Genomic and Biological Characterization of Exon 4 KRAS Mutations in Human Cancer

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Abstract

Mutations in RAS proteins occur widely in human cancer. Prompted by the confirmation of KRAS mutation as a predictive biomarker of response to epidermal growth factor receptor (EGFR)–targeted therapies, limited clinical testing for RAS pathway mutations has recently been adopted. We performed a multiplatform genomic analysis to characterize, in a nonbiased manner, the biological, biochemical, and prognostic significance of Ras pathway alterations in colorectal tumors and other solid tumor malignancies. Mutations in exon 4 of KRAS were found to occur commonly and to predict for a more favorable clinical outcome in patients with colorectal cancer. Exon 4 KRAS mutations, all of which were identified at amino acid residues K117 and A146, were associated with lower levels of GTP-bound RAS in isogenic models. These same mutations were also often accompanied by conversion to homozygosity and increased gene copy number, in human tumors and tumor cell lines. Models harboring exon 4 KRAS mutations exhibited mitogen-activated protein/extracellular signal-regulated kinase kinase dependence and resistance to EGFR-targeted agents. Our findings suggest that RAS mutation is not a binary variable in tumors, and that the diversity in mutant alleles and variability in gene copy number may also contribute to the heterogeneity of clinical outcomes observed in cancer patients. These results also provide a rationale for broader KRAS testing beyond the most common hotspot alleles in exons 2 and 3.

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Introduction

Constitutive mitogen-activated protein kinase (MAPK) activation is frequent in human cancer and is often the result of activating mutations in RAS (1, 2). Mutationally activated forms of RAS were first identified in the Harvey and Kirsten sarcoma viruses, in which they were determined to be oncogenic (3–5). Shortly thereafter, somatic RAS mutations were detected in human tumors (4–6). The most common of these mutations, occurring at the G12, G13, and Q61 positions, result in impaired intrinsic and GTPase-activating protein (GAP)–mediated GTP hydrolysis, leading to elevated levels of cellular RAS-GTP (7).

Despite evidence that oncogenic RAS plays a central role in mediating transformation in a diverse set of human tumors, only recently has limited KRAS mutational testing entered clinical practice. Testing of lung and colorectal tumors for KRAS mutations was prompted by the demonstration that KRAS mutational status is a predictive marker of response to epidermal growth factor receptor (EGFR)–targeted therapies such as erlotinib, cetuximab, and panitumumab (8–13). Clinical testing, however, has been restricted to the identification of mutations involving only few of the most commonly mutated alleles (14, 15).

Recent technological advances have made a more comprehensive assessment of RAS gene alterations feasible, but widespread adoption of broader testing beyond the most commonly mutated alleles at codons 12 and 13 has been limited by a lack of knowledge about the frequency and biological significance of non–exon 2 KRAS mutations (16, 17). We therefore used a multiplatform approach to define the incidence, biological, and prognostic significance of RAS mutations beyond the well-characterized hotspots in KRAS exon 2.

Materials and Methods

Mutation detection

Clinical data were collected on patients under an Institutional Review Board–approved protocol or waiver of authorization. Genomic DNA was obtained by using the DNeasy Tissue kit (Qiagen). Mutations were detected using the iPLEX...
assay (Sequenom, Inc.), which is based on a single-base primer extension assay (18). Briefly, multiplexed PCR and extension primers are designed for a panel of known mutations. After PCR and extension reactions, the resulting extension products are analyzed using a matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometer. For mutation detection by the Sanger method, PCR primer sequences were used for exon amplification as previously reported (19). All primer sequences are available upon request.

**Mass spectrometry–based genetic fingerprinting assay**

Colorectal cancer cell lines and tumors were checked for mislabeling, contamination, and misidentification using a multiplexed PCR/mass spectrometry (MS)–based genetic fingerprinting assay developed specifically for this purpose. Briefly, 42 highly polymorphic single nucleotide polymorphisms, covering all chromosomes, were selected, and a six-well, multiplexed assay was designed. The assays were run on the Sequenom platform as described in the Supplementary Methods.

