KRAS-G12C Mutation Is Associated with Poor Outcome in Surgically Resected Lung Adenocarcinoma

Ernest Nadal, MD, Guoan Chen, MD, PhD,* John R. Prensner, MD, PhD,† Hiroe Shiratsuchi, PhD,‡ Christine Sam, MD,‡ Lili Zhao, PhD,§ Gregory P. Kalemkerian, MD,‡ Dean Brenner, MD,‡‖ Jules Lin, MD,* Rishindra M. Reddy, MD,* Andrew C. Chang, MD,* Gabriel Capellà, MD, PhD,¶ Felipe Cardenal, MD, PhD,# David G. Beer, PhD,* and Nithya Ramnath, MD‡‖*

Introduction: The aim of this study was to examine the effects of *KRAS* mutant subtypes on the outcome of patients with resected lung adenocarcinoma (AC).

Methods: Using clinical and sequencing data, we identified 179 patients with resected lung AC for whom *KRAS* mutational status was determined. A multivariate Cox model was used to identify factors associated with disease-free survival (DFS) and overall survival (OS). Publicly available mutation and gene-expression data from lung cancer cell lines and lung AC were used to assess whether distinct *KRAS* mutant variants have a different profile.

Results: Patients with *KRAS* mutation had a significantly shorter DFS compared with those with *KRAS* wild-type ($p = 0.009$). Patients with *KRAS-G12C* mutant tumors had significantly shorter DFS compared with other *KRAS* mutants and *KRAS* wild-type tumors ($p < 0.001$). In the multivariate Cox model, *KRAS-G12C* remained as an independent prognostic marker for DFS (Hazard ratio = 2.46, 95% confidence interval 1.51–4.00, $p < 0.001$) and for OS (Hazard ratio = 2.35, 95% confidence interval 1.35–4.10, $p = 0.003$). No genes were statistically significant when comparing the mutational or transcriptional profile of lung cancer cell lines and lung AC harboring *KRAS-G12C* with other *KRAS* mutant subtypes. Gene set enrichment analysis revealed that *KRAS-G12C* mutants overexpressed epithelial to mesenchymal transition genes and expressed lower levels of genes predicting KRAS dependency.

Conclusions: *KRAS-G12C* mutation is associated with worse DFS and OS in resected lung AC. Gene-expression profiles in lung cancer cell lines and surgically resected lung AC revealed that *KRAS-G12C* mutants had an epithelial to mesenchymal transition and a KRAS-independent phenotype.

Key Words: *KRAS* mutation, Lung adenocarcinoma, Prognostic markers.

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Mutation in the *KRAS* gene is one of the most frequently detected activating mutations in lung adenocarcinomas (AC).[1] *KRAS* mutations in lung AC usually arise at codon 12, occasionally at codon 13, and rarely at codon 61. *KRAS* mutations are predominantly found in smokers and these smoking-related *KRAS* mutations generally are G→T transversions, in contrast to the G→A transitions found in colorectal cancer and in lung AC patients who never smoked.[2–5] This distinct mutational profile suggests the convergence of diverse carcinogens in the induction of *KRAS* mutation that seems to be an early event of lung tumorigenesis, being detectable in the smoking-related lung AC precursor, atypical alveolar hyperplasia.[6,7]

KRAS belongs to the *RAS* human gene family that acts as signal switch molecule cycling between an active guanosine triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP) state. GDP/GTP cycling is positively regulated by guanine nucleotide exchanging factor and negatively regulated by GTPase-activating proteins (e.g., *NF1*). Oncogenic Ras proteins have defective intrinsic GTPase function and are resistant to GTPase-activating proteins, leading to accumulation of Ras in its active GTP-bound state, which causes constitutive activation of Ras signaling. GTP-bound active Ras recruits and activates downstream pathways such as RAF−MEK−ERK-promoting cell proliferation or PI3K/AKT-inducing inhibition of apoptosis.[8] At least nine specific *KRAS* mutations have been identified in lung cancer, based on the amino acid substitution,[9] and these distinct *KRAS* mutants have differential binding affinity for downstream effector molecules. For example, mutant *KRAS-G12D* demonstrated higher affinity for PI3K, whereas mutant *KRAS-G12C* had higher affinity for RalGDS.[10] In addition, different amino acid substitutions might activate different signaling pathways and indeed, give rise to varying responses rates to chemotherapy and some targeted therapies in lung cancer cell lines and

*Thoracic Surgery, Department of Surgery, University of Michigan Medical School, Ann Arbor, Michigan; †Michigan Center for Translational Pathology, Department of Pathology, University of Michigan, Ann Arbor, Michigan; ‡Division of Hematology/Oncology, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan; §Department of Biostatistics, University of Michigan, Ann Arbor, Michigan; ‖Veterans Administration Medical Center, Ann Arbor, Michigan; ¶Translational Research Laboratory, Catalan Institute of Oncology, L'Hospitalet de Llobregat, Barcelona, Spain; and #Department of Medical Oncology, Thoracic Oncology Multidisciplinary Unit, Catalan Institute of Oncology, L'Hospitalet de Llobregat, Barcelona, Spain

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Address for correspondence: Nithya Ramnath, MD, Division of Hematology/Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, 1500 E. Medical Center Drive, Ann Arbor, MI 48109. E-mail: nithyar@med.umich.edu

