Identical Epidermal Growth Factor Receptor Mutations in Adenocarcinomatous and Squamous Cell Carcinomatous Components of Adenosquamous Carcinoma of the Lung

Shin Myung Kang, MD[1]
Hyun Ju Kang[2,4]
Ju Hye Shin, MS[3]
Hoguen Kim, MD[2-4]
Dong Hwan Shin, MD[2]
Se Kyu Kim, MD[1,3-5]
Joo-Hang Kim, MD[1,4,6]
Kyung Young Chung, MD[7]
Sung Kyu Kim, MD[1,5]
Joon Chang, MD[1,5]

[1] Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea.

[2] Department of Pathology, Yonsei University College of Medicine, Seoul, Korea.

[3] Cancer Metastasis Research Center, Yonsei University College of Medicine, Seoul, Korea.

[4] Brain Korea 21 Projects for Medical Sciences, Yonsei University College of Medicine, Seoul, Korea.

[5] Institute of Chest Diseases, Yonsei University College of Medicine, Seoul, Korea.

[6] Yonsei Cancer Center, Yonsei University College of Medicine, Seoul, Korea.

[7] Department of Cardiovascular and Thoracic Surgery, Yonsei University College of Medicine, Seoul, Korea.

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Address for reprints: Joon Chang, MD, Department of Internal Medicine, Yonsei University College of Medicine, 134 Shinchon-Dong, Seodaemun-Gu, Seoul, 120-752, Korea; Fax: (011) 82 2 393 6884; E-mail: chang@yumc.yonsei.ac.kr

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BACKGROUND. Adenosquamous carcinoma of the lung is composed of adenocarcinomatous and squamous cell carcinomatous components. The epidermal growth factor receptor (*EGFR*) mutations occur mostly in adenocarcinomas and rarely in squamous cell carcinoma of lung. Attempts to investigate the *EGFR* mutation status in each component of adenosquamous carcinoma and to characterize the patients according to mutation status may help to understand the histogenesis of adenosquamous carcinoma.

METHODS. The mutation status of *EGFR* kinase domain from exon 18 to 21 was investigated in 25 Korean patients with adenosquamous carcinoma by polymerase chain reaction–single strand conformation polymorphism using the tissues of each component from the adenosquamous carcinoma tumor. Clinicopathologic characteristics of the patients according to the status of *EGFR* mutations were compared.

RESULTS. *EGFR* mutations were identified in 11 (44%) patients: 9 mutations were in exon 19, 1 in exon 20, and 1 in exon 21. *EGFR* mutations were significantly more frequent ($P = .005$) in women (n = 8, 80%) than men (n = 3, 20%). Never-smokers (n = 8, 62%) had *EGFR* mutations more commonly than smokers (n = 3, 25%; $P = .111$). Identical *EGFR* mutations in both components of adenosquamous carcinoma were confirmed by nucleotide sequencing.

CONCLUSIONS. The frequency of *EGFR* mutation and clinicopathologic characteristics of the *EGFR* mutants in adenosquamous carcinoma are similar to those of Asian patients with adenocarcinomas. Identical *EGFR* mutations in both components suggest the possibility of monoclonality in the histogenesis of adenosquamous carcinoma. ***Cancer*** **2007;109:581–7.** © *2006 American Cancer Society.*

KEYWORDS: epidermal growth factor receptor, mutation, carcinoma, adenosquamous.

Adenosquamous carcinoma is a rare subtype of nonsmall-cell lung cancer (NSCLC), detected in 0.4% to 4% of lung cancer.[1] A notable feature of adenosquamous carcinoma is the heterogeneous nature of the tumors. According to the World Health Organization's histologic classification of lung tumors, adenosquamous carcinoma is defined as a carcinoma showing components of both squamous cell carcinoma and adenocarcinoma, with each comprising at least 10% of the whole tumor.[1] Because phenotypically different 2 types of NSCLC exist in a tumor, 2 different clonal pathways, monoclonality and polyclonality, have been proposed for the histogenesis of

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adenosquamous carcinoma. Monoclonality consists of a transformation from 1 component to the other, and the polyclonality results from the collision of 2 tumors.[2]