**Array comparative genomic hybridization**

For comparative genomic hybridization (CGH) studies, labeled tumor DNA was cohybridized to Agilent 244K aCGH microarrays with a pool of reference normal. Raw copy number estimates were normalized (20), segmented with Circular Binary Segmentation (21), and analyzed with RAE (22), all as previously described. The status of genomic gain was determined for segments spanning the KRAS locus as those with $A_0 > 0.9$ and $A_1 > 0.01$ per the multicomponent model in RAE (22). Regions of significant alteration were excluded as either known or presumed germline copy number polymorphisms if they overlapped previously identified variants (23). Segmented copy number data were visualized in the Integrative Genomics Viewer, and all genome coordinates were standardized to the National Center for Biotechnology Information build 36.1 (hg18) of the reference human genome.

**Site-directed mutagenesis and RAS-GTP measurement**

KRAS mutations were engineered into pcDNA3.1+2XMyr-KRAS4B using QuickChange XLII (Stratagene) as per the manufacturer’s instructions. All constructs were verified by Sanger sequencing. The level of GTP-bound, active RAS was measured using the recombinant Ras binding domain of RAP (Millipore). Briefly, 0.5 mg of lysate was immunoprecipitated using beads containing recombinant Ras binding domain. After washing, the beads were mixed with sample buffer and separated using SDS-PAGE. The membrane was probed with pan-RAS antibody to detect the levels of GTP-bound, active RAS. Total RAS levels were detected using whole-cell lysates.

**Animal studies**

Four to 6-week-old nu/nu athymic BALB/c mice were maintained in pressurized ventilated cages. All studies were performed in compliance with the Institutional Animal Care and Use Committee guidelines. Tumors were established by injecting $1 \times 10^7$ cells suspended 1:1 (vol) with reconstituted basement membrane (Matrigel). Tumor volumes were calculated using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. PD0325901 was formulated in 0.5% hydroxypropyl methylcellulose + 0.2% Tween 80 and administered by oral gavage.

**Results**

**Identification of exon 4 KRAS mutations by mass spectrometric genotyping**

Clinical data showing that KRAS mutations predict for resistance to EGFR-targeted agents has led to the adoption of limited KRAS testing in lung and colorectal cancer patients. Testing beyond the most commonly mutated exon 2 alleles, however, has not been adopted over uncertainty regarding the frequency and clinical significance of lower frequency alleles. To determine the prevalence of nonhotspot KRAS mutations, we genotyped 1,183 human tumor samples that included 415 colorectal tumors and 70 colorectal cell lines for alterations in all three RAS proteins and BRAF. This panel included colorectal tumors from all clinical states including adenomas ($n = 39$), primary invasive colorectal adenocarcinomas ($n = 322$), and lung and liver metastases ($n = 54$).

To facilitate the genomic characterization of large sample sets, we developed a MALDI-TOF MS–based assay to screen for not only the most common hotspot alterations in KRAS, NRAS, and BRAF but also less commonly reported somatic mutations in these genes (16, 17). To validate the sensitivity, specificity, and coverage of the assay, we also sequenced all coding exons of all three RAS isoforms in the 415 colorectal cancers. As shown in Fig. 1A, 49% of the tumors harbored a mutation in KRAS, NRAS, or BRAF. Consistent with prior reports, the majority of these alterations (33%) were at amino acids 12 or 13 of KRAS. Nevertheless, we also detected mutations in exons 3 and 4 of KRAS and in exons 2 and 3 of NRAS in ~10% of tumors. Specifically, 5.5% of tumors harbored exon 4 KRAS mutations (19 A146T, 1 A146V, and 3 K117N). Representative MS and Sanger sequencing traces for three of these mutations (V600E BRAF, Q22K KRAS, and A146T KRAS) are shown in Fig. 1B. Only two mutations (K117N identified in three cases and E31K in a single case) were detected exclusively by Sanger sequencing. The exon 4 (K117 and A146) mutations were confirmed somatic by directly sequencing the corresponding normal tissue DNA (see Supplementary Fig. S1). Nine tumors also harbored mutations in NRAS (three in exon 2 and six in exon 3). All NRAS and exon 3 and 4 KRAS mutations were found in a mutually exclusive pattern with mutations in KRAS exon 2 and BRAF (Figs. 1 and 2). The KRAS, NRAS, and BRAF mutations were not mutually exclusive with mutations in PIK3CA and TP53 (Fig. 2A).

