Lung cancers with acquired resistance to EGFR inhibitors occasionally harbor *BRAF* gene mutations but lack mutations in *KRAS*, *NRAS*, or *MEK1*

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Acquired resistance to EGF receptor (EGFR) tyrosine kinase inhibitors (TKIs) is inevitable in metastatic *EGFR*-mutant lung cancers. Here, we modeled disease progression using *EGFR*-mutant human tumor cell lines. Although five of six models displayed alterations already found in humans, one harbored an unexpected secondary *NRAS* Q61K mutation; resistant cells were sensitive to concurrent EGFR and MEK inhibition but to neither alone. Prompted by this finding and because *RAS*/*RAF*/*MEK* mutations are known mediators of acquired resistance in other solid tumors (colon cancers, gastrointestinal stromal tumors, and melanomas) responsive to targeted therapies, we analyzed the frequency of secondary *KRAS/NRAS/BRAF/MEK1* gene mutations in the largest collection to date of lung cancers with acquired resistance to EGFR TKIs. No recurrent *NRAS*, *KRAS*, or *MEK1* mutations were found in 212, 195, or 146 patient samples, respectively, but 2 of 195 (1%) were found to have mutations in BRAF (G469A and V600E). Ectopic expression of mutant *NRAS* or *BRAF* in drug-sensitive *EGFR*-mutant cells conferred resistance to EGFR TKIs that was overcome by addition of a MEK inhibitor. Collectively, these positive and negative results provide deeper insight into mechanisms of acquired resistance to EGFR TKIs in lung cancer and inform ongoing clinical trials designed to overcome resistance. In the context of emerging knowledge about mechanisms of acquired resistance to targeted therapies in various cancers, our data highlight the notion that, even though solid tumors share common signaling cascades, mediators of acquired resistance must be elucidated for each disease separately in the context of treatment.

NRAS mutation | gefitinib | erlotinib

Mutations in the gene encoding the EGF receptor (*EGFR*) are found in ~10% of white and 30% of Asian non-small cell lung cancers, respectively (1–4). *EGFR*-mutant tumors with exon 19 deletions and L858R substitutions are highly sensitive initially to the EGFR tyrosine kinase inhibitors (TKIs), gefitinib or erlotinib (1–4). Unfortunately, tumor cells eventually acquire resistance, with progression of disease occurring in patients around 10–16 mo after starting treatment (5). Genetic mechanisms of resistance found in tumor samples from patients with acquired resistance include second-site *EGFR* mutations [>50% (6–9)], amplification of the gene encoding an alternative kinase, *MET* [(5–10% (9–12)], and mutations in the downstream signaling lipid kinase, *PIK3CA* [<5% (12)]. The majority of second-site mutations involve a threonine to methionine change at codon 790 of *EGFR*, which alters binding of drug to the ATP-binding pocket (6–9). A few percent seemingly undergo histologic transformation, displaying features of small cell lung cancer (12, 13) or epithelial–mesenchymal transition (EMT) (12). Up to 40% of cases of acquired resistance are mechanistically unexplained.

The RAS/RAF/MEK/MAPK signaling pathway downstream of EGFR plays a significant role in tumorigenesis, including lung cancers. Oncogenic recurrent driver mutations in *KRAS*, *NRAS*, *BRAF*, and *MEK1* and are found in 15–30%, 1%, 3–5%, and 1% of non-small cell lung cancers, respectively (14–18). Unlike *PIK3CA* mutations, which occur concurrently with *EGFR* mutations in individual tumors, genetic alterations in *KRAS*, *NRAS*, *BRAF*, and *MEK1* rarely occur in *EGFR*-mutant tumors (19–22). Mutations in these genes have been associated with primary resistance to targeted EGFR therapy, including EGFR TKIs in lung cancer (23) and anti-EGFR monoclonal antibodies in colon cancer (24). Recently, *KRAS* mutations also have been associated with acquired resistance to the anti-EGFR antibody cetuximab in colorectal cancers (25); *NRAS* and *MEK1* mutations have been shown to mediate acquired resistance to the mutant BRAF kinase inhibitor,

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vemurafenib, in melanomas (26, 27); and *BRAF* mutations have been found in patients with acquired resistance to imatinib in *KIT*/*PDGFRα*-mutant gastrointestinal stromal tumors (GISTs) (28). Previous reports have shown that *RAS* mutations are not found in lung cancers from patients with acquired resistance to EGFR TKIs (7, 12, 29). However, the sample sizes were too small (n = 6, 37, and 14, respectively) to rule them out definitively as mediators of resistance. Only one study looked for *BRAF* mutations, and *MEK1* status was not assessed.

Here, we modeled acquired resistance in vitro and, surprisingly, found that one cell-line model displayed a secondary *NRAS* mutation. Prompted by the data above and by the additional finding that a clinically relevant mouse lung tumor model of acquired resistance also has identified secondary *Kras* mutations (30), we systematically analyzed the frequency of secondary *RAS* pathway gene mutations in samples from patients with acquired resistance. The findings provide deeper insight into mechanisms of acquired resistance, inform ongoing clinical trials designed to overcome resistance, and suggest which mutations should be screened for routinely in samples from patients with acquired resistance.