The epidermal growth factor receptor (EGFR) is a transmembrane receptor with tyrosine kinase activity. NSCLC frequently expresses EGFR.[3–6] Ligand binding to the extracellular domain induces EGFR homo- or heterodimerization, activation of tyrosine kinase activity, autophosphorylation to tyrosine residues, and intracellular signal transduction that leads to cellular proliferation, angiogenesis, metastasis, and inhibition of apoptosis.[7] Somatic gain-of-function mutations in exons encoding the EGFR tyrosine kinase domain have been identified in NSCLC,[8,9] and subsequent studies revealed that *EGFR* mutation could be the responsive marker to the tyrosine kinase inhibitors gefitinib and erlotinib.[10–12] Interestingly, *EGFR* mutations occur more frequently in a subset of patients with NSCLC. Adenocarcinoma, women, never-smokers, and Asians are well-known clinicopathologic characteristics of *EGFR* mutants.[8–10,13–16] On the contrary, *EGFR* mutations in squamous cell carcinomas of the lung are very rare.[10,11,17–19] Therefore, investigation of *EGFR* mutation status in the adenocarcinomatous and squamous cell carcinomatous components separated from individual adenosquamous carcinoma would elucidate whether the occurrence of *EGFR* mutations is histology-specific. This result may help to understand the histogenesis of adenosquamous carcinoma, especially in clonality.

Genetic alterations, chromosomal abnormalities, and immunohistochemical reactions in both components of adenosquamous carcinomas were compared in few studies, which suggested the possibility of monoclonality and similar biological characteristics of both components.[20–23] Using DNA flow cytometry analysis, Ichinose et al.[20] showed a relation between both components in 8 (67%) out of 12 adenosquamous carcinomas. Identical monoclonal pattern in both components of all four cases were shown using clonal analysis of X chromosome.[21] The same p53 overexpression and *p53* mutation, similar loss of heterozygosity at 9p21 and 9q31-32, and no *K-ras* mutation in both components of 12 adenosquamous carcinoma cases were also reported.[22] Recently, Toyooka et al.[23] reported that 3 (27%) of 11 adenosquamous carcinoma had *EGFR* mutations, 2 mutations in exon 19 and 1 in exon 21, and they were identical in each component. They used a laser capture microdissection or manual microdissection for the separation of each component. It was the first report of *EGFR* mutations in the cohort of adenosquamous carcinomas. However, further investigation with a larger sample size is required to clarify the genetic and clinicopathologic characteristics of the patients with adenosquamous carcinomas.

Owing to the poor prognosis of patients with adenosquamous carcinoma,[24–29] other therapeutic strategies to improve patient outcome are needed. Recent studies proved that *EGFR* mutations are associated with response and survival in patients with NSCLC treated with tyrosine kinase inhibitor (TKI).[8–10,12–14,16,30,31] The status of *EGFR* mutations would provide evidence as to whether TKIs could be therapeutic options in adenosquamous carcinoma.

We separated each component from adenosquamous carcinoma to investigate the status of *EGFR* mutation separately and then compared the results of 1 component with the other. To characterize patients according to the status of *EGFR* mutations, we correlated *EGFR* mutation status with clinicopathologic characteristics of the patients.

MATERIALS AND METHODS

Patients and Tissues

One thousand and ten patients underwent surgical resection for the treatment of NSCLC between October 1994 and May 2004 at Severance Hospital, Seoul, Korea. Twenty-eight cases with adenosquamous carcinoma (2.8% of NSCLC) during that period were selected from the database of our institution. Among them, tissue samples were available in 25 cases (Table 1). All cases were reevaluated by 2 pathologists (D.W.S. and H.K.). Formalin-fixed paraffin-embedded tissues of 10 μm thickness were used to extract genomic DNA. Adenocarcinomatous and squamous cell carcinomatous components from each tumor were separated precisely by manual microdissection under microscopic observation to avoid contamination of each sample from different components. To enrich the tumor cell population, areas containing more than 90% of tumor cells were sampled from hematoxylin–eosin-stained slides. Grossly normal tissue remote from the tumor was included as a control and judged to be benign. Clinical data were obtained from the medical record team. Never-smoker was defined as an individual having no lifetime smoking history. The study protocol was reviewed and approved by the Institutional Review Board of Yonsei University College of Medicine, Seoul, Korea.

