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# Clarifying the Spectrum of Driver Oncogene Mutations in Biomarker-Verified Squamous Carcinoma of Lung: Lack of *EGFR/KRAS* and Presence of *PIK3CA/AKT1* Mutations

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## Abstract

**Purpose:** There is persistent controversy as to whether *EGFR* and *KRAS* mutations occur in pulmonary squamous cell carcinoma (SQCC). We hypothesized that the reported variability may reflect difficulties in the pathologic distinction of true SQCC from adenosquamous carcinoma (AD-SQC) and poorly differentiated adenocarcinoma due to incomplete sampling or morphologic overlap. The recent development of a robust immunohistochemical approach for distinguishing squamous versus glandular differentiation provides an opportunity to reassess *EGFR/KRAS* and other targetable kinase mutation frequencies in a pathologically homogeneous series of SQCC.

**Experimental Design:** Ninety-five resected SQCCs, verified by immunohistochemistry as $\Delta Np63^{+}$/TTF-$1^{-}$, were tested for activating mutations in *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, *AKT1*, *ERBB2/HER2*, and *MAP2K1/MEK1*. In addition, all tissue samples from rare patients with the diagnosis of *EGFR/KRAS*-mutant "SQCC" encountered during 5 years of routine clinical genotyping were reassessed pathologically.

**Results:** The screen of 95 biomarker-verified SQCCs revealed no *EGFR/KRAS* [0%; 95% confidence interval (CI), 0%–3.8%], four *PIK3CA* (4%; 95% CI, 1%–10%), and one *AKT1* (1%; 95% CI, 0%–5.7%) mutations. Detailed morphologic and immunohistochemical reevaluation of *EGFR/KRAS*-mutant "SQCC" identified during clinical genotyping ($n = 16$) resulted in reclassification of 10 (63%) cases as AD-SQC and five (31%) cases as poorly differentiated adenocarcinoma morphologically mimicking SQCC (i.e., adenocarcinoma with "squamoid" morphology). One (6%) case had no follow-up.

**Conclusions:** Our findings suggest that *EGFR/KRAS* mutations do not occur in pure pulmonary SQCC, and occasional detection of these mutations in samples diagnosed as "SQCC" is due to challenges with the diagnosis of AD-SQC and adenocarcinoma, which can be largely resolved by comprehensive pathologic assessment incorporating immunohistochemical biomarkers. *Clin Cancer Res; 18(4); 1167–76. ©2012 AACR.*

## Introduction

Adenocarcinoma and squamous cell carcinoma (SQCC) represent the 2 major types of non–small cell lung carcinoma (NSCLC). Historically, the subtype of NSCLC has not been a major factor in determining patient management, and fundamental differences in the molecular pathogenesis of these tumors were not well established. It is only in recent years that it has become apparent that lung adenocarcinoma and SQCC have distinct driver mutation profiles, which underlies their divergent responses to targeted therapies (1). In particular, since the early identification of *EGFR* and *KRAS* mutations in NSCLC—the predictors of sensitivity and resistance, respectively, to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI)—it was noted that these mutations have a remarkable predilection for adenocarcinoma (2–5). Despite the general agreement that *EGFR* and *KRAS* mutations occur almost exclusively in adenocarcinoma, there has been a persistent controversy as to whether these mutations also occur, albeit at a lower frequency, in SQCC. A number of studies have described the detection of activating *EGFR* mutations (range, 1%–15%; refs. 6–10) and *KRAS* mutations (range, 1%–9%; refs. 11, 12) in SQCC, although in most studies, the detailed pathologic analysis of those samples was

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## Translational Relevance

There is significant variability in the reported prevalence of *EGFR*/*KRAS* mutations in squamous cell carcinoma (SQCC) of lung, which has raised a concern about the pathologic homogeneity of the tumors included in molecular studies. Here, we combined mutational analysis with rigorous pathologic verification using immunohistochemistry. We find that *EGFR*/*KRAS* mutations do not occur in pure biomarker-verified SQCC, and occasional detection of these mutations in cases reported as "SQCC" is a result of difficulties in the pathologic diagnosis of adenocarcinoma and adenosquamous carcinoma, which in the majority of cases can be resolved by comprehensive pathologic assessment incorporating immunohistochemistry. The translational relevance is threefold. First, we highlight the value of immunohistochemistry in the diagnosis of SQCC, which clarifies the conflicting data on the spectrum of mutations in this tumor type. Second, we establish a sharp biologic divide in the patterns of oncogenic driver mutations between lung adenocarcinoma (pure or combined) and pure SQCC. Third, we determine the rate of several targetable mutations in a pathologically homogeneous set of SQCC.

not provided. Clarifying this controversy is of interest both as a biologic question pertaining to the degree of lineage restriction of *EGFR*/*KRAS* mutations and as a practical clinical question pertaining to the optimal triage of patient samples for predictive molecular testing based on the pathologic diagnosis.

