CHEST

Original Research

LUNG CANCER

Comprehensive Analysis of Oncogenic Mutations in Lung Squamous Cell Carcinoma With Minor Glandular Component

Yunjian Pan, MD; Rui Wang, MD; Ting Ye, MD; Chenguang Li, MD; Haichuan Hu, MD; Yongfu Yu, MS; Yang Zhang, MD; Lei Wang, MD; Xiaoyang Luo, MD; Hang Li, MD; Yuan Li, MD; Lei Shen, MD; Yihua Sun, MD; and Haiquan Chen, MD, FCCP

Background: **The mutations in oncogenic genes, such as *EGFR*, *ALK*, *BRAF*, *HER2*, *DDR2*, *RET*, and *AKT1*, defined subsets of non-small cell lung cancers (NSCLCs) with potential sensitivity to targeted therapies. At present, the mutational spectrum, prevalence, and clinicopathologic characteristics in squamous cell carcinomas with minor (< 10%) glandular component (SQCC-mGCs) are not well established.**

Methods: **Three hundred ten surgically resected lung squamous cell carcinoma (SQCC) specimens were collected. The histology of all cases was reevaluated using hematoxylin-eosin and immunohistochemistry staining. *EGFR*, *KRAS*, *HER2*, *BRAF*, *PIK3CA*, *AKT1*, and *DDR2* mutations, as well as *ALK* and *RET* rearrangements, were examined in 310 SQCCs by directed sequencing.**

Results: **Ninety-five SQCC-mGCs (30.6%) and 215 pure SQCCs (69.4%) were identified. Of the 95 SQCC-mGCs, 26 (27.4%; 95% CI, 18.7%-37.4%) were found to harbor known oncogenic mutations, including 10 with *EGFR*, seven with *KRAS*, three with *PIK3CA*, one with *BRAF*, one with *HER2*, one each with *EGFR/PIK3CA* and *KRAS/PIK3CA* double mutations, and two with *EML4-ALK* fusions. Ten of 215 pure SQCCs (4.7%; 95% CI, 2.3%-8.4%) harbored mutations, including seven with *PIK3CA*, and each with *AKT1*, *DDR2*, and *EGFR*. No *RET* rearrangements were detected in SQCCs. SQCC-mGCs had a significantly higher rate of mutations in known oncogenic genes than that in pure SQCCs (27.4% vs 4.7%, $P < .001$). All *KRAS* mutations occurred in SQCC-mGCs.**

Conclusions: **Our results demonstrated that oncogenic mutations in *EGFR*, *KRAS*, *BRAF*, *HER2*, and *ALK* were extremely rare or absent in patients with pure SQCC, whereas SQCC-mGC had a relatively high frequency of *EGFR*, *ALK*, or *KRAS* mutations. Prospective identification of these known oncogenic mutations in SQCC-mGC before the initiation of treatment is an essential step to identify which patient could benefit from targeted therapies.** ***CHEST 2014; 145(3):473–479***

Abbreviations: cDNA = complementary DNA; CK = cytokeratin; EGFR-TKI = epidermal growth factor receptor tyrosine kinase inhibitor; IHC = immunohistochemistry; NSCLC = non-small cell lung cancer; OS = overall survival; PCR = polymerase chain reaction; RFS = relapse-free survival; SQCC = squamous cell carcinoma; SQCC-mGC = squamous cell carcinoma with minor glandular component; TTF-1 = thyroid transcription factor-1

Detailed understanding of the genetic alterations that drive subsets of lung cancers has led to the development of targeted agents, which have changed the treatment landscape.[1-6] Oncogenic mutations occur in genes that encode signaling kinases crucial for cellular proliferation and survival.[7] Cancer cells might depend on the mutant kinase for survival and die when it is inactivated. Therefore, these mutant kinases could be exploited for therapeutic target, which is best illustrated by *EGFR*. Treatment with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) leads to dramatic regression of tumors and improved survival in patients whose tumor harbored *EGFR* mutations.[3,8,9] More recently, patients with *ALK* rearrangement demonstrated dramatic responses to crizotinib,[1] which has been recommended by the US Food and

Drug Administration as first-line therapy for patients with advanced-stage non-small cell lung cancer (NSCLC) with *ALK* rearrangement. Except *EGFR* and *ALK*, the established driver genes in NSCLCs also include *KRAS*, *BRAF*, *HER2*, *RET*, *AKT1*, *PIK3CA*, and *DDR2*.[10] *EGFR*, *KRAS*, *HER2*, and *BRAF* mutations, as well as *ALK* and *RET* rearrangements, were found much more frequently in lung adenocarcinomas,[1,7,11-13] whereas mutations in *PIK3CA*, *AKT1*, and *DDR2* were found mainly in squamous cell carcinomas (SQCCs).[7,10] Except for *KRAS*, all the other known mutant kinases could be targeted by agents being used in the clinic or evaluated in clinical trials.[7]

More than 80% of lung adenocarcinomas from East Asian populations can be defined by known oncogenic mutations, such as *EGFR*, *KRAS*, *HER2*, *BRAF*, or *ALK*.[12] However, the mutation profiles in lung SQCCs were still largely unclear.[14] The frequency of *EGFR* and *KRAS* mutations in lung SQCCs ranged from 1% to 15% and 1% to 9%,[15-21] respectively. A study showed that lung SQCCs without any glandular component (pure SQCC) by microscopic examination and immunohistochemistry (IHC) biomarker verification were all negative for *EGFR* and *KRAS* mutations, suggesting that the variation of their mutation rates in SQCCs might be caused by the glandular component in SQCCs.[22] However, its accurate prevalence and clinicopathologic and mutational characteristics remain unknown.

