Predictive Biomarkers and Personalized Medicine

Characteristics of Lung Cancers Harboring NRAS Mutations

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Abstract

Purpose: We sought to determine the frequency and clinical characteristics of patients with lung cancer harboring NRAS mutations. We used preclinical models to identify targeted therapies likely to be of benefit against NRAS-mutant lung cancer cells.

Experimental Design: We reviewed clinical data from patients whose lung cancers were identified at six institutions or reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) to harbor NRAS mutations. Six NRAS-mutant cell lines were screened for sensitivity against inhibitors of multiple kinases (i.e., EGFR, ALK, MET, IGF-1R, BRAF, PI3K, and MEK).

Results: Among 4,562 patients with lung cancers tested, NRAS mutations were present in 30 (0.7%; 95% confidence interval, 0.45%–0.94%); 28 of these had no other driver mutations. 83% had adenocarcinoma histology with no significant differences in gender. While 95% of patients were former or current smokers, smoking-related G:C>T mutations were significantly less frequent in NRAS-mutated lung tumors than KRAS-mutant non–small cell lung cancer [NSCLC; NRAS: 13% (4/30), KRAS: 66% (1772/2733), P < 0.0000001]. Five of 6 NRAS-mutant cell lines were sensitive to the MEK inhibitors, selumetinib and trametinib, but not to other inhibitors tested.

Conclusion: NRAS mutations define a distinct subset of lung cancers (~1%) with potential sensitivity to MEK inhibitors. Although NRAS mutations are more common in current/former smokers, the types of mutations are not those classically associated with smoking. Clin Cancer Res; 19(9): 2584–91. ©2013 AACR.

Introduction

Recent advances have been made in targeting molecularly defined subsets of non-small cell lung cancers (NSCLC) that depend on specific molecular alterations for cell survival. Prime examples include tumors which harbor mutations in the gene encoding the EGFR receptor (EGFR) or translocations in the gene encoding the anaplastic lymphoma kinase (ALK). Patients with these tumors can derive substantial clinical benefit from EGFR (gefitinib, erlotinib) or ALK (crizotinib) tyrosine kinase inhibitors (TKI), respectively (1–8).

To date, many other potential ‘driver mutations’ occurring in genes encoding cellular signaling proteins have also been identified in NSCLCs. Genomic alterations include mutations in the GTPase KRAS (25%; refs. 9, 10), the receptor tyrosine kinase ERBB2 (2%–3%; refs. 11, 12), the lipid kinase PIK3CA (2%–4%; refs. 10, 13, 14), the serine–threonine kinase BRAF (2%–4%; refs. 9, 10, 15), and the serine–threonine kinase MEK1 (1%; ref. 16), as well as translocations in the tyrosine kinases ROS1 (1%–2%; refs. 17–19) and RET (1%; refs. 19–21). A tumor with a mutation in one of these genes rarely harbors a mutation in another (22). Although targeted therapies have not yet been approved for all of these molecular subsets of lung cancer,
Translational Relevance

Recent advances in lung cancer biology and molecular tumor profiling have allowed for rational prioritization of targeted therapies in patients. NRAS mutations have been reported to occur in lung cancers, but as yet, no comprehensive report has focused on the characteristics of patients whose tumors harbor NRAS mutations. Here, we describe clinical characteristics associated with 30 unique patients with NRAS-mutated lung cancers among 4,562 patients tested (0.7%). While 95% of patients were former or current smokers, smoking-related G:C>T:A transversions were significantly less frequent in NRAS-mutated lung tumors than in KRAS-mutant non–small cell lung cancer (NSCLC). NRAS mutations were, for the most part, mutually exclusive with other known driver mutations, suggesting that NRAS mutations define a distinct molecular subset. In preclinical models, 5 of 6 NRAS-mutant NSCLC cell lines were sensitive to MEK inhibitors. Our data suggest the possibility of personalized treatment in this subset of lung cancers.

Materials and Methods

Patient data

Patients with NSCLCs who underwent molecular profiling were identified for review. Clinical characteristics including age, gender, race (reported by the patient), smoking history, and clinical stage were recorded. All chart review/tissue collection was carried out under institutional review board/privacy board–approved protocols or waivers.