There are several well-known limitations in the traditional diagnosis of NSCLC subtypes. In particular, it is well established that poorly differentiated adenocarcinoma and SQCC can appear indistinguishable by routine light microscopy, particularly in small biopsies and cytology samples, leading to a historically high rate of unclassified NSCLC and the potential for erroneous subtype assignment (13). Largely driven by the recent molecular and clinical insights on the significance of NSCLC subtypes, there has been an explosion in research aimed at circumventing the limitations of morphology through the use of immunohistochemical biomarkers to more precisely and objectively determine cell lineage in poorly differentiated NSCLC (14–17). We (14) and others investigators (15) have shown that immunohistochemistry (IHC) for TTF-1 and p63—the developmental transcription factors and master regulators in glandular and squamous cell lineages, respectively—can effectively identify a tumor cell type in clinical samples of NSCLC with equivocal morphology. In particular, recent studies show the value of IHC for ΔN isoform of p63—a highly squamous-specific variant of p63 (18, 19). Several studies have already shown that incorporation of IHC leads to a dramatic decline in the rate of unclassified NSCLC, as well as an increase in the accuracy and reproducibility of adenocarcinoma versus SQCC diagnoses both in the investigational setting (17, 20) and in clinical practice (21, 22). For these reasons, IHC is now widely advocated to be incorporated into routine diagnosis of NSCLC that are difficult to classify by morphology (23). However, to what degree the lack of precision in the morphologic diagnosis of NSCLC subtypes in pre-IHC era may have contributed to atypical molecular findings has not been investigated in detail.

Another well-known confounder in the accurate determination of NSCLC subtype is adenosquamous carcinoma (AD-SQC)—a rare tumor, representing 0.4% to 4% of NSCLC (24), which shows bidirectional differentiation with morphologically and immunophenotypically distinct squamous and glandular components. Preoperative diagnosis of AD-SQC is notoriously difficult because it requires simultaneous sampling of both components, which cannot be reliably achieved in small tissue fragments (14, 21, 23). As a result, this tumor may be diagnosed as "SQCC" or "adenocarcinoma" depending on which component is sampled. Importantly, AD-SQC is known to harbor a spectrum of *EGFR*/*KRAS* mutations that is similar to adenocarcinoma (25–30). Several investigators have previously suggested that the detection of *EGFR* mutations in small samples diagnosed as "SQCC" could be a result of incomplete sampling of AD-SQC (1, 25). However, this possibility has not been formally investigated.

On the basis of these issues, we hypothesized that the reported variability of *EGFR*/*KRAS* mutations in samples diagnosed as SQCC could be a result of the above difficulties in the pathologic distinction of true SQCC from adenocarcinoma and AD-SQC, particularly in samples diagnosed prior to the recent advances in IHC. The goal of the study was therefore to combine mutational analysis with detailed pathologic assessment incorporating IHC. The study design included 2 separate approaches. First, we conducted a screen for *EGFR*/*KRAS* mutations in a large series of surgically resected SQCC ($n = 95$) in which the diagnosis was verified by IHC to exclude the possibility of inadvertent testing of adenocarcinoma that morphologically mimics SQCC. Second, we conducted detailed histologic and immunohistochemical reassessment of cases diagnosed as "SQCC" found to harbor *EGFR*/*KRAS* mutations during 5 years of routine molecular testing at our institution ($n = 16$). In addition, we used this highly validated, pathologically homogenous group of pure resected SQCCs to investigate mutation frequencies in 6 other therapeutically targetable signaling molecules—*BRAF*, *PIK3CA*, *NRAS*, *AKT1*, *ERBB2*/*HER2*, and *MAP2K1*/*MEK1*.

## Materials and Methods

### Study design

This study was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY). For the first part of the study (mutation screen of resected IHC-verified SQCC), a search of the

electronic medical records from the Department of Pathology at MSKCC was conducted to identify 100 lung resections with a diagnosis of SQCC. A representative formalin-fixed, paraffin-embedded (FFPE) tumor block was selected and used for IHC with ΔNp63 and TTF-1, as described later. Sufficient archival material was available for 98 cases. Of those, only cases with immunoprofiles compatible with the diagnosis of SQCC ($n = 95$) were included in the mutation screen, as described later.

For the second part of the study (reassessment of clinical samples), a separate search was conducted to identify specimens with the diagnosis of SQCC which were found to harbor *EGFR* or *KRAS* mutations during routine clinical genotyping at our institution between January 2006 and February 2011 ($n = 16$) by methods described previously (31). Although routine testing for *EGFR*/*KRAS* mutations at out institution during this time period generally excluded tumors with the diagnosis of SQCC, testing of some samples with this diagnosis was conducted at the request of the individual treating oncologists. All available pathologic samples from these patients were evaluated by light microscopy, as well as IHC for ΔNp63/TTF-1 and *EGFR*/*KRAS* mutations if not previously conducted and if sufficient tissue was available.

### Immunohistochemistry

Immunohistochemistry was conducted on a Ventana Discovery XT Automated Stainer (Ventana) as previously described (14, 18). Briefly, primary antibodies included ΔNp63 (p40; CalBiochem/EMD Biosciences, 1:2,000 dilution) and TTF-1 (SPT24 clone, NovoCastra, 1:50 dilution). Antigen retrieval was conducted using CC1 (cell conditioning 1; citrate buffer pH 6.0, Ventana) for 30 minutes. Interpretation of ΔNp63/TTF-1 immunoprofiles was conducted as recently described (18, 19). Briefly, ΔNp63-diffuse/TTF-1–negative profile supported SQCC. Rare cases with diffuse ΔNp63 and weak/focal coexpression of TTF-1 (known to occasionally occur in SQCC with SPT24 clone of TTF-1 antibody) were further confirmed as SQCC by diffuse expression of CK5/6 and negative Napsin A (data not shown) and such TTF-1 reactivity was regarded as nonspecific. Profiles that supported adenocarcinomas were TTF-1–positive/ΔNp63-negative (ΔNp63 reactivity in isolated tumor cells was scored as negative). TTF-1/ΔNp63 double-negative profile was interpreted as indeterminate but favoring adenocarcinoma because negative ΔNp63 is highly unusual for SQCC, whereas TTF-1–negative adenocarcinomas are not uncommon (14, 18, 19). Double-negative carcinomas were further evaluated by Napsin A and mucicarmine, which were noncontributory in all cases and these data are not shown. Reactivity for ΔNp63 and TTF-1 in distinct cell populations was used to support biphenotypic differentiation (i.e., AD-SQC).

