Immunohistochemical Expression of Basic Fibroblast Growth Factor and Fibroblast Growth Factor Receptors 1 and 2 in the Pathogenesis of Lung Cancer

Carmen Behrens,[1] Heather Y. Lin,[2] J. Jack Lee,[2] Maria Gabriela Raso,[3] Waun Ki Hong,[1] Ignacio I. Wistuba,[1,3] and Reuben Lotan[1]

Abstract

Purpose: To identify the patterns of protein expression of basic fibroblast growth factor (bFGF) and FGF receptors 1 and 2 in non-small cell lung carcinoma (NSCLC) and their role in the early pathogenesis of squamous cell carcinoma (SCC) of the lung.

Experimental Design: Archived tissue from NSCLC (adenocarcinoma and SCC; $n = 321$) and adjacent bronchial epithelial specimens ($n = 426$) were analyzed for the immunohistochemical expression of bFGF, FGFR1, and FGFR2, and the findings were correlated with clinicopathologic features of the patients.

Results: High expression of bFGF, FGFR1, and FGFR2 was shown in most NSCLC tumors. The pattern of expression for all markers varied according to tumor histologic type and cellular localization. Cytoplasmic expression scores were significantly higher in tumors than in normal epithelia. Nuclear bFGF ($P = 0.03$) and FGFR1 ($P = 0.02$) levels were significantly higher in women than in men. Although cytoplasmic FGFR1 expression was significantly higher ($P = 0.002$) in ever smokers than in never smokers, nuclear FGFR1 ($P = 0.0001$) and FGFR2 ($P = 0.003$) expression was significantly higher in never smokers. Different prognostic patterns for the expression of these markers were detected for both NSCLC histologic types. Dysplastic changes showed significantly higher expression of all markers compared with squamous metaplasia.

Conclusions: bFGF, FGFR1, and FGFR2 are frequently overexpressed in SCC and adenocarcinoma of the lung. bFGF signaling pathway activation may be an early phenomenon in the pathogenesis of SCC and thus an attractive novel target for lung cancer chemopreventive and therapeutic strategies.

Lung cancer, the leading cause of cancer-related deaths in the United States (1), consists of several histologic types (2), the most common being two non-small cell lung carcinomas (NSCLC): adenocarcinoma and squamous cell carcinoma (SCC; ref. 3). In spite of recent advances, the underlying processes involved in the early pathogenesis of lung cancer remain unclear. NSCLCs are believed to arise after the progression of sequential preneoplastic lesions, including bronchial squamous dysplasias (4). The activation of angiogenesis pathways has been shown to be involved in the development and progression of lung cancer (5–7). A better understanding of the signaling pathways that lead to tumor growth and angiogenesis may help in the development of new and more effective strategies for early detection, targeted chemoprevention, and treatment of lung cancer.

Fibroblast growth factor (FGF) 2, or basic FGF (bFGF), and its transmembrane tyrosine kinase receptors (the FGFRs) make up a large, complex family of signaling molecules involved in several physiologic processes, and the dysregulation of these molecules has been associated with cancer development (8, 9). bFGF belongs to a family of ubiquitously expressed ligands that bind to the extracellular domain of FGFRs, initiating a signal transduction cascade that promotes cell proliferation, motility, and angiogenesis (8–10).

As with some other angiogenesis pathways, the bFGF pathway has been shown to be activated in lung cancer (11–18). Elevated levels of bFGF, FGFR1, and FGFR2 proteins have been detected in NSCLC cell lines (11, 19). Although a few reports discuss the expression of bFGF and FGFRs in NSCLC tumors (12–18), the precise role of these molecules in the early pathogenesis and progression of this tumor is still unknown.

To identify the patterns of expression of bFGF, FGFR1, and FGFR2 proteins in the two major histologies of NSCLC, namely SCC and adenocarcinoma, and their role in the early pathogenesis of SCC of the lung, we investigated the immunohistochemical expression of these three molecules in a large series of archived NSCLC tissue specimens and adjacent

Authors' Affiliations: Departments of [1]Thoracic/Head and Neck Medical Oncology, [2]Biostatistics, and [3]Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

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Note: I.I. Wistuba and R. Lotan contributed equally to this research.

Requests for reprints: Reuben Lotan, Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Unit 432, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-8467; Fax: 713-745-5656; E-mail: rlotan@mdanderson.org.

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Translational Relevance

A better understanding of the signaling pathways that lead to tumor growth and angiogenesis may help in the development of new and more effective strategies for targeted chemoprevention and treatment of lung cancer. Our findings of frequent overexpression of bFGF, FGFR1, and FGFR2 in NSCLC, and in the early pathogenesis of squamous cell carcinomas, suggest that the activation of the bFGF pathway, which has been proposed to facilitate the development of resistance to antiangiogenic therapy targeting the vascular endothelial growth factor pathway, is an attractive novel target for lung cancer therapeutic and chemopreventive strategies.

lung bronchial epithelial foci and constructed tissue microarray (TMA) specimens. We then correlated our findings with clinicopathologic features of patients with lung cancer.

Materials and Methods

Case selection and TMA construction. For this study, which was approved by our institutional review board, we obtained archived formalin-fixed, paraffin-embedded material from surgically resected lung cancer specimens containing tumor and adjacent lung tissues from the Lung Cancer Specialized Program of Research Excellence tissue bank at The University of Texas M. D. Anderson Cancer Center. Tissue specimens collected between 1997 and 2003 from 321 lung cancer tumors (196 adenocarcinomas and 125 SCCs) were classified by using the 2004 WHO classification system (3) and selected specimens were used for construction of TMAs. In addition, detailed histopathologic semiquantitative analysis of lung adenocarcinoma subtypes (acinar, papillary, solid with mucin, and bronchioloalveolar) using the WHO classification (3) was done in 192 lung adenocarcinomas. After histologic examination, the NSCLC TMAs were constructed by obtaining three 1-mm-diameter cores from each tumor.

To assess the immunohistochemical expression of bFGF, FGFR1, and FGFR2 in the early pathogenesis of NSCLC, we studied formalin-fixed, paraffin-embedded material from 426 specimens of bronchial epithelium surgically resected from 130 patients with NSCLC (mean, 3.3 specimens per patient; range, 1-25 specimens). We histologically classified epithelial lesions by using the 2004 WHO classification system for preneoplastic lung lesions (3). The histologic findings from the epithelia were as follows: normal epithelium (n = 150), basal cell hyperplasia (n = 164), squamous metaplasia (n = 26), and squamous dysplasia (n = 86). The squamous dysplasias were arranged into two groups: low-grade (mild and moderate dysplasias; n = 22) and high-grade (severe dysplasia and carcinoma *in situ*; n = 64). For the epithelial foci TMAs, we used single 2-mm cores in an attempt to capture most of the preneoplastic lesions.

