Molecular Cancer Therapeutics

Preclinical Development

Knockdown of Oncogenic KRAS in Non–Small Cell Lung Cancers Suppresses Tumor Growth and Sensitizes Tumor Cells to Targeted Therapy

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Abstract

Oncogenic KRAS is found in more than 25% of lung adenocarcinomas, the major histologic subtype of non–small cell lung cancer (NSCLC), and is an important target for drug development. To this end, we generated four NSCLC lines with stable knockdown selective for oncogenic KRAS. As expected, stable knockdown of oncogenic KRAS led to inhibition of *in vitro* and *in vivo* tumor growth in the *KRAS*-mutant NSCLC cells, but not in NSCLC cells that have wild-type *KRAS* (but mutant *NRAS*). Surprisingly, we did not see large-scale induction of cell death and the growth inhibitory effect was not complete. To further understand the ability of NSCLCs to grow despite selective removal of mutant KRAS expression, we conducted microarray expression profiling of NSCLC cell lines with or without mutant KRAS knockdown and isogenic human bronchial epithelial cell lines with and without oncogenic KRAS. We found that although the mitogen-activated protein kinase pathway is significantly downregulated after mutant KRAS knockdown, these NSCLCs showed increased levels of phospho-STAT3 and phospho–epidermal growth factor receptor, and variable changes in phospho-Akt. In addition, mutant KRAS knockdown sensitized the NSCLCs to p38 and EGFR inhibitors. Our findings suggest that targeting oncogenic KRAS by itself will not be sufficient treatment, but may offer possibilities of combining anti-KRAS strategies with other targeted drugs. *Mol Cancer Ther; 10(2); 336–46. ©2011 AACR.*

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Introduction

The development of non–small cell lung cancer (NSCLC) is a multistep process involving a number of genetic and epigenetic abnormalities acquired over time (1). On basis of the recent successes of molecularly targeted therapy in some cancers, many of these genetic and epigenetic lesions may represent potential therapeutic targets for NSCLC (2, 3). The proto-oncogene *KRAS* is one of the most important of these potential targets because its mutation is common in many cancers and it sits at the apex of multiple growth regulatory cascades (4, 5). *KRAS* encodes a small GTP-binding protein that is involved in many cellular processes including proliferation, differentiation, and apoptosis (4). Wild-type (WT) KRAS has intrinsic GTPase activity, which catalyzes the hydrolysis of bound GTP to GDP thereby inactivating the RAS growth-promoting signal, whereas oncogenic KRAS is locked into the GTP-bound state, leading to constitutive RAS signaling. *KRAS* mutations are detected in more than 25% of lung adenocarcinomas (6), 85% of which affect codon 12 (1), and these mutations are associated with poor prognosis in NSCLC patients (7). Thus, oncogenic mutations of *KRAS* play an important role in the development of NSCLC.

Although several strategies to inhibit KRAS including farnesyltransferase inhibitors have been explored, these approaches tend not to be specific for the mutant form of KRAS, and so lead to inhibition of WT KRAS activity, which is essential for normal growth and development (8, 9). The relative failure of KRAS inhibitors in cancer clinical trials likely derives from specificity issues and from differences in how mutant KRAS controls its downstream effectors in different individual tumors. In

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Table 1. Characteristics of non–small cell lung cancer cell lines and immortalized human bronchial epithelial cell lines

Cell line	*KRAS*			p53 Alteration (15)	Other molecular abnormalities
	Genotype	Mutation type	Copy number		
H23	Mut (missense)	G12C (GGT to TGT)	2.9	Mut (missense)	*PTEN* mut (nonsense; ref. 13), *LKB1* mut (nonsense; ref. 16)
H1792	Mut (missense)	G12C (GGT to TGT)	2	Splice donor site	
H358	Mut (missense)	G12C (GGT to TGT)	6.6	Homozygous deletion	*beta-catenin* mut (T75A missense; ref. 17)
H441	Mut (missense)	G12V (GGT to GTT)	4	Mut (missense)	Overexpression of c-Met and ErbB3 (14, 18)
H1299	WT		1.9	Homozygous deletion	*NRAS* mut (missense; ref. 15)
HBEC3	WT		ND	WT	
HBEC3-K	Mut (missense)	G12V (GGT to GTT)	ND	WT	
HBEC3-p53	WT		ND	Loss of expression[a]	
HBEC3-p53K	Mut (missense)	G12V (GGT to GTT)	ND	Loss of expression[a]	

NOTE: HBEC3 cells were established by retroviral-transfection with CDK4 and hTERT.
Abbreviations: Mut, mutation; ND, not determined.
[a]p53 expression is silenced by the retroviral shRNA vector targeting p53[12].

this study, we use an RNA interference (RNAi)-based approach specifically targeting mutant *KRAS* mRNA to investigate how loss of oncogenic KRAS signaling affects the malignant phenotype of NSCLCs. Recent studies have shown the potential of gene expression profiling analysis along with RNAi technology to uncover oncogenic KRAS-specific gene signatures in lung cancers and other types of cancers (10, 11). In this study, we used a new approach to uncover oncogenic KRAS-specific gene signatures by microarray gene expression profiling with RNAi-mediated mutant-specific KRAS knockdown in NSCLC cells and mutant KRAS-transformed bronchial epithelial cells. Overall, our results show that *KRAS* mutation has canonical mitogen-activated protein kinase (MAPK)-dependent effects on cell proliferation and the malignant phenotype in NSCLC, but that among different NSCLCs, mutant KRAS can lead to different outputs in cellular signaling that affect cell survival. Our findings suggest that by itself, oncogenic KRAS is not an Achilles heel of NSCLC and that treatment of NSCLCs with *KRAS* mutations will require knowledge of other tumor molecular abnormalities, which in turn provide additional targeted therapy opportunities for *KRAS* mutation-positive NSCLC patients.