### Mutational analysis

***DNA extraction.*** Hematoxylin and eosin–stained sections corresponding to the selected FFPE tumor blocks were reviewed to identify areas of tumor. Macrodissection on 10 corresponding 5-μm thick unstained sections was conducted to ensure greater than 50% tumor nuclei for each case. The gDNA was extracted using the DNeasy Tissue kit (QIAGEN) following the manufacturer's recommendations. Extracted DNA was quantified on the NanoDrop 8000 (Thermo Scientific).

***Mutation analysis by Sequenom mass spectrometry genotyping.*** Tumors were genotyped by Sequenom Mass ARRAY system (Sequenom Inc.). Briefly, samples were tested in duplicate using a series of multiplexed assays designed to interrogate the hotspot mutations in 8 oncogenes: *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, *AKT1*, *ERBB2*/*HER2*, and *MAP2K1*/*MEK1*. A total of 92 nonsynonymous mutations were tested in 6 multiplex reactions (see Supplementary Fig. S1 for complete list of tested mutations). Genomic DNA amplification and single base pair extension steps were conducted using specific primers designed with the Sequenom Assay Designer v3.1 software. The allele-specific single base extension products were then quantitatively analyzed using matrix-assisted laser desorption/ionization-time of flight/mass spectrometry (MALDI-TOF/MS) on the Sequenom MassArray Spectrometer. All automated system mutation calls were confirmed by manual review of the spectra.

***EGFR exon 19 fragment analysis.*** Fragment analysis of fluorescently labeled PCR products was conducted in duplicate. Briefly, a 207-bp gDNA fragment encompassing the entire exon 19 was amplified using the following fluorescently labeled primers (FW1: 5′-GCACCATCTCACAATTGCCAGTTA-3′; REV1: 5′-Fam-AAAAGGTGGGCCTGAGGTTCA-3′). PCR products were detected by capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems).

## Results

### Immunohistochemical verification and mutation screen of 95 resected pure SQCCs

Immunohistochemistry for ΔNp63 and TTF-1 was conducted on 98 resected lung tumors with the diagnosis of SQCC. Ninety-five cases had immunoprofiles supporting SQCC ($\Delta Np63^+/TTF\text{-}1^-$), whereas immunoprofiles of 3 tumors were inconsistent with the diagnosis of SQCC due to diffuse expression of TTF-1 and/or lack of ΔNp63 (Supplementary Table S2). Microscopically, the 3 tumors with atypical immunoprofiles were poorly differentiated NSCLCs that had a solid growth pattern, abundant eosinophilic cytoplasm, and sharp cell borders—a morphology resembling SQCC (i.e., "squamoid") but lacking the defining features of true SQCC (i.e., no keratinization or intercellular bridges). In conjunction with biomarker immunoprofile supporting glandular rather than squamous lineage, these tumors were reclassified as solid adenocarcinoma and excluded from further analysis.

Mutation screen was conducted on 95 pure IHC-verified SQCCs. Clinicopathologic characteristics of these patients are summarized in Table 1, and molecular results are shown in Table 2. None of the tested SQCCs harbored *EGFR* or *KRAS* mutations [0% observed incidence; 95% confidence interval (CI), 0%–3.8%], 4 tumors harbored *PIK3CA*

**Table 1.** Clinicopathologic characteristics of 95 IHC-verified resected SQCCs

| Characteristic | *N* (%) or median (range) |
|---|---|
| Age, y | 68 (37–88) |
| Gender | |
| Male | 62 (65) |
| Female | 33 (35) |
| Smoking | |
| Never | 2 (2) |
| Current or former | 93 (98) |
| Smoking pack-years | 58 (0–132) |
| Tumor size | 2.5 (0.7–11.5) |
| Tumor differentiation grade | |
| Well or moderate | 44 (46) |
| Poor | 51 (54) |
| Stage (pathologic)[a] | |
| IA | 48 (51) |
| IB | 9 (10) |
| IIA | 16 (17) |
| IIB | 10 (11) |
| IIIA | 10 (11) |
| IIIB/IV | 2 (2) |
| Surgical procedure | |
| Wedge | 31 (33) |
| Segmentectomy | 2 (2) |
| Lobectomy | 49 (52) |
| Pneumonectomy | 9 (10) |
| *En block* resection of lung and chest wall | 1 (1) |
| Bronchial tumor resection | 3 (3) |
| Immunohistochemistry[b] | |
| $\Delta Np63^+/TTF\text{-}1^-$ | 95 (100) |

[a]American Joint Commission on Cancer Staging Manual, 7th Edition.
[b]See Supplementary Table S2 for details.

**Table 2.** Spectrum of driver oncogene mutations in 95 resected pure SQCCs

| Gene | *N* (%) with mutation | 95% CIs |
|---|---|---|
| *EGFR* | 0 | 0%–3.8% |
| *KRAS* | 0 | 0%–3.8% |
| *PIK3CA* | 4 (4.2) | 1.2%–10.4% |
| *AKT1* | 1 (1.1) | 0%–5.7% |
| *BRAF* | 0 | 0%–3.8% |
| *NRAS* | 0 | 0%–3.8% |
| *ERBB2/HER2* | 0 | 0%–3.8% |
| *MAP2K1/MEK1* | 0 | 0%–3.8% |
| Any mutation | 5 (5.3) | 1.7%–11.9% |

mutations (4.2%; 95% CI, 1.2%–10.4%), and 1 tumor harbored an *AKT1* mutation (1.1%; 95% CI, 0%–5.7%). No mutations were identified in *BRAF*, *NRAS*, *ERBB2/HER2*, or *MAP2K1/MEK1*. *PIK3CA* mutations occurred both in the helical domain encoded by exon 9 (E542K, E545K) and in the catalytic domain encoded by exon 20 (H1047R, $n = 2$). The clinicopathologic features of individual patients with *PIK3CA/AKT1* mutations are summarized in Table 3. *PIK3CA/AKT1* mutations did not show a significant association with age, gender, pack-year smoking history, tumor size, stage, and grade of differentiation (Supplementary Table S3).

