Oncogenic and sorafenib-sensitive *ARAF* mutations in lung adenocarcinoma

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Targeted cancer therapies often induce "outlier" responses in molecularly defined patient subsets. One patient with advanced-stage lung adenocarcinoma, who was treated with oral sorafenib, demonstrated a near-complete clinical and radiographic remission for 5 years. Whole-genome sequencing and RNA sequencing of primary tumor and normal samples from this patient identified a somatic mutation, *ARAF* S214C, present in the cancer genome and expressed at high levels. Additional mutations affecting this residue of ARAF and a nearby residue in the related kinase RAF1 were demonstrated across 1% of an independent cohort of lung adenocarcinoma cases. The ARAF mutations were shown to transform immortalized human airway epithelial cells in a sorafenib-sensitive manner. These results suggest that mutant ARAF is an oncogenic driver in lung adenocarcinoma and an indicator of sorafenib response.

Introduction

Lung adenocarcinomas harbor recurrent activating oncogenic mutations and fusions in receptor tyrosine kinase pathway genes, some of which (*EGFR*, *EML4-ALK*, *CD74-ROS1*) have been associated with clinical response to small-molecule inhibition (1–3). Despite these advances, most lung adenocarcinoma cases lack a clinically actionable genetic alteration and over 50% lack a plausible oncogenic "driver," as demonstrated by recent large-scale genome surveys (3). One approach for the discovery of clinically actionable drivers is genomic analysis of exceptional drug responses (4). We used next-generation sequencing to investigate the genetic basis of a sustained "outlier" response to sorafenib in lung adenocarcinoma.

Results and Discussion

A 66-year-old light former smoker (<5 packs per year smoking history) was diagnosed in April 2002 with stage IV lung adenocarcinoma. She failed multiple therapy regimens (gemcitabine and vinorelbine, gefitinib, bortezomib) between 2002 and 2005 and received a palliative lobectomy in early 2006 for worsening hypoxia. She began treatment with oral sorafenib, a broad-spectrum kinase inhibitor with activity against BRAF, RAF1, RET, PDGFRA, and KIT, among others (5, 6), in June 2006 as part of the ECOG 2501 trial (7). Within 2 months, her CT scans demonstrated a near-complete response (Figure 1). She remained progression free and asymptomatic for the next 5 years while continuing sorafenib treatment. In July 2011, a CT scan demonstrated enlargement of a right lower lobe mass meeting Response Evaluation Criteria In Solid Tumors criteria for progression. Sorafenib was discontinued, and she was started on carboplatin, paclitaxel, and bevacizumab. Therapy was discontinued after 2 cycles due to side effects, worsening fatigue, and oxygen requirements. She was admitted to hospice and died in November 2011. At the time of relapse, she was the last remaining study participant receiving sorafenib and 1 of only 9 responders among 306 evaluable patients. A more detailed time line of her case is shown in Figure 1 and described in the Supplemental Methods (supplemental material available online with this article; doi:10.1172/JCI72763DS1).

We used massively parallel DNA sequencing of this patient's tumor resection and peripheral blood samples before sorafenib treatment (Figure 1) to determine possible genetic alterations underlying her sustained sorafenib response. Whole-genome sequencing (WGS) of primary tumor (37.9X) and normal (37.7X) tissue revealed 25,150 somatic mutations (8.7 mutations per Mb), including 101 nonsynonymous mutations affecting the coding regions of 99 genes. The spectrum of somatic DNA variants (with respect to mutation, rearrangement, and copy number alteration) was consistent with that of other lung adenocarcinomas profiled in large-scale genome surveys (refs. 3, 8–11; Supplemental Figures 1 and 2; Supplemental Tables 1–3; and Supplemental Results).

The genome sequence data were notable for the absence of hot spot mutations in *KRAS*, *EGFR*, *BRAF*, *ERBB2*, or *PIK3CA*; gene fusions involving *ALK*, *ROS1*, or *RET* (a sorafenib target); and any other known oncogenic alterations (Supplemental Results). WGS analysis revealed numerous low-level (1–4 copy) broad DNA gains involving sorafenib targets (Supplemental Figure 1), without high-level focal amplifications. Among these was an approximately 60-Mb alteration, involving 300 genes on chromosome 4 and including 1–2 copy gains of canonical sorafenib targets *PDGFRA*, *KIT*, and *KDR* on 4q12 (6). 4q12 gain has been observed previously

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Conflict of interest: Matthew Meyerson is a founder and equity holder of Foundation Medicine, a for-profit company that provides next-generation sequencing diagnostic services.

