Genetic Predictors of MEK Dependence in Non–Small Cell Lung Cancer

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Abstract

Hyperactivated extracellular signal-regulated kinase (ERK) signaling is common in human cancer and is often the result of activating mutations in BRAF, RAS, and upstream receptor tyrosine kinases. To characterize the mitogen-activated protein kinase/ERK kinase (MEK)/ERK dependence of lung cancers harboring BRAF kinase domain mutations, we screened a large panel of human lung cancer cell lines (n = 87) and tumors (n = 916) for BRAF mutations. We found that non–small cell lung cancers (NSCLC) cells with both V600E and non-V600E BRAF mutations were selectively sensitive to MEK inhibition compared with those harboring mutations in epidermal growth factor receptor (EGFR), KRAS, or ALK and ROS kinase fusions. Supporting its classification as a “driver” mutation in the cells in which it is expressed, MEK inhibition in V600E BRAF NSCLC cells led to substantial induction of apoptosis, comparable with that seen with EGFR kinase inhibition in EGFR mutant NSCLC models. Despite high basal ERK phosphorylation, EGFR mutant cells were uniformly resistant to MEK inhibition. Conversely, BRAF mutant cell lines were resistant to EGFR inhibition. These data, together with the nonoverlapping pattern of EGFR and BRAF mutations in human lung cancer, suggest that these lesions define distinct clinical entities whose treatment should be guided by prospective real-time genotyping. To facilitate such an effort, we developed a mass spectrometry-based genotyping method for the detection of hotspot mutations in BRAF, KRAS, and EGFR. Using this assay, we confirmed that BRAF mutations can be identified in a minority of NSCLC tumors and that patients whose tumors harbor BRAF mutations have a distinct clinical profile compared with those whose tumors harbor kinase domain mutations in EGFR. [Cancer Res 2008;68(22):9375–83]

Introduction

Lung cancer is the leading cause of cancer-specific mortality worldwide, with over 160,000 deaths per year reported in the United States (1). Treatment options are limited for patients with advanced metastatic disease, as traditional cytotoxic chemotherapy confers only a limited survival benefit. Novel treatment strategies are therefore needed for these patients. Molecular profiling studies have shown that activating mutations in the epidermal growth factor receptor (EGFR), HER2, BRAF, and KRAS genes are generally nonoverlapping and identifiable in ~40% of non–small cell lung cancers (NSCLC). Together with the recent discovery of ALK and ROS kinase fusions, potentially targetable “driver mutations” can now be identified in approximately half of all NSCLC patients (2, 3).

In clinical studies, EGFR kinase domain mutations have been shown to strongly predict for response to EGFR tyrosine kinase inhibitors (4–6). Although the response of patients to these agents is often dramatic, resistance invariably develops within the first year. Mechanisms of acquired resistance include selection for the T790M mutation, which increases affinity of the receptor for ATP (7, 8) and amplification of the MET receptor tyrosine kinase (9, 10). KRAS mutation has been shown to confer primary or de novo resistance to EGFR-targeted therapies in both lung and colon cancer patients (11, 12). As extracellular signal-regulated kinase (ERK) activity is high in both EGFR and KRAS mutant tumors, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibition has been proposed as a possible therapeutic strategy for patients whose tumors show resistance to EGFR tyrosine kinase inhibitors.

Although BRAF is the kinase most frequently mutated in human tumors, the reported frequency of BRAF mutations in NSCLC is low (2–3%; refs. 13–15). In melanoma, colon, and thyroid cancers, the tumor types with the highest frequency of BRAF mutation, a single-nucleotide substitution resulting in a glutamic acid for valine substitution within the kinase domain at codon 600 (V600E), accounts for the majority of cases. This mutation results in elevated basal kinase activity, activation of the ERK pathway, and cellular transformation. In melanoma, colon and breast cancer cells harboring V600E BRAF mutation, cyclin D1 expression, and cell cycle progression are MEK-dependent (16). Furthermore, supporting its classification as an oncogene, lung-specific expression of V600E BRAF in mice results to the development of lung cancers with bronchioalveolar carcinoma features similar to those observed in patients (17).

In contrast to the pattern of BRAF mutations observed in most other tumor types, a substantial percentage of the BRAF mutations...
reported to date in lung cancer cell lines and tumors (~90%) are non-V600E (13–15). Many of these non-V600E mutations show only intermediate and low kinase activity, and therefore, their classification as driver mutations remains in doubt (18). The studies described herein were therefore designed to investigate the MEK dependence of lung cancer cell lines harboring V600E and non-V600E BRAF mutations. We show that BRAF mutation in cell lines predicts not only for sensitivity to MEK inhibition but also resistance to EGFR inhibition. Thus, the data suggest that routine testing for BRAF mutation in NSCLC may identify a subset of patients with de novo resistance to EGFR kinase inhibition and enhanced sensitivity to MEK inhibition.