### Reassessment of 16 *EGFR/KRAS*-mutant "SQCCs" identified by routine clinical genotyping

During routine genotyping of clinical samples for *EGFR/KRAS* mutations, 16 samples were encountered which had a pathologic diagnosis of SQCC and were found to harbor *EGFR* ($n = 10$) or *KRAS* ($n = 6$) mutations. *EGFR* mutations included exon 19 deletions ($n = 8$) and L858R mutations ($n = 2$) and *KRAS* mutations included G12C ($n = 2$), G12V ($n = 2$), G12D ($n = 1$), and G12S mutations ($n = 1$). The detailed histologic and immunohistochemical reassessment of the index samples (mutant "SQCC") and all other samples available for these patients is shown in Table 4 and is described next.

In one group of patients (#1–10; $n = 10$), the diagnosis of SQCC in the index sample was verified by morphologic and immunohistochemical reassessment. Remarkably, in all but one of these patients (#1–9; $n = 9$), definitive evidence of glandular differentiation in the form of either adenocarcinoma or AD-SQC was uncovered in second or third tissue samples from other sites or time points. In most cases, the glandular component was apparent on conventional morphology, but in 2 cases (#1 and 8), a minor glandular component was revealed with the aid of IHC. Importantly, despite the distinct histologies, different samples from an individual patient harbored identical *EGFR* or *KRAS* mutations (whenever molecular data were available), indicating that they were clonally related and therefore represented components of a single AD-SQC. Paired samples represented primary tumor versus metastasis/recurrence ($n = 7$), tumors at different metastatic sites ($n = 1$), and preoperative biopsy versus same-site resection ($n = 1$). The latter case (#9) was particularly informative in that it consisted of a preoperative biopsy showing a *KRAS*-mutant "SQCC" with subsequent same-site resection revealing AD-SQC with 80% squamous and 20% glandular components, which directly confirmed that squamous histology in the biopsy represented incomplete sampling of AD-SQC. Interestingly, one case (#7) was a lobectomy in which the completely resected primary tumor was an *EGFR*-mutant SQCC whereas a hilar lymph node metastasis was adenocarcinoma with an identical *EGFR* mutation. A thorough review of the primary tumor failed to uncover the evidence of glandular differentiation, suggesting that glandular metastases arose from a minor glandular component not represented in the sections of primary tumor used for microscopic evaluation (of note, there was no

**Table 3.** Clinicopathologic characteristics of individual patients with *PIK3CA* and *AKT1* mutations in SQCC

| Case ID | Mutation | Age gender | Smoking (pack-years) | Tumor size, cm | Stage (pathologic) | Grade of differentiation | DFS, mo |
|---|---|---|---|---|---|---|---|
| 1 | *PIK3CA* E542K | 81F | 48 | 5.5 | IIA | Poor | 63+ |
| 2 | *PIK3CA* E545K | 51F | 3 | 4 | IIIA | Poor | 12 |
| 3 | *PIK3CA* H1047R | 62M | 35 | 2.6 | IA | Poor | 22+ |
| 4 | *PIK3CA* H1047R | 60M | 120 | 2.5 | IIB | Moderate | 19 |
| 5 | *AKT1* E17K | 65F | 63 | 2 | IA | Moderate | 23+ |

Abbreviations: +, remains disease free; DFS, disease-free survival; F, female; M, male.

pathologic or clinicoradiologic evidence of another primary tumor in this patient). In this group, there was only a single patient (#10; never-smoker) with the diagnosis of *EGFR*-mutant SQCC in 2 small biopsies, in whom evidence of adenocarcinoma could not be identified in either sample. Because a resection of the primary tumor was not conducted, the possibility of an underlying AD-SQC could not be either confirmed or excluded and the final diagnosis was therefore considered "indeterminate." Examples of microscopic findings for this group of patients are illustrated in Fig. 1A–L.

The other group of *EGFR*/*KRAS*-mutant "SQCC" (#11–15; $n = 5$) consisted of poorly differentiated carcinomas with "squamoid" morphology but with ΔNp63/TTF-1 immunoprofiles inconsistent with a diagnosis of SQCC and instead supporting or favoring adenocarcinoma. Similar to "SQCC" excluded from the mutation screen described above, these were poorly differentiated carcinomas with solid histology resembling SQCC but lacking keratinization or intercellular bridges (Fig. 1M–O).

In addition to the above 2 groups, case #16 (*KRAS*-mutant "SQCC") was a tumor with squamous and glandular components, but in which glands occupied only 5% of the tumor mass. This tumor was, as a result, classified as SQCC on the basis of the World Health Organization (WHO) 2004 definition of AD-SQC requiring more than 10% of each component (24).