Materials and Methods

Cell lines

A total of 5 NSCLC cell lines, NCI-H23, H1792, H358, H441, and H1299, and 4 human bronchial epithelial cell (HBEC) lines were obtained from the Hamon Center Collection (University of Texas Southwestern Medical Center). All lines were genotyped by STR analysis in accordance with AACR best practices. HBEC3 cells were established by retroviral-transfection with CDK4, and the catalytic component of telomerase (hTERT) and 4 variants of an isogenic series of HBECs were used in this study: HBEC3, HBEC3/mutant KRAS (HBEC3K), HBEC3/shRNA targeting p53 (HBEC3p53), and HBEC3/mutant KRAS/shRNA targeting p53 (HBEC3K53; ref. 12). Characteristics of these cell lines are summarized in Table 1 (13–18). Cancer cells were cultured with RPMI 1640 medium supplemented with 5% FBS. HBEC3 and its derivatives were cultured with keratinocyte-SFM (Invitrogen) medium with 50 μg/mL bovine pituitary extract (Invitrogen) and 5 ng/mL EGF (Invitrogen).

Construction and use of retroviral vectors

To provide specific oncogenic KRAS knockdown, retroviral vectors producing shRNA against mutant *KRAS* were constructed by inserting annealed 64-mer sense and antisense oligos into pSUPER.retro (pRS; OligoEngine) as described (19). The 64-mer oligos were as follows: pRS-KRAS-V12, 5′-gatccccGTTGGAGCTGTTGGCGTAGttcaagagaCTACGCCAACAGCTCCAACtttttggaaa-3′ (sense) and 5′-agcttttccaaaaaGTTGGAGCTGTTGGCGTAGtctcttgaaCTACGCCAACAGCTCCAACggg-3′ (antisense); pRS-KRAS-C12, 5′- gatccccGTTGGAGCTTGTGGCGTAGttcaagagaCTACGCCACAAGCTCCAACtttttggaaa-3′ (sense) and 5′-agcttttccaaaaaGTTGGAGCTTGTGGCGTAGtctctt-

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gaa- CTACGCCACAAGCTCCAACggg-3′ (antisense). The sequences targeting the mutation in *KRAS* are indicated in capitals in the oligonucleotide sequences. Cells were infected with retroviral vectors (12). Briefly, the pRS vector was cotransfected with pVPack-GP and pVPackVSV-G vectors (Stratagene) into 293T cells by using FuGENE 6 transfection reagent (Roche) according to the manufacturers' protocol. After 48 hours, the culture medium was filtered through a 0.45-μm filter to obtain retroviral supernatants. Cells were then infected with the retroviral supernatants and 4 μg/mL polybrene, and after 10 hours, supernatants were removed and cells were grown with complete growth medium for an additional 24 hours. Infected cells were then selected with 1 μg/mL puromycin. After 7 days of puromycin selection, cells were used for analyses.

Transfection of synthetic small interfering RNA

siRNAs targeting against the *KRAS* mutations were designed and purchased from Dharmacon. The siRNA sequences were 5′-GUUGGAGCUUGUGGCGUAGTT-3′ (sense) and 5′-CUACGCCACAAGCUCCAACTT-3′ (antisense) for the *KRAS* G12C mutation, and 5′-GUUGGAG-CUGUUGGCGUAGTT-3′ (sense) and 5′-CUACGCCAA CAGCUCCAACTT-3′ (antisense) for the *KRAS* G12V mutation. siRNAs were transfected into cells using oligofectamine transfection reagent (Invitrogen) as described (20). Three days after the transfection, cells were harvested for analysis.

Western blot analysis

Western blot was done using whole cell lysates, separated on SDS/polyacrylamine gel, and electroblotted to nitrocellulose membranes (Schleicher & Schuell) as described (20). The membranes were incubated with mouse monoclonal anti-KRAS (Santa Cruz), mouse monoclonal anti-actin (Sigma), rabbit polyclonal anti-MEK1/2 (Cell Signaling), rabbit polyclonal anti–phospho-MEK1/2 (Cell Signaling), rabbit polyclonal anti–extracellular signal-regulated kinase (ERK)1/2 (Cell Signaling), rabbit polyclonal anti–phospho-ERK1/2 (Cell Signaling), rabbit polyclonal anti-Akt (Cell Signaling), rabbit polyclonal anti–phospho-Akt (Thr308; Cell Signaling), rabbit polyclonal anti-signal transducers and activators of transcription (STAT) 3 (Cell Signaling), rabbit polyclonal anti–phospho-STAT3 (Tyr705; Cell Signaling), rabbit polyclonal epidermal growth factor receptor (EGFR; Cell Signaling), and rabbit polyclonal anti–phospho-EGFR (Tyr1068; Cell Signaling) antibodies. The membranes were developed with peroxidase-labeled anti-mouse or anti-rabbit IgG (Amersham Pharmacia) by Super Signal chemiluminescence substrate (Pierce). Actin protein levels were used as a control for adequacy of equal protein loading. Protein expression levels were quantified by densitometry analysis.

RT-PCR and restriction fragment length polymorphism analysis

To detect transcripts of WT or mutant KRAS, PCR–restriction fragment length polymorphism (RFLP) method was done as described (12, 21). Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN) and cDNA was synthesized using 2 μg of total RNA with the SuperScript II First-Strand Synthesis using oligo (dT) primer System (Invitrogen). Aliquots of the reaction mixture were used for the subsequent PCR amplification. The primer sequences for *KRAS* amplification were 5′-GACTGAATATAAACTTGTGGTAGTTGGACCT-3′ (sense) and 5′-TCCTCTTGACCTGCTGTGTCG-3′ (antisense). The sense primer was designed to introduce a base substitution that created a B*st*NI recognition site for the WT codon 12 (GGT), but not for the codon 12 with the *KRAS* mutation. PCR conditions were as follows: a reaction volume of 40 μL for 15 minutes at 95°C for initial denaturation, followed by 25 cycles of 30 seconds at 95°C, 60 seconds at 55°C, 30 seconds at 72°C, and a final extension at 72°C for 10 minutes. PCR products (15 mL) were digested with 30 units of B*st*NI (New England Biolabs) at 60°C for 3 hours, and were visualized on 3% agarose gels stained with ethidium bromide.

KRAS copy number analysis

KRAS copy number was analyzed as described (22). Quantitative real-time PCR was done using the Chromo4 PCR System (Bio-Rad Laboratories). We evaluated *KRAS* copy number in each sample by comparing the *KRAS* locus to the reference *LINE-1*, a repetitive element for which copy numbers per haploid genome are similar among all of the human normal and neoplastic cells (23).

In vivo xenograft growth

In vivo tumor growth was examined by injecting cells into *nu/nu* nude mice. A total of 5×10^6 cells infected with pRS-KRAS-C12 or pRS control vector were injected into nude mice subcutaneously. Five mice were injected for each treatment. Tumor volume was measured for 70 days and 21 days for H358 cells and H1299 cells, respectively.