Results

***NRAS* Q61K Mutation in an *EGFR*-Mutant Cell-Line Model of Acquired Resistance to Erlotinib.** To explore potential modes of drug resistance, we used well-established protocols (31) to develop cell models of acquired resistance to erlotinib. Including two lines previously reported (31), we developed a total of six resistant lines over 3–6 mo from five parental cells (PC-9, HCC827, HCC4006, HCC4011, and 11-18) with drug-sensitive *EGFR* mutations and known sensitivity to drug (Table 1). Using a sensitive assay that can detect mutations at an allele frequency of 5% (32), we found only two resistant lines (PC-9R and HCC827R1) that harbored the EGFR T790M second-site mutation (Table 1 and Fig. S1*A*). PC-9R cells displayed increased amplification of *EGFR* compared with parental cells (31), but HCC827R1 and R2 cells appeared to have no change in *EGFR* gene status. Two lines (HCC4006R and HCC4011R) appeared to lose copies of *EGFR* (Table 1). Two lines (HCC827R2 and HCC4011R) harbored *MET* amplification by fluorescent in situ hybridization (Fig. S2) and increased levels of MET protein by immunoblotting studies (Fig. S1*B*). Consistent with the loss of copies of EGFR, HCC4006R cells displayed loss of dependence on EGFR protein expression for cell growth. Instead, they showed features of EMT, i.e., increased expression of vimentin with loss of E-cadherin (Fig. S1 *B* and *C*). Similar results have been reported previously by us and/or others (10, 31, 33–36). No resistant cells displayed features of small cell lung cancer.

One *EGFR T790M*-negative line, 11-18R cells, had increased levels of EGFR and MET protein but lacked *EGFR* or *MET* amplification and did not display any morphologic changes (Fig. 1*A* and Figs. S1*B*, S2, and S3). Growth-inhibition studies after knockdown of EGFR protein using EGFR-specific siRNAs revealed that, unlike PC-9R cells (Fig. S1*C*), 11-18R cells were no longer dependent solely on EGFR for cell growth (Fig. 1*B*). Consistent with these findings, erlotinib treatment of 11-18R cells inhibited phosphorylation of EGFR but not the downstream signaling protein ERK (Fig. 1*C*). 11-18R cells also were resistant to growth inhibition by the more potent second-generation EGFR TKI afatinib (37), except at high doses (>1 μM) (Fig. S1*D*). These cells were insensitive to a MET TKI, either alone or in combination with erlotinib (Fig. S1*E*).

To determine possible mechanisms of resistance in 11-18R cells, we screened corresponding extracted cell DNA with a platform (a combination of SNaPshot and PCR-based sizing assays) that can detect more than 40 recurrent mutations in nine genes (*AKT1*, *BRAF*, *EGFR*, *HER2*, *KRAS*, *MEK1*, *NRAS*, *PIK3CA*, and *PTEN*) relevant to existing and emerging targeted therapies in lung cancer (32). Surprisingly, we found that, compared with parental cells, DNA from 11-18R cells harbored an acquired *NRAS* Q61K mutation in addition to the primary *EGFR* L858R substitution (Fig. 1*D*, Table 1, and Table S1). None of the other cell lines harbored any additional mutations. Consistent with these results, levels of phospho-ERK decreased after knockdown of EGFR in 11-18 parental cells but not in resistant cells (Fig. 1*B*). Ten individual single-cell clones derived from the resistant cells harbored both mutations (Table S2), suggesting that the *NRAS* and *EGFR* mutations were in the same cell. Because cell-line models of acquired resistance to date have harbored mechanisms of resistance found in human tumors, we performed additional characterization of 11-18R cells.

Functional Role of NRAS Q61K in 11-18R Cells. We assessed whether the acquired NRAS mutation plays a functional role in 11-18R cells. First, we validated the activation status of NRAS in 11-18R cells, using a Ras GTPase-specific pulldown assay. As expected, the expression of NRAS-GTPase was much higher in 11-18R cells than in 11-18 parental cells. Treatment with erlotinib had no effect on NRAS activation (Fig. 2*A*).

Next, we examined the effect of MEK inhibitors with and without erlotinib on 11-18R cell growth. The MEK serine-threonine kinase is downstream of RAS; therefore, its inhibition might be expected to affect cell viability in cells with activated RAS. The MEK inhibitor AZD6244 alone had no effect in either parental or resistant 11-18 cells. Addition of AZD6244 to erlotinib had no additive effect in parental cells, but the combination suppressed growth in the

