse and anti-rabbit (DAKO LSAB2 Systems) horseradish peroxidase-conjugated secondary antibody for HER-2, (e) the addition of a 30-minute streptavidin horseradish peroxidase enzyme complex step after the secondary antibody incubation for HER-2, and (f) a 10-minute automation hematoxylin (DAKO) counterstain step for HER-2. Primary antibodies used were as follows: EGFR (1:10, 15 μ g/mL; Zymed, South San Francisco, CA) mouse monoclonal clone 31G7, HER-2 (1:200; DAKO) rabbit polyclonal, Ki-67 (1:100, 70 μ g/mL; DAKO) clone MIB-1 BM28 mouse monoclonal, and MCM2 (1:500, 250 μ g/mL; Transduction Laboratories, San Diego, CA) mouse monoclonal antibody BM28.

Assessment of immunohistochemical staining. A scoring system that allowed for quantitation independent of morphology was developed to

Fig. 1. Immunohistochemical stains of bronchial biopsy material showing the various staining patterns associated with EGFR, HER-2, Ki-67, and MCM2. There was variability in the distribution and intensity of staining for each of the factors in the normal, low-grade dysplasia (LGD), and high-grade dysplasia (HGD) diagnostic groups. Representative of the overall staining for each of these groups. Magnification, $\times 400$.



assess EGFR and HER-2 expression. EGFR and HER-2 expression were characterized for both distribution and intensity. Lack of expression was occasionally seen with HER-2 immunostains, but EGFR staining was generally found in at least a portion of the basal layer of bronchial epithelia. True negative staining was given a distribution score of 0. Receptor expression confined to the lower half of the epithelium was given a distribution score of 1. This was the most common pattern seen in normal bronchus and all biopsies showing this distribution of staining were considered not to be overexpressors of EGFR or HER-2. A distribution score of 2 corresponded to staining extending into the upper half and/or involving the entire thickness of the epithelium. This score was considered to represent overexpression of the receptor. Although increases in receptor staining intensity (scored from none to

strong: 0-4, respectively) correlated with increased receptor distribution scores (data not shown), taken alone, intensity scores showed a less consistent relationship to histology, clinical variables, and expression of proliferation markers than distribution of receptor expression. Therefore, distribution scores were used in the analyses presented herein. Averages of distribution scores were calculated for EGFR and HER-2 expression levels in biopsies grouped according to histologic grade or clinical variables of smoking status, subject age, and subject gender (Table 1). Correlative studies were done to test for associations between increased EGFR and/or HER-2 receptor expression (distribution score = 2) and expression of Ki-67 and MCM2 (Tables 2 and 3) or within-subject differences in histologic grade (Table 4). Ki-67 and MCM2 were scored as percentage of positive cells (400 cells total counted per biopsy) and

Fig. 2. Summary of percentage of all biopsies with overexpression of EGFR and HER-2 (and corresponding percentages of Ki-67 and MCM2 positivity) for major histologic categories of normal, reactive, and dysplastic bronchial lesions. Note changes of ASD in high-grade dysplasia with vascular structures projecting into overlying dysplastic epithelium (arrow). BCH, basal cell hyperplasia. Asterisk, $n = 103$ for Ki-67 and MCM2 values. Magnification, $\times 400$.

MARKER	HISTOLOGY			
	NORMAL % (N=104*)	BCH % (N=51)	LGD % (N=24)	HGD % (N=64)
EGFR	42	54	58	88
HER2	69	84	92	92
Ki67	11	18	45	52
MCM2	64	64	87	84

Table 1. Marker expression in bronchoscopy tissue (worst tissue specimens)