EGFR Gene Analysis

Genomic DNA from the separate components was prepared using the sodium dodecyl sulfate-proteinase K and phenol-chloroform extraction method. Somatic mutations in exons 18 to 21 of *EGFR* were detected using a polymerase chain reaction (PCR)-based

TABLE 1
Baseline Patient Characteristics

Characteristic	No. of patients (n = 25)	%
Age, y		
Median	61	
Range	40–72	
Sex		
Men	15	60
Women	10	40
Smoking		
Never	13	52
Smoker	12	48
Histologic type		
Acinar	14	56
Solid	1	4
Mixed	10	40
BAC components		
Yes	8	32
No	17	68
Visceral pleural involve		
Yes	12	48
No	13	52
Stage		
I	7	28
II	6	24
III	12	48

BAC indicates bronchioloalveolar carcinoma.

assay as described previously.[32,33] The primer sets were as follows (forward and reverse, respectively): exon 18, (5′-AGCTTGTGGAGCCTCTTACAC-3′ and 5′-CCACCAGACCATGAGAGG-3′), exon 19 (5′-ATGTGG-CACCATCTCACAAT–3′ and 5′-CCACACAGCAAAGCA-GAAAC–3′); exon 20 (20-1, 5′-CACACTGA-CGTGCCT-CTCC-3′ and 5′-GTCTTTGTGTTCCCGGACAT-3′; 20-2, 5′-AGCTCATCACGCAGCTCAT-3′ and 5′-TTATCTCC-CCTCCCCGTATC-3′), and exon 21 (5′-CCTCACAGCA-GGGTCTTCTC–3′ and 5′-CACCTCCTTACTTTGCCT-CCT–3′). PCR was carried out with a 20 μL mixture containing 1.5 mM $MgCl_2$; 20 pmol of primer; 0.2 mM each of dATP, dGTP, and dTTP; 5 mM dCTP; 1 μCi of [α-^{32}P] dCTP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA); 50 ng of sample DNA; 1× PCR buffer; and 1.25 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). After denaturation at 95°C for 5 minutes, DNA amplification was performed for 30 cycles. This consisted of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and elongation at 72°C for 15 seconds. PCR products were separated on 0.5× mutation detection enhancement (MDE, CAMBREX Bioscience, Rockland, ME) gels, followed by autoradiography for single-strand conformation polymorphism (SSCP) analysis. Shifted bands were cut from gel, resuspended in distilled water, resubjected to PCR, and then sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif) in both forward and reverse strand directions.

Statistical Analysis

The association between *EGFR* mutation and clinicopathologic variables were assessed using χ^2, Fisher exact tests, or *t*-test. The SPSS statistical software package (v. 11.5, SPSS, Chicago, Ill) was used for all calculations. Two-sided *P*-values $< .05$ were considered significant.

RESULTS

Molecular Characteristics in *EGFR* Mutations

Eleven (44%) out of 25 cases showed shifted bands on PCR-SSCP analysis. Each component from a tumor displayed the same patterns of shifted band. Nucleotide sequencing the extracted PCR product of a shifted band confirmed the presence of *EGFR* mutations in these cases. Interestingly, adenocarcinomatous and squamous cell carcinomatous components of adenosquamous carcinomas with *EGFR* mutations carried identical *EGFR* mutations (Fig. 1).

Results of the mutational analysis were as follows (Table 2): 9 mutations were in exon 19, 1 in exon 20, and 1 in exon 21. Four types of in-frame deletions were observed: 5 were simple deletions (del E746-A750), 2 were coupled with insertion (del E746-A750, ins IP), 1 with substitution (del L747-E749, A750P), and 1 with duplication/insertion and substitution (indel; del T751-I759, dup/ins EAREA). "indel; del T751-I759, dup/ins EAREA" is a novel mutation found in this study. It resulted from the deletion of nucleotides 2257–2276, duplication or insertion of 2243–2249 at the deleted site, and substitution 2277C→G. "ins D770-G771" is another novel mutation found in exon 20. The nucleotide of 2308 was a common point of insertion in exon 20, but insertion of ACGGCG at this point has never been described before. All the mutations occurred as single mutations in individual samples.