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advanced lung ACs.[10,11] Recent studies have added a new level of complexity because non–small-cell lung cancer (NSCLC) cell lines harboring *KRAS*-activating mutations can be stratified into KRAS dependent and KRAS independent based on the effect of the KRAS ablation on cell proliferation or induction of apoptosis.[12,13]

The prognostic value of *KRAS* mutations remains controversial in lung cancer. Several studies reported that *KRAS* mutations are a marker of poor prognosis in NSCLC.[14] In a recent study, involving 1500 resected NSCLC, *KRAS* mutation was not prognostic for survival and did not predict benefit from adjuvant chemotherapy.[15] In advanced NSCLC, the various amino acid substitutions predicted different clinical outcomes.[10] For example, *KRAS-G12C and KRAS-G12V* predicted worse progression-free survival (PFS), whereas other *KRAS* mutants predicted better PFS. We hypothesized that specific amino acid *KRAS* substitutions are associated with differing outcomes in patients with surgically resected lung ACs.

In this study, we determined the prognostic value of specific amino acid substitutions among 179 resected lung ACs using our data set and comparing and contrasting our results with data from other studies. In addition, we used publicly available data bases to elucidate whether the various codon mutants had different mutational profiles and varying degree of RAS dependency.

PATIENTS AND METHODS

Human Samples

In this retrospective study, we examined 179 primary lung ACs obtained from patients undergoing primary thoracic resection for lung cancer at the University of Michigan Health System, Ann Arbor, Michigan, during 1991–2007 for whom *KRAS* mutation status was known. This cohort encompassed an initial set of tumors from a previous study wherein 86 lung ACs were mRNA profiled[16] and a second set of 93 tumors subsequently used for validating specific genes. None of the patients included in this study received preoperative radiation or chemotherapy. Tissue specimens were banked with informed consent after approval from University of Michigan Institutional Review Board and Ethics Committee and were frozen in liquid nitrogen and stored at −80°C. Regions containing a minimum of 70% tumor cellularity were utilized for DNA and RNA isolation. Clinical data were retrospectively collected from the medical records and all cases were staged according to the revised 7th TNM classification criteria.[17]

DNA Extraction and Sequencing Techniques

Genomic DNA was isolated using the phenol-chloroform method and dissolved in TE buffer. *KRAS* mutations were determined using polymerase chain reaction (PCR) and Sanger sequencing protocol for all samples. PCR was performed using 5 ng genomic DNA with 38 cycles according to the following conditions: 94°C for 30 seconds, 56°C for 30 seconds, and 68°C for 45 seconds. PCR products were subsequently purified using ExoSAP-IT PCR purification product (USB/Affymetrix), according to the manufacturer's instructions. PCR products were then sequenced using the M13 forward primer at the University of Michigan Sequencing Core. Sequence data were analyzed for the presence of canonical activating *KRAS* mutations at codons 12, 13, and 61. Primers used for the PCR reactions were *KRAS* exon 2 forward: TCTTAAGCGTCGATGGAGGAG, *KRAS* exon 2-M13 reverse: GTAAAACGACGGCCAGTTTGAAACCCAAGGTA CATTTCAG, *KRAS* exon 3 forward: CGTCATCTTTGG AGCAGGAAC, *KRAS* exon 3-M13 antisense: GTAAAACGA CGGCCAGTATGCATGGCATTAGCAAAGAC.

Gene Expression and Copy Number Data from Lung Cancer Cell Lines and Patients with Lung AC

Publicly available Affymetrix U133 Plus 2.0 microarray data from lung cell lines were downloaded from the National Cancer for Biotechnology Information website (GSE36133).[18] Cell line mutational status was obtained from the Sanger Institute Catalogue of Somatic Mutations In Cancer (COSMIC) Web site[9] and is shown in Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A673). Publicly available Affymetrix U133A 2.0 microarray and mutation data from 102 lung AC[19] were downloaded from http://cbio.mskcc.org/public/lung_array_data/, and the raw expression data were normalized using the Robust Multichip Average (RMA) normalization method[20] and then was log2-transformed and median centered. Pathways gene lists were developed as previously reported[21]; KRAS dependency genes and epithelial to mesenchymal (EMT) related genes were obtained from primary literature sources.[12,22] The pathway expression data was computed for each tumor as the arithmetic mean of all genes included in each pathway gene list. Mutation information from 623 genes and *KRAS* gene copy number was available from a previous study in lung AC[1] (http://genome.wustl.edu/pub/supplemental/ tsp_nature_2008/). Clinical outcome was available from 67 patients with *KRAS* mutant tumors included in the TCGA–Lung AC study, and these data were downloaded from the TCGA portal (https://tcga-data.nci.nih.gov). Supervised clustering based on genes associated with KRAS dependency[12,13] and EMT analysis was performed using Cluster v3.0[23] and the heat-map plotted using Treeview software.[24]

Statistical Analysis

Chi-square, Fisher's exact tests, or Student's *t* tests were used to identify statistically significant differences between different clinical variables and the type of *KRAS* mutations. For in vitro studies, the significance of difference between the experimental groups was calculated by the Student's *t* test. The outcome variables for survival analysis were disease-free survival (DFS) and overall survival (OS). DFS was measured from the date of surgery to the time of recurrence, death, or censoring at 5 years. OS was measured from date of surgery to the time of death or censoring at 5 years. Survival curves were constructed using the method of Kaplan–Meier, and survival differences were assessed using the log-rank test. The univariate and multivariate Cox proportional hazards model