RNA quality and microarray analysis

The quality of total RNA was analyzed by formaldehyde gel and/or by capillary electrophoresis on the Experion System (Bio-Rad). Total RNA was labeled and amplified by our genomics core facility according to manufacturer's instructions (24). cRNA was reanalyzed after labeling to ensure optimal amplification. cRNA was hybridized to U133 Plus 2.0 (~47,000 transcripts; Affymetrix), and scanned by our microarray core facility (25). Array analysis was done as described (26). Briefly, after scanning, arrays were checked for quality using GeneChip Operating Software and then normalized using Robust Multichip Average. After normalization, all the data were compiled using R and BRB ArrayTools (developed by Dr. Richard Simon and BRB-ArrayTools Development Team). Class comparisons were made using BRB ArrayTools. Bioinformatics were done using NIH-DAVID (27) and BRB ArrayTools.

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Quantitative real-time RT-PCR

The expression of the *DUSP6* and *NT5E* genes was examined by quantitative real-time RT-PCR as described (28). Primers and probes for each gene were purchased from Applied Biosystems. For the quantitative analysis, the *TBP* gene was used as an internal reference gene to normalize input cDNA. PCR was done in a reaction volume of 20 μL, including 2 μL cDNA using the Gene Amp 7700 Sequence Detection System and Software (Applied Biosystems). The comparative Ct method was used to compute relative expression values.

Statistical analysis

Statistical analyses were done using GraphPad Prism version 5.0 software program for Windows (GraphPad Software). Correlations between groups were analyzed by Pearson's correlation coefficient. Differences between groups were analyzed by ANOVA with Bonferroni post hoc test. Differences in tumor growth in nude mice were analyzed by repeated measures ANOVA. $P < 0.05$ was considered to be significant.

Results

Stable knockdown of oncogenic KRAS by retrovirus-mediated shRNA in NSCLC cell lines

We used 4 NSCLC cell lines H23, H1792, H358, and H441, which harbor heterozygous *KRAS* mutations at codon 12, and the H1299 NSCLC cell line with WT *KRAS* (but mutant *NRAS*) as a control (Table 1, Supplementary Fig. S1). We used the H1299 cell line, which has an *NRAS* mutation at codon 61 (15) as a control for the phenotypic effects of mutant *KRAS* knockdown. The *KRAS* mutation-positive NSCLC lines exhibited different levels of KRAS protein expression (Fig. 1A), which were positively correlated with *KRAS* copy number in the NSCLC lines (Fig. 1B).

To examine the effects of loss of oncogenic KRAS on NSCLC, we used a retroviral shRNA strategy to obtain stable and specific knockdown of mutant KRAS by pRS-KRAS-C12 targeting the *KRAS* G12C mutation and pRS-KRAS-V12 targeting the *KRAS* G12V mutation. This system has the advantage that the shRNA vectors can silence expression of mutant KRAS, but retain expression of WT KRAS, which is indispensable for viability of normal cells (8, 9), in the 4 NSCLC cell lines with heterozygous *KRAS* mutation. H23, H1792, and H358 cells were infected with the pRS-KRAS-C12 vector and H441 cells were infected with the pRS-KRAS-V12 vector, and selected with puromycin to generate mutant KRAS-knockdown derivatives of these cell lines. H1299 cells with WT KRAS were infected with pRS-KRAS-C12 or pRS-KRAS-V12 to generate experimental controls. All cell lines were also infected with the pRS non-targeting control vector to generate pRS-infected control cells. In the KRAS-mutant cell lines, KRAS protein expression was reduced in pRS-KRAS–infected cells compared with parental or pRS control vector-infected cells, whereas the infection with pRS, pRS-KRAS-C12, or pRS-KRAS-V12 did not affect KRAS protein expression in H1299 cells with WT KRAS (Fig. 1C). Thus, using the mutant-specific shRNA vector systems, we were able to isolate clones with stable loss of oncogenic KRAS expression in these NSCLC cell lines.

Because there are no commercially available mutant-specific KRAS antibodies, we used PCR-RFLP analysis to verify that our RNAi strategy was specific for only mutant KRAS. In KRAS-mutant NSCLC cells (H23, H1792, H358, and H441), the mutant 186-bp band was detected with higher intensity compared with the WT 156-bp cDNA fragment (Fig. 1D), showing that cells with *KRAS* mutations express more mutant *KRAS* transcripts than WT, as previously reported by us (12) and others (29). In the KRAS-disrupted clones of H23, H1792, H358, and H441 cells, the intensity of the uncut bands was reduced compared with those in parental cells or in the control vector-infected cells (Fig. 1D). Thus, in our hands, retroviral-mediated RNAi substantially depleted expression of mutant *KRAS* mRNA, but not WT *KRAS* mRNA (19).

shRNA-mediated knockdown of mutant KRAS suppresses *in vitro* and *in vivo* growth of NSCLC cell lines

Next, we assessed the effect of shRNA-mediated stable knockdown of mutant KRAS expression on cell proliferation by the MTT assay. shRNA-mediated knockdown of mutant KRAS inhibited cell proliferation in all of the *KRAS*-mutant NSCLC cell lines, but not in H1299, whereas cell proliferation was not affected by the control vector in all cell lines (Supplementary Fig. S2A). The effect of KRAS knockdown on anchorage-independent growth was also assessed by soft-agar colony formation assay. KRAS knockdown led to a marked decrease (≥75%) in colony numbers in the *KRAS*-mutant NSCLC cell lines, but not in H1299 compared with the colony numbers of the parental cells (Supplementary Fig. S2B). Thus, shRNA-mediated knockdown of mutant KRAS significantly inhibited *in vitro* cell growth of *KRAS* mutation-positive NSCLC cells.

