EGFR Tyrosine Kinase Domain Mutations Are Detected in Histologically Normal Respiratory Epithelium in Lung Cancer Patients

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Abstract

To determine whether *EGFR* tyrosine kinase domain mutations are early events in the pathogenesis of lung adenocarcinomas, we tested for the presence of *EGFR* mutations in histologically normal bronchial and bronchiolar epithelia from lung adenocarcinomas bearing the common *EGFR* mutations. DNA was extracted from microdissected tissue obtained from 21 tumors with known *EGFR* mutations, 16 tumors without mutation, and 90 sites of normal bronchial and bronchiolar epithelium from the same surgical specimens. With the use of PCR and direct DNA sequencing, *EGFR* mutations identical to the tumors were detected in the normal respiratory epithelium in 9 of 21 (43%) patients with *EGFR* mutant adenocarcinomas but none in patients without mutation in the tumors. The finding of mutations being more frequent in normal epithelium within tumor (43%) than in adjacent sites (24%) suggests a localized field effect phenomenon. Our findings indicate that mutation of the tyrosine kinase domain of *EGFR* is an early event in the pathogenesis of lung adenocarcinomas, and suggest *EGFR* mutations as an early detection marker and chemoprevention target. (Cancer Res 2005; 65(17): 7568-72)

Introduction

Four major histologic types compose the majority of lung cancers, and adenocarcinoma histology is currently the type most frequently diagnosed (1). It has been established that adenocarcinomas usually arise from the peripheral airway; however, the specific airway structure (bronchus, bronchiole, and alveolus) and the respiratory epithelium cell type (ciliated, goblet, Clara, and type II alveolar cells) from which most adenocarcinomas develop have not been established. Recent findings indicate that clinically evident lung adenocarcinomas are the results of the accumulation of numerous genetic and epigenetic changes, including abnormalities for the inactivation of tumor suppressor genes and the activation of oncogenes (2). Despite these advances, there is extremely limited information available on the early molecular pathogenesis of lung adenocarcinomas.

Somatic mutations of *EGFR*, a tyrosine kinase of the ErbB family, recently have been reported in specific subsets of lung adenocarcinomas (3–9). The mutations are clinically relevant because most of them have been associated with patient tumor sensitivity to small molecule tyrosine kinase inhibitors gefitinib and erlotinib (3–5, 10). About 90% of the mutations detected in *EGFR* are composed either of in-frame deletions in exon 19 or a specific missense mutation in exon 21 (L858R; refs. 3–9). The mutations are significantly associated with adenocarcinoma histology, never or light smoker status, female gender, and East Asian ethnic origin (9). However, there is no information available on the stage of lung adenocarcinoma development when *EGFR* mutation develops. Thus, to investigate the stage of lung adenocarcinoma pathogenesis when *EGFR* mutations commence, we tested for the presence of *EGFR* mutations in peripheral airway respiratory epithelium (small bronchi and bronchioles) obtained from 21 patients with lung adenocarcinoma harboring *EGFR* mutations. We compared the findings with similar samples obtained from 16 lung cancer patients whose tumors had wild-type *EGFR*.

Materials and Methods

Case selection. Tumor tissue specimens obtained from 120 surgically resected lung adenocarcinomas, pathology stages I to IIIA, were obtained from the Lung Cancer Specialized Program of Research Excellence Tissue Bank at the M.D. Anderson Cancer Center (Houston, TX), and were examined for *EGFR* gene mutation in exons 18 to 21 (9). We selected 20 cases of adenocarcinoma and one adenosquamous carcinoma with *EGFR* mutation in exon 19 (n = 13) and exon 21 (n = 8) in which archival formalin-fixed paraffin-embedded tissues from which surgically resected lobectomy specimens were available (Table 1). Most patients were women of East Asian ethnicity and never or former smokers (Table 1). All *EGFR* mutated lung adenocarcinomas were of mixed histologic subtype (WHO classification, 2004; ref. 1). Two patients with *EGFR* mutant lung cancers were current smokers, but with only 5 and 12 pack-year exposures. None of the patients had received prior cytotoxic therapy. Patients who had smoked at least 100 cigarettes in their lifetime were defined as smokers, and smokers who quit smoking at least 12 months before lung cancer diagnosis were defined as former smokers. As a control group, 16 cases of adenocarcinoma without *EGFR* mutation, divided into 8 never and 8 former smokers, were selected. Clinical staging was based on the revised in International System for Stating Lung Cancer (11).