Materials and Methods

**Materials**. PD0325901 was obtained from Pfizer Global Research and Development. Gefitinib was obtained from AstraZeneca. Drugs for *in vitro* studies were dissolved in DMSO to yield 1 and 10 mmol/L stock solutions, respectively, and stored at −20°C.

**Cell culture**. The human cancer cell lines HCC364, H1755, H1666, and H1395 were provided by Adi Gazdar, University of Texas Southwestern. All other obtaines were from American Type Culture Collection. All cell lines were maintained in RPMI with 10 mmol/L HEPES supplemented with 2 mmol/L glutamine, 50 units/ml each of penicillin and streptomycin, and 10% heat-inactivated fetal bovine serum (Gemini Bioproducts) and incubated at 37°C in 5% CO2. For proliferation assays, cells were plated in 96-well plates at a density of 2,000 to 5,000 per well. After 24 h, cells were treated with the inhibitors (PD0325901 or ZD1839) at a range of concentrations prepared by serial dilution. The cells were exposed to Alamar Blue (AccuMed International) 3 to 5 d after drug treatment, and plates were read using a fluorescence spectrophotometer. The dose required to inhibit growth by 50% (IC50) was calculated using the SoftMaxPro ver.5 software. For soft agar studies, 1 to 2 × 104 cells growing in log phase were mixed with agar (0.33%), treated with either DMSO or PD0325901 (1–50 mmol/L), and plated over a bottom layer of 0.5% agar in 60-mm dishes. Cells were incubated at 37°C for 2.5 wk, with 200 μL of medium pipetted over the surface after 1 wk. Colonies were then stained with crystal violet (Sigma-Aldrich) for 1 h, and bright field images were captured using a MZFL3 Stereomicroscope and Velocity 4.3 software (Improvision, Inc.). Images captured within a single experiment were taken at the same magnification and exposure time.

**Western blot analysis**. Treated cells were harvested, washed with PBS, and lysed in NP40 lysis buffer [50 mmol/L Tris (pH 7.4), 1% NP40, 150 mmol/L NaCl, 40 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor] for 30 min on ice. Lysates were centrifuged at 13,200 rpm for 10 min to pellet debris, and the protein concentration of the supernatant was determined by bicinchoninic acid protein assay (Pierce). Equal amounts of total protein were resolved by SDS-PAGE and transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked for 1 h in 5% nonfat milk in TBS-T [0.1% Tween 20, 10 mmol/L Tris (pH 7.4), and 150 mmol/L NaCl] at room temperature and subsequently probed overnight at 4°C with antibody raised against the protein of interest. Anti-p12/44 MAPK, phosphorylated p12/44 MAPK, Akt, phosphorylated Akt (pAkt; Ser473), Bb, cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, and glyceraldehyde-3-phosphate dehydrogenase antibodies were obtained from Cell Signaling Technology. Anti–cyclin D1, cyclin D2, and cyclin D3, and p27 antibodies were obtained from Santa Cruz Biotechnology. Anti–caspase-3, and glyceraldehyde-3-phosphate dehydrogenase antibodies were obtained from Cell Signaling Technology. After incubation with horseradish peroxidase–conjugated secondary antibodies, proteins were detected using chemiluminescence (Amersham).

**Apoptosis**. To measure apoptosis, cells were seeded in 10-cm dishes at a density of 1 × 106 per dish and, on the following day, were treated with the indicated concentration of drug or vehicle (DMSO) for the indicated times. Both adherent and floating cells were harvested and stained with ethidium bromide using the method of Nusse (19). Detection and quantitation of apoptotic cells (sub-G1) was done by flow cytometric analysis, with the results compared with induction of activated caspase-3 and PARP cleavage, as measured by immunoblot.

