KRAS Mutation Status Is Associated with Enhanced Dependency on Folate Metabolism Pathways in Non–Small Cell Lung Cancer Cells

Diarmuid M. Moran¹, Patricia B. Trusk¹, Karen Pry¹, Keren Paz⁴, David Sidransky³,⁴, and Sarah S. Bacus¹,²

Abstract

KRAS gene mutation is linked to poor prognosis and resistance to therapeutics in non–small cell lung cancer (NSCLC). In this study, we have explored the possibility of exploiting inherent differences in KRAS-mutant cell metabolism for treatment. This study identified a greater dependency on folate metabolism pathways in KRAS mutant compared with KRAS wild-type NSCLC cell lines. Microarray gene expression and biologic pathway analysis identified higher expression of folate metabolism– and purine synthesis–related pathways in KRAS-mutant NSCLC cells compared with wild-type counterparts. Moreover, pathway analysis and knockdown studies suggest a role for MYC transcriptional activity in the expression of these pathways in KRAS-mutant NSCLC cells. Furthermore, KRAS knockdown and overexpression studies demonstrated the ability of KRAS to regulate expression of genes that comprise folate metabolism pathways. Proliferation studies demonstrated higher responsiveness to methotrexate, pemetrexed, and other antifolates in KRAS-mutant NSCLC cells. Surprisingly, KRAS gene expression is downregulated in KRAS wild-type and KRAS-mutant cells by antifolates, which may also contribute to higher efficacy of antifolates in KRAS-mutant NSCLC cells. In vivo analysis of multiple tumorgraft models in nude mice identified a KRAS-mutant tumor among the pemetrexed-responsive tumors and also demonstrated an association between expression of the folate pathway gene, methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), and antifolate activity. Collectively, we identify altered regulation of folate metabolism in KRAS-mutant NSCLC cells that may account for higher antifolate activity in this subtype of NSCLC. Mol Cancer Ther; 13(6); 1611–24. ©2014 AACR.

Introduction

Folates play an important role in many cellular biosynthetic processes and are essential for cell growth and proliferation (1). Folate metabolism is critical for the biosynthesis of amino acids such as methionine and nucleotides, including purines and pyrimidines. Folate is reduced to tetrahydrofolate (THF) in steps that are catalyzed intracellularly by dihydrofolate reductase (DHFR). In an interconnected and dependent pathway, thymidylate synthase (TYMS) maintains cellular thymidine pools that are essential for DNA replication and repair using THF cofactors generated from folate metabolism. Mitochondrial metabolism of THF also generates glycine, methylene–THF, and N-10 formyl-THF, which are required for de novo synthesis of purines and thymidylate. Mitochondrial folate metabolism enzymes such as serine hydroxymethyl transferases (SHMT1 and 2), methylenetetrahydrofolate dehydrogenase (MTHFD2), and methylenetetrahydrofolate dehydrogenase 1-like (MTHFD1L) play important roles in the generation of these THF cofactors.

Inhibition of folate metabolism pathways interferes with DNA replication leading to growth arrest and/or cell death in rapidly dividing cells. Methotrexate, a DHFR inhibitor, was introduced in the clinic for the treatment of childhood acute lymphoblastic leukemia and has been subsequently used in the treatment of other cancers (2). Recent cell line studies have demonstrated that higher expression of myc-regulated, proliferation-related, and folate/sulfate–glycine metabolism genes was positively associated with responses to methotrexate (3, 4). More recently, pemetrexed, an inhibitor of TYMS, glycaminide ribonucleotide transformylase (GART—an enzyme involved in the formation of the imidazole ring of purines) and DHFR, has been approved for the treatment of malignant pleural mesothelioma and advanced or metastatic
nonsquamous non–small cell lung cancer (NSCLC; 5, 6). In clinical trials comparing cisplatin/gemcitabine with cisplatin/pemetrexed in patients with advanced-stage NSCLC, pemetrexed was associated with enhanced benefit in terms of overall survival for tumors with adenocarcinoma and large cell histology compared with squamous histology (7). Outside of histology, patient selection biomarkers have not been clinically implemented for pemetrexed treatment in NSCLC or other cancers.