Immunohistochemical staining and evaluation. The following primary antibodies were used for immunohistochemical staining: mouse monoclonal anti-bFGF (BD Biosciences PharMingen; dilution 1:200), rabbit polyclonal anti-FGFR1 (Flg; Santa Cruz Biotechnology; dilution 1:100), and rabbit polyclonal anti-FGFR2 (Bek; Santa Cruz Biotechnology; dilution 1:100). Formalin-fixed, paraffin-embedded tissue sections (5-μm-thick) were deparaffinized and hydrated. For bFGF, antigen retrieval was done by incubating specimens for 5 min in 1% SDS in TBS [100 mmol/L Tris (pH 7.4), 138 mmol/L NaCl, 27 mmol/L KCl] at room temperature. For FGFR1 and FGFR2, antigen retrieval was done by heating specimens in a steamer for 10 min with 10 mmol/L sodium citrate (pH 6.0). We performed protein blocking by incubating specimens for 30 min in 10% bovine serum albumin in TBS with 0.5% Tween 20. Primary antibody incubation was done overnight at 4°C for bFGF and for 2 h at room temperature for FGFR1 and FGFR2.

Specimens were next washed with PBS. They were incubated for 30 min with secondary antibody with Envision Plus Dual Link-labeled polymer (DAKO) and then for 5 min with diaminobenzidine chromogen. Formalin-fixed, paraffin-embedded lung tissues having both tumor and normal tissues were used as a positive control. For a negative control, we used the same specimens that we used for the positive control but replaced the primary antibody with PBS.

The expression was quantified by two observers (C.B. and I.I.W.). Cytoplasmic expression was quantified using a four-value intensity score (0, 1+, 2+, and 3+) and the percentage (0-100%) of the extent of reactivity. Next, the cytoplasmic expression score was obtained by multiplying the intensity and reactivity extension values (range, 0-300). Nuclear expression was quantified using a range of 0 to 100 according to the percentage of positive nuclei present among all tumor or epithelium cells present in the TMA cores.

Statistical analysis. The data were summarized using standard descriptive statistics and frequency tabulations. Associations between the marker expression and patients' clinical demographic variables including age, sex, smoking history, histology type, and pathologic

Table 1. Summary of the clinicopathologic features of lung cancer cases studied

Feature	NSCLC histologic type*		
	SCC (n = 125)	Adenocarcinoma (n = 196)	Total (N = 321)
Mean age (range), y	68.5 (42-90)	64.7 (34-87)	66.3 (34-90)
Sex			
Male	79	74	153
Female	46	122	168
Smoking status †			
Never	6	50	56
Ever	118	146	264
TNM stage			
I	68	130	198
II	39	25	64
III	17	34	51
IV	1	7	8

*Values are number of cases unless otherwise indicated.
† Smoking status was not available in one patient with SCC.

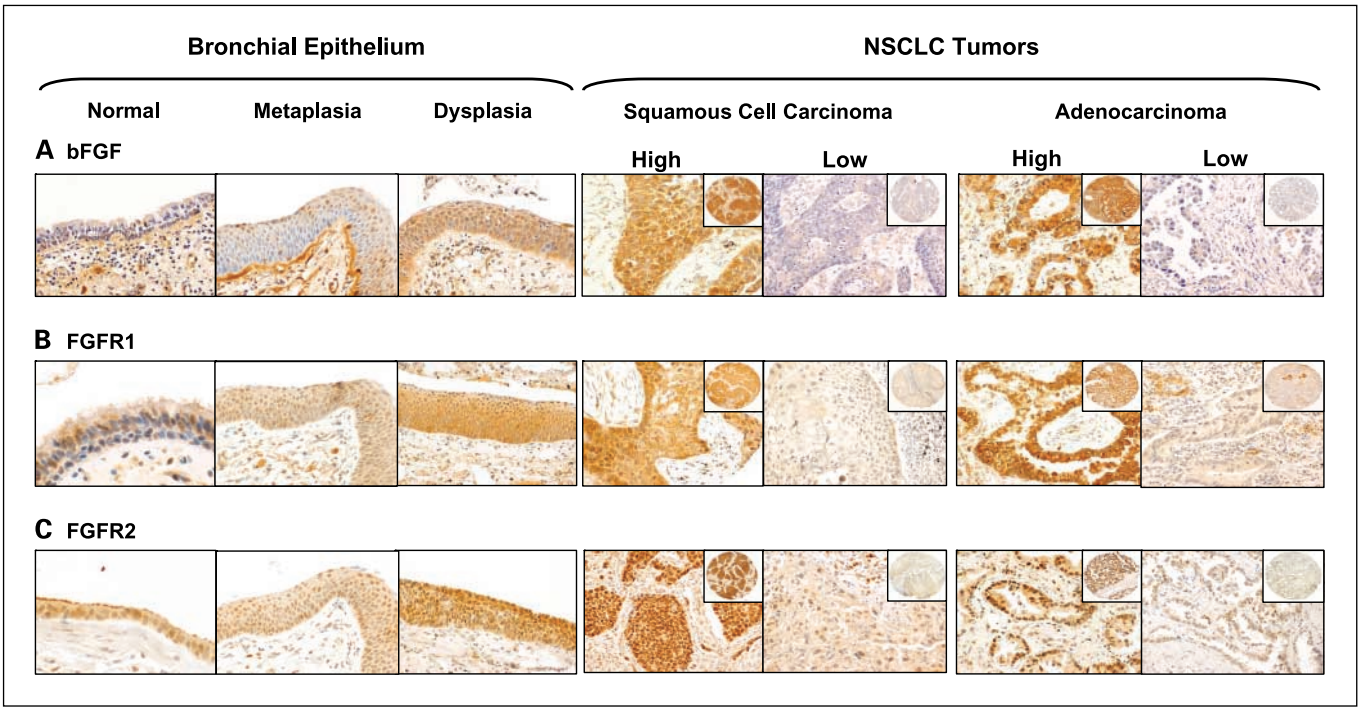

Fig. 1. Microphotographs of immunohistochemical expression of bFGF (*A*), FGFR1 (*B*), and FGFR2 (*C*) in tissue specimens of NSCLC tumor (SCC and adenocarcinoma) and bronchial epithelium (squamous metaplasias, squamous dysplasia, and normal epithelium). For all three markers, immunostaining was preferentially cytoplasmic, but nuclear staining was also detected. In bronchial epithelial specimens, levels of expression were higher in squamous dysplasia than in squamous metaplasia and normal epithelium. For each histologic type and marker in NSCLC tumors, both high and low levels of magnification are shown. *Insets,* tumorTMA core.

stage were assessed using appropriate methods including χ^2 test or Fisher's exact test for categorical variables and Wilcoxon's rank-sum test or Kruskal-Wallis test for continuous variables. Linear mixed-effects model was applied to compare the marker expression level among various histologic progression stage including normal, hyperplasia, metaplasia, dysplasia, and cancer.