Respiratory epithelium foci selection. H&E-stained histology sections of archival specimens having tumor and adjacent normal lung tissues were reviewed to identify available foci of respiratory epithelium containing at least 1,000 cells. From the 21 *EGFR* mutated lung cancers, we identified noncontiguous small bronchial (n = 26) or bronchiolar (n = 38) sites suitable for microdissection. All the foci harbored histologically normal-appearing respiratory epithelium, without identifiable dysplastic or neoplastic cells (Fig. 1). No atypical adenomatous hyperplasias, putative precursors of a subset of lung adenocarcinomas, were detected. The microdissected specimens were obtained from three different locations

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Table 1. Clinicopathologic data of *EGFR* mutant resected lung adenocarcinomas studied

Case no.	Age	Gender	Race	Smoking status	Stage	EGFR mutation	Mutation in normal epithelium
1	55	Female	Caucasian	Never	IIB	15 bp del (746-750)	Yes
2	70	Female	Hispanic	Never	IIIA	15 bp del (746-750)	No
3	64	Male	Caucasian	Never	IA	15 bp del (746-750)	No
4	66	Female	Caucasian	Never	IB	18 bp del (746-751) and S752V	No
5	52	Female	Hispanic	Never	IIIA	L858R	Yes
6	70	Female	Caucasian	Never	IA	L858R	Yes
7	65	Female	Caucasian	Never	IA	L858R	No
8	45	Female	East Asian	Never	IA	L858R	No
9	61	Female	East Asian	Never	IIIA	L858R	No
10	49	Female	East Asian	Never	IIIA	L858R	No
11	35	Female	East Asian	Never	IA	15 bp del (746-750)	Yes
12	62	Female	East Asian	Never	IA	L858R	No
13	66	Female	Caucasian	Former	IV	15 bp del (746-750)	No
14	58	Female	Caucasian	Former	IA	15 bp del (746-750)	Yes
15	65	Male	East Asian	Former	IIB	18 bp del (746-751) and S752I	Yes
16	72	Female	Caucasian	Former	IB	15 bp del (746-750)	Yes
17	69	Male	East Asian	Former	IIIA	L858R	No
18	56	Male	East Asian	Former	IIB	18 bp del (747-752) and P753Q	Yes
19	69	Male	East Asian	Former	IB	15 bp del (747-751)	No
20	73	Male	East Asian	Current	IIB	15 bp del (746-750)	Yes
21	68	Female	Caucasian	Current	IIIA	15 bp del (746-750)	No

based on their relationship to the tumors: within the tumor (21 foci), <5 mm apart from the tumor margin (adjacent to tumor; 29 sites), and from sites located >5 mm from the tumor margin ("distant" lung; 14 sites). From the 16 non-*EGFR* mutated lung adenocarcinoma cases, we selected 26 (10 small bronchi and 16 bronchioles) sites of histologically normal respiratory epithelium (Table 2). Averages of 3.1 (range, 2-6) and 1.6 (range, 1-3) respiratory epithelium foci were examined from *EGFR* mutated and nonmutated cases, respectively. Small bronchi were identified as having well-defined smooth muscle and discontinuous cartilage layers. Bronchioles were defined as small conducting airways lacking well-defined smooth muscle wall or cartilage layers. As internal negative controls, stromal tissue obtained from bronchial walls was also selected for microdissection. As these were retrospectively collected specimens, location of the small bronchial and bronchiolar respiratory epithelium examined for mutations was assessed based on their location with respect to tumor tissue in the corresponding histology sections.

Microdissection and DNA extraction. Approximately 1,000 cells (tumor, respiratory epithelium, or stromal) were precisely microdissected from sequential 8-μm-thick H&E-stained, formalin-fixed paraffin-embedded histology sections, using laser capture microdissection (Arcturus Engineering Laser Microdissection System, Mountain View, CA; Fig. 1). To avoid possible nonspecific binding of mutant tumor cells to the microdissection cap film, the specifically microdissected epithelial cells were redissected from the film under stereomicroscope visualization using fine needles (25G5/8). DNA was extracted using 25 μL of Pico Pure DNA Extraction solution (Arcturus) containing proteinase K and incubated at 65°C for 24 hours. Subsequently, proteinase K inactivation was done by heating samples at 95°C for 10 minutes.