RAS proteins are small GTPases that are activated in response to various cellular stimuli (8). RAS activation initiates downstream signaling in multiple cellular pathways (PI3K–AKT, RAF–MEK–ERK, and RAL–GEF) that regulate cellular processes such as proliferation, survival, and differentiation. Four highly homologous RAS proteins have been identified: HRAS, NRAS, KRAS4A, and KRAS4B (KRAS 4B is the most common splice variant of KRAS and is referred to throughout this study as KRAS). KRAS mutations are most frequently observed in pancreatic, colorectal, lung, endometrial, and biliary tract cancers (8). Oncogenic KRAS mutations are present in 15% to 30% of the adenocarcinoma subtype of NSCLC and less frequently in squamous cell carcinomas (<5%; ref. 9). Missense mutations primarily result in amino acid substitutions at residues G12, G13, or Q61 in KRAS leading to its constitutive activation (8). KRAS mutations are negative prognostic indicators in NSCLC (10) and have been associated with poor response to drugs targeting EGFR in NSCLC and colorectal carcinoma (10–12). Despite many attempts, pharmacologic targeting of KRAS remains a challenge (13–18). Recent studies have identified alterations in metabolic programming that are specific to RAS–mutant cells such as enhanced glucose uptake, decreased tricarboxylic acid (TCA) cycle activity and higher dependency on glutamine for biosynthetic reactions than RAS wild-type cells (19–21). Little is known about the relationship between folate metabolism and KRAS mutation status. One study examined the influence of dietary one-carbon nutrients such as folate on KRAS mutation in wild-type cells (19–21). Little is known about the relationship between folate metabolism and KRAS mutation status. In this study, we focused on understanding the relationship to differ according to KRAS mutational status. In this colon cancer (22). However, influence of dietary one-carbon nutrient intake on colon cancer risk did not seem to differ according to KRAS mutational status. In this study, we focused on understanding the relationship between folate metabolism and KRAS mutation status in NSCLC.

Materials and Methods

Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC) and expanded upon delivery into numerous vials of low-passage cells for cryopreservation. Cells were passaged for up to 6 months after resuscitation. Cell line characterization by the ATCC is conducted through short tandem repeat typing. Reauthentication was not conducted. A549 cells were cultured in Iscove’s Modified Dulbecco Minimum Essential Medium; Calu6 cells were cultured in Eagle’s minimal essential medium; H460, H2122, H358, H1792, H1754, H661, H2126, H1993, H1299, H1395, H23, H1975, and Calu3 cells were cultured in RPMI-1640. All media were purchased from Mediatech and supplemented with 10% FBS (Life Technologies) and antibiotics (Mediatech). Cells were cultured in a 37°C, 5% CO2 humidified environment.

Cell proliferation assay

Of note, 6,000 cells per well were seeded into 96-well culture plates and left to attach overnight. Overnight culture media were removed and cells were treated with 0 to 10 μM/L of either methotrexate (Sigma-Aldrich) or pemetrexed (LC Laboratories) or the appropriate vehicle in 100 μL of treatment media. Cells were left in culture for 72 hours. Cyquant Direct Cell Proliferation Assay (Life Technologies) was used to assess cell proliferation. Of note, 100 μL of diluted Cyquant reagent was added to each well and incubated at 37°C for 1 hour. Plates were read on a Bio-Tek Synergy 2 plate reader.

RNA isolation

Following treatment, total RNA was isolated using the miRNeasy Kit (Qiagen Inc.) according to the manufacturer’s instructions. Quality and concentration were assessed using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

Gene expression

Gene expression levels were assessed on isolated RNA by real-time reverse transcription PCR (RT-PCR) analysis using the High Capacity cDNA Reverse Transcription Kit and TaqMan Gene Expression Master Mix (both Life Technologies). Relative expression was calculated using the ΔΔCt method on an ABI 7900HT Fast Real-Time PCR System (Life Technologies). TaqMan gene expression assays from Life Technologies included: KRAS (Hs00364284_g1), DHFR (Hs0075828_s1), TMY5 (Hs00426586_m1), MTHFD2 (Hs00759197_s1), SLC19A1 (Hs00953344_m1), GART (Hs00894582_m1), SHMT2 (Hs01052963_s1), Huj-actin (4326315E), and MYC (Hs00153408-m1). Huj-2M control (4326319E) was used for normalization.

KRAS overexpression

K-RAS2MYC–tagged vector (cloneID, RASK20MN00; a gift from Dr. David Solit’s laboratory) or pcDNA3.1+ vector alone (Life Technologies) was transfection into the cells using Lipofectamine 2000 (Life Technologies). Briefly, 500 ng or 1 μg of DNA was incubated with the Lipofectamine 2000 in serum and antibiotic-free media on the cells for 4 hours at 37°C. Transfection media were removed without washing the cells and replaced with regular growth media. Cells were harvested after 48 hours for gene expression analysis as described above.

siRNA knockdown

KRAS and MYC levels were knocked down using siRNA-targeting KRAS and MYC, Dharmacon ON-TARGET SMARTpool, Human KRAS (cat# L-005069) or...
KRAS activity assay

A549 cells were treated for 48 hours with 0.5 μmol/L of either methotrexate (Sigma-Aldrich) or pemetrexed (LC Laboratories) or the appropriate vehicle in the appropriate media. Following treatment, changes in KRAS activity were analyzed using the K-Ras Activation Assay Kit (Cell Biolabs, Inc.). Briefly, cells were harvested in the supplied lysis buffer, protein concentration was determined and equivalent amounts of protein were pulled down as per the manufacturer’s instructions. Samples were electrophoresed on a 4% to 15% TGX gel (Bio-Rad Laboratories), transferred to polyvinylidene difluoride membrane and blotted for KRAS protein levels using the supplied KRAS-specific antibody. In addition, 30 μg of total protein lysate was electrophoresed and blotted in the same manner.