We performed overall survival (OS) and recurrence-free survival (RFS) analyses for the expression of bFGF, FGFR1, and FGFR2 by using specimens from 321 NSCLC patients with a median follow-up of 3.78 years. Survival curves were estimated using the Kaplan-Meier method. OS was defined as the time from surgery to death or to the end of the study and RFS as the time from surgery to recurrence or to the end of the study. The effect of the marker expression on patients' OS and RFS was tested for both histologic types separately and adjusted for age, sex, smoking status, and pathologic TNM stage. The binary cutoff points of biomarkers were identified using the Classification and Regression Tree algorithm, in which a cutoff point is determined for each predictor variable such that the two resulting subgroups are the most different in OS. The same cutoffs were used in the RFS analysis. Univariate and multivariate Cox proportional hazards models were used to assess the effect of covariates on OS and RFS. Two-sided P values < 0.05 were considered statistically significant. Any significant findings can serve as hypothesis generation and require further confirmation in future studies. All analyses were conducted in SAS 9.1 (SAS Institute) and S-PLUS (Insightful Corp.).

Results

Patients' characteristics. We studied 321 surgically resected lung cancers representing the two major NSCLC histologies using archival tissue specimens. Detailed clinical and pathologic information was available in most cases (Table 1) and included patients' demographic data, smoking history [never smokers or ever smokers (patients who had smoked at least 100 cigarettes in their lifetime)], pathologic TNM staging (20), OS time, and RFS time.

Immunohistochemical expression of bFGF and receptors in NSCLC compared with that in normal epithelium. bFGF, FGFR1, and FGFR2 were detected in the cytoplasm and nuclei of bronchial epithelium and tumor cells (Fig. 1). Overall, NSCLC tumor cells showed higher levels of bFGF, FGFR1, and FGFR2 protein expression than histologically normal bronchial epithelium did. No significant difference in the expression of these three markers was detected in normal epithelium obtained from patients with adenocarcinoma or SCC. Specimens from both histologic types of NSCLC, however, had significantly higher cytoplasmic expression scores for all three markers than did the histologically normal specimens of bronchial epithelium obtained from patients with lung cancer (Figs. 1 and 2). Significant higher FGFR1 and FGFR2 (but not bFGF) nuclear expression scores were also found in tumor than in normal epithelium for patients with adenocarcinoma. On the other hand, significantly higher bFGF (but not FGFRs) nuclear expression score was found in tumor than in normal epithelium in patients with squamous carcinoma (Fig. 2).

Markers of immunohistochemical expression in the sequential pathogenesis of SCC. We investigated the expression of bFGF, FGFR1, and FGFR2 in 426 epithelial specimens containing histologically normal, hyperplastic, squamous metaplastic, or squamous dysplastic bronchial epithelia adjacent to NSCLC obtained from 130 patients. Similar levels of cytoplasmic and nuclear expression were detected in normal and hyperplastic epithelia for all three markers. For cytoplasmic FGFR1 and FGFR2 localizations, squamous metaplastic epithelia showed

significantly lower levels of the markers than did normal (FGFR1, $P = 0.022$; FGFR2, $P = 0.022$) and hyperplastic (FGFR1, $P = 0.047$; FGFR2, $P = 0.049$) epithelia. For nuclear localization, no significant differences in the scores of any markers were detected between normal, basal cell hyperplastic, and squamous metaplastic epithelia. Of interest, squamous dysplastic lesions showed significantly higher levels of expression than did squamous metaplastic lesions for all three markers (Fig. 3). The monotone increase pattern in the expression of these markers from squamous dysplasia to invasive SCC is consistent with the progression model of this tumor type.

Correlation between markers of immunohistochemical expression in NSCLC and clinicopathologic features and disease outcomes. We correlated bFGF and FGFR scores and levels of expression with tumor histologic characteristics, age, sex, smoking history, and TNM pathologic stage. The expression of these markers at the cytoplasmic level was similar in both NSCLC histologic types, except for FGFR2, which was significantly higher ($P = 0.006$) in SCC (Fig. 2). At the nuclear level, SCC showed significantly higher ($P = 0.016$) expression of bFGF than did adenocarcinoma, whereas the latter showed significantly higher expression of both receptors (FGFR1, $P < 0.0001$; FGFR2, $P = 0.0007$; Fig. 2).

One striking association observed was the correlation between the immunohistochemical expression of bFGF and the receptors with sex and smoking history in patients with adenocarcinoma (Table 2). Expression of nuclear bFGF and nuclear FGFR1 were significantly higher ($P = 0.03$ and $P = 0.02$, respectively) in women than in men. Although cytoplasmic FGFR1 expression was significantly higher ($P = 0.002$) in ever smokers than in never smokers, expression of nuclear FGFR1 ($P = 0.0001$) and nuclear FGFR2 ($P = 0.003$) was significantly higher in never smokers than in ever smokers. No significant associations between all three markers and gender or smoking status were found in patients with squamous carcinoma. For both NSCLC types, no correlation was detected between marker expression and age or TNM pathologic stage. In lung adenocarcinoma, FGFR1 nuclear expression positively and significantly (Spearman's correlation coefficient $r = 0.29$;