To further evaluate the effects of reduced mutant KRAS expression on the tumorigenic phenotype, and in particular its contribution to *in vivo* tumor growth, we used the H358 cell line because this cell line consistently produces tumors in nude mice, (30) and the effects of KRAS knockdown on *in vitro* growth inhibition was most prominent among 4 KRAS-mutant NSCLC lines (Supplementary Fig. S2A and B), indicating that H358 cells seem to be dependent on oncogenic KRAS signaling. There was a significant difference in *in vivo* tumor growth between H358 cells with pRS-KRAS-C12 and those with the control vectors (Supplementary Fig. S2C). H1299 cells infected with pRS-KRAS-C12 or the control vector were also injected into mice to verify whether there were any off-target effects of the pRS-KRAS-C12 infection that might inadvertently suppress tumor growth. In fact, the pRS-KRAS-C12–positive

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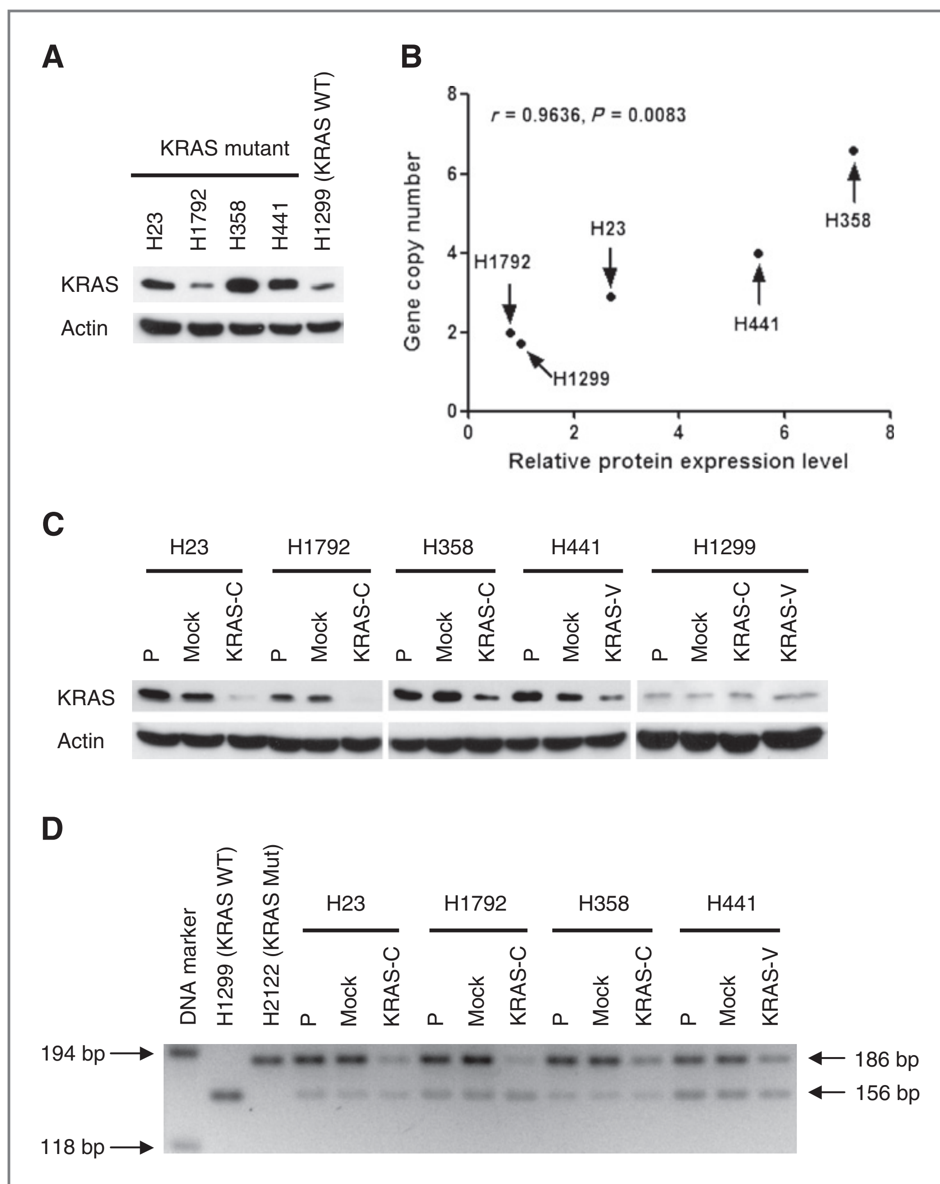

Figure 1. A, immunoblot of KRAS protein expression in H23, H1792, H358, H441, and H1299 NSCLC cell lines. Whole cell lysate (30 μg) was loaded per lane and Western blot was done. B, positive correlation between KRAS copy number and the protein expression levels in NSCLC cell lines (Pearson's $r = 0.9636$, $P = 0.0083$). The protein expression levels were determined by densitometry. C, stable knockdown of mutant KRAS protein by retroviral-mediated shRNA in NSCLC cell lines. P, parental cells; Mock, pRS control vector-infected cells; KRAS-C, pRS-KRAS-C12–infected cells; KRAS-V, pRS-KRAS-V12–infected cells. D, specific reduction of mutant *KRAS* transcripts by retroviral shRNA vectors (pRS-KRAS-C12 or pRS-KRAS-V12), but not by the pRS control vector in the NSCLC cells. BstNI digestion cuts the WT *KRAS* allele (e.g., H1299 cells) to produce a 156-bp DNA fragment, whereas the mutant *KRAS* allele remains uncut to produce a 186-bp DNA fragment (e.g., the H2122 cell line that has a homozygous *KRAS* G12C mutation was used as a control).

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H1299 cells grew better than the vector controls, although this difference was not significant (Supplementary Fig. S2C). These results show that *in vivo* tumor growth was partially inhibited by shRNA-mediated knockdown of mutant KRAS expression in *KRAS*-mutant NSCLC cells.

Effects of mutant KRAS knockdown on expression profiles in NSCLC cell lines

KRAS sits at the hub of multiple signaling cascades and activating mutations of *KRAS* can lead to diverse aberrant signal transduction events. As a first step, to elucidate how oncogenic KRAS affects downstream pathways and to discover what might account for the residual tumorigenicity observed after KRAS knockdown, we conducted microarray expression profiling (Affymetrix HG-U133-Plus2 array) of NSCLCs with and without oncogenic KRAS, and compared the expression profiles. As a control, we profiled H1299 cells (WT KRAS) after infection with the 2 vectors. Importantly, there were almost no differences in gene expression in H1299 with either vector (Supplementary Fig. S3A). Within the mutant KRAS NSCLC cell lines, microarray analysis revealed considerable heterogeneity in genes whose expression changed more than 2-fold with knockdown of oncogenic KRAS (Supplementary Fig. S3B). Thus, although knockdown of oncogenic KRAS has a clear and consistent effect on cell growth in NSCLC, there is significant diversity in its effect at the level of gene transcription.