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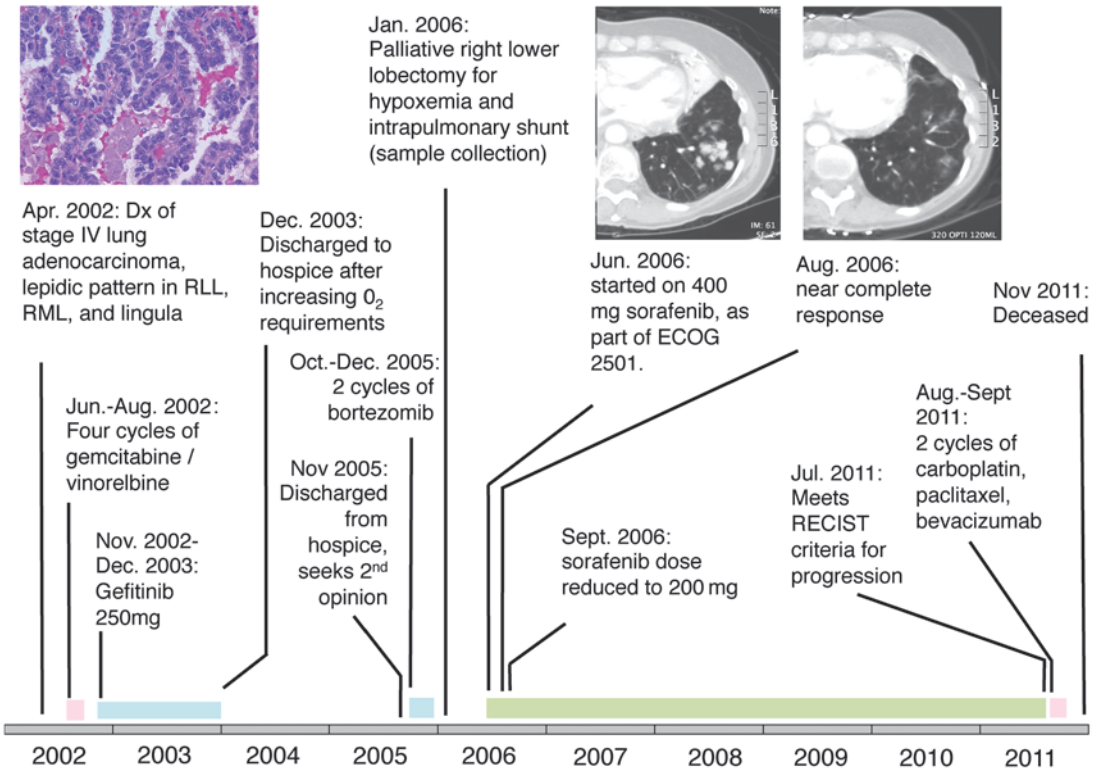


Figure 1
Time line of patient's lung adenocarcinoma diagnosis, treatment, and response. Colored rectangles near the time line represent durations of targeted therapy (blue, green) and chemotherapy (pink). Original magnification, ×200 (Apr. 2002). Dx, diagnosis; RLL, right left lobe; RML, right middle lobe.

in non–small-cell lung cancer lines, in which it was not associated with sensitivity to tyrosine kinase inhibition (imatinib, sunitinib) (12). Germ line variant analysis of peripheral blood WGS data did not reveal any rare deleterious germ line coding mutations in 29 known sorafenib target genes (ref. 5 and Supplemental Table 4).

Among 101 somatic coding mutations and 2 in-frame fusions predicted by WGS analysis, only 15 variants were detected in RNA sequencing (RNA-seq) data with more than 2 supporting reads (Table 1). Among expressed coding variants, the most likely candidate oncogenic driver was *ARAF* S214C. *ARAF* encodes a