Exon 4 KRAS mutations were identified not only in primary invasive colorectal tumors, but also adenomas, suggesting that these alterations occur early within the natural history of the disease (Supplementary Table S1; Fig. 2B). Notably, the clinical outcome of patients with non-G12/G13 KRAS mutations and NRAS mutations was more favorable than that of...
patients with mutations in KRAS at the G12 and G13 positions ($P = 0.006$; Fig. 2C). In our series, not a single patient with stage 1 to 3 colorectal cancer whose tumor expressed an exon 3 or 4 KRAS mutation or an NRAS mutation died of colorectal cancer (median clinical follow-up of 6.5 y). Furthermore, all four patients who underwent curative intent liver resection and whose liver metastases harbored A146T KRAS mutation were disease free following liver resection (follow-up intervals of 1.7, 12.5, 13, and 19.5 y; see Supplementary Table S1). This latter observation, if confirmed in larger data sets, would suggest that exon 4 KRAS mutation might identify a population of patients more amenable to aggressive surgical treatment of low-volume metastatic disease. Consistent with some but not all prior studies, a trend toward improved disease-specific survival was observed in a pairwise comparison of the KRAS wild-type cohort versus the cohort of patients with G12/G13 KRAS mutations, but this was not statistically significant ($P = 0.07$; Supplementary Fig. S2; refs. 24–26). Notably, patients whose tumors harbored G12/G13 KRAS mutations did exhibit a worse disease-specific survival versus those wild-type for KRAS when the non-G12/G13 KRAS mutants and NRAS mutants were included in the wild-type cohort ($P = 0.02$; Supplementary Fig. S2).

**Cells harboring exon 4 KRAS mutations exhibit elevated RAS-GTP expression and KRAS dependence**

The mutually exclusive distribution of the A146T/V and K117N KRAS mutations with those in BRAF and exons 2 and 3 of KRAS suggests that these alterations confer overlapping downstream effects. To biologically characterize the A146T and K117N mutations, we transiently expressed them as myc-tagged constructs in HEK-293FT cells. Expression of K117N and A146T KRAS mutants in HEK-293FT cells resulted in elevated RAS-GTP and phosphorylated extracellular signal-regulated

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**Figure 1. Prevalence of KRAS, NRAS, and BRAF mutations in patients with colorectal cancer.** A, 415 colorectal tumors were screened for mutations in RAS and BRAF. Light blue, exon 2 KRAS mutations; dark blue, exon 3 KRAS mutations; red, exon 4 KRAS mutations (K117 and A146); yellow, NRAS mutations; green, BRAF mutations. B, representative MS and Sanger sequencing traces are shown for tumors harboring a V600E BRAF, Q22K KRAS, and A146T KRAS mutation.
kinase (ERK) expression compared with wild-type RAS (Fig. 3A). In contrast, the E31K KRAS allele exhibited RAS-GTP levels similar to the wild-type construct (data not shown). The level of RAS-GTP expression induced by the K117N and A146T mutants was, however, lower than that observed in cells transfected with the G12D and Q22K KRAS mutants.