	Marker expression			
	EGFR, average score (n)	HER-2, average score (n)	Ki-67, average % positivity (n)	MCM2, average % positivity (n)
Histology*				
1	1.47 (16)	1.69 (16)	10.94 (16)	72.94 (16)
2	1.53 (16)	1.91 (16)	20.31 (16)	63.09 (16)
3	1.86 (7)	2.00 (7)	26.14 (7)	79.43 (7)
4	1.67 (18)	1.94 (18)	46.28 (18)	86.61 (18)
5	1.84 (38)	1.87 (38)	52.27 (37)	80.47 (38)
6	1.90 (21)	1.90 (21)	48.24 (21)	87.86 (21)
7	2.00 (4)	2.00 (4)	69.50 (4)	86.00 (4)
8	2.00 (14)	2.00 (14)	66.75 (14)	96.04 (14)
<i>P</i> [†]	<0.001	0.102	<0.001	0.001
Smoking				
Current	1.79 (50)	1.92 (50)	49.64 (50)	84.64 (50)
Former	1.81 (68)	1.88 (68)	40.19 (67)	78.00 (68)
Never	1.54 (14)	1.82 (14)	33.68 (14)	82.61 (14)
<i>P</i> [†]	0.16	0.41	0.04	0.42
Age				
37-49	1.62 (13)	1.77 (13)	36.00 (13)	78.54 (13)
50-59	1.70 (32)	1.92 (32)	44.84 (32)	81.22 (32)
60-69	1.84 (51)	1.83 (51)	41.58 (50)	79.05 (51)
70-79	1.79 (31)	1.98 (31)	41.34 (31)	85.37 (31)
80-84	1.71 (7)	2.00 (7)	58.86 (7)	83.57 (7)
<i>P</i> [†]	0.22	0.17	0.42	0.47
Gender				
Female	1.68 (36)	1.81 (36)	38.10 (35)	76.46 (36)
Male	1.80 (98)	1.92 (98)	44.31 (98)	82.96 (98)
<i>P</i> [†]	0.13	0.13	0.29	0.21

*1 = normal, 2 = basal cell hyperplasia, 3 = squamous metaplasia, 4 = mild dysplasia, 5 = moderate dysplasia, 6 = severe dysplasia, 7 = carcinoma-in-situ, 8 = carcinoma.

† *P* values test the linear trend across histologic grade, smoking status, age, or gender.

average scores were calculated for groups of biopsies as defined by histology, clinical variable, or receptor expression.

Statistical analysis. The primary analyses focused on the relationship between histologic grade and the expression of EGFR and HER-2. These analyses were based on the bronchial biopsies having the most severe diagnosis for each subject because those represented the greatest range of histologic grades. Linear regression models were used to test the significance of correlations between these variables. Secondary analyses examined the association between EGFR and/or HER-2 expression and other measures of proliferation (Ki-67 and MCM2) using a *t* test of difference between EGFR and/or HER-2 positive (score = 2) and negative (score = 0/1) samples. Extent of marker expression across the lung was examined by analyzing the difference in marker expression levels between the bronchial biopsy with best and worst histologic diagnosis within each subject; again, linear regression methods were used to determine if there was a relationship between levels of marker expression and differences in histologic grade. Finally, testing for a potential relationship between receptor (EGFR or HER-2) expression and ASD morphology was analyzed by the χ^2 method. Results are reported as two-sided *P*s and/or 95% confidence intervals.

Results

Immunohistochemical staining patterns and patient population. Alterations in the distribution of expression for EGFR and HER-2 were noted (Fig. 1). The majority of normal

bronchial biopsies showed EGFR staining that was confined to the lower half of the epithelium (score = 1; Fig. 1, *Normal, EGFR*). Increased thickness of positive cells with expression in either the lower half (score = 1; Fig. 1, *LGD, EGFR*) or entire thickness of the epithelium (score = 2; Fig. 1, *HGD, EGFR*) was more commonly seen in dysplasias. HER-2 expression was most frequently present throughout the full thickness of dysplastic epithelium (Fig. 1, *HER2, LGD and HGD*), whereas, in normal bronchial epithelium, expression was sometimes limited to the lower portion of the mucosa (Fig. 1, *Normal, HER2*). Faint surface expression of HER-2 as shown in the normal section of Fig. 1 was occasionally noted, but these biopsies were only scored as staining pattern 2 when detectable full circumference membrane staining extending to the epithelial surface was seen. Levels of nuclear staining for Ki-67 and MCM2 were quantified, and an increased percentage of positive epithelial cell staining was generally associated with extension of expression into upper levels of epithelium. These antigens showed immunostaining patterns that were associated with higher scores in higher grades of dysplasia or invasive carcinoma compared with normal or nondysplastic bronchial epithelium (Fig. 1, *Ki67 and MCM2*). The patient population studied in these analyses was predominantly male (74%) and older (average age, 63 years) with a spectrum of associated

smoking histories (10% never, 52% previous, and 38% current; Table 1).