Recently, Sweet-Cordero and colleagues (11) attempted to identify an oncogenic KRAS-specific gene expression signature in NSCLC using cell lines and primary tumors. However, due to the heterogeneity of the KRAS expression phenotype in NSCLC, they used a KRAS mouse model to identify a robust signature. In our study, we used an isogenic series of HBECs (HBEC3, HBEC3-K, HBEC3-p53, and HBEC3-p53K) that contain various oncogenic changes, including forced expression of mutant KRAS-V12 (K) and shRNA-dependent knockdown of p53 (p53) for similar purposes (Supplementary Fig. S4A; ref. 12).

When NSCLC cell lines and HBECs were grouped according to whether they expressed mutant KRAS (H23-vector, H1792-vector, H358-vector, H441-vector, HBEC3-K, and HBEC3-p53K) or not [H23-KRAS knockdown (KD), H1792-KRAS (KD), H358-KRAS (KD) H441-KRAS (KD), HBEC3, and HBEC3-p53; Table 1], we identified 53 unique (several of which were poorly annotated) genes that distinguished these groups in a statistically significant manner (Supplementary Fig. S4B; Supplementary Table S1). Among these genes, 2 genes, *ecto-5′-nucleotidase* (*NT5E*, also known as *CD73*) and *dual specificity phosphatase 6* (*DUSP6*, also known as *MKP-3*), were identified as significantly downregulated genes. Although relationship between Ras and NT5E remains unknown, recent studies by us and others have elucidated that DUSP6 is upregulated through ERK-MAPK signaling pathway on oncogenic activation of EGFR (12, 31) or Ras (32), and controls the pathway as a negative feedback mediator (31). The reproducibility of transcriptional regulation of *DUSP6* and *NT5E* was validated by transient knockdown of mutant KRAS by synthetic siRNAs. siRNAs targeting against mutant KRAS were transfected into H23, H1792, H358, and H441 cells, and expression levels of *NT5E* and *DUSP6* were examined at 3 days posttransfection by quantitative RT-PCR analysis. Log ratios of mRNA expression changes by siRNA-mediated KRAS knockdown were significantly correlated with those by the retroviral shRNA-mediated knockdown in the 4 NSCLC lines (Supplementary Fig. S4C), confirming that the expression of *DUSP6* or *NT5E* expression was downregulated by mutant KRAS knockdown.

Functional annotation analysis of the 53 genes revealed that many of these genes fell into the MAPK cascade as both positive and negative regulators of cell proliferation and survival (Supplementary Table S2). Importantly, we found that 3 dual specificity phosphatase activities (*DUSP-4*, *-5*, and *-6*) were downregulated, and *TGFß*, an important activating ligand for SMAD/p38 signaling, was upregulated by oncogenic KRAS knockdown (Supplementary Table S3). These data suggest that the cells responded to loss of oncogenic KRAS signaling by reducing levels of negative feedback MAPK regulators and upregulating autocrine signals to establish compensatory progrowth stimuli through alternative, but related signal transduction cascades. In addition, a comparison of the gene expression changes between HBEC cells with ectopic oncogenic KRAS and the NSCLC knockdown experiments suggest that the early effectors of mutant RAS-dependent signaling are similar between cell lines (underscored by the fact that nearly all members of each group either overexpressed or underexpressed each of the 53 genes).

Effects of mutant KRAS knockdown on phosphoproteins in EGFR and RAS pathways in KRAS-mutant NSCLC cell lines

The principal finding of our array analysis was that genes involved in MAPK regulation were transcriptionally altered by oncogenic KRAS. Although this is not surprising, we decided to further explore these changes at the protein level because many components of this pathway regulate protein phosphorylation. Before knockdown, it should be noted that the levels of KRAS protein expression varied among the KRAS mutation-positive NSCLC cell lines (Fig. 1A). The effects of KRAS knockdown on phosphorylation levels of MEK, ERK, AKT, and EGFR protein are summarized (Fig. 2A and B). Consistent with our array analysis, KRAS knockdown reduced the levels of pMEK and pERK in all cell lines. Conversely, pEGFR levels were upregulated in all cell lines after KRAS knockdown. Of note, the effects of KRAS knockdown on pAKT levels were different among these 4 lines. We also examined the effects of KRAS knockdown on the levels of phosphorylated STAT3 because it is an important downstream mediator of the EGFR signaling. STAT3 is activated by tyrosine phosphorylation, acting as a transcription factor to regulate gene expression (33), and the phosphorylation is mediated by oncogenic activation of EGFR in NSCLC cells (34). We found that pSTAT3 levels were upregulated by KRAS knockdown in all cell lines, which seems to be a result from EGFR phosphorylation induced by KRAS knockdown. These results indicate that oncogenic KRAS positively regulates the MEK-ERK activation, but negatively regulates activation of STAT3 and EGFR. On the other hand, AKT activation is differentially regulated by oncogenic KRAS among NSCLC cells carrying *KRAS* mutations. Our data, along with those recently reported by Singh and colleagues (10), reflect the complexities of oncogenic KRAS-induced regulation of downstream pathways and led us to consider the possibility that oncogenic KRAS by itself does not explain all of the activity driving oncogenesis through the MAPK pathway in established tumors.