Correlations of *EGFR* Mutations With Clinicopathologic Characteristics

EGFR mutations were significantly more frequent in women (80%) than in men (20%; $P = .005$) (Table 3). Never-smokers (62%) had *EGFR* mutations more commonly than smokers (25%), but there was no statistic significance ($P = .111$). All the female patients with *EGFR* mutations were never-smokers. The classification of histologic subtypes in this study was based on histologic characteristics of the adenocarci-

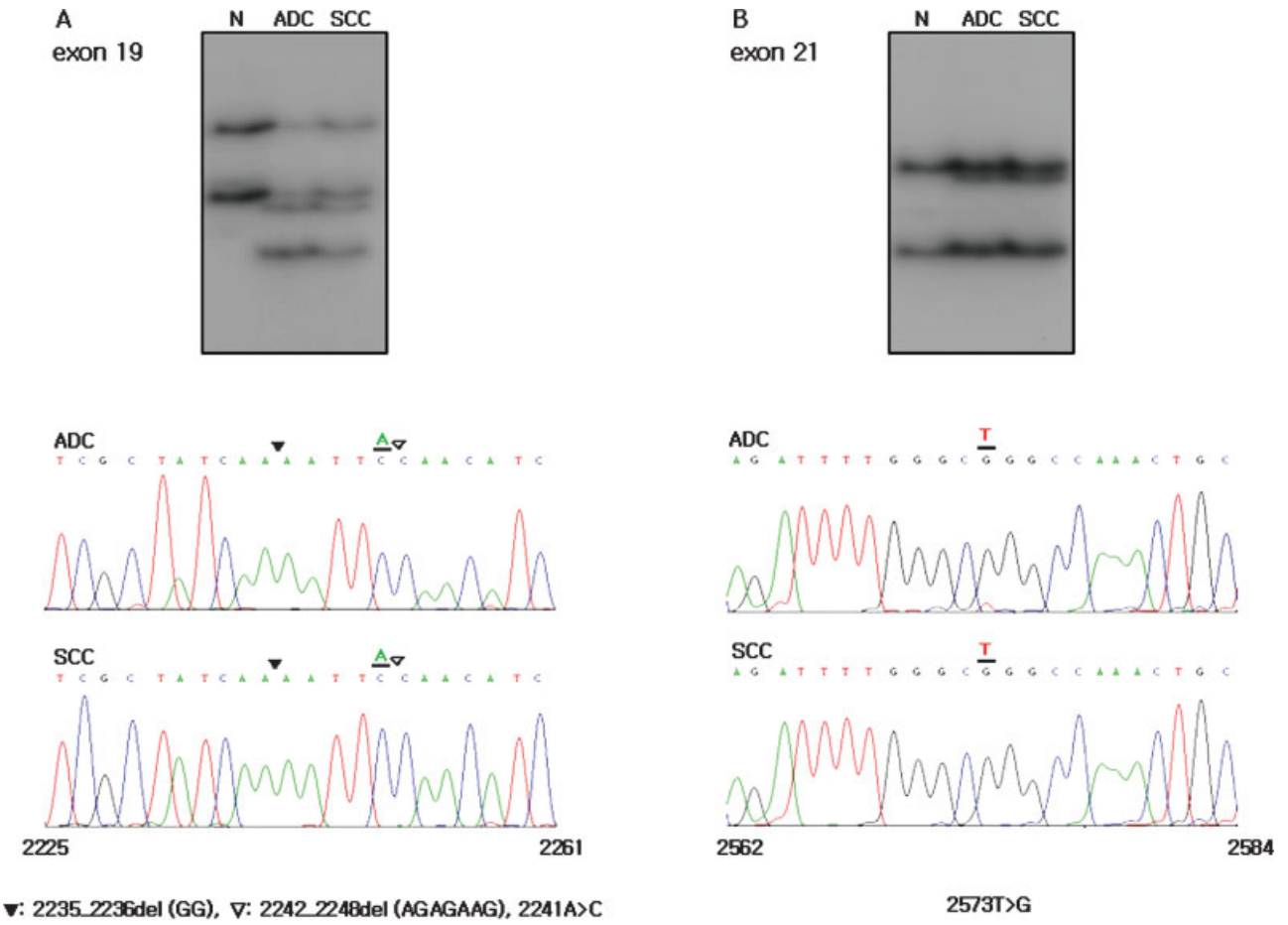

FIGURE 1. Single-strand conformation polymorphisms and electropherograms demonstrating identical *EGFR* mutations in ADC and SCC. (A) in-frame deletion with insertion (del E746-A750, ins IP) in exon 19. (B) amino acid substitution (L858R) in exon 21. The sites of deletion (black and white inverted triangles) and substitution (line) are shown. N, normal tissue; ADC, adenocarcinomatous component; SCC, squamous cell carcinomatous component.