To further define the biological importance of the K117N and A146T/V KRAS mutations, we used our Sequenom assay to screen 351 cancer cell lines of which 70 were derived from colorectal cancers for RAS pathway mutations (Supplementary Table S2 and Fig. S3). To exclude the possibility of redundancy among the colorectal cancer cell lines due to mislabeling or cross-contamination, DNA from each cell line was characterized for 42 highly polymorphic single nucleotide polymorphisms using a MS-based assay generated specially for this purpose (see Supplementary Materials and Methods for a detailed description of the assay methods and validation). Seven unique colorectal cancer cell lines were identified, which harbored exon 4 KRAS mutations (4 A146T, 2 A146V, and 1 K117N). Matched tumor tissue was available for two cell lines (CCCL-18 and CCCL-23; ref. 27), and in both cases, we were able to confirm the presence of A146T KRAS alterations in the primary tumors from which the cell lines were derived (Supplementary Fig. S1C). None of the 281 noncolorectal cancer cell lines were found to harbor exon 4 KRAS mutations.

Figure 2. Concordance of KRAS, NRAS, BRAF, PIK3CA, and TP53 mutations. A, exon 4 KRAS mutations were nonoverlapping in distribution with mutations in KRAS exons 2 and 3, and BRAF. B, A146 KRAS mutations were identified in samples derived from all clinical states including adenomas, invasive primary colorectal adenocarcinomas, and liver metastases. C, Kaplan-Meier plot of disease-specific survival of 186 patients with stages 1 to 3 colorectal cancer as a function of KRAS/NRAS mutational status \((P = 0.006, \text{log-rank test})\). No patients with stage 1 to 3 colorectal cancer whose tumor expressed an exon 3 or 4 KRAS or NRAS mutation died of colorectal cancer. D, Sanger and MS traces of three tumors resected from a 75-y-old woman who presented with synchronous primaries in the rectosigmoid (primary 1), cecum (primary 2), and four liver metastases. She is without evidence of disease 20 mo following surgical resection of all disease sites. Primary 1 and the liver metastasis harbored A146T KRAS and R306* TP53 mutations. Primary 2 was G12V KRAS mutant and TP53 wild-type.
Cell lines expressing A146T KRAS exhibited elevated RAS-GTP expression compared with those with V600E BRAF mutation or EGFR amplification (Fig. 3B). To characterize the KRAS dependence of cells expressing an A146T KRAS mutation, we used small interfering RNA to knock down KRAS expression in LS1034 (A146T KRAS) cells. Transfection of KRAS but not nontargeting control small interfering RNA effectively downregulated KRAS expression (>90%) and led to the inhibition of colony formation (Fig. 3C).

Whereas expression of A146T KRAS in the HEK-293FT model was associated with lower RAS-GTP expression than G12D KRAS, three of four A146T KRAS cell lines expressed...
levels of RAS-GTP comparable with that expressed in models harboring G12D and G12V KRAS mutations (Fig. 3B). We hypothesized that the variable RAS-GTP expression in the A146T KRAS mutant cell lines could be the result of increased gene dosage due to KRAS gene amplification or conversion to homozygosity. In fact, we found two of the four A146T KRAS cell lines (LS1034 and CCCL-23) were homozygous for the mutant allele.

To explore the hypothesis that focal KRAS amplification may be common in tumors harboring less potent KRAS alleles, we performed DNA copy number profiling on 128 colorectal tumors and three of the A146T mutant cell lines using the Agilent 244K aCGH platform. We identified a profile of statistically significant copy number alterations using the RAE framework (22), with results consistent with previous genome-wide characterization efforts (Fig. 4A). A detailed listing of the focal areas of copy number gain and loss, and genes of interest located within these regions is included in Supplementary Tables S3 and S4 (28, 29). In our analysis, focal KRAS amplification was uncommon (2.3%, all KRAS mutant samples) and was not identified as a statistically significant region of focal copy number gain (Supplementary Table S3; Fig. 4A). We did, however, detect broader copy number gains at the KRAS locus in 24% of tumors. Although focal KRAS amplification was rare in the overall data set, the percentage of samples with KRAS copy number gain was significantly higher among tumors harboring A146T KRAS mutations versus those with exon 2 or 3 KRAS mutations or those wild-type for KRAS (36% versus 14% and 4%, respectively; \( P = 0.014 \); Fig. 4B).
was also observed in two of the three A146T KRAS mutant cell lines. As shown by fluorescence in situ hybridization (FISH) for the LS1034 cell line, the mechanism responsible for increased KRAS copy number was complex. LS1034 exhibits a hypertriploid karyotype with three normal copies of chromosome 12 and two copies of an isochromosome for 12p. In summary, these data suggest that the lower potency exon 4 KRAS alleles are more frequently associated with increased KRAS gene dosage.