***EGFR* mutation analysis.** Exons 19 and 21 of *EGFR* were PCR amplified using intron-based primers as previously described (10). From microdissected formalin-fixed paraffin-embedded cells, ~100 cells were used for each PCR amplification. Each amplification was done in 25 μL volume containing 2.5 μL DNA, 0.5 μL each primer (20 pmol/L), 12.5 μL HotStarTaq Master Mix (Qiagen, Valencia, CA), and 9 μL DNase-free water. DNA was amplified for 38 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 45 seconds, followed by 7-minute extension at 72°C. All PCR products were directly sequenced using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA). All sequence variants were confirmed by independent PCR amplifications from at least two independent microdissections, and sequenced in both directions.

Statistical analysis. For data in which there is one record per patient, all relationships between categorical variables were assessed via the Fisher's exact test (12). For continuous outcomes, differences between cohorts were assessed via the Wilcoxon rank-sum test. Data in which there are multiple records per patient, relationships between binary variables were assessed via generalized estimating equation models.

Results

***EGFR* mutation in histologically normal epithelium.** Mutation analysis of the microdissected tumor tissue confirmed the mutational pattern originally identified using frozen and non-microdissected formalin-fixed paraffin-embedded tissues in all 21 cases (Table 1). Of interest, *EGFR* mutations were detected in at least one sample of corresponding histologically normal small bronchial and bronchiolar epithelia in 9 of 21 (43%) *EGFR* mutant lung adenocarcinoma patients (Tables 1 and 2). By contrast, no mutations in exons 19 and 21 of the *EGFR* gene were detected in 26 respiratory epithelium foci obtained from 16 lobectomy specimens of cancers having wild-type *EGFR* ($P = 0.005$). In *EGFR* mutant lung adenocarcinomas, 16 of 64 (25%) normal respiratory epithelium foci microdissected showed *EGFR* mutations. Five of nine cases showing mutations in normal respiratory epithelium showed two or three microdissected sites with *EGFR* mutation. In all cases, the mutational patterns in the tumors and corresponding nonmalignant respiratory epithelial specimens were identical. All mutations detected were confirmed using additional microdissected samples and multiple independent sequencing experiments in both sense and antisense directions. Importantly, no mutations were detected in stromal cells microdissected from bronchial walls in five cases in which adjacent lung normal epithelium and tumor showed *EGFR* mutations.

The frequency of mutations in the histologically normal respiratory epithelium was higher in samples microdissected within the tumor (9 of 21, 43%) than samples obtained from tissue adjacent to tumor (distance of <5 mm from the tumor margin; 7 of 29, 24%; $P = 0.013$; Table 2). No mutation was detected in 14 distant bronchial and bronchiolar samples. Although not statistically significant, a higher incidence of mutation was detected in small bronchial (9 of 26, 35%) compared with bronchiolar structures (7 of 38, 18%; $P = 0.093$). More frequent mutations affecting normal epithelium were found in *EGFR* exon 19 (14 of 16, 54%) compared with exon 21 (2 of 28, 7%; $P = 0.02$). There was no correlation noted between mutations in the normal epithelium and age, gender, ethnic background, former or never smoker status, or lung cancer clinical stage in our patients.

Discussion

This is the first report of the presence of *EGFR* mutations in histologically normal bronchial and bronchiolar epithelium in patients with lung adenocarcinomas. Our findings of identical *EGFR* mutations in normal-appearing respiratory epithelium in 9 of 21 (43%) patients with mutant tumors suggest that the mutations occur as early events in the pathogenesis of a subset of lung adenocarcinomas, commencing in histologically normal peripheral airways. These findings impact our understanding of the early pathogenesis of *EGFR* mutant lung adenocarcinomas, and may lead to clinical applications, such as targeted early detection and chemoprevention strategies.