Genotype analysis

gDNA was extracted from patient samples (>70% tumor cells) and cell lines using standard procedures. Tumor specimens were obtained as standard of care for clinical management or with patients’ consent under Institutional Review Board–approved protocols. A mass spectrometry–based (Sequenom; ref. 22) or SNapShot assay (32, 33) was conducted for genotyping as described. Cell lines were genotyped using SNapShot and/or direct sequencing.

Statistical analysis

Fisher’s exact tests (for small sample size) were applied to test associations among NRAS mutations, smoking history, and race. The \( \chi^2 \) tests were applied to compare the frequency of transversions in KRAS- versus NRAS-mutant cancers.

Cell culture

H1299, H2347, H2087, and SW1271 were purchased from American Type Culture Collection. HCC15 were obtained as described before (34). HCC1195 was kindly provided by Dr. Roman Thomas. H1299, H2347, HCC15, and HCC1195 cells were cultured in RPMI-1640 media (Mediatech) supplemented with 10% heat-inactivated FBS (Atlanta Bio) and pen–strept solution (Mediatech; final concentration 100 U/mL penicillin, 100 \( \mu \)g/mL streptomycin). H2087 and SW1271 cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM; Mediatech) with the same supplements. Cells were grown in a humidified incubator with 5% CO2 at 37°C.

Growth inhibition assay

Cells were seeded in 96-well plates at a density of 500 to 5,000 cells per well and exposed to drugs alone or in combination the following day. At 120 hours after drug addition, Cell Titer Blue Reagent (Promega) was added, and fluorescence was measured on a Spectramax spectrophotometer (Molecular Devices), according to the manufacturer’s instructions. All experimental points were set up in hextuplicate replicates and were conducted at least 3 independent times. Erlotinib was synthesized by the MSKCC Organic Synthesis Core. Selumetinib, trametinib, vemurafenib, GDC-0941, crizotinib, linsitinib, and SGX-523 were purchased from Selleck Chemicals.

Table 1. The frequency of NRAS mutations in lung cancers from 6 institutions and the COSMIC database

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</tbody>
</table>

NOTE: Case 18 had a mediastinal lymph node aspiration with cell block showing squamous cell carcinoma, with immunohistochemistry (IHC) positive for CK5/6 and p63 and negative for TTF-1. This was the sample that was genotyped. The patient also had a surgical resection after neoadjuvant chemoradiation that showed areas of residual squamous cell with IHC positive for p63, CK5/6, and CK903 and negative for CEA, TTF-1, synaptophysin, and chromogranin. Case 21 had metastatic disease with a couple of biopsies. Mediastinoscopy was conducted with bronchoscopic biopsies and lymph node dissection—there were read as squamous cell carcinoma but no IHC was ordered. This is the sample that was genotyped. The patient also had a liver biopsy with IHC positive for CK7 and negative for CK20, positive for p63, and negative for TTF-1. Case 22 received docetaxel, gemcitabine, and pemetrexed as salvage chemotherapies. Case 24 received pemetrexed as second-line treatment and gemcitabine as third-line treatment. A pem<br>Abbreviations: asterisk, deceased; †, COSMIC database; ‡, Asian; ‡‡, African American; Adeno, adenocarcinoma; bev, bevacizumab; Carbo, carboplatin; C, Caucasian; chemo, chemotherapy; CRT, chemoradiotherapy; F, female; na, not available; Large, large cell carcinoma; M, male; na, not available; NOS, not otherwise specified histologic type; OS, overall survival; pem, pemetrexed; PFS, progression-free survival; Sq, squamous carcinoma; WT, wild-type; Y, Yes. Smoking history positive but details were unknown.
Antibodies and immunoblotting

The following antibodies were obtained from Cell Signaling Technology: phospho-EGFR, EGFR, MET, phospho-ERK, ERK, phospho-AKT, AKT, actin, HRP-conjugated anti-mouse, and HRP-conjugated anti-rabbit. NRAS antibody was purchased from Santa Cruz. For immunoblotting, cells were harvested, washed in PBS, and lysed in 50 mmol/L Tris-HCl, pH 8.0/150 mmol/L sodium chloride/5 mmol/L magnesium chloride/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/40 mmol/L sodium fluoride/1 mmol/L sodium orthovanadate and complete protease inhibitors (Roche Diagnostics). Lysates were subjected to SDS-PAGE followed by blotting with indicated antibodies and detection by Western Lightening ECL reagent (Perkin Elmer).

siRNA experiment

NRAS and negative control oligos (Dharmacon) were used at a concentration of 10 nmol/L and transfected with Lipofectamine RNAiMAX according to the manufacturer’s protocol (Invitrogen).