Immunohistochemistry

Formalin-fixed paraffin-embedded 5-μm sections were stained for MTHFD2 levels by immunohistochemistry (IHC). Antigen retrieval was performed in the Dako PTLink in Envision FLEX Target Retrieval Solution, low pH (Dako). Staining was carried out in a Dako Link 48 Autostainer. The sections were stained with a primary antibody to MTHFD2 (Novus) at a 1:500 dilution for 30 minutes. All other reagents used in the staining process were Dako Envision Flex Reagents (Dako). Sections from a Hodgkin’s nodular sclerosis lymph node were used as a positive control. Hematoxylin (Surgipath/Leica) was used as the counterstain. Image analysis was performed on an Aperio Scanscope XT using Spectrum Software version 10.2.5.2352 and Aperio Image Scope software. Average optical density for each tissue was determined using the color deconvolution algorithm on images from at least five regions in each tissue.

Tumorgraft studies

In vitro experiments were performed at the Champions Oncology (Champions Oncology), which is accredited and regulated by the Institutional Animal Care and Use Committee (IACUC). Pemetrexed sensitivity testing was conducted using TumorGraft models established from resected tumors of the lung. Explants were previously implanted in immunodeficient mice for the purpose of propagating the tumor to provide sufficient tumor models for testing. NSCLC tumorgrafts were implanted in the flanks of nude mice and allowed to grow. At approximately 150 mm³ tumor volume, animals were randomized and dosing with pemetrexed (n = 5/tumorgraft, 150 mg/kg, i.p. everyday × 5 × 2) or saline control (n = 10/tumorgraft) was initiated. Pemetrexed was formulated in saline. Tumor dimensions were measured twice weekly by digital caliper and mean estimated tumor volumes ± SEM were graphed for each tumorgraft. Tumor volume was calculated using the formula: TV = width² × length × π/2.

Ion torrent AmpliSeq analysis

DNA was isolated from snap-frozen TumorGraft samples using the PureLink Genomic DNA Mini Kit exactly as the manufacturer describes (Life Technologies). Sequencing libraries were generated from isolated DNA using the Ion Ampliseq Cancer Hotspot Panel v2 Primer Kit and the Ion AmpliSeq Library Kit 2.0 reagents exactly as the manufacturer describes (Life Technologies). Sequencing was performed on the Ion Torrent Personal Genome Machine device and analysis was performed using Torrent Suite Software v3.0 and Variant Caller Plugin as the manufacturer describes (Life Technologies).

Ingenuity pathway analysis

Gene chip robust multiarray averaging-normalized (as described in ref. 23) basal gene expression microarray data for nonsquamous NSCLC cell lines from the study of Shankavaram and colleagues (23) were compiled and grouped according to KRAS mutation status. Genes showing significant differences (Student t test P < 0.05, >1.5-fold cutoff) in expression between KRAS-mutant and KRAS wild-type cells were analyzed using ingenuity pathway analysis (IPA) software (Ingenuity Systems). Canonical pathways that differed most significantly between KRAS-mutant and KRAS wild-type NSCLC cells across the entire dataset were determined from IPA. The P value (determined by Fisher exact test right-tailed) and the ratio of genes in a given pathway that meet cutoff criteria relative to the genes that make up that pathway were reported from IPA. Overlapping canonical pathways from IPA that share common genes in the dataset were also analyzed by IPA and reported (including Fisher exact test P value). Upstream regulator analysis (IPA) was performed to predict the transcriptional regulators most associated with differential gene expression in KRAS-mutant cells. The Z-score and P value (Fisher exact test) were reported for each regulator.

Statistical analysis

Tumor sizes and gene expression were compared using Student t tests. Proliferation studies were analyzed using Mann–Whitney U tests. For all the tests conducted, the P value of <0.05 was considered statistically significant, and all tests were two-tailed. The statistical analyses were conducted using GraphPad Prism 5.0 software.