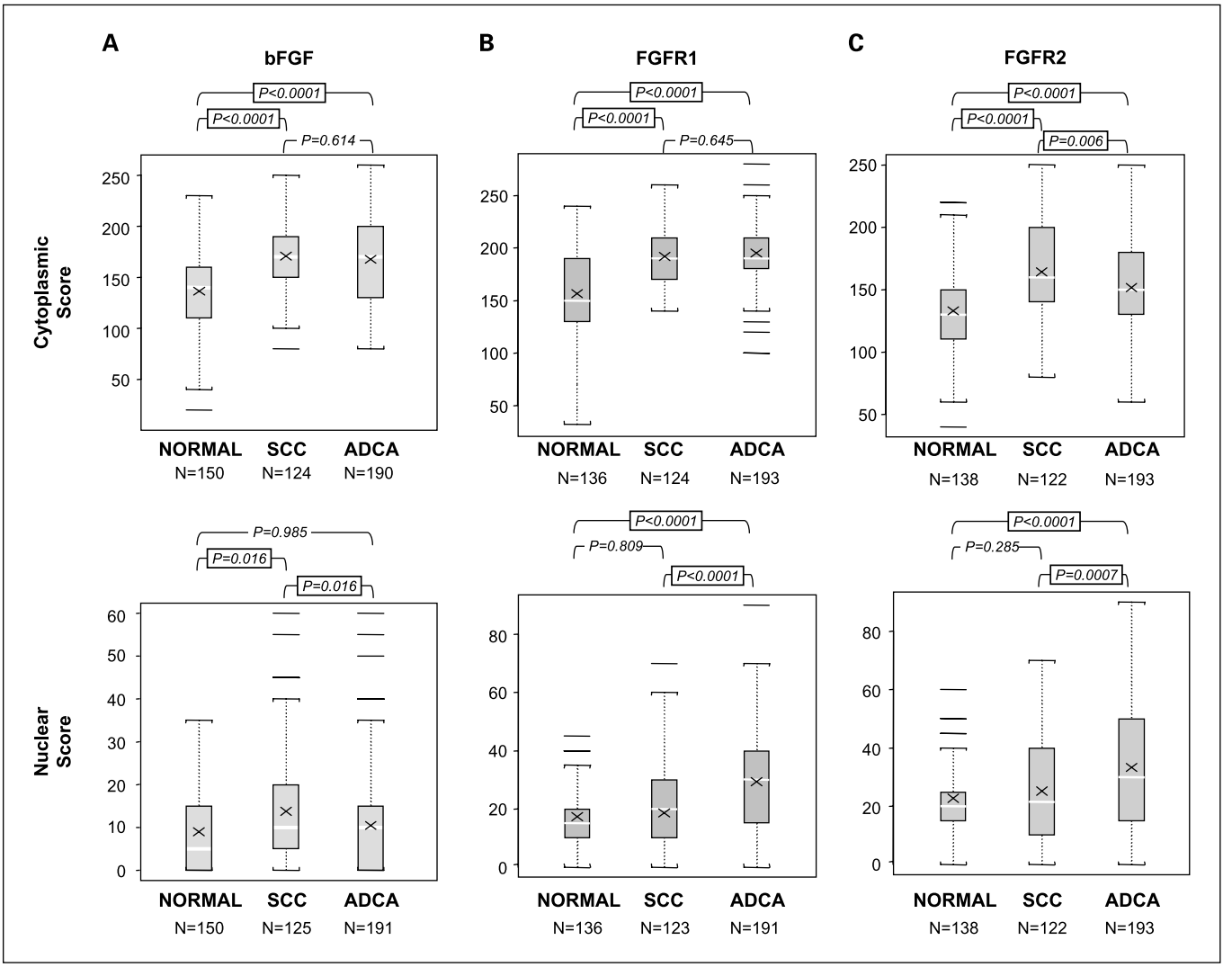

Fig. 2. Cytoplasmic (*top*) and nuclear (*bottom*) scores of immunohistochemical expression of bFGF (*A*), FGFR1 (*B*), and FGFR2 (*C*) in normal bronchial epithelia (*NORMAL*) obtained from lung cancer patients, SCC, and adenocarcinoma (*ADCA*) of the lung. The number of samples is indicated for each histologic group and marker. *P* values comparing normal epithelial and tumor histologic types are shown for all comparisons. Boxes around *P* values indicate statistical significance. *Bars,* 95% CI.

$P < 0.0001$) correlated with the presence of bronchioloalveolar subtype. Of interest, this correlation was independent of gender and remained significant ($P < 0.003$) after adjusting by smoking history in multivariate analysis (data not shown).

We analyzed the prognostic effects of the markers on disease outcomes using both a continuous score and a dichotomized score of the marker expressions using the cutoffs identified by Classification and Regression Tree algorithm. In patients with adenocarcinoma, both scores of cytoplasmic FGFR1 show a trend, although nonsignificant, effect on OS ($P = 0.070$ and $P = 0.068$, respectively, for the continuous and binary score, >195) and RFS ($P = 0.087$ and $P = 0.12$, respectively, for the continuous and binary measure) in the univariate setting. The Kaplan-Meier curves for the binary score shown in Fig. 4A indicate that high cytoplasmic FGFR1 was associated with a worse OS and a worse RFS. After adjusting for age, gender, smoking history, and pathologic stage, the effects of the cytoplasmic overexpression of FGFR1 on OS and on RFS remained marginally significant. Patients with high cytoplasmic FGFR1 had a worse OS [$P = 0.07$; hazard ratio (HR), 1.51; 95% confidence interval (95% CI), 0.97-2.33] and a worse RFS ($P = 0.05$; HR, 1.63; 95% CI, 1.00-2.67). Of interest in these patients, when treating as a continuous variable, each one-unit increase in the FGFR1 cytoplasmic score conferred a nonsignificant trend to a better OS ($P = 0.071$; HR, 1.01; 95% CI, 1.00-1.01) and a significantly worse RFS ($P = 0.02$; HR, 1.01; 95% CI, 1.00-1.02).

In patients with SCCs, we detected a more complex pattern of prognostic association. In the univariate analyses, nuclear FGFR1 (score > 17.5) and nuclear FGFR2 (score > 55) showed close to significant or significant effects on OS ($P = 0.061$ and 0.031 for nuclear FGFR1 and FGFR2, respectively). Nuclear FGFR1 and FGFR2 had a significant effect on RFS ($P = 0.027$ and 0.021, respectively). As the Kaplan-Meier curves for the binary score in Fig. 4B show, high nuclear expression of FGFFR1 was associated with a worse OS and a worse RFS. When age, gender, smoking history, and pathologic stage are compared, the nuclear overexpression of FGFR1 (score > 17.5) and FGFR2 (score > 55) significantly correlated with worse outcome in RFS and OS, respectively. The nuclear overexpression of FGFR1 conferred to patients a worse RFS ($P = 0.04$; HR, 1.97; 95% CI, 1.04-3.72), and the nuclear overexpression of FGFR2 correlated with worse OS ($P = 0.02$; HR, 2.54; 95% CI, 1.18-5.47) and RFS ($P = 0.02$; HR, 2.84; 95% CI, 1.18-6.86). In contrast, the cytoplasmic overexpression of bFGF (score > 175) and FGFR2 (score > 155) significantly correlated with better OS (bFGF: $P = 0.02$; HR, 0.55; 95% CI, 0.33-0.92 and FGFR2: $P = 0.008$; HR, 0.51; 95% CI, 0.31-0.83). When they are analyzed as a continuous variable, they also show trend, although nonsignificant, effects on OS and RFS. Again, high cytoplasmic bFGF is associated with a better OS ($P = 0.068$; HR, 0.99; 95% CI, 0.99-1.00 per unit increment). High cytoplasmic FGFR2 is associated with a better OS ($P = 0.086$; HR, 0.99; 95% CI, 0.99-1.00 per unit increment).