**BRAF mutation analysis**. We previously analyzed over 800 NSCLC tumors from patients undergoing surgical resection in four different countries (Japan, Taiwan, the United States, and Australia) to determine the EGFR and KRAS mutational status (20–22). Clinical information, including age, gender, smoking status, histology, and clinical stage, was available. Of these, DNAs from 689 NSCLC tumors (Japan (n = 375), Taiwan (n = 88), the United States (n = 125), and Australia (n = 101)) were available to perform further analyses for BRAF exons 11 and 15 mutations. Institutional Review Board permission and informed consent were obtained at each collection site. Genomic DNA was obtained from primary tumors by standard phenol-chloroform (1:1) extraction followed by ethanol precipitation or by using the DNeasy Tissue kit (Qiagen). For BRAF mutational analysis, the intron-based PCR primer sequences for BRAF (exons 11 and 15) were (forward and reverse, respectively) 5′-TCC CTC TCA GGC ATA AGG TAA-3′ and 5′-CGA ACA GTG AAT ATT TTC TTT GAT-5′ for exon 11 and 5′-TCA TAA TGC TTG CTC TCA TAG GA-3′ and 5′-GGG CAA AAA TTT AAT CAG TGG A-3′ for exon 15, with PCR product lengths of 313 and 224 bp, respectively. All PCR reactions were carried out in 25 μL volume containing 100 ng of genomic DNA using HotStarTaq DNA polymerase (Qiagen). PCR conditions were as follows: 95°C (12 min) for one cycle; 94°C (30 s), 56°C (30 s), and 72°C (30 s) for 36 cycles, and a final extension step of 72°C (7 min). All PCR products were incubated using exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences) and sequenced directly using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp.). All the sequence variants were confirmed by independent PCR amplifications and sequenced in both directions. For all the mutant cases, corresponding nonmalignant tissue DNA was available to confirm that mutations were somatic mutations.

**Mass spectrometry (Sequenom)–based mutation screens**. A separate set of 227 NSCLC tumors from patients undergoing surgical resection at Memorial Sloan-Kettering Cancer Center (MSKCC)11 were screened for five common point mutations in exons 11 and 15 of BRAF (G466V, G469A, L597V, L597F, and V600E) using a mass spectrometry (Sequenom)–based genotyping assay. The Sequenom MassARRAY system is based on matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). In these assays, the mutant and germline alleles for a given point mutation produce single-allele base extension reaction products of different masses, which are then resolved by MALDI-TOF MS. Both the amplification and extension primers were designed using Sequenom Assay Design v3.1 software. The amplification primers were designed with a 10mer tag sequence to increase their mass so that they fall outside the range of detection of the MALDI-TOF MS. Results were generated using the SpectroTyPER v3.4 software (Sequenom). All the positive cases were confirmed by visually reviewing the spectra. For the PCR amplification, 15 ng of genomic DNA (in 1 μL) were amplified in a 5-μL reaction mixture containing 0.1 μL (0.5 units) HotStarTaq enzyme (Qiagen), 0.625 μL of 10× HotStar buffer, 0.325 μL of 25 mmol/L (total) MgCl2, 0.25 μL of 10 mmol/L (each) deoxynucleotide triphosphate, 1 μL of 100 mmol/L of each forward and reverse primers, and 1.7 μL of water. The PCR step was initiated with a 95°C soak for 15 min, followed by 45 cycles, consisting of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, and a final extension of 3 min at 72°C. After PCR, the remaining unincorporated deoxynucleotide triphosphates were dephosphorylated by adding 2 μL of the SAP cocktail, containing 1.33 μL of water, 0.17 μL of reaction buffer (Sequenom), and 0.5 μL of SAP (Sequenom). The 384-well plate was then sealed and placed in a thermal cycler with the following conditions: 37°C for 40 min, 85°C for 5 min, and then held at 4°C

indefinitely. After the SAP treatment, a 2-μL cocktail [consisting of 0.755 μL water, 0.2 μL iPlex 10x buffer (Sequenom), 0.2 μL iPlex terminator mix (Sequenom), 0.804 μL of 7 or 14 μmol/L (depending on the low versus high mass primers) extension primer mixture, and 0.041 μL iPlex enzyme (Sequenom)] was added. After the iPlex cocktail addition, the plate was again sealed and placed in a thermal cycler with the following program: 94°C for 2 min followed by 40 cycles of 94°C for 5 s, [5 cycles (52°C or 5 s, 80°C for 5 s) and 72°C for 5 s]. The reaction mixture was then desalted by adding 16 μL of water and 6 mg cationic resin mixture, SpectroCLEAN (Sequenom). The plate was then sealed and placed in a rotating shaker for 20 min to desalt the iPlex solution. Completed genotyping reactions were spotted in nanoliter volumes onto a matrix-arrayed silicon chip with 384 elements (Sequenom SpectroCHIP) using the MassARRAY Nanodispenser. SpectroCHIPs were analyzed using the Bruker Autoflex MALDI-TOF MS, and the spectra were processed using the SpectroTYPER v3.4 software (Sequenom).