Results

RAS-mutant cancer cells display altered metabolism compared with their nonmutant counterparts, which includes increased activity of various biosynthetic pathways (19–21). To explore the therapeutic consequences of KRAS-mutant–specific metabolic alterations, we initially
analyzed microarray data from the publically available NCI-60 dataset from the study of Shankavaram and colleagues (23). In this study, basal gene expression across all NCI-60 cell lines was determined using gene expression microarrays (Affymetrix HGU-95). GCRMA-normalized microarray gene expression data were downloaded from the NCI Cellminer website (http://discover.nci.nih.gov/cellminer; ref. 24). For our purposes, basal gene expression data for nonsquamous NSCLC cell lines in this study were compiled and grouped according to KRAS mutation status. Genes showing significant differences ($P < 0.05$, $>1.5$-fold cutoff) in expression between KRAS-mutant (KRAS-mut) and KRAS wild-type (KRASwt) cells were analyzed using IPA, which assigns genes into biologic pathways and networks. Analysis of the canonical pathways that differed most significantly between KRAS-mut and KRASwt NSCLC cells identified upregulation of multiple metabolic pathways related to de novo purine biosynthesis and folate metabolism in KRASmut cells (Fig. 1A; Supplementary Table S1). Genes associated with 5-aminoimidazole ribonucleotide biosynthesis, a key intermediate in the biosynthesis of purine nucleotides and thiamine, were also elevated in KRASmut cells. Analysis of the relatedness of canonical pathways that share common genes in this dataset also identified a network of interrelated metabolic canonical pathways in KRASmut NSCLC cells that converge on purine biosynthesis, folate metabolism, and to a lesser extent glycine biosynthesis (Fig. 1B); and identified genes that are shared by these pathways (Table 1). IPA upstream regulator analysis was next used to predict which upstream regulators may be activated or inhibited to explain the gene expression patterns observed in KRASmut cells. Upstream regulator analysis predicted both upregulation and downregulation of various upstream regulators in KRASmut compared with KRASwt cells (Supplementary Table S2). Of these regulators, MYC was predicted to be the strongest transcriptional regulator in KRAS-mutant cells based on altered expression of its target genes (Fig. 1C; Supplementary Table S3). Furthermore, KRAS-mutant–related metabolic genes were also identified as transcriptional targets of MYC (Fig. 1C, highlighted in red box). Among others, IPA also predicted increased activity of MEK and cyclin D1 in KRASmut cells, which have been previously identified as upregulated regulators in KRASmut cells, and which adds confidence to the predictive capacity of this approach.

To further investigate the relationship between KRAS, MYC, and the expression of folate metabolism–related genes, A549 (KRASmut) cells were transfected with siRNA-targeting KRAS (siKRAS), siRNA-targeting MYC (siMYC), or control siRNA (siCTRL). Quantitative RT-PCR (qRT-PCR) using primers specific to key folate metabolism–related genes was performed on RNA isolated from cells 48 hours after siRNA transfection. Analysis of KRAS gene expression demonstrated potent knockdown of KRAS gene expression in siKRAS-transfected cells (Fig. 2A). KRAS knockdown also significantly decreased $MTHFD2$, $GART$, $SHMT2$, and the folate transporter, $SLC19A1$ (Fig. 2A). DHFR and TYMS expression as assessed by qRT-PCR was not significantly decreased by KRAS knockdown. Similarly, MYC gene expression was strongly inhibited in siMYC-transfected cells and concomitant decreases in $MTHFD2$, $GART$, $SHMT2$, $SLC19A1$, and to a lesser extent DHFR and TYMS were observed (Fig. 2B). Conversely, transfection of A549 cells with a KRAS expression vector followed by qRT-PCR 48 hours after transfection using primers specific to folate pathway genes demonstrated significant upregulation of gene expression for all folate pathway genes studied (Fig. 2C). Collectively, these data suggest that KRAS partially regulates the expression of key folate genes in NSCLC cells and suggests that MYC plays a role in the regulation of these genes in KRAS-mutant NSCLC cells. Furthermore, although KRAS overexpression can drive the expression of DHFR and TYMS, siRNA experiments suggest that these genes may not be basally regulated by KRAS in KRASmut cells.

Because of our observation of elevated folate pathway gene expression in KRASmut NSCLC cells, we proposed that KRASmut NSCLC cells may have a greater dependency on folate metabolism pathways. To investigate this, we treated NSCLC cell lines (Table 2) with methotrexate or pemetrexed (0–10 μmol/L) and assayed for proliferation 72 hours later. Mutation status of KRAS in each cell line was determined from the Sanger Catalogue of Somatic Mutations in Cancer database (COSMIC; http://www.sanger.ac.uk/genetics/CGP/cosmic) and was confirmed by KRAS pyrosequencing (data not shown). KRASwt cell lines were relatively resistant to methotrexate and pemetrexed with average IC$_{50}$ values greater than 10 μmol/L (Fig. 3A and B). Conversely, KRASmut cell lines were significantly more sensitive to both methotrexate and pemetrexed with average IC$_{50}$ values less than 0.3 μmol/L. Furthermore, an NRAS-mutant (Q61K) cell line (H1299) was sensitive to both methotrexate and pemetrexed. To corroborate our findings, we next analyzed data from two independent datasets. First, we interrogated the Sanger Institute Genomics of Drug Sensitivity in Cancer project database, a study in which over 130 drugs were tested on 639 cancer cell lines (4). Methotrexate drug response data from this database were compiled for 63 nonsquamous NSCLC cell lines and grouped according to KRAS mutation status. In support of our findings, KRASmut NSCLC cells were significantly more responsive to methotrexate than KRASwt NSCLC cells in this study (Fig. 3C). Next, we interrogated the NCI Developmental Therapeutics Program cancer drug screen database to identify compounds that selectively inhibit the proliferation of KRASmut NSCLC cell lines (25). Data from this study demonstrated that methotrexate and other antifolates (trimetrexate and soluble Baker antifol) have significantly lower GI$_{50}$ values in KRASmut versus KRASwt NSCLC cells (Fig. 3D). Collectively, these data demonstrate a greater susceptibility to
Folate inhibition by multiple folate inhibitors in KRAS-mut cells, thereby suggesting a greater dependency on folate pathways for KRASmut versus KRASwt NSCLC cells.