Correlations of markers' expression. In tumor specimens, we analyzed the correlation between the expression of all markers at cytoplasmic and nuclear localizations. Comparing all three markers, we detected a complex pattern of correlations of immunohistochemical expression in NSCLC that differed between the adenocarcinoma and SCC histologic types. A positive significant correlation between the cytoplasmic and nuclear overexpression of bFGF was detected in adenocarcinoma and of FGFR2 in both adenocarcinoma and SCC. In adenocarcinoma specimens, the correlation between bFGF and FGFR1 and between FGFR1 and FGFR2 was observed at cytoplasmic and nuclear localizations, whereas these correlations were found only at cytoplasmic localizations in specimens of SCC.

Discussion

As with some other angiogenesis pathways, the bFGF pathway has been shown previously to be activated in lung cancer (11–18). Although several reports showed high levels of

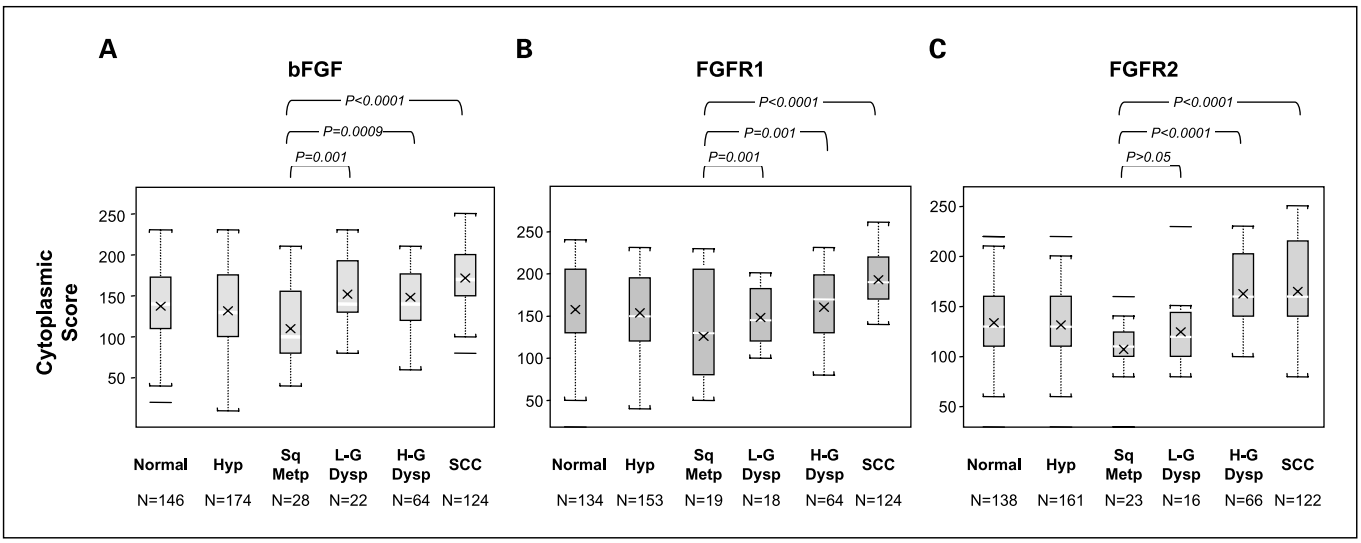

Fig. 3. Scores for cytoplasmic immunohistochemical expression of bFGF (*A*), FGFR1 (*B*), and FGFR2 (*C*) in bronchial respiratory epithelial lesions related to the pathogenesis of SCC of the lung: normal epithelium (*Normal*), hyperplasia (*Hyp*), squamous metaplasia (*Sq Metp*), low-grade dysplasia (*L-G Dysp*), high-grade dysplasia (*H-G Dysp*), and SCC. The number of samples is indicated for each histologic group and marker. Only significant *P* values for comparisons between squamous metaplastic and squamous dysplastic lesions and SCC are shown.

Table 2. bFGF, FGFR1, and FGFR2 immunohistochemical expression in NSCLC types by histology, sex, and smoking status

	No.	Immunohistochemical markers, mean (SD)					
		bFGF		FGFR1		FGFR2	
		Cytoplasmic expression score*	Nuclear expression score †	Cytoplasmic expression score*	Nuclear expression score †	Cytoplasmic expression score*	Nuclear expression score †
Adenocarcinoma							
Sex							
Male	74	169.6 (47.6)	8.1 (9.4)	196.1 (27.7)	25.1 (18.9)	151.4 (37.4)	32.4 (23.1)
Female	122	166.1 (46.3)	11.9 (12.7)	194.6 (28.6)	31.8 (19.7)	151.9 (35.0)	33.9 (23.0)
P		0.62	0.03	0.73	0.02	0.92	0.65
Smoking status							
Never	50	162.5 (45.0)	11.6 (9.6)	184.7 (24.2)	38.4 (17.6)	148.4 (33.6)	41.5 (24.1)
Ever	146	169.1 (47.3)	10.1 (12.3)	198.8 (28.6)	26.2 (19.4)	152.9 (36.6)	30.5 (22.1)
P		0.40	0.44	0.002	0.0001	0.45	0.003
SCC							
Sex							
Male	79	174.5 (33.1)	12.9 (13.2)	193.3 (24.9)	18.3 (14.2)	166.1 (36.0)	24.2 (17.3)
Female	46	164.3 (37.0)	15.1 (12.8)	189.1 (26.5)	19.0 (13.5)	160.9 (36.6)	26.9 (18.8)
P		0.12	0.36	0.36	0.80	0.44	0.43
Smoking status							
Never	6	176.7 (46.3)	5.8 (13.3)	185.0 (15.2)	17.5 (11.7)	141.7 (43.1)	12.3 (9.9)
Ever	118	170.2 (35.1)	14.1 (13.2)	192.3 (25.5)	18.5 (14.1)	165.6 (35.6)	25.8 (18.0)
P		0.66	0.13	0.48	0.86	0.11	0.07

*Cytoplasmic immunostaining expression was quantified using a four-value intensity score (0, 1+, 2+, and 3+) and the percentage (0-100%) of the extent of reactivity. The cytoplasmic expression score was obtained by multiplying both intensity and reactivity extension values (range, 0-300).

† Nuclear immunostaining expression was quantified using a range of 0 to 100 according to the percentage of positive nuclei.

expression of bFGF and FGFR1 in NSCLC, the precise role of these signaling molecules in the pathogenesis and progression of this tumor is still unclear (12–18). Eight studies that conducted immunohistochemical expression analyses of NSCLCs (including adenocarcinomas and SCCs) described frequent bFGF expression in lung tumors, ranging from 49% to 77% of lung tumors (12–17, 21, 22). In four studies about FGFR1 immunohistochemical expression in NSCLC tissue specimens, these frequencies ranged from 50% to 81% (12–15). For FGFR2, only one immunohistochemical study was done involving 61 NSCLC specimens, 50% of which showed receptor expression (21). All of these studies used different arbitrary cutoffs to assess positive immunohistochemical expression; thus, the data are difficult to interpret.