Table 1. EGFR mutant cell-line models of acquired resistance to erlotinib

Cell line	Primary EGFR mutation	Drug selection	Erlotinib IC_{50} (μM)	*EGFR* T790M	*EGFR* amp	*MET* amp	Other
PC-9	Exon19 deletion	N/a	0.01	No	Yes*	No	N/a
PC-9R	Exon19 deletion	Erlotinib	>5	Yes	Yes*	No	N/a
HCC827	Exon19 deletion		0.005	No	Yes†	No	N/a
HCC827R1	Exon19 deletion	Erlotinib	>5	Yes	Yes†	No	N/a
HCC827R2	Exon19 deletion	Erlotinib	>5	No	Yes†	Yes	N/a
HCC4006	Exon19 deletion	N/a	0.02	No	Yes	No	N/a
HCC4006R	Exon19 deletion	Erlotinib	>5	No	No	No	EMT
HCC4011	L858R	N/a	0.03	No	Yes	No	N/a
HCC4011R	L858R	Erlotinib	>5	No	No	Yes	N/a
11-18	L858R	N/a	0.1	No	No	No	N/a
11-18R	L858R	Erlotinib	>5	No	No	No	*NRAS* Q61K

Six resistant cell lines were established from five parental cell populations. PC-9R and HCC827R1 cells harbored *EGFR* T790M. HCC827R2 and HCC4011R cells displayed *MET* amplification (amp) and increased levels of MET protein (Figs. S1*A* and S2). HCC4006R cells showed features of EMT (Fig. S1*A*). 11-18R cells harbored an *NRAS* Q61K mutation. N/a, not applicable.
*PC-9R cells showed further EGFR amplification.
†HCC827R1and HCC827R2 cells showed no further EGFR amplification.

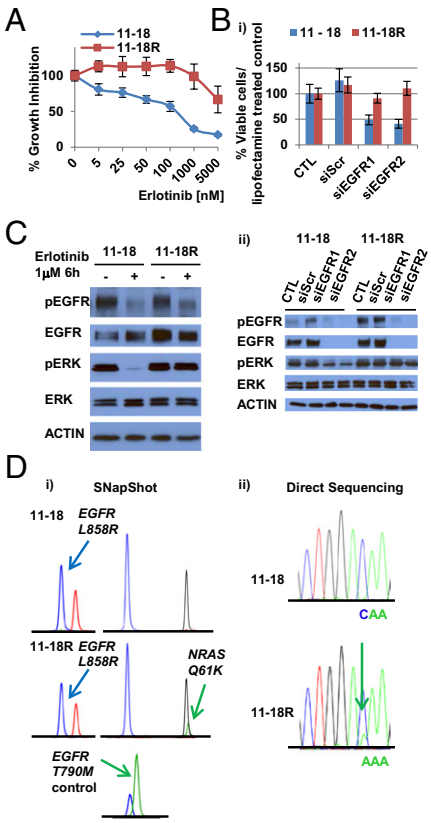

Fig. 1. Characterization of 11-18R cells. (*A*) Cell growth-inhibition assays show the relative sensitivity of 11-18 and 11-18R cells to erlotinib. Data are expressed as percentage of viability compared with vehicle by the cell titer blue assay. Data shown are mean ± SD of three independent experiments performed in hextuplicate. (*B*) (*i*) EGFR knockdown experiments using siRNAs show that, compared with parental cells,11-18R cells are no longer dependent upon EGFR for survival. Data shown are mean ± SD of three independent experiments performed in hextuplicate. Scr, Scramble. Two different siRNAs against EGFR were used. (*ii*) Immunoblotting studies using the indicated antibodies show that downstream signaling is inhibited in parental but not resistant cells after knockdown of EGFR. (*C*) The effect of erlotinib on EGFR pathway signaling in 11-18/11-18R cells. (*D*) (*i*) SNaPshot assay reveals that, in addition to a baseline *EGFR* L858R mutation, 11-18R cells have acquired an *NRAS* Q61K mutation but not an *EGFR* T790M mutation. (*ii*) Direct sequencing confirms presence of the *NRAS* 181C > *A* (Q61K) mutation in 11-18R cells.

resistant cells (Fig. 2*B*). Similar results were obtained in vitro as well as in vivo with another MEK inhibitor, GSK1120212 (Fig. S4*A* and Fig. 2*C*). By contrast, addition of AZD6244 to erlotinib had no additive effect against PC-9 parental or resistant cells, the latter of which harbor EGFR T790M (Fig. 2*B*). Consistent with these findings, only the combination of erlotinib and AZD6244 strongly inhibited levels of phospho-ERK in 11-18R cells (Fig. S4*B*). Interestingly, the MEK inhibitor alone was able to inhibit the growth of H1299 cells, which harbor no drug-sensitive *EGFR* mutations but do have an *NRAS* Q61K mutation (Fig. S4*C*). This discrepancy suggests that signaling pathways in *NRAS* Q61K mutant cells are different in the context of wild-type and mutant *EGFR*. A selective PI3K inhibitor, GDC-0941, did not restore sensitivity of 11-18R cells to erlotinib (Fig. S4*D*). These data suggest that the MEK rather than the PI3K pathway is active downstream of mutant *NRAS* in these cells.