We next examined KRAS gene expression in the presence of antifolates. Representative KRASmut and KRASwt NSCLC cell lines were treated with methotrexate (0.1 μmol/L), pemetrexed (0.1 μmol/L), or vehicle.
When tumorgrafts reached approximately 150 mm$^3$, animals were randomized and dosing with pemtrexed was initiated. After 30 days of treatment (150 mg/kg i.p. everyday x 5 x 2), two of five tumorgrafts demonstrated significantly lower tumor volumes with pemtrexed treatment compared with saline-treated controls (Fig. 5A and B). Molecular profiling of pemtrexed-responsive tumorgrafts using the Ion Torrent Ampliseq Cancer Hotspot Panel V2 identified a KRAS G12V mutation (CTG0164) in one of these tumors, whereas no oncogenic mutations from the Ion Torrent Ampliseq Cancer Hotspot Panel V2 were detected in the other pemtrexed-responsive tumorgraft (CTG0159). In nonresponsive tumorgrafts, mutational analysis identified a MET T1010I and a TP53 Y220C mutation in one of the tumorgrafts (CTG0160) studied and a KIT M541L mutation in another of these tumorgrafts (CTG0163); however, no oncogenic mutations from the Ion Torrent Ampliseq Cancer Hotspot Panel V2 were detected in the remaining tumorgraft (CTG0162). To further explore the relationship between pemtrexed response and folate pathway expression, we examined the protein expression of the critical folate pathway member, MTHFD2, in tumorgrafts. We selected this gene because it was a member of the folate pathway that had one of the highest fold differences in gene expression between KRAS-mutant and KRAS wild-type cells because these cells are typically more dependent on the presence of active KRAS for survival and growth than wild-type cells (26, 27).

To further investigate the biomarkers of response to antifolates in NSCLC tumors, we examined the in vivo activity of pemtrexed on five tumorgrafts (patient derived xenografts) that were obtained from primary resected tumors of 5 patients with NSCLC. Each tumorgraft, which was previously expanded in nude mice, was implanted in the flanks of nude mice and allowed to grow. When tumorgrafts reached approximately 150 mm$^3$, animals were randomized and dosing with pemtrexed was initiated. After 30 days of treatment (150 mg/kg i.p. everyday x 5 x 2), two of five tumorgrafts

<table>
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<tr>
<th>Gene symbol</th>
<th>Entrez gene name</th>
<th>P value KRASmut vs. KRASwt</th>
<th>Fold difference KRASmut vs. KRASwt</th>
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NOTE: Normalized basal gene expression microarray data for nonsquamous NSCLC NCI60 cell lines were grouped according to KRAS mutation status. Genes showing significant differences in expression between KRAS-mutant and KRAS wild-type cells were analyzed using IPA and significantly different pathways were identified.
negative (CTG0160 and CTG0162) or low tumoral expression of MTHFD2 (CTG0163). Collectively, these data highlight the activity of pemetrexed in KRASmut tumors and provide preliminary correlations between folate pathway gene expression and response to antifolate drugs.

Figure 2. KRAS regulates folate pathway gene expression in NSCLC cells. A549 (KRAS mutant) NSCLC cells were treated with transfection reagent only or transfected with control siRNA (siControl), siRNA-targeting KRAS (siKRAS; A) or siRNA-targeting MYC (siMYC; B). RNA was harvested 48 hours after treatment and was subsequently analyzed by qPCR using primers specific to KRAS, MYC, DHFR, TYMS, SHMT2, MTHFD2, GART, and SLC19A1. Gene expression was normalized to internal β-2-microglobulin control and expressed as fold change versus transfection reagent only control. (ˌ, \( P < 0.05 \) by Student \( t \) test, \( n = 3; \) siKRAS or siMYC vs. siControl). C, A549 NSCLC cells were transfected with pcDNA3.1 empty vector (1,000 ng) or pcDNA3.1 KRAS (500 and 1,000 ng). RNA was harvested 48 hours after treatment and was subsequently analyzed by qPCR using primers specific to KRAS, DHFR, TYMS, SHMT2, MTHFD2, GART, and SLC19A1. Gene expression was normalized to internal β-2-microglobulin control and expressed as fold change versus pcDNA3.1 empty vector control. (ˌ, \( P < 0.05 \) by Student \( t \) test, \( n = 3; \) pcDNA 3.1 KRAS vs. pcDNA 3.1 empty vector).
Pharmacologic targeting of mutant KRAS cancer cells has proven to be difficult. Farnesyltransferase inhibitors, that prevent the anchoring of KRAS to the plasma membrane, were previously developed as drug candidates for KRAS-mutant cancers but failed because of resistance that was primarily mediated through the activity of the redundant geranylgeranylation pathway as an alternative means to mediate this anchoring step (13, 14, 28). More recently, genes such as \textit{PLK1} (15), \textit{STK33} (16), \textit{TBK1} (17), and \textit{CDK4} (18) have been identified as synthetically lethal in cells harboring KRAS mutation and, thus, these proteins may be pharmacologically tractable targets in KRAS-mutant cancer cells; however, the clinical relevance of these approaches remains to be validated.