The above findings are summarized in Table 5. Overall, of 16 cases with the diagnosis of "SQCC" harboring *EGFR*/*KRAS* mutations, 10 (63%) were reclassified as AD-SQC (including the tumor with 5% glandular component), 5 (31%) as adenocarcinoma, and one case (6%) was indeterminate. The samples in which these diagnostic issues were encountered were primarily (75%) small specimens (biopsy/cytology), and they were divided equally between sampling of the primary tumor (50%) or metastatic/recurrent sites (50%). Notably, the majority (7 of 10; 70%) of patients with *EGFR*-mutant "SQCC" were never-smokers.

## Discussion

In this study, we combined mutational analysis of lung cancers diagnosed as SQCC with rigorous IHC-assisted pathologic verification in an attempt to resolve the persistent controversy about the occurrence of *EGFR*/*KRAS* mutations in this tumor type. The main finding of this study is that *EGFR*/*KRAS* mutations do not occur in pure biomarker-verified SQCC and that the main culprits responsible for the detection of these mutations in samples diagnosed as "SQCC" are (i) incomplete sampling of AD-SQC and (ii) poorly differentiated adenocarcinoma morphologically mimicking SQCC. In addition to addressing the above controversy, we used a highly homogeneous series of IHC-verified SQCC to investigate the rate of mutations in other signaling molecules, for which targeted agents are either available or are at various stages of clinical development.

While the possibility that incomplete sampling of an underlying AD-SQC is a potential culprit for the detection of *EGFR*/*KRAS* mutations in samples diagnosed as "SQCC" has been suggested by several investigators in the past (1, 25), our data provide direct supporting evidence for this hypothesis. AD-SQC is uncommon but harbors a similar rate of *EGFR*/*KRAS* mutations as adenocarcinoma (25–30), with our data supporting previous observations that these mutations are present uniformly in both the glandular and squamous components of this tumor type (25–29). These findings indicate that squamous histology *per se* is not incompatible with *EGFR*/*KRAS* mutations but that these mutations are restricted to squamous histology that represents a component of AD-SQC whereas they are not a feature of pure SQCC. Notably, this situation in SQCC is remarkably similar to the findings in small cell lung carcinoma (SCLC). While *EGFR*/*KRAS* mutations are not a feature of SCLC in general, these mutations may be present in SCLC that has a clonal relationship with adenocarcinoma, as seen in rare instances when SCLC is combined with adenocarcinoma or in recently described small cell transformation of adenocarcinoma after prolonged treatment with EGFR-TKIs (32–36). These findings suggest that *EGFR*/*KRAS*-mediated tumorigenesis is specific to progenitor/stem cells giving rise to adenocarcinoma but that subsequent (or inherent) clonal phenotypic divergence accounts for the occasional detection of these mutations in unexpected histologies. Other than the presence of adenocarcinoma-specific mutations, both SQCC and SCLC arising from adenocarcinoma are otherwise morphologically and immunophenotypically identical to

**Table 4.** Reassessment of 16 *EGFR/KRAS*-mutant SQCCs identified by routine clinical genotyping

| Pt ID | Age gender | Pack-years | Pathology | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Sample no[a]; site (time in mo) | Specimen type | Diagnosis (original → Δ post-review[b]) | IHC | | Mutation | Interpretation |
| | | | | | | TTF-1 | ΔNp63 | | |
| 1 | 61M | 0 | (i) Spine (0) | Bx | SQCC | nd | nd | EGFR ex 19 18 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) Lung (−26) | Bx | SQCC → AD-SQC | nd | nd | EGFR ex 19 18 bp Δ | |
| 2 | 71F | 0 | (i) Lung, RLL (0) | Bx | SQCC | − | + | EGFR ex 19 15 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) Lung, RLL (−23) | Bx | ADC | + | nd | EGFR ex 19 15 bp Δ | |
| 3 | 58F | 0 | (i) Bronchus (0) | Bx | SQCC | − | + | EGFR ex 19 15 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) Lung, RUL (−9) | Bx | AD-SQC | nd | nd | EGFR ex 19 15 bp Δ | |
| | | | (iii) Lung, LLL (+21) | Bx | ADC | + | − | EGFR ex 19 15 bp Δ | |
| 4 | 45F | 0 | (i) Sacrum (0) | FNA | SQCC | − | + | EGFR ex 19 15 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) Lung (−17) | Pleural fluid | ADC | + | − | EGFR ex 19 15 bp Δ | |
| 5 | 46M | 0 | (i) Lung (0) | FNA | SQCC | − | + | EGFR ex 19 9 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) SCLN (+1) | Bx | ADC | + | − | EGFR ex 19 9 bp Δ | |
| 6 | 73M | 25 | (i) Adrenal (0) | Resection | SQCC | − | + | EGFR ex 19 18 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) SCLN (−9) | Bx | AD-SQC | f[c] | f[c] | nd | |
| 7 | 68M | 45 | (i) Lung (0) | Lobectomy | SQCC | − | + | EGFR ex 19 15 bp Δ | "SQCC" = component of AD-SQC |
| | | | (ii) Hilar LN (0) | Dissection | ADC | + | − | EGFR ex 19 15 bp Δ | |
| 8 | 50M | 63 | (i) Skin (0) | FNA | SQCC | nd | nd | KRAS G12C | "SQCC" = component of AD-SQC |
| | | | (ii) Lung (−5) | FNA | ADC | nd | nd | nd | |
| | | | (iii) SCLN (−5) | Bx | SQCC → AD-SQC | − | f[c] | KRAS G12C | |
| 9 | 63F | 0 | (i) Lung, LUL (0) | Bx | SQCC | − | + | KRAS G12D | "SQCC" = component of AD-SQC |
| | | | (ii) Lung, LUL (0) | FNA | ADC | nd | nd | nd | |
| | | | (iii) Lung, LUL (+5) | Lobectomy | AD-SQC | f[c] | f[c] | KRAS G12D | |
| 10 | 58M | 0 | (i) Bronchus (0) | Bx | SQCC | − | + | EGFR L858R | Indeterminate |
| | | | (ii) Spine (+1) | Bx | SQCC | − | + | nd | |
| 11 | 89F | 0 | Lung | Bx | SQCC → ADC | + | − | EGFR L858R | "SQCC" = PD ADC |
| 12 | 53F | 31 | Lung | Lobectomy | SQCC → ADC | + | − | EGFR ex 19 18 bp Δ | "SQCC" = PD ADC |
| 13 | 49F | 33 | Bronchus | Bx | SQCC → ADC | + | − | KRAS G12V | "SQCC" = PD ADC |
| 14 | 69F | 34 | Hilar LN | FNA | SQCC → ADC | + | − | KRAS G12V | "SQCC" = PD ADC |
| 15 | 63M | 80 | Bone | Bx | SQCC → favor ADC | − | − | KRAS G12S | "SQCC" = favor PD ADC |
| 16 | 58M | 51 | Lung | Bronchial excision | SQCC with 5% glands | f (5%)[c] | f (95%)[c] | KRAS G12C | "SQCC" = biphasic tumor with <10% glands |