Third, we examined the effect of NRAS protein knockdown using siRNAs against *NRAS*. siRNA knockdown modestly inhibited the growth of 11-18R but not parental cells (Fig. 2*D*). When combined with erlotinib, NRAS knockdown led to an even greater growth reduction in resistant cells (Fig. 2*D*). Conversely, when combined with AZD6244, EGFR knockdown led to similar levels of growth inhibition (Fig. 2*D*). Consistent with these results, only the combinations of erlotinib plus siRNA knockdown of NRAS (Fig. 2*E*) or AZD6244 plus siRNA knockdown of EGFR (Fig. S4*E*) led to significant inhibition of phospho-ERK in resistant cells. Finally, to extend these results further, we performed growth-inhibition assays using siRNAs and kinase inhibitors as above with two separate 11-18R single-cell clones (C1 and C4) and observed essentially the same results (Fig. S4 *F* and *G*). Collectively, these data indicate that in 11-18R cells NRAS Q61K functions downstream of EGFR L858R and mediates resistance to erlotinib via the MEK signaling pathway.

***RAS* Signaling Pathway Gene Mutations in Tumors from Patients with Acquired Resistance.** Given the RAS-related findings in the murine (30) and cell-line models of acquired resistance and the reported data regarding *KRAS*/*NRAS*/*BRAF*/*MEK1* mutations in other cancers that grow after responding to targeted therapies (25–28), we reexamined the frequency of mutations occurring in *KRAS*, *NRAS, BRAF,* and *MEK1* in 195, 212, 195, and 146 tumor samples, respectively, from patients with acquired resistance to EGFR TKIs. Tumors were screened by a variety of methods including a SNaPShot/sizing platform (32, 38), a Sequenom mass spectrometry-based assay (39), and/or direct sequencing. Although we did not detect any *NRAS*, *KRAS*, or *MEK1* mutations, we did identify two BRAF mutations. One tumor harbored simultaneous *EGFR* exon19 deletion, *EGFR* T790M, and *BRAF* V600E mutations, and another tumor harbored EGFR exon 19 deletion and *BRAF* G469A (Table 2 and Fig. S5). Both patients were found at diagnosis to have tumors with an *EGFR* exon19 deletion that initially responded radiographically to erlotinib monotherapy. Lack of sufficient tissue precluded further analysis of the acquired-resistance specimens. A pretreatment tumor sample was unavailable for the first patient. This patient also had no history of melanoma, a disease in which *BRAF* V600E mutations are common. In the second case, the *BRAF* G469A mutation was confirmed to be absent before treatment. Collectively, these data demonstrate that *RAS* pathway gene mutations are rare, but *BRAF* mutations can occur in 1% of tumors with acquired resistance to EGFR TKIs.

Ectopic Expression of *NRAS* Q61K, *BRAF* V600E, or *BRAF* G469A Confers Resistance to Cells Harboring Drug-Sensitive *EGFR* Mutations. To confirm that mutations in genes in the RAS signaling pathway could confer acquired resistance to EGFR-TKIs, we introduced cDNAs encoding *NRAS* Q61K or *BRAF* G469A in PC-9 cells and *BRAF* V600E in both PC-9 and PC-9R cells and treated cell transfectants with various inhibitors. We did not express mutant forms of *KRAS* or *MEK1*, because we did not find correlative human data. Ectopic expression of these mutants but not their wild-type counterparts led to constitutive pERK activation, even in the presence of erlotinib or afatinib (Fig. 3 *A* and *B* and Fig. S6 *A* and *B*). Consistent with these data, *EGFR*-mutant cells with stable expression of either mutant were resistant to growth inhibition by erlotinib (Fig. 3 *C* and *D*), and phospho-ERK activation was maintained in the presence of drug (Fig. S6 *C* and *D*). In the stable transfectants, addition of MEK inhibitors to erlotinib led to greater growth inhibition (Fig. 4 *A* and *C*) and enhanced reduction of phospho-ERK levels compared with either drug alone (Fig. 4 *B* and *D*). In the mutated *BRAF* transfectants, addition of the BRAF inhibitor vemurafenib to erlotinib induced greater growth inhibition than either drug alone (Fig. S7).

Discussion

Elucidating how patients with *EGFR*-mutant lung tumors develop acquired resistance to EGFR TKIs has been an active area of investigation. More than half of patients develop a second-site *EGFR* mutation, i.e., the T790M amino acid change, which results in altered binding of drug to the ATP-binding pocket (6–9). Rarer mechanisms (1–10%) include *MET* amplification, *PIK3CA* muta-