In this study, we explored the possibility of exploiting inherent differences in KRAS-mutant cell metabolism for treatment. Our study identifies a greater dependency on folate metabolism pathways in KRAS-mutant compared with KRAS wild-type NSCLC cell lines. Furthermore, we have demonstrated a relationship between KRAS mutation status and folate pathway gene expression. In prior studies, KRAS-mutant tumors have been recognized to be associated with altered metabolic processes versus their wild-type counterparts. This includes higher glucose uptake, decreased TCA cycle activity and higher dependency on glutamine for biosynthetic reactions than RAS wild-type cells (19–21, 29). Previous studies have also shown that mutant KRAS can direct glucose into the nonoxidative arm of the pentose phosphate pathway, resulting in increased ribose-5-phosphate production (20, 29). Moreover, blocking the ribose-5-phosphate pathway decreases KRAS-dependent tumorigenesis, indicating that the role of KRAS in ribose biosynthesis is critical to its oncogenicity. In our study, we have identified elevated expression of genes associated with purine biosynthesis and folate metabolism in KRAS-mutant NSCLC cells. We also identify an increase in the 5-aminoimidazole ribonucleotide biosynthesis pathway, which converts ribose-5-phosphate into 5-aminoimidazole ribonucleotide, a key intermediate in purine synthesis. Purine synthesis requires factors generated from other metabolic reactions, including ribose-5-phosphate generated from the pentose phosphate pathway, THF cofactors generated from folate metabolism, glycine, and the amide nitrogen groups generated from glutamine and aspartate metabolism (30). We propose that KRAS mutation drives increased purine synthesis activity and as a result an elevated dependency on the factors needed to feed this biosynthetic pathway such as those generated by folate metabolism. Thus, antifolates can indirectly inhibit purine synthesis through the depletion of folate cofactors and may account for the stronger response to these agents in KRAS-mutant cells.

In our analysis, we identify the ability of KRAS to modulate the expression of genes related to folate metabolism and have established a role for MYC in the regulation of these genes in KRAS-mutant NSCLC cells. Although it is possible that increased metabolic gene expression may reflect a higher proliferative index, and thus increased metabolic demand in KRAS-mutant cells, previous studies have provided convincing evidence that KRAS can actively control metabolic gene expression in the absence of other phenotypic changes (20). Our study highlights that this may also be the case for genes associated with purine synthesis and folate metabolism. Interestingly, transcriptomic analysis predicted MYC as the

**Table 2.** NSCLC cell lines used in this study

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 NOTE: Mutation status of each cell line in KRAS, NRAS, BRAF, and EGFR was determined from the Sanger Catalogue of Somatic Mutations in Cancer database (COSMIC, http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Abbreviation: WT, wild-type.

Discussion

Pharmacologic targeting of mutant KRAS cancer cells has proven to be difficult. Farnesyltransferase inhibitors, that prevent the anchoring of KRAS to the plasma membrane, were previously developed as drug candidates for KRAS-mutant cancers but failed because of resistance that was primarily mediated through the activity of the redundant geranylgeranylation pathway as an alternative means to mediate this anchoring step (13, 14, 28). More recently, genes such as \textit{PLK1} (15), \textit{STK33} (16), \textit{TBK1} (17), and \textit{CDK4} (18) have been identified as synthetically lethal in cells harboring KRAS mutation and, thus, these proteins may be pharmacologically tractable targets in KRAS-mutant cancer cells; however, the clinical relevance of these approaches remains to be validated.

In this study, we explored the possibility of exploiting inherent differences in KRAS-mutant cell metabolism for treatment. Our study identifies a greater dependency on folate metabolism pathways in KRAS-mutant compared with KRAS wild-type NSCLC cell lines. Furthermore, we have demonstrated a relationship between KRAS mutation status and folate pathway gene expression. In prior studies, KRAS-mutant tumors have been recognized to be associated with altered metabolic processes versus their wild-type counterparts. This includes higher glucose uptake, decreased TCA cycle activity and higher dependency on glutamine for biosynthetic reactions than RAS wild-type cells (19–21, 29). Previous studies have also shown that mutant KRAS can direct glucose into the nonoxidative arm of the pentose phosphate pathway, resulting in increased ribose-5-phosphate production (20, 29). Moreover, blocking the ribose-5-phosphate pathway decreases KRAS-dependent tumorigenesis, indicating that the role of KRAS in ribose biosynthesis is critical to its oncogenicity. In our study, we have identified elevated expression of genes associated with purine biosynthesis and folate metabolism in KRAS-mutant NSCLC cells. We also identify an increase in the 5-aminoimidazole ribonucleotide biosynthesis pathway, which converts ribose-5-phosphate into 5-aminoimidazole ribonucleotide, a key intermediate in purine synthesis. Purine synthesis requires factors generated from other metabolic reactions, including ribose-5-phosphate generated from the pentose phosphate pathway, THF cofactors generated from folate metabolism, glycine, and the amide nitrogen groups generated from glutamine and aspartate metabolism (30). We propose that KRAS mutation drives increased purine synthesis activity and as a result an elevated dependency on the factors needed to feed this biosynthetic pathway such as those generated by folate metabolism. Thus, antifolates can indirectly inhibit purine synthesis through the depletion of folate cofactors and may account for the stronger response to these agents in KRAS-mutant cells.