In this study, we described high levels of immunohistochemical expression of bFGF, FGFR1, and FGFR2 in a large series of NSCLC specimens, including the two most frequent histologic types, SCC and adenocarcinoma, similarly to previous studies (12–17, 21, 22). To our knowledge, this is the first comprehensive analysis of bFGF, FGFR1, and FGFR2 in the same set of NSCLC specimens. Overall, we found higher levels of bFGF and FGFRs expression in tumor cells than in adjacent normal bronchial epithelia at cytoplasmic localization in both SCC and adenocarcinoma. These findings are consistent with the postulated mitogenic and angiogenic effects of the bFGF signaling pathway activation in human tumors, including NSCLC (8).

Lung cancers are believed to develop through a series of progressive pathologic changes (preneoplastic or precursor lesions) in the respiratory mucosa that result in an accumulation of genetic abnormalities (23). These abnormalities are frequently extensive and multifocal throughout the respiratory epithelium, indicating a field effect or field cancerization phenomenon (23). Although sequential preneoplastic changes have been defined for centrally arising squamous carcinomas, they have been poorly documented for lung adenocarcinomas (4). Mucosal changes in the large airways that may precede invasive SCC include squamous dysplasia and carcinoma *in situ* in the central bronchial airway (24, 25). In lung cancer pathogenesis, the genetic changes commence in histologically normal epithelium and are present in a similar frequency in mildly abnormal epithelia, including basal cell hyperplasia and squamous metaplasia (23). In our study, we describe, to our knowledge for the first time, a widespread expression of bFGF, FGFR1, and FGFR2 adjacent to histologically normal, mildly abnormal, and preneoplastic bronchial respiratory epithelium in NSCLC specimens. Our findings of a significant increase in the immunohistochemical expression of bFGF and FGFR proteins at cytoplasmic (bFGF, FGFR1, and FGFR2) and nuclear (FGFR2) localizations in respiratory epithelium with low-grade (cytoplasmic bFGF) and high-grade (cytoplasmic bFGF and FGFRs and nuclear FGFR2) squamous dysplastic, compared with metaplastic, bronchial epithelia, suggest that the activation of the bFGF pathway is an early event in the pathogenesis of SCC of the lung and could play a role in the angiogenic switch characteristic of tumor promotion.

Whereas most of the previous studies reported either cytoplasmic protein expression only (12, 13, 16) or did not indicate immunostaining cell localization (14, 15, 17, 22), we described the expression of bFGF, FGFR1, and FGFR2 at both

cytoplasmic and nuclear localizations in our NSCLC specimens. These findings are consistent with previous *in vitro* studies showing that bFGF and FGFR1 translocate to the cell nucleus (26). In addition, the nuclear localization of bFGF in cells has been known for many years (27, 28), and there is some evidence that this translocation is required for the induction of cell proliferation (29). More recently, it has been shown that on cell stimulation with bFGF, FGFR1, a plasma membrane-associated protein, is undergoing endocytosis to the cytosol and translocates to the cell nucleus along with its ligand bFGF (26). Within the nucleus, FGFR1 serves as a general transcriptional regulator that activates structurally distinct genes located on different chromosomes and stimulates multigene programs for cell growth and differentiation (26).

In the correlation analysis of the immunohistochemical expression of bFGF and its receptors and patients' clinicopathologic characteristics, we found, somewhat surprisingly, a significantly higher level of nuclear bFGF and FGFR1 expression in tumor specimens obtained from female patients than in specimens from male patients with adenocarcinoma. These findings link the phenomenon of angiogenesis with sex-related oncogenic mechanisms, including sex steroid hormones. In women, the effects of estrogen and progesterone in angiogenesis pathways such as those associated with vascular endothelial growth factor have been established in normal endometrial and breast tissues (30–33) and are being investigated in the corresponding tumors (34, 35). In the bFGF pathway, much less is known about whether sex steroids are involved with the regulation of normal and malignant tissues. In the normal endometrium, bFGF mRNA has shown a cyclic variation, suggesting that at least mRNA levels may be regulated by sex steroids (36). In breast cancer, bFGF levels seem to be up-regulated compared with normal adjacent tissue and associated with a high expression of the estrogen receptor, suggesting a correlation between them (35). The role of hormones, particularly estrogen, as a risk factor for the development of lung cancer among women is an area of vigorous investigation (37), and in NSCLC, there is evidence of cross-talk between the estrogen receptor and other growth factor signaling pathways, including the epidermal growth factor receptor (38).

In addition, in adenocarcinoma specimens, we detected differences in the expression of the three markers and patients' smoking status, with cytoplasmic FGFR1 expression being significantly higher in smokers and nuclear FGFR1 and FGFR2 significantly higher in never smokers. To our knowledge, these associations have not been reported