Clinical data collection. MSKCC tumor specimens were obtained from an institutional tumor bank of patients who had undergone NSCLC resections between 2002 and 2007. Retrospective review of clinical data, including smoking history, was obtained by the review of a patient-completed smoking questionnaire and the medical record. Never-smokers had smoked <100 cigarettes. Former smokers had previously smoked cigarettes but quit smoking >1 y before diagnosis of lung cancer. Pack-years of smoking was defined as [(average number of cigarettes per day / 20) × years smoking]. Smoking history and clinical information were collected independently, using similar smoking questionnaires, at each site that provided clinical DNA samples.

Results

Genetic predictors of MEK dependence in NSCLC cell lines. Activation of the classic MAPK cascade (RAS-RAF-MEK-ERK) is a common event in lung cancer and often results from mutations in BRAF, KRAS, and upstream receptor tyrosine kinases, such as EGFR. We hypothesized that the dependence of lung cancer cells on MEK-ERK pathway activity would vary as a function of the genetic alterations responsible for pathway activation. To compare

Figure 1. BRAF mutant cell lines are selectively sensitive to MEK inhibition. A, a panel of cell lines with mutant BRAF (blue), mutant KRAS (red), concomitant BRAF and NRAS mutations (blue/red), or wild-type for both (black) were grown in the presence of the MEK inhibitor PD0325901 at a range of concentrations, and day 5 IC₅₀ values were determined using the Alamar Blue assay. The BRAF and RAS wild-type NSCLC cell lines H2228 (EML4-ALK fusion), HCC78 (SCLC4A2-ROS fusion), H1703 (PDGFR-overexpression), and six cell lines with EGFR mutation (H820, PC9, H3255, 11-18, H1975, and H1650) were all resistant to PD0325901. B, immunoblot of pERK1/2 (Thr202/Tyr204) and total ERK1/ERK2 after administration of the MEK inhibitor in selected cell lines from A, showing that resistance to MEK inhibition was not the result of the inability of the drug to inhibit ERK activity.
the MEK/ERK dependence of lung cancer cell lines with BRAF, RAS, and RTK mutations, we screened a large panel \( (n = 87) \) of human lung cancer cell lines for exons 11 and 15 BRAF mutations. In total, we identified five NSCLC cell lines with BRAF mutation (see Supplementary Fig. S1). Only one of these, HCC364, harbored the V600E kinase domain mutation in exon 15, which is, by far, the most frequently observed BRAF mutation in melanoma, thyroid, and colon cancers (13, 23, 24). Two additional cell lines, H1755 and H1395, harbored high-activity \( G^{600A} \)BRAF mutations, and one cell line, H1666, harbored a \( G^{466E} \)BRAF low-activity mutation. A fifth cell line, H2087, harbored both \( Q^{61K} \)NRAS and \( L^{597V} \)BRAF intermediate-activity mutations (18). All of these cell lines were derived from patients with a histologic diagnosis of adenocarcinoma, and at least three of the five were former smokers (no data on smoking history were available for the patients from which the cell lines HCC364 and H1666 were derived).

To determine the dependence of these cell lines on MEK/ERK for proliferation, we used PD0325901, an allosteric inhibitor of MEK that inhibits MEK1 and MEK2 kinase activity by locking the enzyme in a closed and catalytically inactive conformation (25–27). Consistent with our prior results in melanoma and colon cancer cell lines, we found that HCC364 cells (\( V^{600E} \)BRAF) were exquisitely MEK-dependent, with an IC\( _{50} \) of 3.2 nmol/L. The other four BRAF mutant cell lines were also MEK-dependent for proliferation, with IC\( _{50} \)s for PD0325901 ranging from 2.7 to 24 nmol/L. In contrast, all six NSCLC cell lines with EGFR kinase domain mutations were resistant to MEK inhibition (IC\( _{50} \), >300 nmol/L; Fig. 1A). Similarly, H1703 cells, a cell line with PDGFR\( \alpha \) overexpression, HCC78 cells, which express the SLC34A2-ROS fusion protein, and H2228 cells, which express the EML4-ALK fusion protein (2), were also resistant to MEK inhibition. Resistance was not a result of the failure of PD0325901 to inhibit MEK1/MEK2; in all cell lines examined, the drug was effective in inhibiting the pathway as measured by decreased expression of phosphorylated ERK (pERK) after drug exposure (Fig. 1B and data not shown). The sensitivity of the RAS mutant class was more variable, with IC\( _{50} \)s ranging from 2.7 to >300 nmol/L. Notably, the RAS mutant tumor demonstrating the greatest MEK dependence was the H2087 cell line, which also coexpressed the L597V BRAF mutation.