Abbreviations: +, diffusely positive; −, negative; Δ, deletion or change; ADC, adenocarcinoma; Bx, biopsy; ex, exon; f, focally positive; FNA, fine-needle aspiration; LLL, left lower lobe; LN, lymph node; LUL, left upper lobe; nd, not done (tissue insufficient or unavailable); PD, poorly differentiated; Pt, patient; RLL, right lower lobe; SCLN, supraclavicular lymph node.

[a]Sample order is nonchronologic: sample 1 represents an index case (*EGFR/KRAS*-mutant "SQCC").

[b]Review diagnosis was based on a combination of morphology and IHC; in the absence of IHC, tumor type was considered verified if morphology was diagnostic.

[c]TTF-1- and ΔNp63-labeled distinct cell populations.

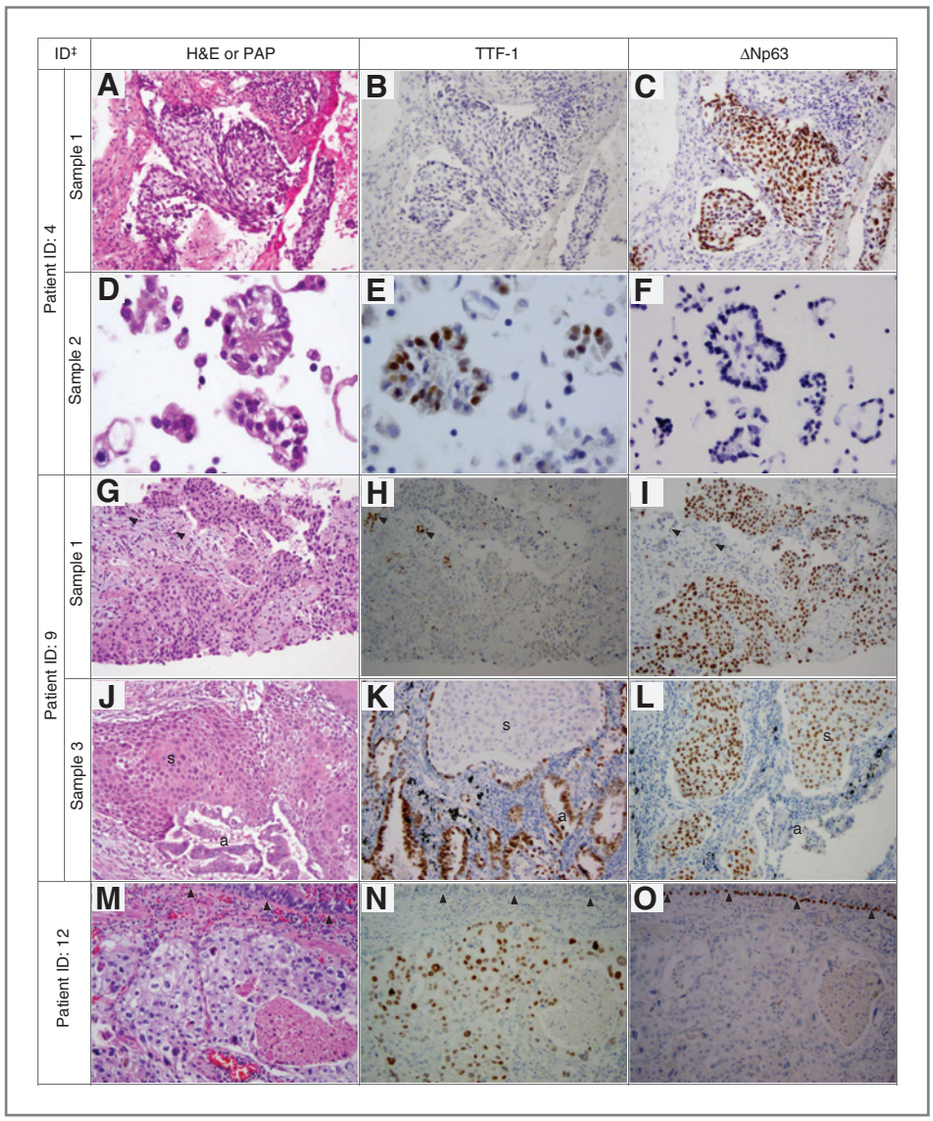


**Figure 1.** Microscopic features of *EGFR*/*KRAS*-mutant "SQCC" with a revised diagnosis of AD-SQC or solid adenocarcinoma. Patient 4 and patient 9 illustrate incomplete sampling of AD-SQC leading to the diagnosis of "SQCC" with *EGFR* or *KRAS* mutations, respectively. While only squamous component was present in the index sample (bone FNA A–C; lung core biopsy G–I), glandular component was manifested in a cytology sample from another site (pleural fluid; D–F) or subsequent same-site resection (lung lobectomy; J–L). Glandular and squamous components have distinct morphology and ΔNp63/TTF-1 immunoprofiles as seen in different small samples (A–C vs. D–F) or different areas of a resected tumor (J–L). Patient 12 illustrates a solid growth pattern in *EGFR*-mutant adenocarcinoma which resembles SQCC morphologically ("squamoid" or "pseudo-squamous" appearance), but it is readily identified as being of a glandular rather than squamous lineage by IHC. ‡, ID corresponds to patient and sample numbers in Table 4. a, adenocarcinoma component; s, squamous component; H&E, hematoxylin and eosin stain; PAP, Papanicolaou stain; arrowheads in G–I—benign pneumocytes (TTF-1$^+$), arrowheads in M–O—benign bronchial basal cells (ΔNp63$^+$).

their pure counterparts. The analysis of early driver mutations thus provides an insight into the cell of origin and clonal relationship of divergent histologies in these settings.

As mentioned above, a key issue with the diagnosis of AD-SQC is that small biopsy or cytology specimens may contain only 1 of the 2 histologic components. A further diagnostic challenge is that metastases derived from AD-SQC sometimes consist entirely of a single histology (37, 38), as exemplified by case 6 in this study. These factors make it impossible to determine whether squamous histology in a small biopsy or even a metastasectomy represents a true SQCC versus a component of AD-SQC. On the other hand, because AD-SQC is a rare tumor, the diagnosis of SQCC in a small specimen is statistically very likely to represent true (i.e. pure) SQCC (as discussed below, a patient's clinical characteristics, particularly the never-smoker status, may serve as a clue to an underlying AD-SQC). Although these challenges in the diagnosis of AD-SQC in clinical samples are well known (24), that they are the main cause of conflicting molecular data in SQCC has not been previously well documented. This study illustrates that a comprehensive pathologic analysis of all specimens from different sites and time points, when available, can reveal both squamous and glandular differentiation in the majority of patients, thereby clarifying the atypical molecular findings. Furthermore, we show that while IHC cannot circumvent the issue of incomplete sampling, it can improve the diagnosis of AD-SQC in a subset of cases.