nomatous component only. The acinar component was observed in all the patients, except 1 with solid subtype. Four patients showing a solid component in their tumors had no *EGFR* mutations. All mixed subtypes of the *EGFR* mutant were a mixture of acinar and bronchioloalveolar carcinoma (BAC) subtypes. The occurrence of the *EGFR* mutation according to the presence or absence of BAC components did not differ ($P = .389$).

DISCUSSION

Adenosquamous carcinomas comprised 2.8% of 1010 NSCLC patients treated by surgical resections. This frequency is similar to that of the previous reports.[25,27,29,34] In the present study we detected *EGFR* mutations in 11 (44%) of 25 Korean adenosquamous carcinoma patients. Also, we found that both components of adenosquamous carcinoma, adenocarcinomatous and squamous cell carcinomatous component, carried identical *EGFR* mutations.

We demonstrated that adenosquamous carcinoma was another subtype of NSCLC that frequently harbors *EGFR* mutations (44%), which was comparable to the frequency observed in Asian patients with adenocarcinomas.[9,13,14,16,17,35,36] *EGFR* mutations have been found mostly in adenocarcinomas. On the contrary, the frequency of *EGFR* mutation in squamous cell carcinoma of the lung is very low. Only 8 cases (1.1%) out of 732 of squamous cell carcinomas had *EGFR* mutations in our review of published reports.[10,11,13–15,17–19,30,35] The clinical characteristics of *EGFR* mutants of adenosquamous carcinoma patients were similar to those of adenocarcinomas, but not squamous cell carcinomas. Asian ethnicity, adenocarcinoma histology, female and never-smoker are well-known clinicopathologic characteristics of patients with *EGFR* mutations in NSCLC.[8–10,13–16] High frequency, female predominance, and never-smoking status of *EGFR* mutations in Korean patients with adenosquamous carcinoma

TABLE 2
Mutations in *EGFR*

Sex	Age	Smoking	Exon	Nucleotide changes	Amino acid changes
W	40	Never	19	2235_2249del	del E746-A750
W	64	Never	19	2235_2249del	del E746-A750
W	44	Never	19	2235_2249del	del E746-A750
W	57	Never	19	2235_2249del	del E746-A750
W	72	Never	19	2235_2249del	del E746-A750
W	72	Never	19	2239_2247del, 2248G>C	del L747-E749, A750P
M	63	Smoker	19	2235_2236del, 2242_2248del, 2241A>C	del E746-A750, ins IP
W	52	Never	19	2235_2236del, 2242_2248del, 2241A>C	del E746-A750, ins IP
W	61	Never	19	2252_2276del, 2243_2249dup/ins, 2277C>G	indel; del T751-I759, dup/ins EAREA
M	48	Smoker	20	2308insACGGCG	ins D770-G771
M	57	Smoker	21	2573T>G	L858R

TABLE 3
Comparison of Clinicopathologic Characteristics With *EGFR* Mutations

	Epidermal growth factor receptor gene				
	Mutant (n = 11)		Wild type (n = 14)		
	No.	%	No.	%	*P*
Age, y					.207
Median	57		64		
Range	40 – 72		49 – 72		
Sex					.005
Men	3	20	12	80	
Women	8	80	2	20	
Smoking					.111
Never	8	62	5	38	
Smoker	3	25	9	75	
Histologic type					.827
Acinar	6	43	8	57	
Solid	0	0	1	100	
Mixed	5	50	5	50	
BAC components					.389
Yes	5	63	3	37	
No	6	35	11	65	
Stage					.238
I, II	4	31	9	69	
III	7	58	5	42	

BAC indicates bronchioloalveolar carcinoma.