The difference in MEK dependence in the EGFR and BRAF mutant cell lines could not be explained by differences in the basal expression of pERK, as shown in Fig. 2. However, there was an inverse correlation between the level of pAKT (Ser\( ^{473} \)) expression and MEK dependence (\( P = 0.0012 \)). In resistant cells, high pAKT was associated with EGFR or KRAS mutations, whereas the majority of BRAF mutant cell lines had low or undetectable pAKT (Fig. 2A and B).

To further characterize the consequences of MEK inhibition in NSCLC cell lines with BRAF, RAS, and EGFR mutations, we performed immunoblot and fluorescence-activated cell sorting (FACS) analysis as a function of both PD0325901 dose and time posttreatment. As shown in Fig. 3A for the HCC364 (\( V^{600E} \)BRAF) and H1666 (\( G^{466E} \)BRAF) cell lines, MEK inhibition decreased the expression of pERK, which led to a decline in D-cyclin expression and an increase in p27 expression. This was followed by RB hypophosphorylation and accumulation of cells in G1. These changes in cell cycle–related proteins were not seen in the EGFR mutant, MEK inhibitor–resistant cell lines (see Supplementary Fig. S2A, PC9). Notably, the full effects of drug on D-cyclin and p27 expression and RB phosphorylation were not observed until 24 to 48 hours posttreatment. The kinetics of the effect of MEK inhibition on RB likely explain the delayed effect of PD0325901 on cell growth, which was most prominent in cells, such as H1666, that show a slow doubling time. As shown in Fig. 3B, MEK inhibition had no inhibitory effect on H1666 proliferation at 48 hours; rather, there was a modest acceleration in H1666 cell growth at 48 hours, which was followed by the accumulation of cells in G1 and the induction of cell death at later time points (4 days and beyond; Fig. 3B and C and data not shown). HCC364 cells are nonadherent in two-dimensional culture, and therefore, the effect of MEK inhibition in soft agar was also assessed. PD0325901 treatment resulted in a dose-dependent inhibition of colony formation at the IC\( _{50} \) of the drug for MEK1 and MEK2. In contrast, MEK inhibition had no effect on the growth of PC9 (\( d^{47E746-A^{750}} \)EGFR; ref. 28) colonies (Fig. 3C and D).

**Figure 2.** The MEK dependence of NSCLC cell lines was inversely correlated with the level of pAKT (Ser\( ^{473} \)) expression. A, whole cell lysates from untreated cell lines harvested at 70% to 80% confluence were analyzed by immunoblot for pAKT (Ser\( ^{473} \)), total AKT, pERK (Thr\( ^{202} \)/Tyr\( ^{204} \)), and total ERK protein expression. Band intensity was quantified using Science Lab 2003 Image Gauge software (Fujifilm). B, cells were grouped as either sensitive (IC\( _{50} \), <50 nmol/L) or resistant (IC\( _{50} \), ≥50 nmol/L) to the MEK inhibitor PD0325901. Relative pAKT and pERK are shown for the two groups. pAKT (Ser\( ^{473} \)) levels were variable in the resistant cell lines but low in the sensitive cell lines. Only the difference in pAKT between the groups was statistically significant (\( P = 0.0012 \), Wilcoxon test). There was no correlation between pERK levels and MEK dependence.
In the \( V_{600}^{E600} \) BRAF HCC364 model, MEK inhibition resulted in profound apoptosis as measured by the induction of PARP cleavage (Figs. 3A and 4B) and the accumulation of cells in the sub-G1 fraction, as measured by FACS (Fig. 4A). Notably, in assays of apoptosis, PD0325901 had variable effects in H1395 and H1755, two cell lines that express the same high-activity G469A BRAF mutation, suggesting that additional genetic heterogeneity within the cell lines conditions their response to MEK inhibition. Apoptosis was not observed after MEK inhibition in EGFR mutant cell lines and was either modestly or not at all induced after MEK inhibition in the RAS mutant cell lines (Fig. 4A and B).