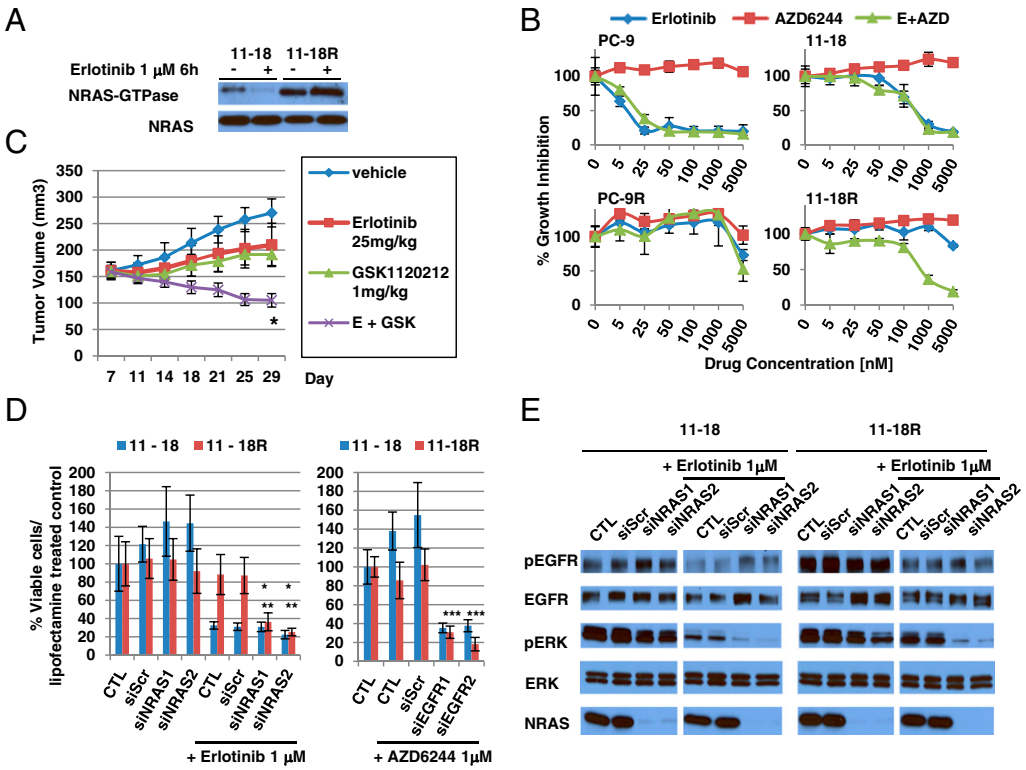

Fig. 2. Functional role of *NRAS* Q61K in 11-18R cells. (*A*) RAS GTPase-specific pulldown assay shows increased activated NRAS in 11-18R cells compared with parental cells. Erlotinib has no effect on activated NRAS activity in 11-18R cells. (*B*) Cell growth-inhibition assays show the relative sensitivity of 11-18/11-18R and PC-9/PC-9R cells to erlotinib, the MEK inhibitor AZD6244, or the combination of erlotinib with AZD6244. Data shown are mean ± SD of three independent experiments performed in hextuplicate. (*C*) Athymic nude mice with 11-18R tumors were administered vehicle, erlotinib, MEK inhibitor, GSK1120212, or erlotinib plus GSK1120212. Tumor volume was determined at the indicated times after the onset of treatment. $n = 5$ mice per group. Error bars indicate SE. $^*P < 0.05$ (Student's t test) for the combination of erlotinib plus GSK1120212 versus either erlotinib or GSK1120212 alone. (*D*) siRNA-mediated knockdown of NRAS combined with erlotinib or siRNA-mediated knockdown of EGFR combined with AZD6244 inhibits growth of 11-18R cells. Scr, Scramble. Two different siRNAs against NRAS were used. Data shown are mean ± SD of three independent experiments performed in hextuplicate. *, $^{**}P < 0.05$ (Student's t test) for the combination of erlotinib plus siNRAS knockdown versus erlotinib or siNRAS alone in 11-18R cells. $^{***}P < 0.05$ (Student's t test) for the combination of siEGFR plus AZD6244 knockdown versus AZD6244 alone in 11-18R cells. (*E*) Immunoblotting with the indicated antibodies demonstrates that siRNA-mediated knockdown of NRAS combined with erlotinib inhibits ERK activation in 11-18R cells. These samples were run on the same gel but were noncontiguous.

tion, and changes in tumor morphology (i.e., transformation to small cell lung cancer or development of features of EMT) (9–13). Up to 40% of cases are still unexplained.

Knowledge of these mechanisms coupled with prospective testing in the relevant clinical samples has led to the creation of rational strategies to overcome acquired resistance. For example, using mouse lung tumor models of drug-sensitive and -resistant *EGFR*-mutant alleles, we previously showed that dual inhibition of EGFR with the second-generation EGFR TKI afatinib plus the anti-EGFR antibody cetuximab could eradicate *T790M*-driven tumors (40). A trial based on these data has now shown a 40% partial response rate in patients with acquired resistance (41).

Here, we performed in vitro modeling to determine additional mechanisms of resistance. Surprisingly, we found that one human EGFR-mutant tumor cell line acquired a new mutation in *NRAS*. Because these models have recapitulated findings in humans (notably T790M mutations and MET amplification), and because a variety of *RAS* signaling pathway genes have been associated with acquired resistance to other targeted therapies in other solid tumors, we systematically screened for recurrent mutations in *KRAS*/*NRAS*/*BRAF*/*MEK1* in nearly 200 tumor samples from patients with acquired resistance to EGFR TKIs. Although no *KRAS*, *NRAS,* or *MEK1* mutations were detected, we did find one case with concurrent *EGFR* exon19 deletion and *EGFR* T790M and *BRAF* V600E mutations and another case with *EGFR* exon19 deletion and the *BRAF* G469A mutation (2/195, 1.0%). Although our cell-line models indicated that the *NRAS Q61K* and *EGFR L858R* mutations were in the same cell, we could not determine if the *BRAF* and *EGFR* mutations were in the same or different tumor cells in the patient samples. In case they were present in the same cells, we studied further the biological and therapeutic consequences of acquired *NRAS* and *BRAF* mutations in *EGFR*-mutant lung tumor cells and showed that these tumor cells were resistant to erlotinib alone but were sensitive to combination treatment with EGFR and MEK inhibition. Transfectants with mutant *BRAF* were sensitive to the combination of EGFR and BRAF inhibition as well. Collectively, these data suggest that *RAS* pathway gene mutations are rare but do occur, with *BRAF* gene mutations found in 1% of these patients.