In our analysis, we identify the ability of KRAS to modulate the expression of genes related to folate metabolism and have established a role for MYC in the regulation of these genes in KRAS-mutant NSCLC cells. Although it is possible that increased metabolic gene expression may reflect a higher proliferative index, and thus increased metabolic demand in KRAS-mutant cells, previous studies have provided convincing evidence that KRAS can actively control metabolic gene expression in the absence of other phenotypic changes (20). Our study highlights that this may also be the case for genes associated with purine synthesis and folate metabolism. Interestingly, transcriptomic analysis predicted MYC as the
strongest regulator of gene expression in KRAS-mutant NSCLC cells. Moreover, siRNA experiments targeting MYC in KRAS-mutant cells confirmed a regulatory role for MYC in the expression of folate- and purine synthesis–related genes. MYC has been shown to be required for RAS-dependent tumor maintenance and is associated with metabolic reprogramming in other KRAS tumor models (20, 31, 32). Furthermore, knockdown experiments have shown that MYC plays a role in the effect of KRAS on the pentose phosphate pathway (20). Vazquez
and colleagues reported a correlation between MYC gene expression signatures and methotrexate sensitivity. Moreover, a correlation between overexpression of mitochondrial folate pathway genes and response to methotrexate in cancer cells has been described previously (3). We show for the first time that a similar antifolate-response signature, which includes dominance of MYC transcriptional activity and enhanced expression of folate pathway genes, is associated with a KRAS-mutant phenotype. Collectively, this evidence supports the hypothesis that KRAS drives metabolic gene expression, potentially through the transcriptional activity of MYC, which may account for the greater dependency of KRAS-mutant cancer cells on folate metabolism.

In our study, we also report a robust downregulation of KRAS expression at the RNA level with antifolate treatment in both KRAS-mutant and wild-type cells. Furthermore, total KRAS protein expression and KRAS activity were decreased in KRAS-mutant cells. In our study, KRAS protein levels in KRAS wild-type cells were below the level of detection by Western blots in our activity assays and, thus, changes in KRAS activity were not detected (data not shown). However, due to the decrease in KRAS gene expression levels detected by quantitative PCR (qPCR) in KRAS wild-type cells, we expect that similar decrease in total KRAS protein levels occur. KRAS downregulation using siRNA is growth inhibitory in KRAS-mutant cells with little to no effect in KRAS wild-type counterparts highlighting the dependency of KRAS-mutant cells on KRAS activity for survival, proliferation, and tumorigenicity (26, 27). Furthermore, downregulation of mutant KRAS has been shown to potentiate responses to other drugs (27, 33). Methotrexate has also been implicated in the inhibition of isoprenylcysteine carboxyl methyltransferase, an enzyme necessary for KRAS membrane localization and activation. Although these effects on KRAS localization may contribute to the potent activity of antifolates in KRAS-mutant cells, we...
propose that KRAS downregulation will have a greater impact than localization on KRAS activity. In summary, the downregulation of KRAS RNA expression may also contribute to the enhanced response to pemetrexed and methotrexate observed in KRAS-mutant cells. The mechanism leading to KRAS downregulation by antifolates is unclear. Folate deficiency has been previously shown to modulate specific miRNA expression in cells (34). Preliminary data in our laboratory suggest that treatment with antifolates alters the expression of specific miRNA families in target cells irrespective of KRAS mutation status (data not shown). Cellular stress in antifolate-treated cells may induce changes in miRNA expression (targeting KRAS) that account for KRAS downregulation observed; however, this mechanism requires further study.

In support of our in vitro findings, we examined pemetrexed response in NSCLC tumorgrafts. Tumorgrafts typically maintain the fundamental genotypic and phenotypic features of the original tumor and have been shown to be highly predictive of patient response to therapies (35, 36). Even in the limited number of tumorgrafts included in this study, initial findings are revealing. In this study, we demonstrate that two of five tumorgrafts responded to pemetrexed treatment. Interestingly, one of the responsive tumorgrafts was KRAS-mutant demonstrating that KRAS-mutant tumors are responsive in vivo.