**BRAF mutant cell lines are resistant to EGFR inhibition.** We have shown that inhibition of MEK/ERK alone is insufficient to block proliferation or induce cell death in NSCLC cell lines expressing kinase domain mutations in EGFR. As the expression of activating mutations in KRAS has been shown to predict for de novo resistance to EGFR inhibitors in patients with NSCLC (11, 29–31), we examined whether cell lines expressing high-activity (\( V_{600}^{E600} \) and G469A) BRAF mutations were also resistant to EGFR inhibition. As shown in Fig. 5, PC9 \((E758K_{EGFR})\) cells were sensitive to gefitinib with an IC\(_{50}\) of 43 nmol/L, whereas both HCC364 \((V_{600}^{E600})\) and H2030 \((G12C_{KRAS})\) cells were resistant to gefitinib at doses of up to 5 \(\mu\)mol/L. Consistent with the work of others, gefitinib was variably effective in inducing apoptosis in EGFR mutant NSCLC cell lines (32, 33). Treatment of H3255 \((L858R_{EGFR})\) cells with gefitinib resulted in profound induction of apoptosis as measured by FACS, whereas H1975 cells were resistant to gefitinib due to the presence of the T790M mutation (7). Although both PC9 and H3253 cells have similar IC\(_{50}\) values for gefitinib, the response of PC9 cells was primarily cytostatic (G1 increase from 72% to 95%), with gefitinib inducing only a modest induction of apoptosis (sub-G1 increase from 1.6% to 7.8%). Gefitinib had no effect on the survival of the BRAF mutant and RAS mutant cell lines tested (Fig. 5B). These data suggest that BRAF mutations may predict for de novo resistance to EGFR kinase inhibitors in patients with NSCLC.

**Frequency of BRAF mutations in NSCLC.** As our preclinical models suggest that BRAF mutations may be predictive of sensitivity to MEK inhibitors and resistance to EGFR inhibitors, we studied the frequency of BRAF mutations in two large tumor repositories of patients diagnosed with NSCLC. In an initial series of 689 patients from five centers (University of Texas, M. D. Anderson Cancer Center, Okayama University, Chiba University, The Prince Charles Hospital, Chung Shan Medical University),
sequencing of exons 11 and 15 of \textit{BRAF} identified 11 mutant cases or 1.6%. Of note, in none of these cases were patients previously exposed to the tyrosine kinase inhibitors gefitinib or erlotinib. Six of 11 patients were smokers, and 9 of 11 had adenocarcinoma histology (see Supplementary Table S1). Despite the availability of agents that target both RAF and MEK, this low frequency of \textit{BRAF} mutation in NSCLC has discouraged widespread efforts to prospectively test NSCLC patients for mutations in this gene. With the goal of facilitating the identification of low-frequency driver mutations in genes such as \textit{BRAF}, we developed a MALDI-TOF MS assay to screen for mutations in \textit{EGFR}, \textit{KRAS}, \textit{BRAF}, \textit{HER2}, and \textit{PIK3CA}. Using this assay, we identified an additional six patients (2.7%) harboring exon 11 or exon 15 \textit{BRAF} mutations (Supplementary Table S1). Combining these two data sets, we observed a total incidence of 17 cases out of 916 patients or 1.9%. The clinical characteristics of this combined data set are shown in Table 1 and Supplementary Table S1 and include a predominance of adenocarcinoma histology and female gender. Notably, in contrast to the clinical variables associated with \textit{EGFR} mutations in lung cancer, the majority of patients harboring \textit{BRAF} mutations were former or current smokers.

**Discussion**

Recent advances in our understanding of the genetic alterations responsible for the development of cancer have been paralleled by an increasing capacity for the development of drugs with selectivity for specific protein targets. Such advances have led to the hope that more effective cancer therapies that work by selectively inhibiting the specific molecular alterations responsible for cancer initiation and progression can be developed. Thus far, this paradigm has been most effective with inhibitors of proteins that are mutation-ally activated in tumors. Examples include imatinib in patients with CML (ABL), GIST (KIT), and dermatofibrosarcoma protuberans (PDGFR; refs. 34, 35), and gefitinib/erlotinib in NSCLC (EGFR; refs. 4–6).

In CML, ABL translocations, most commonly \textit{BCR-ABL}, are the hallmark of this disease; therefore, patient selection beyond traditional pathologic criteria is not needed to identify a target population predicted to be sensitive to ABL kinase inhibitors. In contrast, it is now apparent that solid tumors, traditionally classified by tissue of origin and histologic subtype, can have a diversity of mutations that confer similar selective advantage. For example, in NSCLCs, \textit{EGFR}, \textit{KRAS}, and \textit{BRAF} mutations are nonoverlapping\textsuperscript{11} and all activate the MAPK cascade (36–38). In NSCLC, the presence or absence of mutations in the \textit{EGFR} and \textit{KRAS} genes has been shown to correlate strongly with response to \textit{EGFR} inhibitors (4–6, 11). Whereas an activating mutation in \textit{EGFR} is a positive predictor of response to gefitinib and erlotinib, \textit{KRAS} mutation confers negative predictive value for the same class of agents (11). Recent data have also confirmed that \textit{KRAS} mutation is sufficient to confer resistance to \textit{EGFR}-targeting antibodies, such as cetuximab and panitumumab (12, 39). These observations suggest that the response of patients to a particular targeted agent will depend strongly upon the complement of mutations within an individual patient’s tumor and that such predictors (both positive and negative) can be identified. The experience with gefitinib and erlotinib also suggests that it would be valuable to know the tumor genotype of the patients prospectively and use this information to select the appropriate patients for clinical trial. This is particularly important if the frequency of mutation in the population tested is low.