In previous smaller studies, with only 6, 37, and 14 patients, respectively, we and others showed that *RAS* mutations are not found in lung cancers from patients with acquired resistance to EGFR TKIs (7, 12, 29). The sample sizes were too small to be definitively conclusive. Only 37 tumors have been examined for *BRAF* mutations (7, 12, 29), and *MEK1* status has not been assessed. By contrast, *PIK3CA* mutations have been found with other drivers in lung cancers, including in tumors from patients with acquired resistance (12, 19, 20). Why *RAS* signaling pathway gene mutations are infrequent in lung cancers as opposed to other types of solid tumors treated with targeted therapies is unclear. One possibility is that introduction of mutant *RAS* or *BRAF* into *EGFR* mutant lung cells is toxic in most instances, whereas other cell types are more per-

Table 2. RAS signaling pathway gene mutations in tumor samples from 212 patients with EGFR-mutant lung cancer and acquired resistance to EGFR TKIs

Institution	*EGFR* T790M	*NRAS*	*KRAS*	*BRAF*	*MEK1*
MSKCC*	57/103	0/103	0/103	0/103	0/103
Vanderbilt-Ingram Cancer Center†	8/8	0/8	0/8	1/8	0/8
Massachusetts General Hospital‡	42/84	0/84	0/84	1/84	0/35
Others§	n/d	0/17	n/d	n/d	n/d
Total	107/195 (54.9%)	0/212	0/195	2/195 (1.0%)	0/146

n/d, no data.

*Sixty-eight MSKCC patient samples were assessed by a mass spectrometry-based (Sequenom) assay (ref. 39), and 35 samples were assessed using a SNaPshot-based assay (ref. 32).

†Eight samples were analyzed at Vanderbilt-Ingram Cancer Center by the SNaPshot assay.

‡Eighty-four samples were analyzed at Massachusetts General Hospital by SNapshot assay (ref. 38).

§Seventeen samples were analyzed for NRAS mutations by direct sequencing at other institutions (nine patients at Okayama University, Japan, five patients at National Taiwan University Hospital, and three patients at the Max Planck Institute, Cologne, Germany).

missive. Another possibility is that other, unexamined alterations in the pathway, such as amplification (e.g., involving *KRAS*) or loss of downstream regulatory genes (e.g., *NF1*) either by genetic or epigenetic mechanisms, occur specifically in lung cancer but not in other cancers.

In the 11-18R cell-line model of acquired resistance, we found from biochemical and pharmacologic studies that the *NRAS* Q61K mutation acts in the ERK pathway downstream of mutant *EGFR*. Consistent with these data, a recent report showed that chronic exposure of cells harboring *EGFR* T790M to an EGFR T790M-selective irreversible pyrimidine EGFR kinase inhibitor, WZ4002, led to the development of *ERK2* amplification. Combined treatment of resistant cells with WZ4002 and a MEK inhibitor or ERK2 knockdown suppressed cell growth (42). These results demonstrate that activation of the RAS signaling pathway can mediate resistance; such activation may become more frequent in patients as better ways to inhibit *EGFR* T790M are developed. Thus, it will be interesting to determine if patients who develop acquired resistance to afatinib/cetuximab or *EGFR* T790M-specific inhibitors harbor activation of the RAS signaling pathway via either gene mutation or amplification.