The synergistic effect of p38 and EGFR inhibitors with KRAS knockdown on cell growth of NSCLC

To be able to tolerate the "stress" of oncogenic KRAS expression, lung epithelial cells likely require secondary changes to adapt to their altered signaling homeostasis. On the basis of our finding that different NSCLCs responded differently to knockdown of oncogenic KRAS in terms of mRNA expression profile and MAPK-related signal transduction, we hypothesized that each of these lines had acquired different secondary changes

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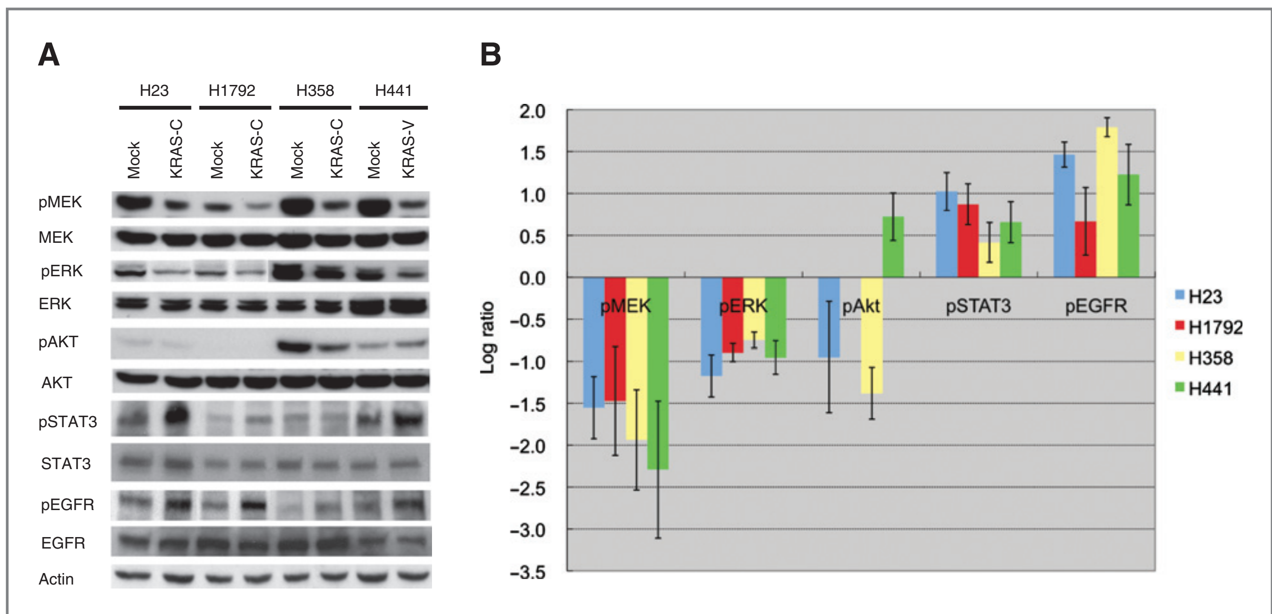

Figure 2. A, the effects of KRAS knockdown on the phosphorylation of MEK, ERK, Akt, STAT3, and EGFR in the *KRAS* mutation-positive NSCLC cell lines. Whole cell lysate (30 μg) was loaded per lane for Western blot analysis. Mock, pRS vector–infected cells; KRAS-C, pRS-KRAS-C12–infected cells; KRAS-V, pRS-KRAS-V12–infected cells. B, the effect of KRAS knockdown on the phosphoprotein levels. The data were obtained from 3 independent experiments.

potentially in KRAS-controlled signaling pathways and/or, through some feedback mechanism to compensate for loss of oncogenic KRAS. On the basis of these considerations and the Western blot data, we considered whether NSCLCs in which oncogenic KRAS was targeted (in our case with stable knockdown) acquired sensitivity to other targeted therapies. To test this hypothesis, we examined the effect of inhibitors to MEK1/2 (U0126), p38 (p38 V), PI3K (LY294002), AKT 1/2 (Akt VIII), EGFR tyrosine kinase (gefitinib) and an anti-EGFR antibody (cetuximab) on cell growth of KRAS-mutant NSCLC cells. The treatment of p38 V in combination with KRAS knockdown resulted in significant growth inhibition in H23 at 2.5 μmol/L or more, in H1792 at 0.025 μmol/L or more, and in H441 at 2.5 μmol/L compared with control treatments of p38 in the parental cells without KRAS knockdown (Fig. 3A). Thus, KRAS knockdown enhanced sensitivity to the p38 inhibitor markedly in H1792, and less so in H23 and H441. The treatment of gefitinib with KRAS knockdown resulted in significant growth inhibition in H23 at 10 μmol/L, in H1792 at 0.1 to 10 μmol/L, and in H358 at 0.01 to 1 μmol/L compared with the treatment of gefitinib without KRAS knockdown (Fig. 3B). Also, the treatment of cetuximab with KRAS knockdown resulted in significant growth inhibition in H1792 at 0.5 μg/mL or more (Fig. 3C). Thus, KRAS knockdown significantly enhanced sensitivity to gefitinib in H23, H1792, and H358, and cetuximab in H1792, although the effects of sensitization were modest in some cases. In contrast, KRAS knockdown did not significantly affect the sensitivity to U1026, LY294002, and Akt VIII in these NSCLC lines (data not shown). These results suggest that inhibitors of EGFR and p38 could be effective for treatment of KRAS mutation-positive NSCLC in combination with KRAS knockdown.

Discussion

In this report, we used shRNA methodology to stably silence the expression of oncogenic KRAS in 4 NSCLC cell lines. We found that stable knockdown of oncogenic KRAS led to reduced proliferation rates and anchorage independent growth *in vitro*, and decreased *in vivo* tumorigenicity. However, we found that knockdown of oncogenic KRAS did not lead to apoptosis or complete loss of tumorigenicity, which is in contrast to previously reported results in the pancreatic cancer cell line CAPAN-1 carrying homozygous *KRAS* mutations (19). One possible explanation for the differences between their study and ours is that the NSCLC cell lines used in our study carry both mutant and WT *KRAS* alleles. Hence, the mutant-specific KRAS shRNA vectors did not affect WT KRAS expression, which seems to be indispensable for cell viability under normal conditions. Although it is possible that the lack of complete loss of cell viability by mutant KRAS knockdown was not significant due to the remaining WT KRAS, it is also possible that retained expression of WT KRAS in the oncogenic KRAS-disrupted clone permits survival of the NSCLC cells through normal regulation of signal transduction. Of note, Brummelkamp and colleagues (19) also reported that oncogenic KRAS knockdown had no significant effect on the ability of CAPAN-1 cells to proliferate adherently. Thus, it is also possible that knocking down oncogenic KRAS does not have striking

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effects on cell proliferation in contrast to the effects on anchorage-independent growth.