Results

Characteristics of patients whose NSCLCs harbor NRAS mutations

At multiple centers, NSCLCs undergo routine multiplexed mutational profiling for recurrent driver mutations. From 6 institutions [Memorial Sloan-Kettering Cancer Center (MSKCC), Massachusetts General Hospital (MGH), University of Colorado Cancer Center (UCCC), John Hopkins University (JHU), University of California at Los Angeles (UCLA), and Vanderbilt-Ingram Cancer Center (VICC)], we identified 18 patients with NSCLCs with NRAS mutations from a total of 3,698 tested (0.5%; MSKCC: 2, MGH: 10, UCCC: 1, JHU: 2, UCLA: 1, VICC: 2). The spectrum of mutations (not including ALK fusions) from patients with NSCLCs at VICC (Supplementary Fig. S1) shows a distribution of driver mutations consistent with the literature (EGFR 17%, ERBB2 1%, KRAS 21%, BRAF 3%, PIK3CA 3%, MEKI 0.5%, and NRAS 0.25%; ref. 10). Another 12 NRAS-mutant NSCLCs were listed in the COSMIC database, among 864 lung cancers reported (including small cell lung cancers; 1.4%); 83% of these were adenocarcinoma histology (Supplementary Table S1). The 30 NRAS-mutant NSCLCs were in the COSMIC database, among 864 lung cancers reported (including small cell lung cancers; 1.4%); 83% of these were adenocarcinoma histology (Supplementary Table S1). There was no overlap between the 2 datasets. Thus, in total, we identified 30 NRAS-mutant cases among 4,562 tested (0.7%; 95% confidence interval 0.45%–0.94%) (Table 1). One of the tumors also had a KRAS G12A, whereas another had MET amplification. Only NRAS mutations were found in the other 28 tumors (Table 2).

Clinical characteristics of patients with NRAS mutations are summarized in Tables 2 and 3 and Supplementary Table S2. Among the 21 patients for whom smoking history was known, 20 were current or former smokers (95%) with a median smoking history of 34 pack-years (Table 3). In a cohort of 3,247 patients with lung cancer (from MSKCC, MGH, UCCC, JHU, and UCLA) for which there was detailed clinical information, there was no significant correlation with NRAS mutations and gender, histology, or clinical stage, but there was a significant association of NRAS mutations with smoking history [current smoker (1.5%), former (0.3%), never smoker (0.1%); Fisher’s exact test: never smoker vs. current smoker (P = 0.0065), former smoker vs. current smoker (P = 0.0043)] and race [Caucasian (0.5%), African American (4.1%), Asian (0%), Hispanic (0%); Caucasian vs. African American (P = 0.0274), African American vs. Asian (P = 0.0603); Supplementary Table S2].

NRAS mutation genotypes

The 30 NRAS mutations corresponded to 9 different amino acid substitutions: Q61H/K/L/R (exon 3) and G12A/C/D/R/S (exon 2). Codon Q61 was the most frequently mutated (80%), and half of mutations were NRAS Q61L (Fig. 1A). Although NRAS and KRAS are related genes, the
distribution of KRAS mutations (n = 2,733) in NSCLCs as reported in COSMIC was completely different; more than 90% of KRAS mutations involved codons 12 or 13 (Fig. 1B). The types of mutations were also distinct. G:C > T:A transversions, thought to be associated with direct exposure to tobacco carcinogens (35–37), were found in 1,772 of 2,733 (66%) KRAS-mutant lung cancers. In contrast, among the 30 NRAS mutations, only 4 (13%) were G:C > T:A transversions (χ² test; P < 0.00000001; Fig. 1C). Even among the 21 patients with NRAS mutations and known smoking histories, only 3 of the 20 former/current smokers had such transversions.