Characteristics of EGFR, HER-2, Ki-67, and MCM2 expression in bronchial dysplasia and lung cancer. Expression of EGFR, Ki-67, and MCM2 but not HER-2 showed a statistically significant positive association with histologic grade in analyses of the most atypical biopsies from each subject (Table 1). HER-2 expression was lowest in normal bronchial mucosa (average score, 1.69), and the average score tended to increase to 2.0 as histology increased; however, the overall trend was only marginally significant ($P = 0.102$). In contrast, strongly significant, progressive increases in marker expression were noted as histologic class increased from 1 to 8 for EGFR (1.47-2.0), Ki-67 (10.94-66.75), and MCM2 (72.94-96.04). A statistically significant association between Ki-67 expression and smoking status was noted, but no other statistically significant relationships between demographic characteristics (smoking status, age, and gender) and the expression of EGFR, HER-2, Ki-67, and MCM2 were identified (Table 1). Age- and gender-adjusted analyses indicated that these factors were not confounding the association between marker expression and histology (data not shown). Figure 2 shows representative H&E-stained sections from bronchial biopsies of normal, basal cell hyperplasia, low-grade dysplasia, and high-grade dysplasia with the corresponding data correlating degree of dysplasia with frequency of EGFR, HER-2, Ki-67, and MCM2 overexpression. Figure 2 includes expression data from all biopsies regardless of whether they represent the worst or best histology from a given subject. Comparisons of 28 lesions subclassified as ASD (see Fig. 2, HGD) with non-ASD specimens from the same histologic categories did not reveal statistically significant differences in frequency of EGFR or HER-2 overexpression (data not shown).

Correlation between increased receptor expression and Ki-67 and MCM2 expression. As shown in Table 2, specimens that expressed high levels (score = 2) of EGFR or HER-2 also showed significantly elevated expression of Ki-67 ($P < 0.001$ for both receptors) and MCM2 ($P = 0.004$ for both receptors) compared with Ki-67 and MCM2 levels in specimens with low EGFR or HER-2 expression (score = 0/1). Furthermore, the highest levels of Ki-67 and MCM2 expression were seen when both EGFR and

HER-2 were overexpressed, suggesting a potential synergistic effect (Table 3).

Comparison of marker expression in intrasubject samples of differing histologic grade. We hypothesized that if alterations in the expression of any of these markers were specifically associated with histologic change, then their expression levels would be significantly higher when comparing biopsies of the highest-grade with the lowest-grade lesions from each subject. We investigated this hypothesis by testing for an association between the difference in histologic grade and the difference in levels of marker expression for the worst and best biopsies within the same subject. Results from this analysis for EGFR and HER-2 are shown in Table 4. A progressive trend toward increased expression of HER-2 and Ki-67 when comparing subjects who had a spectrum of differences in histologic grade ranging from 1 to 7 was found, although this did not quite reach statistical significance for HER-2 ($P = 0.07$, HER-2, Table 4; $P < 0.001$, Ki-67, data not shown). This association was not present for expression of EGFR or MCM2 ($P = 0.74$, EGFR, Table 4; $P = 0.59$, MCM2, data not shown). The combination of an absence of an association in Table 4 and a significant association in Table 1 implies that expression of these latter two markers was influenced by the worst histology in the airways of the subject regardless of the histologic appearance of the tissue used for analysis.

Discussion

The ErbB signaling pathway has received great attention in recent years due to the clinical effects associated with inhibition of ErbB family receptors. Significant responses have been seen with compounds, such as the monoclonal antibodies Herceptin (Genentech, South San Francisco, CA) in breast cancer (37) and Cetuximab (Bristol-Myers Squibb/ImClone, New York, NY) in colorectal cancer (38) and the tyrosine kinase inhibitors erlotinib (Tarceva, OSI Pharmaceuticals, Inc., Melville, NY) and gefitinib (Iressa, AstraZeneca, Macclesfield, United Kingdom) in lung cancer (30). The purpose of the current study was to determine whether increased expression of the ErbB family receptor members, EGFR and HER-2, might contribute to the development of bronchial preneoplasia and to provide insight

Table 2. Proliferation indices by EGFR and/or HER-2 positivity (worst tissue samples)

	Receptor positivity, average % positivity (SD)*	Receptor negativity, average % positivity (SD) †	Difference	
			Difference (95% confidence interval)	P ‡
EGFR §				
Ki-67	48.8 (28.0)	25.5 (28.1)	23.3 (12.3-34.4)	<0.001
MCM2	85.9 (23.0)	68.0 (32.2)	17.8 (5.9-29.7)	0.004
HER-2				
Ki-67	46.2 (28.8)	10.0 (14.1)	36.2 (26.5-45.9)	<0.001
MCM2	84.6 (23.6)	50.1 (34.8)	34.5 (13.2-55.8)	0.004

*Samples in which EGFR or HER-2 is overexpressed (score = 2).