In this study, tumor with $< 10\%$ glandular component is classified as SQCC with minor glandular component (SQCC-mGC), and SQCC without any glandular component is identified as pure SQCC. The mutational status of known oncogenic genes, including *EGFR*, *KRAS*, *HER2*, *BRAF*, *PIK3CA*, *AKT1*, *DDR2*, *ALK*, and *RET*, were examined in 310 SQCCs.

Manuscript received November 1, 2012; revision accepted September 16, 2013.
Affiliations: From the Department of Thoracic Surgery (Drs Pan, R. Wang, Ye, C. Li, Hu, Zhang, L. Wang, Luo, H. Li, Sun, and Chen), and the Department of Pathology (Drs Y. Li and Shen), Fudan University Shanghai Cancer Center; and the Department of Oncology (Drs Pan, R. Wang, Ye, C. Li, Hu, Zhang, L. Wang, Luo, H. Li, Y. Li, Shen, Sun, and Chen), Shanghai Medical College, and the Department of Biostatistics (Mr Yu), School of Public Health, Fudan University, Shanghai, China.
Drs Pan, R. Wang, Ye, and C. Li contributed to this work equally and should be considered co-first authors. Drs Chen and Sun contributed to this work equally.
Funding/Support: This study was supported by the National Natural Science Foundation of China [Grant 81101761, 81172218] and the Key Construction Program of the National "985" Project Grant [985-YFX0102].
Correspondence to: Haiquan Chen, MD, FCCP, Department of Thoracic Surgery, Fudan University Shanghai Cancer Center, 270 Dong'An Rd, Shanghai 200032, China; e-mail: hqchen1@yahoo.com

DOI: 10.1378/chest.12-2679

Materials and Methods

Specimen Collection

A total of 324 cases reported as SQCC were included, and the frozen samples and corresponding formalin-fixed, paraffin-embedded tumor blocks (at least two blocks for each case) were consecutively collected from August 2007 to August 2011 in Fudan University Shanghai Cancer Center. Written informed consent was obtained from each patient. Each specimen contained at least 50% tumor cells. The study was approved by the Committee for Ethical Review of Research (Fudan University Shanghai Cancer Center IRB# 090977-1). Only cases with confirmed SQCC diagnosis by hematoxylin-eosin and IHC staining were selected for mutational analysis. Clinicopathologic data were obtained from electronic medical records.

Pathologic Reassessment

Tumor blocks were cut into 4-μm-thick sections. All sections were stained with hematoxylin-eosin and IHC (at least two for each case). Morphologic examinations were performed by two pathologists (Y. Li and L. Shen), as shown in Figure 1. Well-differentiated tumors with typical keratinization and/or intercellular bridges were only stained with thyroid transcription factor-1 (TTF-1) and cytokeratin (CK) 7. Moderate and poorly differentiated tumors were stained with a panel of P63, CK5/6, TTF-1, and CK7.

Primary antibodies included mouse anti-human P63 monoclonal antibody (4A4 clone; Maixin Technology Co, Ltd) (working concentration 1 μg/mL), mouse anti-human TTF-1 monoclonal antibody (8G7G3/1 clone; Shanghai Long Island Biotec Co, Ltd) (working concentration 1 μg/mL), mouse anti-human CK5/6 monoclonal antibody (D5/16 B4 clone; Dako) (working concentration 10 μg/mL), and mouse anti-human CK7 monoclonal antibody (OV-TL 12/30 clone; Maixin Technology Co, Ltd) (working concentration 2 μg/mL). Antigen retrieval was performed using sodium citrate buffer (10 mM, pH = 6.0) (Solarbio). Slides were treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. Primary antibodies were applied and incubated for 60 min. The DAB Envision Kit was used (Real Envision Detection Kit; Gene Tech). For TTF-1 and CK7 staining, intensity (0, 1+, 2+, 3+) and percentage of immunoreactive tumor cells were recorded as follows: 3+, strong staining intensity in $< 10\%$ tumor cells; 2+, moderate staining intensity in $< 10\%$ tumor cells; 1+, faint or weak staining intensity in $< 10\%$ tumor cells; and 0, no staining. Tumors with 3+ and 2+ intensity in $< 10\%$ tumor cells were defined as TTF-1 or CK7-positive. Tumors with $> 10\%$ TTF-1 and/or CK7-positive tumor cells were excluded in the following study. Pure SQCC was defined by p63 and/or CK5/6-diffuse with TTF-1/CK7 double-negative staining.

Reverse Transcription Polymerase Chain Reaction and Mutation Analysis

Frozen tumor specimens were dissected, and RNA/DNA was coextracted following the standard instructions of the RNA/DNA isolation kit (Tiangen Biotech Co, Ltd). Single-stranded RNA of each sample is reverse transcribed into complementary DNA (cDNA) by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc). *EGFR* (exons 18-21), *HER2* (exons 18-21), *KRAS* (exons 2-3), *BRAF* (exons 11-15), *AKT1* (exons 2-3), *PIK3CA* (exon 9 and exon 20), and *DDR2* (whole coding exons) were amplified with KOD Plus Neo DNA polymerase (Toyobo Co, Ltd). For detection of *EML4-ALK*, *KIF5B-RET*, and *CCDC6-RET* fusions, primers were designed to cover all known fusion variants. Polymerase chain reaction (PCR) was performed in a 25-μL reaction tube on Mastercycler pro PCR apparatus (Eppendorf AG). RNase-free water was used as a PCR-negative control. Thermocycler