\textit{BRAF} missense mutations, the vast majority of which are V600E, are the most common kinase domain mutations in human tumors.

![Figure 4. Induction of apoptosis in a panel of cell lines with \textit{EGFR}, \textit{RAS}, and \textit{BRAF} mutations in response to MEK inhibition. A, percentage of cells in the sub-G\textsubscript{1} population, as determined by FACS analysis in the presence or absence of MEK inhibitor (50 nmol/L PD0325901 for 72 h). Bars, SD of replicate experiments. MEK inhibition induced a significant increase in sub-G\textsubscript{1} fraction in four of five BRAF mutant cell lines. The variable response of H1996 and H1755 cells, which both express the G469ABRAF mutation, suggests that additional genetic heterogeneity within these lines conditions MEK dependence in NSCLC. B, immunoblot of cleaved PARP and caspase-3 in representative \textit{EGFR}, \textit{KRAS}, and \textit{BRAF} mutant cell lines in the absence (–) or presence (+) of 50 nmol/L PD0325901 for 24 h.](image-url)
These mutations, found in ~8% of all tumors, are nonoverlapping in distribution with RAS mutations. Supporting its classification as an oncogene, V600E BRAF stimulates ERK signaling, induces proliferation, and is capable of promoting transformation and inducing tumors in transgenic mice (41, 42). To study the biology of BRAF mutation in NSCLC, we screened a large panel of cell lines for exons 11 and 15 BRAF mutations. We identified five cell lines with known hotspot mutations within the BRAF kinase domain, one of which, HCC364, harbors the V600E mutation. We observed that NSCLC with BRAF mutations were selectively sensitive to MEK inhibition compared with cell lines expressing EGFR mutations, the SLC34A2-ROS fusion, the EML4-ALK fusion, and those with amplification of PDGFRα or MET. Notably, we did not assess the MEK dependence of cell lines in which no identifiable driver mutation has been identified. As such cell lines may contain occult mutations in genes, such as NF1 or MEK1 (MAP2K1), that activate the MAPK pathway, we would expect that a subset of these BRAF wild-type cell lines may also be dependent upon MEK for proliferation or survival. Validating this possibility, MEK1 mutations were recently identified with low frequency in NSCLC (43).

We observed that MEK inhibition in the V600E BRAF-expressing HCC364 NSCLC cells induced levels of apoptosis comparable to those seen with EGFR inhibition in the EGFR mutant H3255 model. However, as seen with EGFR inhibitors in EGFR mutant models, the apoptotic response of BRAF and KRAS mutant cell lines to MEK inhibition was variable. These data suggest that additional genetic heterogeneity within the BRAF and KRAS mutant classes likely conditions the response of these cells to ERK pathway inhibition. Future studies are underway to determine which genetic and epigenetic alterations commonly coexist with EGFR, RAS, and BRAF mutations in lung cancer tumors and their effect on MEK/ERK pathway dependence.

Using a cohort of over 900 lung cancer tumors, we identified a total of 17 patients whose lung tumors harbored BRAF exon 11 or exon 15 mutations, representing 1.9% of the total cases analyzed, an incidence consistent with previous reports (13, 14, 21). Notably, in contrast to the clinical profile of patients with EGFR kinase