†Samples in which EGFR or HER-2 is not overexpressed (score = 0/1).

‡P values test the differences between the levels of Ki-67 and MCM2 in biopsies with high (receptor positivity) versus low (receptor negativity) receptor expression.

§n = 99 for receptor-positive samples (except Ki-67, n = 98) and n = 35 for receptor-negative samples.

||n = 121 for receptor-positive samples (except Ki-67, n = 120) and n = 13 for receptor-negative samples.

Table 3. Proliferation indices by EGFR and/or HER-2 positivity (worst tissue samples)

	EGFR/HER-2 status, average % positivity (SD)*			<i>P</i> _{trend} [†]
	EGFR-/HER-2- (<i>n</i> = 10)	Mixed (<i>n</i> = 28) [‡]	EGFR+/HER-2+ (<i>n</i> = 96) [§]	
Ki-67	8.70 (11.37)	30.27 (29.60)	49.91 (27.51)	0.00001
MCM2	58.90 (30.84)	66.23 (36.03)	87.91 (19.50)	0.00056

*EGFR+ (EGFR-) denotes a sample that is positive (negative) for EGFR overexpression; HER-2+ (HER-2-) denotes a sample that is positive (negative) for HER-2 overexpression.

[†]Test for a linear trend across the HER-2-/EGFR-, mixed, and HER-2+/EGFR+ categories.

[‡]Mixed denotes either HER-2+/EGFR- or HER-2-/EGFR+.

[§]*n* = 95 for Ki-67.

regarding the potential role of these receptors as targets for chemoprevention. Our results show a significant positive association between levels of EGFR expression and increasing degrees of dysplasia in bronchial airway epithelium. High levels of HER-2 expression were found in the majority of both normal and dysplastic bronchial biopsies, and no definite association was found between HER-2 expression levels and degree of dysplasia. Previously, a few immunohistochemical studies have also shown increased EGFR expression in bronchial dysplasia (31, 33, 35). However, because of small numbers of specimens and/or subjects, these studies were unable to convincingly show a correlation between levels of EGFR expression and degree of atypia associated with dysplastic bronchial lesions. In the current study, analysis of 134 separate lesions, each of which was derived from a different subject, allowed for the demonstration of such a relationship.

Interaction between EGFR and HER-2 in the induction of a proliferative state in bronchial dysplasia has not been investigated previously, and our results suggesting potential synergy between EGFR and HER-2 in epithelium with concomitant overexpression of both of these receptors may

have important clinical implications. A recent report from our group has shown that, in non-small cell lung cancer patients treated with a tyrosine kinase inhibitor of EGFR (gefitinib), the presence of increased HER-2 gene copy number is associated with significantly better objective response, disease control rate, and time to progression and a strong trend toward longer overall survival in patients with EGFR-positive tumors (39). Taken together, our results suggest that chemopreventive effects via inhibition of EGFR activity in bronchial dysplasia may be most successful in patients with dual overexpression of EGFR and HER-2, a frequent finding in our analysis of these lesions.

A further characteristic of EGFR expression revealed in our analysis provides insight regarding the stimulus for increased expression of this receptor. A comparison of EGFR expression levels between worst and best histologic biopsies from within individual subjects showed that significant increases in expression of EGFR could not be shown in association with increased differences in histologic grade. This result indicates that levels of EGFR expression are most influenced by the worst histology in the airways of a given patient regardless of the histologic appearance of the bronchial epithelium in which

Table 4. Difference in marker expression (worst minus best tissue specimens)

Histology difference*	Marker expression [†]									
	EGFR overexpression					HER-2 overexpression				
	<i>n</i>	- (%)	+/- (%)	-/+ (%)	+/+ (%)	<i>n</i>	- (%)	+/- (%)	-/+ (%)	+/+ (%)
1	21	33.3	0.0	28.6	38.1	21	4.8	0.0	9.5	85.7
2	13	7.7	7.7	38.5	46.2	13	0.0	0.0	7.7	92.3
3	22	31.8	0.0	36.5	31.8	23	4.3	0.0	30.4	65.2
4	36	16.7	0.0	38.9	44.4	36	8.3	5.6	27.8	58.3
5	7	0.0	0.0	42.9	57.1	7	0.0	0.0	28.6	71.4
6	6	0.0	0.0	16.7	83.3	6	0.0	0.0	33.3	66.7
7	9	0.0	0.0	44.4	55.6	9	0.0	0.0	22.2	77.8
<i>P</i> [‡]				0.74					0.07	

