Title:

Characteristics of Lung Cancers Harboring NRAS Mutations

Running Head:

NRAS mutated lung cancer

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Conflict of interest statement

CMR has received consulting fees from AVEO Pharmaceuticals, and Oncothyreon. SD has received consulting fee from Tragara Pharmaceuticals. GJR has received consulting fees from Chugai, Tragara, ARIAD, Daiichi and Abbott and research funding for other projects from Pfizer, Merck, GlaxoSmithKline and Bristol-Myers Squibb. DD received consulting fees from Bio-reference laboratories. MGK has received consulting fees from Pfizer, Genentech and Boehringer Ingelheim and research funding for other projects from Pfizer and Boehringer Ingelheim. DD received consulting fees from Bio-reference laboratories. PAB has received consulting fees from AMGEN, Bristol-Myers Squibb, Merck, Boehringer Ingelheim, GlaxoSmithKline, Novartis, Roche, Pfizer, Eli Lilly, Sanofi Aventis, Astelas, AstraZeneca and Bayer. WP has received consulting fees from MolecularMD, AstraZeneca, Bristol-Myers Squibb, Symphony Evolution, Clovis Oncology and research funding for other projects from Enzon, Xcovery, AstraZeneca, and Symphogen. We also acknowledge that WP is a part of a patent regarding EGFR<sup>T790M</sup> mutation testing that was licensed by Memorial Sloan-Kettering Cancer Research.
Center to Molecular MD. Each patent holder received a total of $500.00 (five hundred dollars) and no royalties. There are no other conflicts to report.

**Statement of 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 4562 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 compared to *KRAS*-mutant NSCLCs. *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 suggests the possibility of personalized treatment in this subset of lung cancers.
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.

Patients and Methods

We reviewed clinical data from patients whose lung cancers were identified at 6 institutions or reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) to harbor NRAS mutations. 6 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 4562 patients with lung cancers tested, NRAS mutations were present in 30 (0.7%; 95% confidence interval, 0.45% to 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:A transversions were significantly less frequent in NRAS mutated lung tumors compared to KRAS-mutant NSCLCs (NRAS: 13% (4/30), KRAS: 66% (1772/2733), p<0.00000001). 5 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. While NRAS mutations are more common in current/former smokers, the types of mutations are not those classically associated with smoking.
Introduction

Recent advances have been made in targeting molecularly defined subsets of non-small cell lung-cancers (NSCLCs) that depend on specific molecular alterations for cell survival. Prime examples include tumors which harbor mutations in the gene encoding the epidermal growth factor 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 (TKIs), 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%) (9, 10), the receptor tyrosine kinase ERBB2 (2-3%) (11, 12), the lipid kinase PIK3CA (2-4%) (10, 13, 14), the serine-threonine kinase BRAF (2-4%) (9, 10, 15), and the serine-threonine kinase MEK1 (1%) (16), as well as translocations in the tyrosine kinases ROS1 (1-2%) (17-19) and RET (1%) (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, pre-clinical and emerging clinical data suggest that molecular subtyping will allow for the rational prioritization of treatment options for lung cancer patients (23).

NRAS is a GTPase related to KRAS, originally identified in neuroblastoma cell lines as a third RAS family member following KRAS and HRAS (24). RAS GTPases regulate cell growth, proliferation and differentiation. Although the three family members
share conserved sequences, their protein products generate distinct signal outputs (25, 26) and have distinct roles in development (27, 28) and tumorigenesis in mice (29, 30).

NRAS mutations have been reported to occur in lung cancers, (31) but as yet no comprehensive report has focused on the characteristics of patients whose tumors harbor NRAS mutations. Here, we used retrospective clinical data as well as preclinical models to define the clinical relevance of NRAS mutations in lung cancer.

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 NSCLC patients with NRAS mutations from a total of 3698 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 NSCLC at VICC (Figure S1) shows a distribution of driver mutations consistent with the literature (EGFR 17%, ERBB2 1%, KRAS 21%, BRAF 3%, PIK3CA 3%, MEK1 0.5%, and NRAS 0.25%) (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 (Table S1). There was no overlap between the two datasets. Thus, in total, we
identified 30 NRAS mutant cases among 4562 tested (0.7%; 95% confidence interval 0.45% to 0.94%) (Table 1). One of the tumors also had a KRAS G12A, while another had a 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, 3 and 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 3247 lung cancer patients (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))] (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=2733) in NSCLC 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,(32-34) were found in 1772 of 2733 (66%) KRAS mutant lung cancers. By contrast, among the 30 NRAS mutations, only 4 (13%) were G:C>T:A transversions (Chi-square 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 (Table S3) against a variety of kinase inhibitors in in vitro cell growth inhibition assays (Fig. 2A). None of the lines were sensitive (with lower than 1 micromolar IC50s) to the EGFR TKI, erlotinib, the ALK/MET/RON/ROS1 inhibitor, crizotinib, or the IGF-1R inhibitor, linsitinib. By contrast, 5 of 6 lines were sensitive to two different MEK inhibitors, selumetinib and trametinib. Consistent with these data, the MEK inhibitors inactivated ERK phosphorylation in the NRAS mutated cells while erlotinib did not (Fig. 2B). In order to verify further the dependency of these cells on NRAS, we performed siRNA-mediated knockdown experiments. As expected, NRAS knockdown 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 PI3 kinase 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 a MET inhibitor, SGX-523, since a recent report showed that melanomas with mutant NRAS displayed activated MET (35). However, none of the NRAS mutant lung lines were sensitive to MET inhibition, either alone or in combination with MEK inhibitors (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 (36). 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 (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 4562 patients tested (0.7%). The actual frequency of NRAS mutations in NSCLC could be lower than in this study, because over 80% of tumors were adenocarcinomas in the cohorts examined. Although the frequency of NRAS mutations in NSCLC is relatively rare, NSCLC is a common disease with 230,000 new cases in the US. Thus, about 1,500 patients in the US 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 NSCLC 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 has 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 (37). Furthermore, oncogenic K-Ras promotes the activation of wild-type H- and N-Ras (38). Why certain lung tumors harbor NRAS vs KRAS mutations is unclear (39). 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 (32, 33), and G:C >T:A transversions are found in 70-90% of KRAS mutations in smoking-related lung cancers (33, 34). This relationship has also been observed for TP53 mutations in lung cancers from smokers (40). By contrast, more than 50% of NRAS mutations involve A:T >T:A transversions (Fig. 1C). Carcinogens known to induce A:T >T:A transversions include 7,12-dimethylbenz[a]anthracene (DMBA), which is released into the environment through the
The outcomes of NSCLC patients with early stage or metastatic disease remain poor (42). 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 (43). By contrast, KRAS mutant lines display much greater variability in sensitivity to this class of drugs (44, 45), 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 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 in order to prioritize targeted therapies most likely to be of maximal benefit.
Materials and Methods