The second culprit that we identified as a cause for the conflicting data surrounding *EGFR*/*KRAS* mutations in SQCC is the ability of poorly differentiated adenocarcinomas to grow in a solid pattern closely mimicking SQCC (i.e., "squamoid" or "pseudo-squamous" appearance). We show that the cell lineage in areas with this ambiguous appearance is readily clarified by IHC for ΔNp63 and TTF-1 but that

**Table 5.** Summary of reassessment of 16 *EGFR/KRAS*-mutant SQCCs identified by routine clinical genotyping

| Characteristics[a] | *N* (%) |
|---|---|
| *Specimen type* | |
| Small specimen (biopsy or cytology) | 12 (75) |
| Surgical resection | 4 (25) |
| *Tumor site* | |
| Lung primary | 8 (50) |
| Metastasis (lymph node, adrenal, bone, skin) | 6 (38) |
| Recurrence | 2 (11) |
| *Interpretation after morphologic and immunohistochemical reassessment* | |
| Reclassified as AD-SQC[b] | 10 (63) |
| Reclassified as solid adenocarcinoma by IHC | 5 (31) |
| Indeterminate | 1 (6) |
| *Smoking status by mutation* | |
| *EGFR*-mutant "SQCC" | 10 (63) |
| Never | 7 |
| Current or former | 3 |
| *KRAS*-mutant "SQCC" | 6 (37) |
| Never | 1 |
| Current or former | 5 |

[a]Data for index samples (*EGFR/KRAS*-mutant "SQCC"), corresponding to samples #1 in Table 4.

[b]Includes patients with a small biopsy/cytology diagnosis of "SQCC" in an index sample, but evidence of glandular differentiation in other (non-index) tissue sample(s) ($n = 9$), and one resected tumor with 5% glandular component.

interpretation in the absence of IHC may lead to an erroneous diagnosis of "SQCC" harboring *EGFR*/*KRAS* mutations. This is an underrecognized morphologic feature in lung adenocarcinoma, which may be present either focally or as a predominant pattern, and we show here that it may present a diagnostic pitfall in both small specimens and resected tumors. These findings highlight the value of IHC, which is becoming widely incorporated into clinical practice in recent years, for the diagnosis of morphologically challenging NSCLC and for assuring homogeneity of tumor types included in molecular studies. It is of note that while immunomarkers used in this study are not new, their application to clinical samples was characterized in detail only recently (15–19). We should also emphasize that IHC is not required for the diagnosis of all SQCC; in the majority of cases, the line of differentiation is evident based on morphology and IHC is only needed for a minority of cases that are poorly differentiated and have equivocal morphology.

The third and least common cause of conflicting molecular data is the definition of AD-SQC itself, which requires that both glandular and squamous components represent at least 10% of the tumor mass (24). This arbitrarily selected criterion predates the new molecular data showing that any amount of glandular differentiation, even less than 10%, appears to be a harbinger of adenocarcinoma-specific driver mutations. Similar to our *KRAS*-mutant "SQCC" in which glands represented 5% of the tumor (case #16), detection of *EGFR* mutation in "SQCC" with less than 10% glands has been described by Ohtsuka and colleagues (26). Therefore, any amount of glandular differentiation should qualify a tumor for *EGFR*/*KRAS* genotyping.