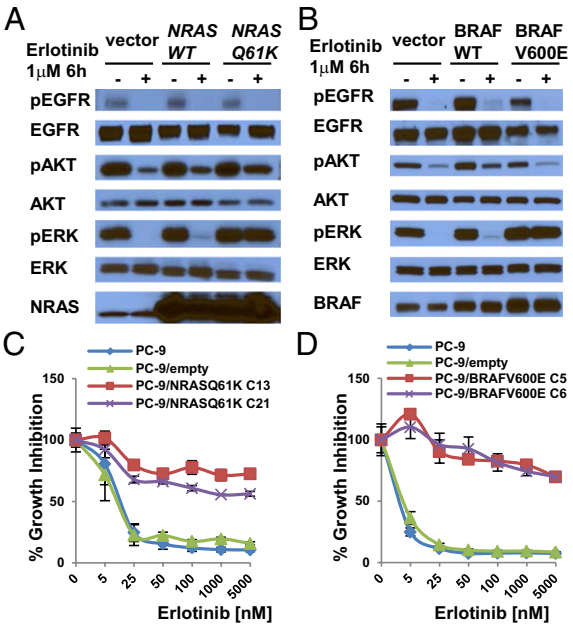

Fig. 3. Ectopic expression of *NRAS* Q61K or *BRAF* V600E in *EGFR* mutant cells mediates resistance to EGFR TKIs. (*A* and *B*) PC-9 cells were transiently transfected with expression plasmids encoding *NRAS* wild type or *NRAS* Q61K (*A*) or *BRAF* wild type or *BRAF* V600E (*B*) and were cultured in the absence or presence of erlotinib for 6 h. Corresponding cell lysates were subjected to immunoblotting with the indicated antibodies. Lysates from cells harboring *NRAS* wild type or *NRAS* Q61K displayed higher levels of total NRAS than seen in control-transfected cells, but only cells transfected with *NRAS* Q61K displayed enhanced phospho-ERK expression in the presence of erlotinib. Similarly, lysates from cells harboring *BRAF* wild type or *BRAF* V600E displayed higher levels of total BRAF than seen in control-transfected cells, but only cells transfected with *BRAF* V600E displayed enhanced phospho-ERK expression in the presence of erlotinib. (*C* and *D*). PC-9 cells were stably transfected with control plasmids or expression plasmids encoding *NRAS* Q61K (*C*) or *BRAF* V600E (*D*). Ectopic expression of *NRAS* Q61K (*C*) or *BRAF* V600E (*D*) mediated resistance of PC-9 cells to erlotinib. C13, clone 13; C21, clone 21; C5, clone 5; C6, clone 6. Data shown are mean ± SD of three independent experiments performed in hextuplicate.

In summary, preclinical modeling coupled with emerging data about acquired resistance to targeted therapies in solid tumors led us to reexamine, in the largest collection of tumor samples to date, the contribution of mutations in *RAS* signaling pathway genes as mediators of acquired resistance to EGFR TKIs in lung cancer. Such samples occasionally harbored *BRAF* mutations but lacked recurrent mutations in *KRAS*, *NRAS*, or *MEK1*. Although the percent of cases with *BRAF* mutation is small (1%), the positive findings coupled with the negative results provide deeper insight into mechanisms of acquired resistance to EGFR TKIs in lung cancer, inform ongoing clinical trials designed to overcome resistance, and narrow the list of genes that should be routinely screened for in samples from patients with acquired resistance. As more data emerge on mechanisms of resistance to targeted therapies in various cancers, the findings reported here further demonstrate that, even though colorectal cancers, melanomas, GISTs, and lung cancers share common signaling cascades, each disease must be examined independently to determine disease-specific mediators of acquired resistance.

Materials and Methods

Cell Culture. *EGFR*-mutant PC-9 (*EGFR* exon19del E746–A750), HCC827 (*EGFR* exon19del E746–A750), HCC4006 (*EGFR* exon19del L747–E749), HCC4011 (*EGFR* L858R), 11-18 (*EGFR* L858R), and H1299 (*NRAS* Q61K) cells were cultured in RPMI 1640 medium (Mediatech) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) and penicillin-streptomycin solution (final concentration 100 U/mL penicillin, 100 μg/mL streptomycin) (Mediatech). Cells were grown in a humidified incubator with 5% CO_2 at 37 °C. To create EGFR TKI-resistant lines, parental cells were cultured with increasing concentrations of TKIs starting with the IC_{30}. Doses were increased in a stepwise pattern when normal cell proliferation patterns resumed. Fresh drug was added every 72–96 h. Resistant cells that grew in 5 μM erlotinib were derived after 3–6 mo of culturing with drug. 11-18R cells were maintained initially as polyclonal populations under constant TKI selection. DNA identity testing on both the parental and resistant cells confirmed that the cells were derived from the same origin. Clonal resistant cells were isolated by limiting dilution.