Patient data

Patients with NSCLC 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

Genomic DNA 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)(22) or SNapShot assay(46, 47) was performed 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. Chi-squared tests were applied to compare the frequency of tranversions in KRAS vs NRAS mutant cancers.

Cell culture
H1299, H2347, H2087 and SW1271 were purchased from ATCC. HCC15 were obtained as described before (36). 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 fetal bovine serum (Atlanta Bio) and pen-strep solution (Mediatech; final concentration 100U/mL penicillin, 100μg/mL streptomycin). H2087 and SW1271 cells were cultured in 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 5000 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 performed 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.

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 the 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 nM and transfected with Lipofectamine RNAiMAX according to the manufacturer's protocol (Invitrogen).

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References


Figure Legends

Figure 1. Distribution of the types of mutations in NRAS and KRAS mutated lung cancers. A. Q61 was the most frequently mutated codon in 30 NRAS mutated lung cancers (80%). B. The type of mutations in KRAS (COSMIC). 92% of mutations occurred at codon G12. C. Comparison of the types of mutations in KRAS (COSMIC) and NRAS. G:C >T:A transversions were significantly more common in KRAS (1772/2733, 66%) than NRAS (4/30, 13%) mutated lung cancers (Chi-square test; p<0.00000001).

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 linsitinib (see text and Figure 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 three independent experiments performed 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.
Figure 1

A

NRAS n=30

Q61: 20%
G12: 80%

B

KRAS n=2733 (COSMIC)

Q61: 6%
G12: 92%
G13: 2%

C

% types of mutation

KRAS
NRAS

G>T transitions
C>A transitions
G>C transitions
G>T transitions
G>A transitions
Figure 2

A

Target

- Erlotinib
- Crizotinib
- SGX-523
- Linsitibin
- Vemurafenib
- selumetinib
- Trametinib
- GDC-0941

B

Erlotinib
Selumetinib
Trametinib

pEGFR
EGFR
pAKT
AKT
pERK
ERK

C

% Viable cells/
lipofectamine-treated control

- HCC1195
- H1299
- PC-9
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Table 1. The frequency of *NRAS* mutations in lung cancers from 6 institutions and the COSMIC database. No – number, pts – patients, mts – mutations.
**Table 2. Characteristics of individual patients with NRAS mutant tumors.**

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**Early stage**

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9* 51 M A Adeno T1N2M0 IIIA Q61L WT WT WT na Current 53 CRT/surgery 28 38
10* na na na Adeno na na Q61L WT WT WT na Current 105 RT 14
11* na na na Adeno IIII Q61L WT WT WT na Current 30 Surgery 1 1
12* na na na Adeno IIII Q61L WT WT WT na Current 40 RT 20

**Metastatic stage**

21 91 M C Sq T1aN2M1 IV Q61K WT G12A na na Former 30 Supportive care 1
22 48 F AA Adeno T4N2M1 IV Q61K WT WT N Current 15 Carbo/paclitaxel 2 23
23* na na na Adeno T3N0M1 IV Q61L na na na na Y na na na
24 53 M C Adeno na IV Q61L WT WT WT na Former 30 Carbo/paclitaxel/bev 26
25 79 F C Adeno T4N2M1 IV Q61L WT WT WT na Current 163 Supportive care 18
26 51 M C NOS T2aN3M1 IV Q61R WT WT WT na Current 55 Carbo/pem 7
27 69 M na Adeno T2aN3M1 IV Q61R WT WT WT na Current 55 Carbo/pem 4 4
28 50 F C Adeno T4N3M1 IV Q61R WT WT WT na Former 32 Carbo/pem 1 3
29 58 F C Adeno T3N1M1 IV Q61R WT WT WT Y na Current 75 Pem 3
30 30 M AA Adeno T2bN2M1 IV Q61R WT WT WT Y Never 0 Carbo/pem 2 8

* deceased, * COSMIC database. 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 TTF1. This was the sample that was genotyped. The patient also had a surgical resection after neo-adjuvant 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 performed with bronchoscopic biopsies and lymph node dissection - these 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 TTF1. Case number 22: This patient received docetaxel, gemcitabine and pemetrexed as salvage chemotherapies. Case number 24: This patient received pemetrexed as second line treatment and gemcitabine as third line treatment.
### Table 3. Clinical Characteristics of patients with NRAS mutant lung cancers.
No. - number of patients, NOS - not otherwise specified histologic, Yes- smoking history positive but details were unknown.

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Clinical Cancer Research

Characteristics of Lung Cancers Harboring NRAS Mutations

Kadoaki Ohashi, Lecia V. Sequis, Maria E. Arcila, et al.

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