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Sensitivity of EGFR mutant and BRAF mutant cell lines to the selective EGFR inhibitor gefitinib (Iressa). **A,** the BRAF mutant cell line HCC364 (blue) was sensitive to PD0325901, whereas H2030 (KRAS mutant, red) and PC9 (EGFR mutant, black) cells were resistant. In contrast, only the EGFR mutant cell lines (PC9 is shown) showed sensitivity to gefitinib, whereas BRAF mutant cell lines were resistant. **B,** percentage of cells in the sub-G1 population, as determined by FACS analysis in the presence or absence of gefitinib (2 μmol/L for 72 h). Bars, SE of replicate experiments. Gefitinib treatment of H3255 (L858R-EGFR) and PC9 (E746-A750EGFR) NSCLC cell lines resulted in an increase in sub-G1 fraction. H1975 cells, which express both the L858R and T790M EGFR kinase domain mutations, were resistant to gefitinib, as previously reported (7). Both BRAF mutant and RAS mutant lines were resistant to EGFR inhibition.
domain mutations, the majority of patients with BRAF mutations were current or former smokers. Our observed incidence of V600E mutations (1.2%) was also higher than would have been predicted based upon prior reports. One explanation for the higher observed frequency of V600E mutations may have been the use of mass spectrometry genotyping in the MSKCC series, a technology with greater sensitivity than Sanger sequencing.

Overall, our data suggest that BRAF is a driver mutation in patients in which it is mutated and that targeting MEK may be a useful therapeutic strategy in this subset of patients. Such a targeted strategy, however, has not been pursued to date in the clinic, and no ongoing or completed phase 1 or phase 2 trial of a MEK-selective inhibitor has yet enriched for NSCLC patients with BRAF mutations. The primary hurdle has been that testing for BRAF mutation is not prospectively performed in patients with NSCLC. Routine testing of NSCLC patients for KRAS mutation, on the other hand, is becoming more widespread, as the presence of a KRAS mutation predicts for de novo resistance to the EGFR inhibitors gefitinib and erlotinib. We now show that BRAF mutant cell lines are also resistant to EGFR inhibition. Based upon these data, we propose clinical studies to determine whether a BRAF mutation has similar value in predicting for de novo resistance to EGFR inhibition. If confirmed, we believe that routine clinical testing of all NSCLC for BRAF mutation would be justified. Such an effort would also have the secondary benefit of accelerating the development of BRAF-selective and MEK-selective inhibitors by aiding in the identification of the minority of lung cancer patients likely to respond to such agents. As shown by our use of the MALDI-TOF MS assay to screen simultaneously for EGFR, KRAS, and BRAF mutations, we believe that such efforts are now technically feasible. Based upon these results, we have initiated routine prospective genotyping of NSCLC patients for BRAF mutation and have proposed clinical studies of MEK inhibitors with enrollment restricted exclusively to those with an activating BRAF mutation.

Table 1. Clinical characteristics of lung cancer patients with BRAF mutations

<table>
<thead>
<tr>
<th>BRAF mutation</th>
<th>Total</th>
<th>BRAF mutant (n = 17), n (%)</th>
<th>Total (n = 916), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 11</td>
<td>4 (24)</td>
<td>0.4</td>
<td>11</td>
</tr>
<tr>
<td>Exon 15 (non-V600)</td>
<td>2 (12)</td>
<td>0.2</td>
<td>8</td>
</tr>
<tr>
<td>Exon 15 (V600E)</td>
<td>11 (65)</td>
<td>1.2</td>
<td>72</td>
</tr>
<tr>
<td>Wild-type</td>
<td>—</td>
<td>0.0</td>
<td>98.1</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6 (35)</td>
<td>37</td>
<td>83</td>
</tr>
<tr>
<td>Female</td>
<td>11 (65)</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>15 (88)</td>
<td>68</td>
<td>87</td>
</tr>
<tr>
<td>Other</td>
<td>2 (12)</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Smoker*</td>
<td></td>
<td>67</td>
<td>3</td>
</tr>
<tr>
<td>Former/current</td>
<td>12 (71)</td>
<td>67</td>
<td>80</td>
</tr>
<tr>
<td>Never</td>
<td>5 (29)</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>Median pack-years</td>
<td>50</td>
<td>41%</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: A total of 916 NSCLC patient samples were analyzed, and 17 mutations in BRAF were identified. Percentages were calculated as a function of the total number of BRAF cases (17) and as a function of the total number of cases analyzed (916).

*Smoking history is defined as follows: current smoker, former smoker (previously smoked but quit >1 y before diagnosis of lung cancer), or never-smoker (has smoked <100 cigarettes in a lifetime).

NOTE: A total of 916 NSCLC patient samples were analyzed, and 17 mutations in BRAF were identified. Percentages were calculated as a function of the total number of BRAF cases (17) and as a function of the total number of cases analyzed (916).

Disclosure of Potential Conflicts of Interest

A. Gazdar: consultant, AstraZeneca. N. Rosen: commercial research support and speakers bureau/honoraria from Pfizer and AstraZeneca. D.B. Solit: commercial research support and speakers bureau/honoraria from Pfizer and AstraZeneca.

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References


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