Figure 5. KRAS-mutant tumorgrafts respond to pemetrexed in vivo. Tumorgrafts that show response to pemetrexed (responders; A) and tumorgrafts that lack response to pemetrexed (nonresponders; B) were identified in nude mice models. NSCLC tumorgrafts were implanted in the flanks of nude mice and allowed to grow. At approximately 150 mm³ tumor volume, animals were randomized and dosing with pemetrexed (n = 5/tumorgraft, 150 mg/kg, i.p.; everyday × 5 × 2) or saline control (n = 10/tumorgraft) was initiated. Mean estimated tumor volumes ± SEM were graphed for each tumorgraft. **, P < 0.01; ***, P < 0.001 by Student t test analysis. Mutations detected using the Ion Torrent AmpliSeq Cancer Hotspot Panel V2 on DNA isolated from each tumorgraft are identified in parentheses under each tumorgraft.
Importantly, we also report preliminary evidence, suggesting a correlation between the protein expression of the folate pathway gene, MTHFD2, and response to pemetrexed. MTHFD2 is a mitochondrial enzyme that contains methylenetetrahydrofolate dehydrogenase and methenylltetrahydrofolate cyclohydrolase activity. This enzyme is critical to the synthesis and use of methylene-THF, N-10 formyl-THF, and glycine for de novo purine and thymidylate synthesis. The expression of this gene was one of the most significantly correlated folate pathway genes with the KRAS-mutant phenotype and has been associated with methotrexate sensitivity in cell lines in prior studies (3). Collectively, we propose that tumoral expression of MTHFD2 and other folate genes should be further explored as possible biomarkers for folate pathway activity. Expression of these genes may have important clinical utility in determining inclusion and exclusion criteria for pemetrexed.

Figure 6. MTHFD2 is expressed at higher levels in pemetrexed-responsive tumorgrafts. A, representative images of H&E (hematoxylin and eosin) staining and MTHFD2 immunostaining on NSCLC tumorgrafts (n = 3). Hodgkin nodular sclerosis lymph node was used as a positive control for MTHFD2 staining. B, optical density (OD) of MTHFD2 staining was determined in each tumorgraft (at least 5 regions/tissue) using Spectrum image analysis software. Mean estimated OD ± SEM were graphed for each tumorgraft and compared by Student t test analysis (P < 0.05, responders vs. nonresponders).
therapy. We have initiated studies to further explore these correlations in an expanded set of clinical samples.

Multiple studies have demonstrated an association between TYMS expression levels and pemetrexed response (37–39). In these studies, NSCLC tumors and cells with lower expression of TYMS demonstrated enhanced response to pemetrexed. The lack of correlation between KRAS mutation status and TYMS expression levels in our study highlights the possibility of distinct functional mechanisms accounting for enhanced responsiveness to pemetrexed in these subpopulations of NSCLC (KRAS-mutant tumors and low TYMS expressing tumors). We propose that tumors expressing low levels of TYMS are likely more sensitive to pemetrexed because of a favorable ratio between drug levels and the target (TYMS), which allows for potent inhibition of the target. As demonstrated in our study, KRAS-mutant tumors are associated with enhanced dependency on folate- and purine-related pathways rendering these cells more susceptible to pemetrexed. As such, although some overlap may occur between these populations, we propose that TYMS expression alone may not be sufficient to account for all pemetrexed responders and, thus, additional mechanisms of response and resistance should also be taken into consideration.

Clinical evidence is emerging to support our findings. In the recent abstract of Levy and colleagues (40), retrospective analysis suggests that KRAS mutation is associated with significant improvement in progression-free survival for patients with stage IV NSCLC treated with platinum/pemetrexed-based therapy. Furthermore, in the article of Camidge and colleagues (41), KRAS-mutant NSCLC patients trended toward an enhanced benefit from pemetrexed compared with their triple-negative (EGFR, ALK, and KRAS wild-type) or EGFR-mutant counterparts (median progression-free survival, 7 months for KRAS-mutant vs. 4 months for triple negative and 5.5 months for EGFR mutant). This trend did not reach statistical significance in this study, which could be related to the small cohort size (HR, 0.55; 𝑃 = 0.0952, 21 KRAS-mutant patients vs. 37 triple negative patients) and/or the possible presence of two subtypes within the KRAS-mutant population; one of which responds well to pemetrexed treatment and another that responds poorly. This suggests that further stratification of the KRAS-mutant population may be beneficial in the identification of the most suitable subpopulation of patients for antifolate treatment.

Collectively, our findings highlight that a better understanding of the molecular mechanisms underlying the dependency of cancer cells on specific metabolic pathways may result in more effective metabolic targeting and new approaches in treating specific cancers. Currently, patient selection for pemetrexed is based on histology alone (42). Our study suggests that prospective trials and retrospective analysis focusing on KRAS status and folate pathway expression as criteria for patient selection for antifolates are warranted.

Disclosure of Potential Conflicts of Interest

D. Sidransky is a consultant/advisory board member of Quintiles. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.B. Trusk, K. Pry

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.M. Moran, K. Paz, S.S. Bacus

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