were similar features observed in adenocarcinomas. Thus, we found that common characteristics were associated with the occurrence of *EGFR* mutations in patients with adenocarcinomatous histology in their tumors. About half of patients who were smokers in our study did not correspond to the previous studies, which reported that about 80% of patients with adenosquamous carcinomas were smokers.[25,34] Although higher responses to TKI in individuals with adenocarcinomas with BAC features have been reported,[37] the association between the presence of *EGFR* mutations and BAC components of adenocarcinomas was unclear. In some studies, *EGFR* mutations were observed more commonly in adenocarcinomas with BAC features.[15,35] However, no association between the presence of *EGFR* mutation and BAC features in adenocarcinomas was reported in other studies.[16,36] In our study with adenosquamous carcinomas, there was no association between features of BAC histology in adenocarcinomatous components and the presence of *EGFR* mutations. There was no relation between *EGFR* mutations and pathologic stage, as described in previous reports with NSCLC.[14–17,35] It is unusual that most of *EGFR* mutations (82%) occurred in exon 19 because mutation in exon 21 is as common as in exon 19. A study showed that in-frame deletions in exon 19 accounted for 44% of all *EGFR* mutations and missense mutation in exon 21 for 41% of lung cancer.[38] Because SSCP is more sensitive than direct sequencing to detect *EGFR* mutations, especially point mutations,[15] it is unlikely to miss a mutation in exon 21 in our samples.

Identical *EGFR* mutations, despite distinct phenotypic differences between adenocarcinomatous and squamous cell carcinomatous components, were detected simultaneously in both components of a tumor in this study. It was not only an interesting finding, but could be the clue to the clonal pathway of adenosquamous carcinoma because the occurrence of *EGFR* mutations in squamous cell histology was very rare. In addition to frequent mutations in the squamous cell component, collision of both components with identical *EGFR* mutations seemed very unlikely to happen. Therefore, we thought that a monoclonal pathway that each component of adenosquamous carcinoma originated from common progenitor cells was more probable than a polyclonal pathway in the histogenesis of adenosquamous carcinoma.

Patients with adenosquamous carcinoma have been reported to have a poor prognosis.[24–27,29] In a

series of studies including more than 40 cases, 5-year postoperative survival rates of patients with adenosquamous carcinoma ranged from 18.5% to 36.7%, which were lower than patients with either adenocarcinomas or squamous cell carcinomas.[24,25,28,29] Stage, peripheral location, visceral pleural involvement, and component predominance are the factors associated with poor prognosis in adenosquamous carcinoma.[25,28] Shigematsu et al.[16] reported no relation between *EGFR* mutation status and patient survival in the absence of EGFR kinase inhibitor therapy. However, patients with deletions in exon 19 had worse survival, with borderline significance, than those with L858R missense mutation in exon 21, and those without *EGFR* mutations. Considering the predominance of deletional types in *EGFR* mutation in adenosquamous carcinoma patients, the specific type of *EGFR* mutation, deletion in exon 19, could be another factor associated with the poor prognosis. Because of the low incidence and difficulties in diagnosing adenosquamous carcinoma, data available on *EGFR* mutation status in adenosquamous carcinoma were very limited. Seven cases of adenosquamous carcinoma showing *EGFR* mutations have been reported.[13,14,18,23,31] Among them, a male patient with a recurring large chest-wall mass showed a dramatic response to gefitinib.[31] Therefore, trial with TKI that targets both components of adenosquamous carcinoma would be a reasonable therapeutic option to adenosquamous carcinoma patients with *EGFR* mutations.

Surgical resection is required for the diagnosis of adenosquamous carcinoma due to its histologic heterogeneity. It is impossible to diagnose adenosquamous carcinoma with certainty in small biopsy specimens obtained by transbronchial biopsy because they cannot represent all the histologic characteristics of adenosquamous carcinoma. If histologic diagnosis of squamous cell carcinoma is made on a bronchoscopic or transbronchial biopsy specimen, and the specimen is found to have *EGFR* mutation, one could consider the possibility of adenosquamous carcinoma.

In conclusion, the frequency of the *EGFR* mutation and clinicopathologic characteristics of the *EGFR* mutants of adenosquamous carcinoma are similar to those of Asian patients with adenocarcinomas. Identical *EGFR* mutations in both adenocarcinomatous and squamous cell carcinomatous components suggest the possibility of monoclonality in the histogenesis of adenosquamous carcinoma.

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