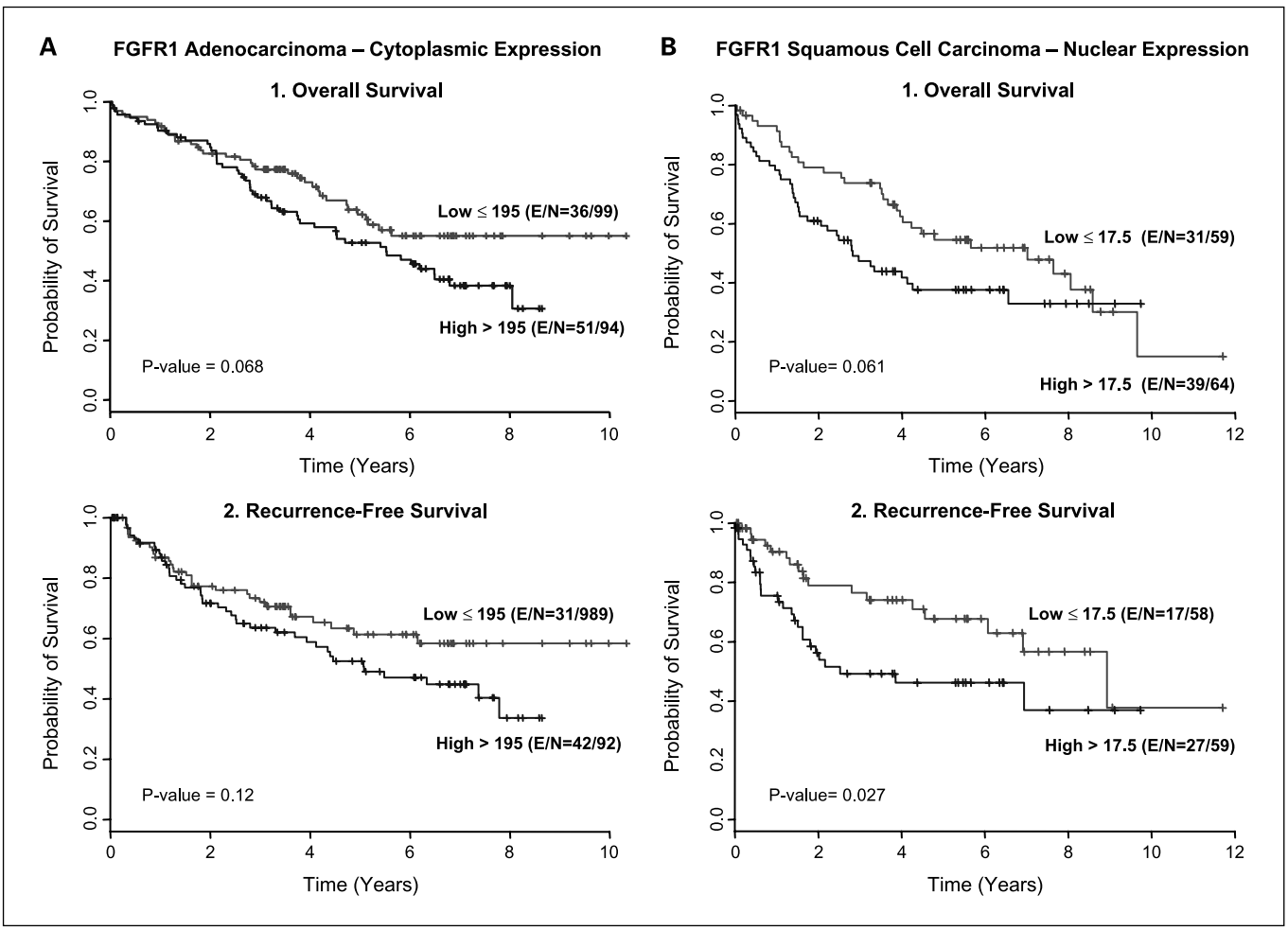

Fig. 4. Kaplan-Meier curves illustrating FGFR1 cytoplasmic protein expression for adenocarcinoma (*A*) and FGFR1 nuclear expression for SCC (*B*) patients.

previously. These differences highlight the potential differential role of these proteins in the pathogenesis of both smoking and non-smoking-related lung cancers. There is some evidence that links the bFGF pathway with smoking in lung diseases. It has been suggested that the bFGF pathway plays a role in regulating airway wall remodeling, especially in individuals with smoking-induced chronic obstructive peripheral disease, which is related to inflammation of the small airway (39). Of interest, Kranenburg et al. (40) conducted an immunohistochemical analysis of bronchial specimens obtained from patients with chronic obstructive peripheral disease and showed that FGFR1 immunolocalized in the cytoplasm of bronchial epithelial cells and in other cell components of the bronchial wall. We hypothesize that the inflammatory phenomenon occurring in smoking-damaged airway may be associated with the predominantly cytoplasmic localization of these receptors. Our finding of the differential expression of cytoplasmic FGFR2 and of both receptors at the nuclear level with smoking status in tumor specimens needs further study in epithelial lesions.

The prognostic effect of the expression of bFGF, FGFR1, and FGFR2 proteins in lung cancer has been studied previously in a limited number of tumor cases (14, 16, 17, 21). In NSCLC, high levels of bFGF expression, determined by ELISA in tissue extracts (n = 71; ref. 17) and by immunohistochemical expression in the cytoplasm of tumor cells (n = 119; ref. 16), correlated with worse OS. Similarly, high levels of FGFR1 and FGFR2 expression correlated with shorter OS in patients with NSCLC (n = 206; ref. 14) and lung adenocarcinomas (n = 30; ref. 21). When we investigated the correlation of the expression of these markers with OS and RFS, our findings suggested that the overexpression of FGFR1 in the cytoplasm of tumor cells correlated with worse OS only in patients with adenocarcinoma, similar to findings reported by Yamayoshi et al. (21). In contrast, for SCC, we found that the nuclear overexpression of FGFR1 and FGFR2 correlated significantly with worse outcome in both OS and RFS. Somewhat surprisingly, the cytoplasmic overexpression of bFGF and FGFR2 in squamous tumor cells correlated with better OS. Although it seems paradoxical that a tumor that expresses high levels of bFGF and FGFR exhibits a better prognosis than a tumor that expresses low levels, we hypothesize that high levels of cytoplasmic expression may prevent nuclear localization of the proteins. Our data confirmed the notion that adenocarcinoma and SCC are not a homogeneous group of tumors and that they should always be examined separately for molecular targets and outcome.

We identified multiple correlations between the immunohistochemical expression of bFGF, FGFR1, and FGFR2 and their cytoplasmic and nuclear tumor cell localizations. All correlations but one were positive. Of interest, we detected a correlation between bFGF and FGFR1 expression in both NSCLC histologic types at the cytoplasmic level. These findings suggest that the cytoplasmic overexpression of the ligand bFGF in tumor cells may influence the expression and cell internalization of FGFR1. In lung adenocarcinomas, this association was also found at the nuclear level, emphasizing the role of bFGF in FGFR1 translocation to the cell nucleus (26). A similar pattern of association was detected between both FGFRs, a phenomenon that could be associated with preferential receptors dimerization and subsequent cytoplasmic internalization and nuclear translocation.