Abbreviations: -, best and worst samples both negative (no overexpression of receptor); +/-, best sample positive (overexpression of receptor) and worst sample negative; -/+, best sample negative and worst sample positive; +/+, both samples positive.

*Difference between numeric histologic identifiers for worst and best biopsies in each subject.

[†]Values for marker expression are percentage of samples with particular receptor expression pattern (i.e., +/-, -/+, etc.) within each histologic difference group.

[‡]*P* values for testing linear trend in the (-/+) with increasing histology difference. Test is adjusted for within-subject average histology (average of the best and worst histologies). A significant *P* value implies the absence of a field effect (i.e., a significant *P* value implies that the difference in marker expression is related to difference in histology). If marker expression is elevated across the entire lung, then there would be no difference between worst and best samples; in particular, the difference would not change with increasing difference in histology between best and worst samples.

EGFR expression is measured. We propose that a field effect occurs in patients at higher risk for the development of premalignant and invasive lesions of the airways and that increased EGFR expression may be a central molecular alteration associated with this effect. This observation suggests a potential role for an EGFR-mediated proliferative state in permitting the development of increasingly dysplastic lesions with their associated increased risk for subsequent progression to invasive carcinoma.

HER-2 expression has only been assessed in bronchial dysplasia in one previous study. This previous work suggested that HER-2 expression was increased in dysplasia compared with nondysplastic bronchial epithelium, although the numbers of specimens studied were not adequate to show a statistically significant difference (40). Our results did not show an association between HER-2 levels and degree of dysplastic change, but the *P* for this relationship (0.102) was only slightly more than twice the value used to determine statistical significance. Although normal bronchial tissue seems to have slightly lower levels of HER-2 expression than the remaining histologic classes (group 2, basal cell hyperplasia; group 8, invasive carcinoma; Table 1), these latter lesions all show very similar, high levels of HER-2 expression. In contrast, EGFR maintains a trend toward higher levels of expression with increased histologic class when evaluating groups 2 to 8. Because basal cell hyperplasia and squamous metaplasia without dysplastic change are considered to be reactive lesions without associated increased risk for the development of lung cancer, these observations suggest that HER-2 expression is not a specific marker of premalignant change in the airways and may not be central to the development of these lesions. Despite these findings, our results suggesting a potential synergy between EGFR and HER-2 with respect to associated increased expression

of proliferation markers could indicate that, in the presence of EGFR overexpression, increased levels of HER-2 may contribute to epithelial proliferation in bronchial dysplasia.

Angiogenic activity was not directly quantified in our study, but analysis of EGFR and HER-2 expression in ASDs was done. Although some recent publications have shown an association between activation of EGFR and induction of vascular endothelial growth factor-mediated angiogenic activity (17), we did not find any significant differences in the expression of EGFR or HER-2 between ASDs and non-ASD lesions of similar levels of dysplasia. Given that recent work by our group has shown increased levels of vascular endothelial growth factor expression in ASD (41), more quantitative analyses and in-depth characterization of receptor signaling pathway activation may be needed to determine whether EGFR or HER-2 is involved in the induction of angiogenesis in these lesions. A previous finding that is confirmed by our data is the demonstration of an association between EGFR expression and induction of epithelial proliferation in these dysplasias (31, 35, 39, 42, 43). Although increased proliferating cell nuclear antigen expression in association with EGFR expression has been reported, our data extend the demonstration of an association between EGFR expression and increased epithelial proliferation to include induction of the proliferation markers Ki-67 and MCM2. More importantly, we show for the first time (a) a direct correlation between levels of EGFR expression and degree of dysplasia, (b) a field effect in the induction of EGFR expression in the airways of subjects with bronchial dysplasia, and (c) a potential synergistic effect on epithelial proliferation in lesions with increased expression of both EGFR and HER-2. These findings suggest that EGFR plays a prominent role in the development and progression of bronchial dysplasia and could provide a promising target for chemoprevention of lung cancer.

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