**MAP/ERK kinase dependence and EGFR inhibitor resistance of colorectal cancers with exon 4 KRAS mutation**

One strategy for treating RAS-mutant tumors is to inhibit the signaling cascades downstream of RAS that mediate RAS-dependent transformation. We previously reported that cells harboring BRAF mutations are selectively sensitive to MAP/ERK kinase (MEK) inhibition, whereas tumors harboring G12/13 KRAS and Q61 NRAS mutations are variably dependent on MEK/MAPK (19, 30). To determine the MEK/ERK dependence of cell lines expressing exon 4 KRAS mutations, we used PD0325901, a selective allosteric inhibitor of MEK1/2. As shown in Fig. 5, treatment of exon 4 KRAS–expressing cells with PD0325901 resulted in the inhibition of MAPK signaling as assessed by the downregulation in the expression of phosphorylated ERK1/2 (Fig. 5C). All seven exon 4 KRAS mutant colorectal cancer cell lines were MEK dependent for proliferation (IC_{50} < 100 nmol/L; Fig. 5A and B). In BRAF-mutant tumors, MEK/ERK signaling is required for both D-cyclin expression and assembly of the cyclin D/cdk4 complex (30). Similarly, treatment of LS1034 and CCCL-18 (both A146T KRAS) cells with PD0325901 caused a marked decline in D-cyclin protein levels, induction of p27, hypophosphorylation of retinoblastoma, and a profound G_{1} cell cycle arrest (Fig. 5C). G_{1} arrest was accompanied by apoptosis in the LS1034 cell lines, but not in the other three A146T KRAS mutant models, suggesting that additional genetic or epigenetic alterations exist in these tumors that likely diminish KRAS dependence (data not shown). One candidate would be P1K3CA, which is concurrently mutated in both CCCL-23 and CCCL18, both of which show a purely cytostatic response to MEK inhibition. Consistent with prior data showing that exon 2 KRAS mutation confers resistance to EGFR-directed therapies, cell lines harboring A146T/V KRAS mutations were also resistant to the selective EGFR inhibitor gefitinib (Fig. 5D).

To explore the potential clinical utility of MEK inhibition in tumors driven by A146T KRAS mutation, mice with established A146T KRAS xenografts were treated with PD0325901. Treatment of mice bearing established LS1034 (A146T KRAS) xenografts with a single 25 mg/kg dose of PD0325901 resulted in >95% downregulation of phosphorylated ERK expression at 6 hours (Fig. 6A). MAPK pathway inhibition was associated with the downregulation of cyclin D1, and a durable increase in p27 expression and hypophosphorylation of retinoblastoma. Furthermore, chronic treatment of established LS1034 xenografts with nontoxic doses of PD0325901 was associated with complete growth suppression (Fig. 6B). In contrast to the marked sensitivity of the LS1034 model to the MEK inhibitor, this model was resistant to the EGFR-targeted antibody cetuximab (Fig. 6C). These data suggest that exon 4 KRAS mutations may predict for sensitivity to MEK inhibition and resistance to EGFR-targeted inhibitors.