In summary, our findings indicate that bFGF, FGFR1, and FGFR2 are frequently overexpressed in NSCLC, although different patterns of expression are detected in its two major types. Our findings further suggest that bFGF signaling pathway activation is an early phenomenon in the pathogenesis of SCC of the lung. In addition, the frequent and early overexpression of bFGF and FGFR markers in patients with NSCLC suggests that the activation of the bFGF pathway, which has been proposed to facilitate the development of resistance to antiangiogenic therapy targeting the vascular endothelial growth factor pathway (41), is an attractive novel target for lung cancer therapeutic and chemopreventive strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.
2. Minna JD, Gazdar A. Focus on lung cancer. Cancer Cell 2002;1:49–52.
3. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC. Tumours of the lung. In: Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, editors. Pathology and genetics: tumours of the lung, pleura, thymus and heart. Lyon: IARC; 2004. p. 9–124.
4. Wistuba I. Genetics of preneoplasia: lessons from lung cancer. Curr Mol Med 2007;7:3–14.
5. Gazdar AF, Minna JD. Angiogenesis and the multistage development of lung cancers. Clin Cancer Res 2000;6:1611–2.
6. Keith RL, Miller YE, Gemmill RM, et al. Angiogenic squamous dysplasia in bronchi of individuals at high risk for lung cancer. Clin Cancer Res 2000;6:1616–25.
7. Merrick DT, Haney J, Petrunich S, et al. Overexpression of vascular endothelial growth factor and its receptors in bronchial dypslasia demonstrated by quantitative RT-PCR analysis. Lung Cancer 2005;48:31–45.
8. Dailey L, Ambrosetti D, Mansukhani A, Basilico C. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev 2005;16:233–47.
9. Ribatti D, Vacca A, Rusnati M, Presta M. The discovery of basic fibroblast growth factor/fibroblast growth factor-2 and its role in haematological malignancies. Cytokine Growth Factor Rev 2007;18:327–34.
10. Mohammadi M, Olsen SK, Ibrahimi OA. Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 2005;16:107–37.
11. Kuhn H, Kopff C, Konrad J, Riedel A, Gessner C, Wirtz H. Influence of basic fibroblast growth factor on the proliferation of non-small cell lung cancer cell lines. Lung Cancer 2004;44:167–74.
12. Takanami I, Tanaka F, Hashizume T, et al. The basic fibroblast growth factor and its receptor in pulmonary adenocarcinomas: an investigation of their expression as prognostic markers. Eur J Cancer 1996;32A:1504–9.
13. Takanami I, Tanaka F, Hashizume T, Kodaira S. Tumor angiogenesis in pulmonary adenocarcinomas: relationship with basic fibroblast growth factor, its receptor, and survival. Neoplasm 1997;44:295–8.
14. Volm M, Koomagi R, Mattern J, Stammler G. Prognostic value of basic fibroblast growth factor and its receptor (FGFR-1) in patients with non-small cell lung carcinomas. Eur J Cancer 1997;33:691–3.
15. Guddo F, Fontanini G, Reina C, Vignola AM, Angeletti A, Bonsignore G. The expression of basic fibroblast growth factor (bFGF) in tumor-associated stromal cells and vessels is inversely correlated with non-small cell lung cancer progression. Hum Pathol 1999;30:788–94.
16. Shou Y, Hirano T, Gong Y, et al. Influence of angiogenetic factors and matrix metalloproteinases upon tumour progression in non-small-cell lung cancer. Br J Cancer 2001;85:1706–12.
17. Iwasaki A, Kuwahara M, Yoshinaga Y, Shirakusa T. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) levels, as prognostic indicators in NSCLC. Eur J Cardiothorac Surg 2004;25:443–8.
18. Bremnes RM, Camps C, Sirera R. Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. Lung Cancer 2006;51:143–58.
19. Berger W, Setinek U, Mohr T, et al. Evidence for a role

of FGF-2 and FGF receptors in the proliferation of non-small cell lung cancer cells. Int J Cancer 1999; 83:415–23.
20. Mountain CF. Revisions in the international system for staging lung cancer. Chest 1997;111:1710–7.
21. Yamayoshi T, Nagayasu T, Matsumoto K, Abo T, Hishikawa Y, Koji T. Expression of keratinocyte growth factor/fibroblast growth factor-7 and its receptor in human lung cancer: correlation with tumour proliferative activity and patient prognosis. J Pathol 2004;204:110–8.
22. Ito H, Oshita F, Kameda Y, et al. Expression of vascular endothelial growth factor and basic fibroblast growth factor in small adenocarcinomas. Oncol Rep 2002;9:119–23.
23. Wistuba II. Genetics of preneoplasia: lessons from lung cancer. Curr Mol Med 2007;7:3–14.
24. Colby TV, Wistuba II, Gazdar A. Precursors to pulmonary neoplasia. Adv Anat Pathol 1998;5:205–15.
25. Kerr KM. Pulmonary preinvasive neoplasia. J Clin Pathol 2001;54:257–71.
26. Stachowiak MK, Maher PA, Stachowiak EK. Integrative nuclear signaling in cell development—a role for FGF receptor-1. DNA Cell Biol 2007;26:811–26.
27. Friesel R, Maciag T. Internalization and degradation of heparin binding growth factor-I by endothelial cells. Biochem Biophys Res Commun 1988;151:957–64.
28. Walicke PA, Baird A. Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. J Neurosci 1991;11:2249–58.
29. Wiedlocha A, Falnes PO, Madshus IH, Sandvig K, Olsnes S. Dual mode of signal transduction by externally added acidic fibroblast growth factor. Cell 1994; 76:1039–51.
30. Hyder SM, Murthy L, Stancel GM. Progestin regulation of vascular endothelial growth factor in human breast cancer cells. Cancer Res 1998;58:392–5.
31. Fujimoto J, Sakaguchi H, Hirose R, Ichigo S, Tamaya T. Progestins suppress estrogen-induced expression of vascular endothelial growth factor (VEGF) subtypes in uterine endometrial cancer cells. Cancer Lett 1999;141:63–71.
32. Hyder SM, Huang JC, Nawaz Z, et al. Regulation of vascular endothelial growth factor expression by estrogens and progestins. Environ Health Perspect 2000;108:785–90.
33. Welter H, Wollenhaupt K, Einspanier R. Developmental and hormonal regulated gene expression of fibroblast growth factor 2 (FGF-2) and its receptors in porcine endometrium. J Steroid Biochem Mol Biol 2004;88:295–304.
34. Fujimoto J, Toyoki H, Jahan I, et al. Sex steroid-dependent angiogenesis in uterine endometrial cancers. J Steroid Biochem Mol Biol 2005;93:161–5.
35. Smith K, Fox SB, Whitehouse R, et al. Upregulation of basic fibroblast growth factor in breast carcinoma and its relationship to vascular density, oestrogen receptor, epidermal growth factor receptor and survival. Ann Oncol 1999;10:707–13.
36. Nakamura J, Lu Q, Aberdeen G, Albrecht E, Brodie A. The effect of estrogen on aromatase and vascular endothelial growth factor messenger ribonucleic acid in the normal nonhuman primate mammary gland. J Clin Endocrinol Metab 1999;84:1432–7.
37. Stabile LP, Siegfried JM. Estrogen receptor pathways in lung cancer. Curr Oncol Rep 2004;6:259–67.
38. Stabile LP, Lyker JS, Gubish CT, Zhang W, Grandis JR, Siegfried JM. Combined targeting of the estrogen receptor and the epidermal growth factor receptor in non-small cell lung cancer shows enhanced antiproliferative effects. Cancer Res 2005;65:1459–70.
39. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 2004; 364:709–21.
40. Kranenburg AR, Willems-Widyastuti A, Mooi WJ, et al. Chronic obstructive pulmonary disease is associated with enhanced bronchial expression of FGF-1, FGF-2, and FGFR-1. J Pathol 2005;206:28–38.
41. Jubb AM, Oats AJ, Holden S, Koeppen H. Predicting benefits from anti-angiogenic agents in malignancy. Nat Rev Cancer 2006;6:626–35.

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