Analysis of the transcriptional changes associated with knockdown of oncogenic KRAS suggest that the cells responded by downregulating negative regulators of MAPK, in particular those of ERK1/2 and JNK, and by upregulating Ras-related GTPases, and ligands for EGFR and SMAD. The biological relevance of downregulating phosphatase activity and upregulating autocrine ligands show the adaptability of NSCLCs of finding alternative signal transduction cascades-–through MAPK-related pathways-–to reestablish oncogenic signaling. Most importantly, we show that mutant KRAS knockdown in these NSCLCs led to sensitization to molecularly targeted therapeutics: inhibitors of EGFR and p38, especially for H1792 cells, although the effect of KRAS knockdown was modest and required high concentrations of these inhibitors in some cases. To our knowledge, this is the first report showing that loss of oncogenic KRAS activity leads to upregulation of phospho-EGFR and sensitizes formerly resistant NSCLCs to inhibitors of EGFR.

In NSCLC, mutations in the *EGFR* and *KRAS* genes are major molecular abnormalities that seem to be mutually exclusive (5, 35). Thus, it will be essential to develop therapeutic modalities for NSCLC carrying either *EGFR* or *KRAS* mutations. Although EGFR-tyrosin kinase inhibitor (TKI) therapy is effective in *EGFR* mutation-positive patients (36–39), they provide no benefit for KRAS-mutant lung cancer (40). Currently, there are no effective treatment strategies for *KRAS*-mutant NSCLC. In our study, shRNA-mediated knockdown of mutant KRAS in NSCLCs led to upregulation of pEGFR and significantly increased sensitivity to gefitinib. Our findings may explain, at least in part, why NSCLCs with *KRAS* mutations are resistant to EGFR-TKIs: oncogenic activation of KRAS obviates the need for signaling via ligand-dependent activation and phosphorylation of EGFR. However, our data also show that NSCLCs, which probably evolved through a KRAS-dependent process, retain the capacity to upregulate other signals, which feed through MAPK. This conclusion is supported by a recent study showing that exogenous expression of mutant KRAS in HBECs reduced the phosphotyrosine immunoprecipitates of EGFR in the phosphoproteomic studies (41).

The present study indicates, as expected, that activation of the MAPK pathway is a critical event in the initiation of KRAS-mutant NSCLC, whereas additional molecular abnormalities resulting in deregulation of other signaling pathways such as the PI3K-Akt pathway could play a role in the maintenance of the oncogenic phenotype. These findings suggest that multiple molecular targets exist for combination therapy in NSCLC patients carrying *KRAS* mutations. Our results show that inhibiting oncogenic KRAS activity alone is not sufficient to completely inhibit tumor growth of KRAS-mutant NSCLC. By applying the results from 4 NSCLC cell lines carrying additional distinct molecular abnormalities to a broader genetic analysis of *KRAS* mutation-positive patients, it might be possible to identify the critical molecules to target in combination with mutant KRAS knockdown to improve treatment for patients.

We found that mutant KRAS knockdown enhanced sensitivity to the p38 inhibitor p38V in NSCLC cells. This unexpected result might be due to autocrine upregulation of TGFβ, which was significantly upregulated after knockdown of oncogenic KRAS (Supplementary Table S3; Supplementary Fig. S4B), and cross-talk between ERK and p38. p38 is involved in many cellular processes including cell proliferation and apoptosis in response to stress stimuli and cytokines. Although the role of p38 in tumor development is not as well defined as ERK, recent evidence suggests that the 2 pathways, ERK and p38, play opposing roles in tumor cells (42). It is possible that p38 signaling substitutes for deactivation of ERK signaling caused by loss of mutant KRAS; thus, dual inhibition of KRAS and p38 activities could be effective for the treatment of *KRAS* mutation-positive NSCLC.

Recent advances in molecular biology have made considerable progress in molecularly targeted cancer therapy. A number of molecularly targeted drugs are clinically available or being tested for clinical use in lung cancer on the basis of growing knowledge of the molecular abnormalities relevant to lung carcinogenesis (3). Considering that *KRAS* mutations are observed in more than 25% of lung adenocarcinomas (6), therapeutic strategies targeting oncogenic KRAS and its signaling pathway would be very valuable in treating NSCLCs with activating *KRAS* mutations. The present study suggests that targeting oncogenic KRAS alone does not seem to be an Achilles heel of mutant KRAS NSCLC. Recently, several studies using genome-wide RNAi screen with human cancer cell lines have identified potential molecular targets that are synthetically lethal with oncogenic KRAS, including PLK1 (43), TBK1 (44), and STK33 (45). Although these molecules are potential targets in NSCLC cells with KRAS mutations, the synthetic lethal interactions are influenced by their oncogenic KRAS dependency; thus, the extent of the "synthetic lethality" seems to be cell type–dependent. For instance, Singh and colleagues (10) found that only some KRAS-mutant cells retained a requirement for this oncogene, which led the authors to stratify KRAS-mutant tumors into 2 broad subgroups: dependent or independent of sustained mutant KRAS signaling. Added to this, other studies reported dependent but not independent KRAS-mutant tumors are sensitive to suppression of STK33 (45) and TBK1 (44). Our findings, together with other recent reports, do point to the fact that mutant KRAS oncogenic signaling is likely, more complex than previously perceived. Tumor cells might be more robust than we thought in that they can have versatile mechanisms to overcome "targeted inhibition" and that even knocking down mutant KRAS itself does not seem to be an effective Achilles heel. The observance of these diverse subsets of KRAS-mutant NSCLCs—with distinct molecular pro-