**Discussion**

Several decades have passed since oncogenic RAS was first identified as the transforming factor in the Harvey and Kirsten strains of the Mouse Sarcoma Virus (1, 3–5). Since these discoveries, all three RAS family genes (KRAS, NRAS, and HRAS) have been shown to be somatically mutated in human cancer, most commonly as a result of single point mutations at codons 12, 13, and 61. Despite overwhelming evidence that oncogenic RAS plays a central role in mediating transformation in human tumors, only recently has limited testing for somatic RAS mutations entered routine clinical practice. Widespread adoption of mutational profiling in the clinic has been delayed for several reasons. First, before recent advances in sequencing technology, RAS mutational testing was expensive and time intensive. Second, until recently, there was no definitive evidence that routine testing for RAS mutations would meaningfully affect clinical practice. This changed with the identification of KRAS mutations as a predictor of resistance to EGFR kinase inhibitors in patients with lung adenocarcinoma (8). Similar data soon followed in patients with colorectal cancer, in which mutations in exon 2 of KRAS were associated with a lack of clinical benefit with panitumumab and cetuximab (9–13). Based on these data, routine testing of patients with lung and colorectal cancers has become increasingly common, and some clinical practice guidelines and regulatory agencies have proposed the restriction of anti-EGFR therapy to patients whose tumors lack G12 or G13 KRAS mutations.

In the vast majority of studies to date, tumors have been genotyped only for KRAS mutations at the most commonly altered G12 and G13 positions (31–34). The frequency, predictive, and prognostic value of other RAS mutations has therefore remained poorly defined (16, 17). To facilitate the identification of low-frequency RAS pathway mutations, we developed a multiplexed MALDI-TOF–based genotyping assay using the Sequenom platform. Consistent with prior studies, we observed that approximately one third of colorectal tumors harbored mutations at the G12 and G13 codons. Notably, an additional 10%, which would have been characterized as KRAS wild-type in clinical practice, harbored mutations in exons 3 or 4 of KRAS or in NRAS. These latter mutations were mutually exclusive with those at G12 and G13, suggesting overlapping roles in tumorigenesis.

Our data set suggests that the underrepresentation of these mutations in the literature and their low reported frequency in the COSMIC database (0.002% of the KRAS mutations reported in the large intestine) is the result of detection bias (35). To explore this possibility further, we used our MALDI-TOF assay to characterize the frequency of exon 4 KRAS mutations in several additional lineages. In an analysis of 698 noncolorectal cancer tumors and cell lines, we identified...
only two additional samples with A146 mutations (one ovarian and one endometrial cancer). A146 mutations in KRAS were also not identified in two recent analyses comprising 449 non–small cell lung cancers in which the entire coding region of the gene was sequenced (36, 37). The basis for the higher relative frequency of exon 4 KRAS mutations in colorectal cancer versus other cancers such as lung cancer is unknown, but may be the result of differences in the underlying mutagenic insults responsible for cancer initiation at these sites. We also sequenced exon 4 of both NRAS and HRAS, but detected no mutations in these exons in our colorectal tumors and cell lines. NRAS A146T mutation has, however, been reported in the leukemic cell lines NALM6 and ML-2 (16), and germline HRAS mutations at the K117 and A146 codons have been reported in few patients with Costello’s Syndrome (38).

The RAS family proteins function as small GTPases that cycle between an inactive GDP-bound and an active GTP-bound state. The slow intrinsic GTPase activity of RAS is enhanced by several orders of magnitude by GTPase-activating proteins (RAS GAPs), which include p120 GAP and NF1, which facilitate GTP hydrolysis by stabilizing an intermediate high-energy transitional state (7). The most common site of...
RAS mutation located at position 12 results in the substitution of glycine for a residue with a side chain. Crystal structure modeling predicts that this substitution is associated with steric interference with GAP-mediated GTP hydrolysis. As glycine is the only amino acid lacking a side chain, a diversity of mutations at this position confers similar phenotypic effects. Mutations at the codon 61 position also impair RAS GTPase activity but, in this case, by disrupting a hydrogen bond between the glutamine residue at position 61 of RAS and Arg789 of GAPp120 (39, 40).