Sensitivity profiles of 6 NRAS-mutant lung cancer cell lines tested against various kinase inhibitors

To identify potential therapies for patients with NRAS-mutant tumors, we tested the sensitivity of 6 NRAS-mutant NSCLC cell lines (Supplementary Table S3) against a variety of kinase inhibitors in vitro cell growth inhibition assays (Fig. 2A). None of the lines were sensitive (with lower than 1 µmol/L IC₅₀) to the EGFR-TKI, erlotinib, the ALK/MET/RON/ROS1 inhibitor, crizotinib, or the insulin-like growth factor-1 receptor (IGF-1R) inhibitor, linsitinib. In contrast, 5 of 6 lines were sensitive to 2 different MEK inhibitors, selumetinib and trametinib. Consistent with these data, the MEK inhibitors inactivated ERK phosphorylation in the NRAS-mutant cells whereas erlotinib did not (Fig. 2B). To verify further the dependency of these cells on NRAS, we carried out siRNA-mediated knockdown experiments. As expected, NRAS knockdown with 2 different siRNA constructs led to growth inhibition in the NRAS-mutant cell lines, H1299 and HCC1195, but not in PC-9 cells, which harbor an EGFR mutation (Fig. 2C).

Like MEK, the phosphoinositide 3-kinase (PI3K) is reported to be a signaling protein activated downstream of RAS. We found that the selective PI3 kinase inhibitor, GDC0941, had little effect in the NRAS mutant lines. We also tested the efficacy of a MET inhibitor, SGX-523, as a recent report showed that melanomas with mutant NRAS displayed activated MET (38). However, none of the NRAS mutant lung lines were sensitive to MET inhibition, either alone or in combination with MEK inhibitors (Supplementary Fig. S2A and data not shown). HCC15 cells were the only NRAS-mutant line insensitive to MEK inhibition alone. We previously reported that these cells displayed high levels of IGF-1R (34). Therefore, we assessed the effect of an IGF-1R inhibitor, linsitinib, together with trametinib. The combination showed a greater effect on cell growth than either drug alone (Supplementary Fig. S2B), suggesting that resistance to MEK inhibition could be overcome by linsitinib in these cells.

Discussion

To our knowledge, this is the largest study of NRAS-mutant lung cancer to date, describing clinical characteristics associated with 30 unique patients among 4,562...
NRAS-Mutated Lung Cancer

Figure 2. Sensitivity profiles of 6 NRAS-mutant lung cancer cell lines tested against various kinase inhibitors. A, IC₅₀ values derived from growth inhibition assays were plotted for each drug and each cell line. HCC15 cells were resistant to MEK inhibitors but sensitive to the combination of a MEK inhibitor plus linalinib (see text and Supplementary Fig. S2 for details). B, MEK inhibitors but not erlotinib led to de-phosphorylation of ERK in NRAS-mutated cells. Erlotinib inhibited phosphorylation of EGFR, AKT, and ERK in PC-9 cells, which harbor an EGFR mutation. C, siRNA-mediated knockdown of NRAS inhibits growth of the NRAS-mutated HCC1195 and H1299 cells but not of PC-9 cells. Mean ± SD of 3 independent experiments conducted in hextuplicate replicates is shown; *, **, P < 0.01 (Student’s t test) for the comparison of siRNAs against NRAS versus scrambled controls in HCC1195 and H1299. Lipo, Lipofectamine control; scr, scrambled siRNA control.

patients tested (0.7%). The actual frequency of NRAS mutations in NSCLCs could be lower than in this study, because more than 80% of tumors were adenocarcinomas in the cohorts examined. Although the frequency of NRAS mutations in NSCLCs is relatively rare, NSCLC is a common disease with 230,000 new cases in the United States. Thus, about 1,500 patients in the United States would develop lung cancer harboring NRAS mutations every year. NRAS mutations were most significantly associated with smoking and potentially African American race, although the numbers for the latter association were too small to make meaningful conclusions. NRAS mutations were also, for the most part, mutually exclusive with other known driver mutations, including EGFR, KRAS, and ALK, etc. Of course, the probability has to be considered that these driver mutations could exist simultaneously in a single tumor at low frequency but, collectively, these data suggest that NRAS mutations in NSCLCs define a distinct molecular subset.