It has been proposed that lung cancer cells with mutant *EGFR* might become physiologically dependent on the continued activity of the gene for the maintenance of their malignant phenotype (13). Mutant *EGFR* selectively transduces survival signals, specifically Akt, and signal transduction and activator of transcription signaling pathways, on which lung cancer tumor cells become dependent (14). Our finding of identical *EGFR* mutations (15 or 18 bp in-frame deletion and L858R mutation) in lung adenocarcinoma cells and in 25% of the corresponding adjacent histologically normal epithelial sites examined indicates that *EGFR* tyrosine kinase mutations also may play an important role in the initiation of the malignant phenotype. This notion is further supported by the absence of *EGFR* mutations in normal-appearing epithelium from 16 lung adenocarcinomas with wild-type *EGFR* from never and former smokers.

Clinically and pathologically (1), most adenocarcinomas of the lung are considered to arise from the peripheral lung airway compartment (small bronchi/bronchioles and alveoli; ref. 15), which arise by division of the tertiary bronchi (16). Whereas bronchi are lined by pseudostratified ciliated epithelium with occasional mucin-producing cells, bronchioles contain ciliated cells and secretory Clara cells (16). The latter are believed to be

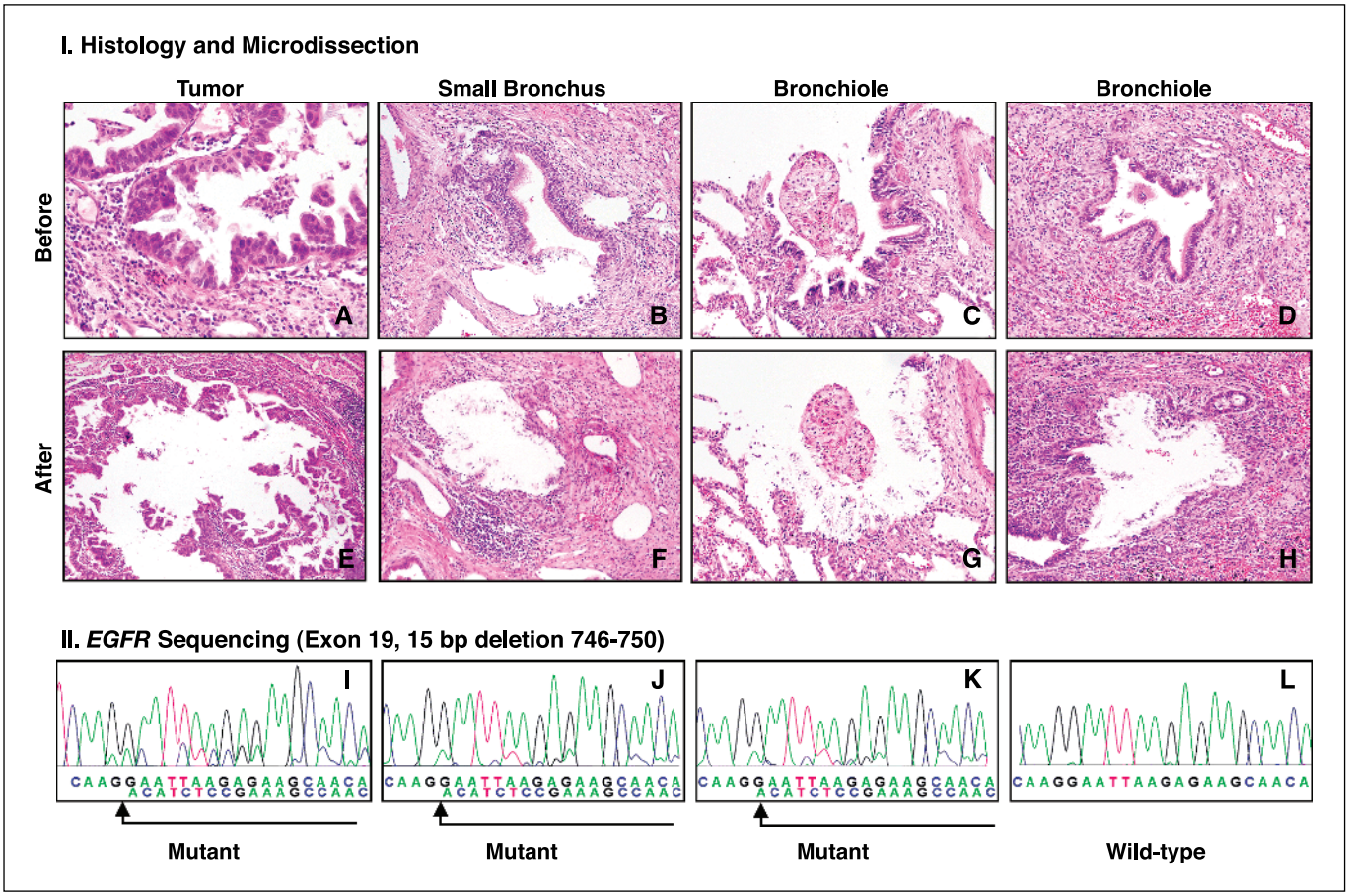

Figure 1. *I,* examples of tumor and histologically normal airway structures harboring the mutations are illustrated before (*A-D*) and after microdissection (*E-H*). *II,* sequencing chromatograms showing the presence of wild-type and mutant forms in the tumor (*I*), a small bronchus (*J*), and bronchiole (*K*), whereas only wild-type form is present in another bronchiole (*L*). *Arrow,* in-frame deletion mutation sequence.

Table 2. Frequency of *EGFR* tyrosine kinase domain mutations in microdissected histologically normal bronchial and bronchiolar epithelium in lung adenocarcinoma patients

Cases/samples	Mutated tumors			Wild-type tumors
	Exon 19 deletion	Exon 21 mutation	Total mutated	
Patients by smoking status (n = 30)				
Never	2 of 5	2 of 7	4 of 12	0 of 8
Former	4 of 6	0 of 1	4 of 7	0 of 8
Current	1 of 2	—	1 of 2	—
Total patients	7 of 13 (54%)	2 of 8 (25%)	9 of 21 (43%)*	0 of 16*
Foci by location compared with tumor (n = 90)				
Within	8 of 14 (57%)	1 of 7 (14%)	9 of 21 (43%)†	0 of 8
Near (<5 mm)	6 of 13 (46%)	1 of 16 (6%)	7 of 29 (24%)†	0 of 13