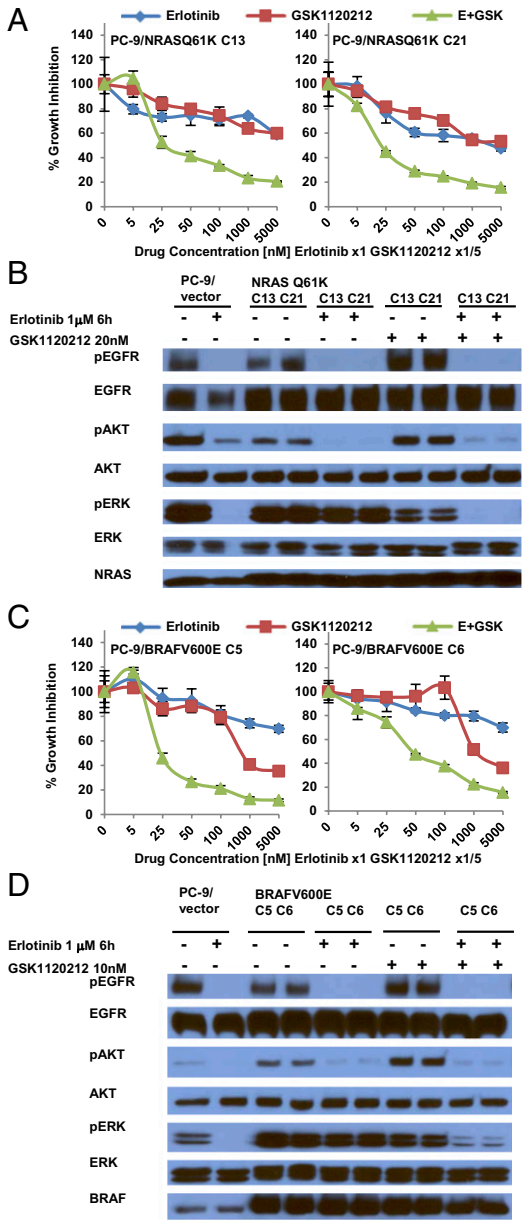

Fig. 4. MEK inhibition restores the sensitivity of PC-9/*NRAS* Q61K or PC-9/*BRAF* V600E stable clones to erlotinib. C13, clone 13; C21, clone 21; C5, clone 5; C6, clone 6. (*A* and *C*) The combination of erlotinib and GSK1120212 leads to greater inhibition of cell growth in PC-9 cells stably expressing *NRAS* Q61K (PC-9/*NRAS* Q61K cells) (*A*) or *BRAF* V600E (PC-9/*BRAF* V600E cells) (*C*) than seen either drug alone. Data shown are mean ± SD of three independent experiments performed in hextuplicate. (*B* and *D*) In stable transfectants the combination of erlotinib plus GSK1120212 leads to a greater reduction in phospho-ERK levels than either drug alone. PC-9/*NRAS* Q61K (*B*) or PC-9/*BRAF* V600E (*D*) cells were cultured in the absence or presence of erlotinib or/and GSK1120212 for 6 h; corresponding cell lysates were subjected to immunoblotting with the indicated antibodies.

Growth Inhibition Assay. For cell growth-inhibition experiments, cells were seeded in 96-well plates at a density of 3,000 cells per well and on the following day were exposed to drugs alone or in combination. Cell Titer Blue Reagent (Promega) was added 72 h after drug addition, 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 three independent times. Erlotinib and afatinib were synthesized by the Memorial Sloan-Kettering Cancer Center (MSKCC) Organic Synthesis Core. AZD6244, GSK1120212, vemurafenib, SGX-523, and GDC-0941 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, HRP-conjugated anti-mouse, and HRP-conjugated anti-rabbit. NRAS and BRAF antibody were 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 Lightning ECL reagent (Perkin-Elmer).

RAS Activation Assay. 11-18 and 11-18R cells were serum starved overnight and supplemented with 1 μM of erlotinib for 6 h. RAS activity was measured using the Ras-binding domain of Raf-1 to pull down active Ras according to the manufacturer's protocol (Cell BioLabs). Following separation by SDS PAGE, proteins were transferred to membranes which were probed with an anti-NRAS antibody.

Patient Samples and Data. Tumor specimens were obtained with patients' consent under Institutional Review Board (IRB)-approved protocols in each institution. Samples were frozen and stored at −80 °C in institutional tumor banks or in formalin-fixed, paraffin-embedded blocks. Mutational profiling results were performed within Clinical Laboratory Improvement Amendments-certified laboratories on multiple samples as part of routine standard of care and/or on IRB-approved protocols.

Sequencing of *NRAS* and Systematic Mutation Screening. Genomic DNA was extracted from patient samples (>70% tumor cells) and cell lines using standard procedures. NRAS exons 2 and 3 were amplified from genomic DNA and were sequenced directly. NRAS mutations, along with other mutations, also were screened using a SNapShot-based (32, 38) or mass spectrometry-based (Sequenom) assays (39).

siRNA Experiments. *EGFR, 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).

Expression Constructs and Transfections. A cDNA for *NRAS* was purchased from Origene and subcloned into a Flag-*N*-CMV6 entry vector (Origene). A cDNA for *BRAF* was kindly provided by David Solit (MSKCC) and was subcloned into a pcDNA3.1 vector (Invitrogen). The *NRAS* Q61K and *BRAF* V600E and *BRAF* G469A mutations were introduced into the cDNAs using site-directed mutagenesis (Agilent) with mutant-specific primers according to the manufacturer's instructions. The cDNAs were fully resequenced to ensure that no additional mutations were introduced. Plasmid transfections into PC-9 or PC-9R cells were performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Selection of cells was started 48 h later in 96-well plates with appropriate antibiotics.

Xenograft Studies. Nude mice (nu/nu; Harlan Laboratories) were used for in vivo studies and were cared for in accordance with guidelines approved by the MSKCC Institutional Animal Care and Use Committee and Research Animal Resource Center. Eight-week-old female mice were injected s.c. with 15 million 11-18R cells together with Matrigel. Once tumors reached an average volume of 150 mm^3, mice were randomized and dosed via oral gavage with either erlotinib (MSKCC Organic Synthesis Core), GSK1120212 (Selleck Chemicals), or the combination at the indicated doses. A uniform volume for administration (200 μL) was used for each group. Mice were observed daily throughout the treatment period for signs of morbidity/mortality. Tumors were measured twice weekly using calipers, and volume was calculated using the formula length × $width^2$ × 0.52. Body weight also was assessed twice weekly. The experiment was terminated after 4 wk of treatment.

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