NRAS and KRAS both encode GTPases involved in cell growth, proliferation, and differentiation. They share conserved sequences, but their protein products lead to differential downstream signaling events (25, 26) and have different roles in development (27, 28) and tumorigenesis in mice (29, 30). Recent data have suggested that oncogenic and wild-type RAS isoforms play independent and nonredundant roles within cancer cells. Oncogenic RAS regulates basal effector pathway signaling, whereas wild-type RAS mediates signaling downstream of activated receptor tyrosine kinases (39). Furthermore, oncogenic K-Ras promotes the activation of wild-type H- and N-Ras (40). Why certain lung tumors harbor NRAS versus KRAS mutations is unclear (41). One clue may involve the types of mutations that occur in each gene. Tobacco components, particularly benzo[a]pyrene, are believed to be strong carcinogens for KRAS-mutated lung cancer (35, 36), and G:C>T:A transversions are found in 70% to 90% of KRAS mutations in smoking-related lung cancers (36, 37). This relationship has also
been observed for TP53 mutations in lung cancers from smokers (42). In contrast, more than 50% of NRAS mutations involve A:T>TA transversions (Fig. 1C). Carcinogens known to induce A:T>TA transversions include 7,12-dimethylbenz[a]anthracene (DMBA), which is released into the environment through the combustion of fossil fuels (43). Perhaps the combination of smoking and such a carcinogen are associated with the etiology of NRAS-mutated lung cancer.

The outcomes of patients with NSCLCs with early-stage or metastatic disease remain poor (44). Here, we were able to determine relapse-free survival after resection of early-stage disease for 7 patients (33 months) and overall survival in the metastatic setting after treatment with systemic chemotherapy for 7 patients (8 months). Although the number of patients in each cohort was small, these preliminary data suggest at least for patients with advanced-stage disease that NRAS mutations may be a poor prognostic marker, relative to EGFR and ALK alterations, which have been associated with better prognosis (9). These data will need to be verified in independent datasets.

Recent advances in lung cancer biology and molecular tumor profiling have allowed for rational prioritization of targeted therapies in patients with improved outcomes (5–8). Using preclinical models, we showed that 5 of 6 NRAS-mutant NSCLC cell lines (83%) were sensitive to MEK inhibitors but not to other kinase inhibitors. These data are consistent with previous reports using some but not all related compounds (45). In contrast, KRAS-mutant lines display much greater variability in sensitivity to this class of drugs (46, 47), suggesting that NRAS-mutant lines display a greater dependence upon the MEK pathway for tumor maintenance in lung cancers. To our knowledge, no patient with NRAS-mutant lung cancer has yet been treated with a MEK inhibitor, but our data would suggest that such patients are likely to benefit from this class of agents.

In summary, NRAS mutations occur in about 1% of NSCLCs (mostly those with direct tobacco exposure), are mostly exclusive of other known driver mutations, have a nucleotide transversion profile different from that of KRAS mutations, and may be associated with sensitivity to MEK inhibitors. Such patients should be prospectively identified to prioritize targeted therapies most likely to be of maximal benefit.

References


Disclosure of Potential Conflicts of Interest

C.M. Rudin has received consulting fees from AVEO Pharmaceuticals and Oncothyreon. S.M. Dubinett has received a consulting fee from Tragata Pharmaceuticals. G.J. Riely has received consulting fees from Chugai, Tragra, ARIAD, Daiichi, and Abbott and research funding for other projects from Pfizer, Merck, GlaxoSmithKline, and Bristol-Myers Squibb. M.G. Kris has received consulting fees from Pfizer, Genentech, and Boehringer Ingelheim and research funding for other projects from Pfizer and Boehringer Ingelheim. D. Dias-Santagata received consulting fees from Bio-reference laboratories. P.A. Bunn has received consulting fees from AMGEN, Bristol-Myers Squibb, Symphogen, and Clovis Oncology and research funding for other projects from Enzon, Xcovered, AstraZeneca, and Symphogen. We also acknowledge that W. Pao is a part of a patent regarding EGFR mutant mutation testing that was licensed by Memorial Sloan-Kettering Cancer Center to Molecular MD. No potential conflicts of interest were disclosed by the other authors.

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