The potential contribution of the above diagnostic challenges to the previous reports of *EGFR*/*KRAS* mutations in SQCC is difficult to estimate. The vast majority of reported patients with *EGFR*-mutant SQCC had advanced disease (where the diagnosis is typically based on small specimens) and many patients were never-smokers (6, 8, 10), suggesting that similar to this study, those cases may have represented incompletely sampled AD-SQC. Interestingly, one recent study used a similar approach of combining IHC-based diagnosis verification with *EGFR* mutation testing in a series of 85 resected SQCCs (9). A total of 5 *EGFR*-mutant tumors were identified, of which 2 were reclassified by IHC as AD-SQC and adenocarcinoma whereas 3 tumors were verified as SQCC. These latter cases may be similar to case #7 in our series, in which whole tissue sections of the primary tumor showed only SQCC (by morphology and IHC). Remarkably, a hilar lymph node metastasis showed adenocarcinoma that harbored an identical *EGFR* mutation as the primary tumor, supporting their clonal relationship. This suggests that in rare instances undersampling of AD-SQC is possible even in whole tissue sections, which may occur because large tumors are examined representatively in pathology laboratories and therefore a minor glandular component may not be represented in microscopically scrutinized tissue. In the above case (#7), we hypothesize that the minor unsampled component with a distinct histology had an enhanced metastatic potential, and was therefore solely represented in the metastasis.

A potential criticism of this study is that although no *EGFR*/*KRAS* mutations were identified in the screen of 95 IHC-verified pure SQCC, one cannot exclude the possibility of low-frequency mutations in these genes falling within the CIs of this study (95% CI, 0%–3.8%). However, our conclusions are, in equal part, supported by the findings that none of the "SQCC" with *EGFR*/*KRAS* mutations identified in our clinical practice could be confirmed to represent true (pure) SQCC. Detailed pathologic review, incorporating the modern immunohistochemical methods, of *EGFR*/*KRAS*-mutant carcinomas diagnosed as SQCC at other institutions will be needed to validate the conclusions reached in this study.

What are the implications of our findings for clinical practice? The current recommendation in the National Comprehensive Cancer Network guidelines is to exclude SQCC from *EGFR*/*KRAS* mutation testing (39). Our findings support this recommendation for SQCC diagnosed in a surgically resected primary tumor (where undersampling of AD-SQC is highly unlikely). On the other hand, testing patients with SQCC diagnosed in small biopsy or cytology specimens (where the possibility of an underlying AD-SQC

cannot be excluded) should be guided by clinical parameters. In particular, similar to adenocarcinoma, *EGFR* mutations in AD-SQC are associated with a never-smoker status (25, 30), whereas the lack of smoking history is unusual for patients with a true SQCC [2% in this study and 1%–3% in other studies (refs. 5, 9, 40)]. Indeed, the majority of patients with *EGFR*-mutant "SQCC" with a revised diagnosis of adenocarcinoma or AD-SQC in this study were never-smokers. We therefore suggest that patients that receive a diagnosis of SQCC based on a small biopsy and who do not have a history of smoking are likely to have an underlying mixed tumor and should be tested for *EGFR*/*KRAS* mutations. Detailed clinical analysis of the patients in this series, including their EGFR-TKI sensitivity, will be reported separately (Paik and colleagues, manuscript in preparation).

The specificity of *EGFR*/*KRAS* mutations for carcinomas with glandular differentiation (adenocarcinoma and AD-SQC) and strict exclusion of these mutations from pure SQCC provide an interesting insight into the histogenetic relationship of these tumors. In the past, it has been questioned whether lung adenocarcinoma and SQCC represent truly distinct entities versus a spectrum of related tumors (41). The sharp divide in tumor-initiating mutations provides a strong support for the distinct molecular pathogenesis of pure SQCC compared with tumors with glandular differentiation. The biologic underpinnings of specificity for adenocarcinoma of *EGFR*/*KRAS* and several other driver mutations in NSCLC, including *BRAF* (42) and *EML4-ALK* (43), remain to be elucidated, but these findings are in line with lineage restriction of many other somatic genetic alterations across human tumors (44).

Finally, in this study, we used a rigorously verified series of pure SQCCs to investigate the rate of mutations in other important signaling molecules. We found a low frequency of *PIK3CA* (4%) and *AKT1* (1%) mutations, which is in agreement with prior studies showing a 2% to 4% rate of *PIK3CA* mutations (45–47) and an approximately 1% rate of *AKT1* mutations (48, 49) in NSCLC. Prior studies have shown that *PIK3CA* mutations occur in both adenocarcinoma and SQCC but are more common in SQCC, and *AKT1* mutations are found primarily in SQCC. Importantly, several phosphoinositide 3-kinase (PI3K) and AKT1 inhibitors are in early clinical development (50), and screening of lung SQCC for these mutations may be used in the future to select patients for targeted therapies.

In conclusion, we present compelling evidence that *EGFR*/*KRAS* mutations have a strong specificity for carcinomas with glandular differentiation, whereas they are not a feature of pure SQCCs. We describe several pitfalls in the traditional pathologic diagnosis of NSCLC subtypes that can lead to conflicting genotype data and illustrate how comprehensive pathologic assessment using biomarker expression can clarify the tumor lineage and resolve atypical molecular findings. Our results support incorporating this approach into routine clinical practice and future clinicopathologic and molecular studies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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