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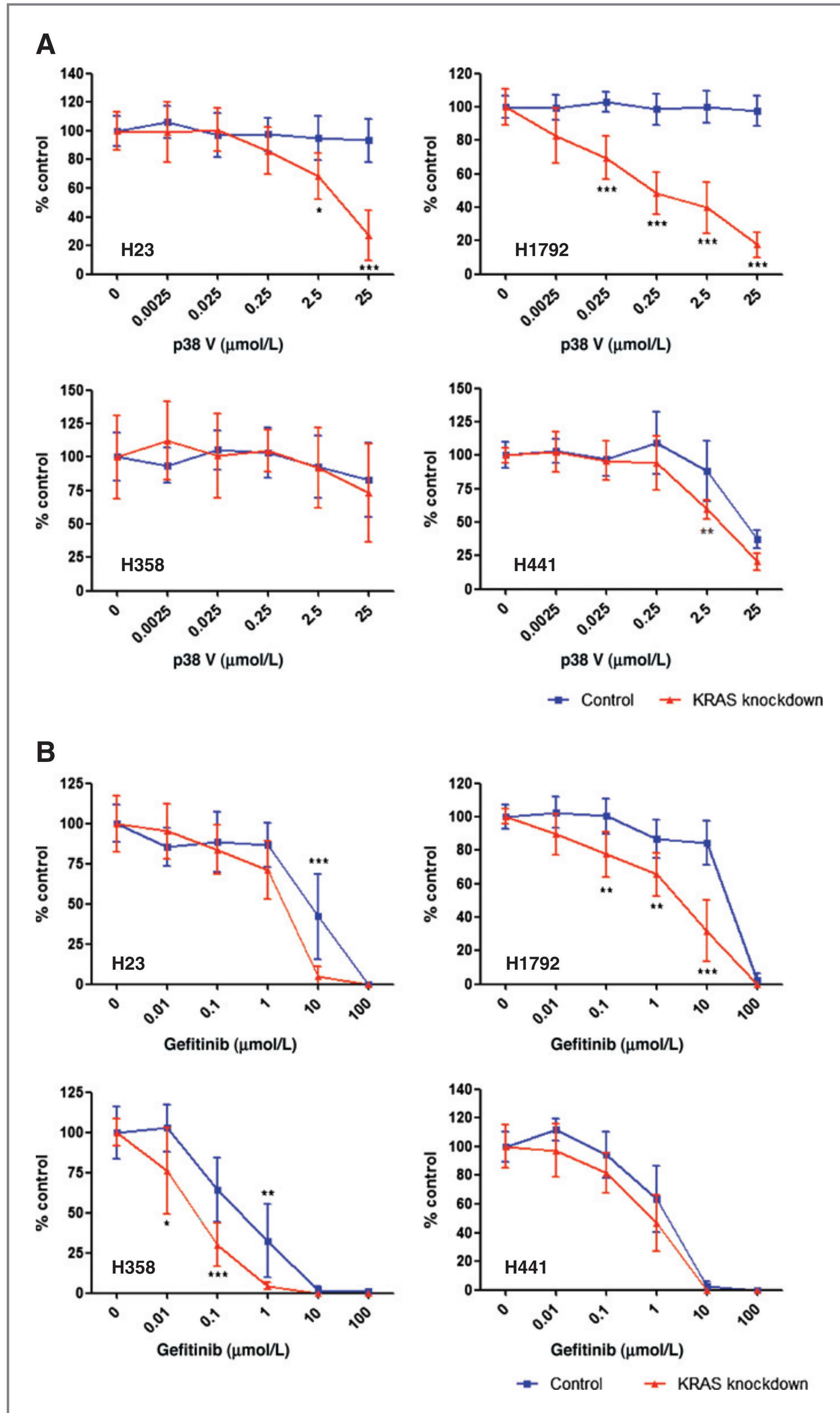

Figure 3. The effects of p38 V (A), gefitinib (B), and cetuximab (C) on colony formation in H23, H1792, H358, and H441 cells with KRAS knockdown (red line) or without KRAS knockdown (blue line) at various concentrations. Number of colonies in untreated cells was set at 100%. The data were obtained from 6 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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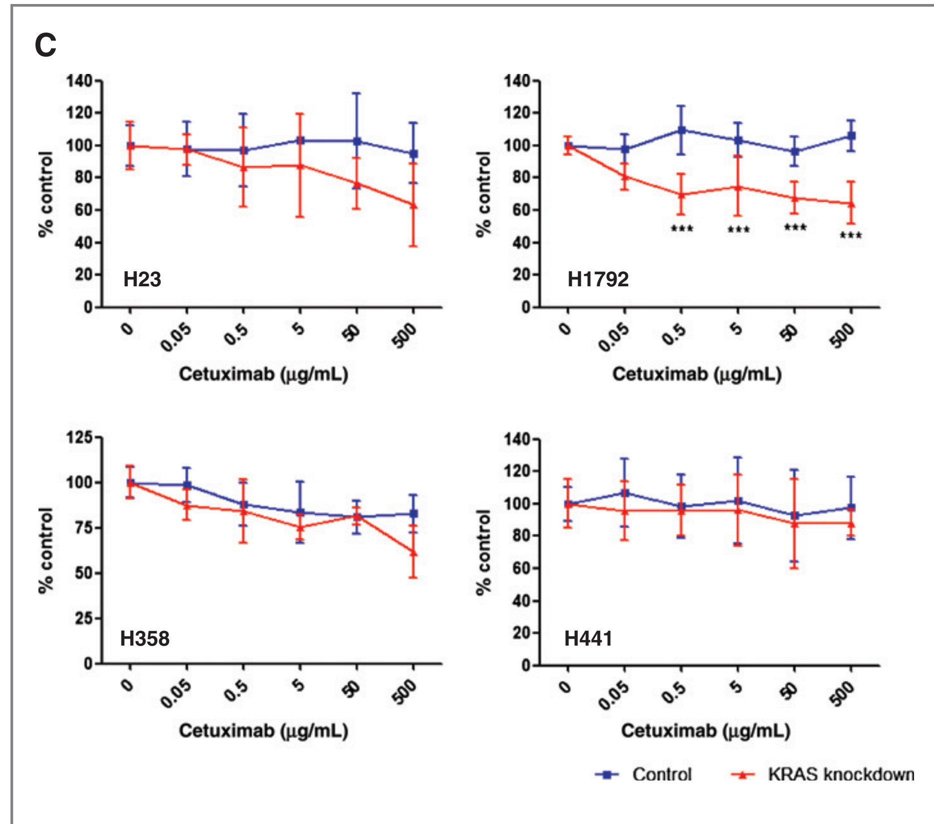

Figure 3. *(Continued).*

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files, signaling pathway activation, drug sensitivity, epithelial-to-mesenchymal status, and molecular aberrations-–supports what we envision as a more effective treatment strategy for KRAS-mutant NSCLCs: the combined targeting of oncogenic KRAS in addition with other therapeutic agents specific to the molecular profile of the tumor. Thus, the treatment of KRAS mutation-positive NSCLC patients will require *a priori* knowledge of other molecular abnormalities in the tumors to achieve more effective therapies for NSCLC. Further studies focusing on the cells' compensatory mechanisms could also be quite fruitful.

Disclosure of Potential Conflicts of Interest

D.S. Shames is employed by Roche/Genentech. All other authors have no financial or personal relationships with other people or organizations that could inappropriately influence our work.

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