Distant	0 of 9	0 of 5	0 of 14 †	0 of 5
Foci by respiratory structure (n = 90)				
Small bronchus	8 of 17 (47%)	1 of 9 (11%)	9 of 26 (35%)	0 of 10
Bronchiole	6 of 19 (32%)	1 of 19 (5%)	7 of 38 (18%)	0 of 16
Total foci	14 of 36 (39%)‡	2 of 28 (7%)‡	16 of 64 (25%)	0 of 26

*P value of comparison: 0.0046.
†P value of comparison: 0.013.
‡P value of comparison: 0.021.

the progenitor cells of the bronchiolar epithelium. Respiratory bronchioles terminate in alveolar ducts and alveolar sacs, which are lined by type I and II pneumocytes (16). Our finding of *EGFR* mutations in microdissected histologically normal epithelial cells obtained from small bronchi and bronchioles supports the concept of adenocarcinomas arising from the peripheral lung airway compartment. The tendency of higher frequency of *EGFR* mutations in normal epithelium obtained from small bronchi (35%) compared with bronchioles (18%) may correlate with different cell types populating those epithelia, which could represent the site of the cell of origin for *EGFR* mutant adenocarcinomas. However, the possibility that common stem or progenitor cells for both bronchial and bronchiolar epithelia are the cell type bearing *EGFR* mutation cannot be excluded.

The finding of *EGFR* mutations in small bronchial and bronchiolar epithelium obtained from sites within (43%) and adjacent (24%) to tumors, but none in the distant peripheral lung sites, suggests that a localized type of field effect phenomenon may exist for *EGFR* mutations in the lung respiratory epithelium. A widespread field effect phenomenon with several molecular changes affecting histologically normal and abnormal bronchial and bronchiolar epithelium has been previously shown by us (17, 18) and others (19) in the smoking-damaged respiratory epithelium from lung cancer patients and from smokers without lung cancer. Therefore, our findings extend the field theory from centrally arising squamous carcinomas to peripheral occurring adenocarcinomas arising both in smokers and never smokers.

Our findings of *EGFR* mutations present in histologically normal epithelium of patients with lung adenocarcinomas bearing identical mutations open new avenues of investigation in the early pathogenesis of lung adenocarcinoma, including the identification of specific epithelial cell types hit by crucial genetic abnormalities involved in lung tumorigenesis.

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