Although the mutual exclusivity of the exons 2, 3, and 4 mutations in KRAS suggest significant functional overlap, the cohort of patients with non–exon 2 mutations in KRAS exhibited a better prognosis than patients whose tumors expressed G12 or G13 KRAS mutations. The most common site of KRAS mutation in exon 4 in our series was at amino acid A146. This site is within an evolutionarily conserved region which in structural modeling is predicted to interact with the guanine base of GDP (41). In contrast to mutations at codons 12 and 13, mutations at codon 146 do not impair RAS GTPase activity (42). Rather, the transforming potential of the A146 HRAS mutations has been attributed to an increase in guanine nucleotide exchange (42). As discussed above, mutations of A146 and of the biologically conserved K117 positions of HRAS have been reported in Costello’s syndrome. The most common mutant allele in Costello’s syndrome is G12S, and notably, its transforming effects are lower than that of the G12V mutation, which is among the most common mutant alleles in human cancer (43). Based on this observation, it has been speculated that only low activation alleles of RAS may be compatible with viability when found in the germline.

Given the favorable prognosis of colorectal cancer patients with exon 4 KRAS mutations and the observation that the K117 and A146 mutations are found in Costello’s syndrome, we hypothesized that these mutations may be less potent than mutations at codons 12 and 13. Although we found lower RAS-GTP expression in an isogenic model of K117 and A146T KRAS, in some A146T KRAS–expressing cancer cell lines, we observed levels of RAS-GTP comparable with that of cell lines harboring the G12D/V mutations. Our data suggest that whereas A146T KRAS mutation may confer lower intrinsic RAS activity, this may be augmented in part by frequent conversion to homozygosity and low-level copy number gain of the KRAS gene locus.

Our analysis suggests that a broader assessment of RAS mutations beyond the most common mutations in exon 2 is warranted and would lead to the identification of a mutation predicted to confer EGFR inhibitor resistance in close to 50% of patients with colorectal cancer (12, 19). Although such testing would decrease the use of toxic and expensive agents in this population unlikely to derive benefit, it would also further limit the available treatment options in a disease in which few currently exist. This and the inability to date to identify a clinically effective inhibitor of RAS have contributed to the reluctance of many clinicians to advocate broader RAS mutation testing. It should be noted, however, that the prospective identification of RAS mutations could also have the secondary benefit of accelerating the clinical development of novel therapies in this class of patients by facilitating the identification of those most likely to benefit. Promising therapeutic approaches include targets that function as synthetic lethals in RAS mutant tumors and inhibitors of downstream effectors such as MEK (30, 44–47). Our data showing complete growth inhibition in A146T KRAS–expressing xenografts with the selective MEK inhibitor PD0325901 support the clinical feasibility of this latter approach.

In summary, our data support a more comprehensive assessment of RAS mutational status beyond the most frequently mutated alleles at positions 12, 13, and 61. The ability to use a multiplexed platform makes such an approach feasible even in the case of low-frequency alleles.

Figure 6. The growth of A146T KRAS mutant xenografts was MEK dependent. A, treatment of mice bearing established LS1034 xenografts with a single oral dose of PD0325901 (25 mg/kg) resulted in downregulation of pERK and cyclin D1 expression at 6 h and upregulation of p27 at 24 h. UnTx, untreated control. B, mice with established LS1034 (A146T KRAS) xenografts were treated with PD0325901 (25 mg/kg 5×/wk× 3 wk) or vehicle only as control. C, mice were treated with 10 mg/kg of cetuximab or vehicle only as control twice weekly for 3 wk.
Our data also support the hypothesis that different RAS alleles have overlapping but not identical biological activities and may thus confer differential prognostic effects. These differences may affect the choice of therapy in individual cases and may be exploited to therapeutic advantage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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