Table 1
Summary of genes harboring expressed nonsilent somatic coding variants

					Variant read fraction		
Gene	**Somatic CN**	**Tumor gene expression (FPKM)**	**Genomic variant**	**Protein variant**	**Tumor DNA**	**Normal DNA**	**Tumor RNA**
ACADVL	4	190.53	g.chr17:7125396A>G	p.I250V	11/23	0/26	47/805
ARAF	5	24.04	g.chrX:47426121C>G	p.S214C	11/23	0/18	48/56
ATP8B1	3	4.49	g.chr18:55319937G>A	p.R1014*	4/31	0/41	3/37
C3orf10	3	35.03	g.chr3:10157495A>G	p.N37S	5/13	0/13	90/332
DICER1	3	3.26	g.chr14:95557552G>A	p.R1839W	6/36	0/39	4/23
EXT1	6	6.15	g.chr8:118819526G>A	p.R605W	22/48	0/35	46/75
LRP12	6	1.69	g.chr8:105503431C>A	p.A684S	22/45	0/22	4/12
NACC1	2	0.73	g.chr19:13249152G>T	p.V506L	4/4	0/10	11/11
PPIL6	6	0.00	g.chr6:109752383C>T	p.A133T	52/74	0/39	3/5
RAD54L2	3	0.00	g.chr3:51690015C>T	p.P1019S	15/33	0/29	4/5
SH3YL1	4	30.16	g.chr2:229997->GAT	p.250_250S>SS	7/39	0/33	124/404
TOMM70A	3	11.10	g.chr3:100087931T>C	p.K501E	10/29	0/32	48/81
UBE2A	5	12.94	g.chrX:118717207C>T	p.R150C	7/35	0/37	29/178
UBQLN2	5	6.90	g.chrX:56592088->T	p.F594fs	11/42	0/20	25/108
ZNF189	3	6.10	g.chr9:104171019A>T	p.K323N	5/39	0/31	9/34

CN, copy number; FPKM, fragments per kilobase of exon per million fragments mapped.

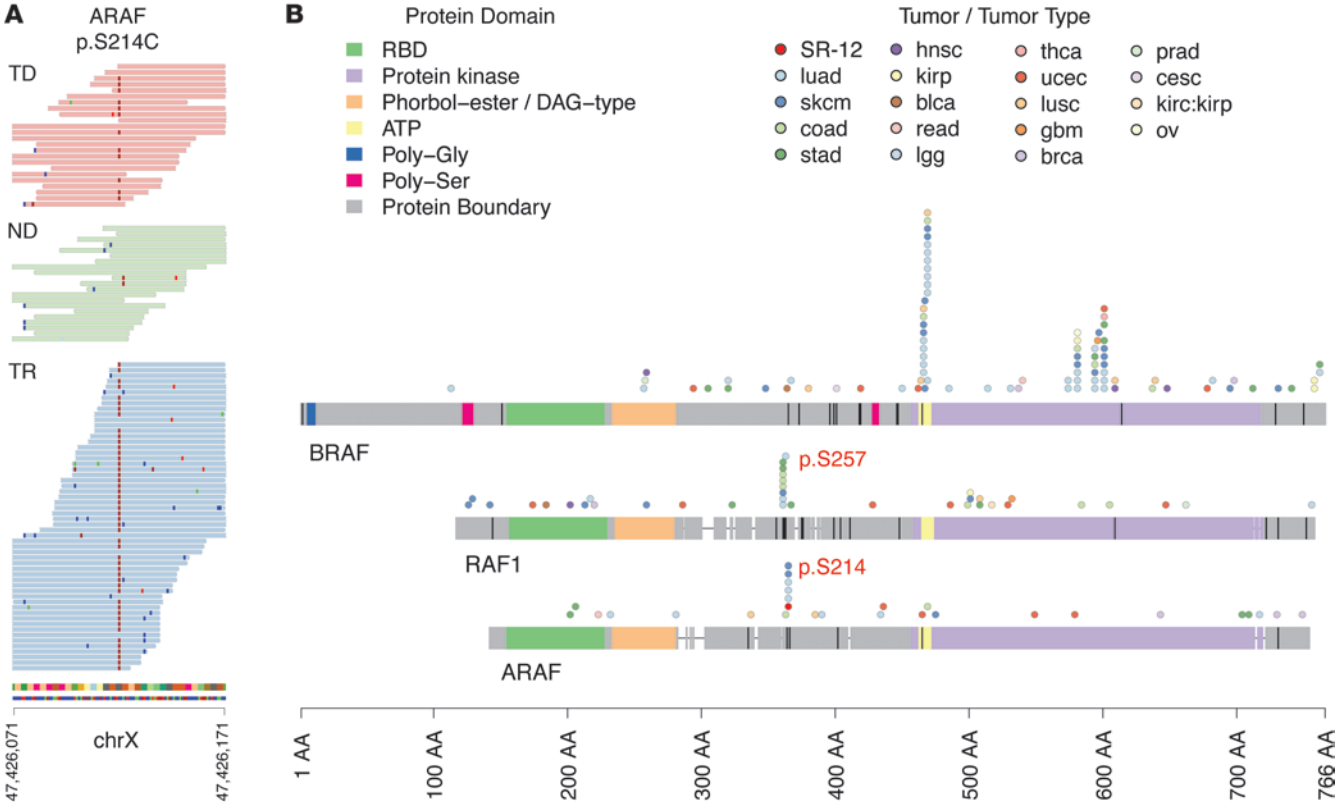


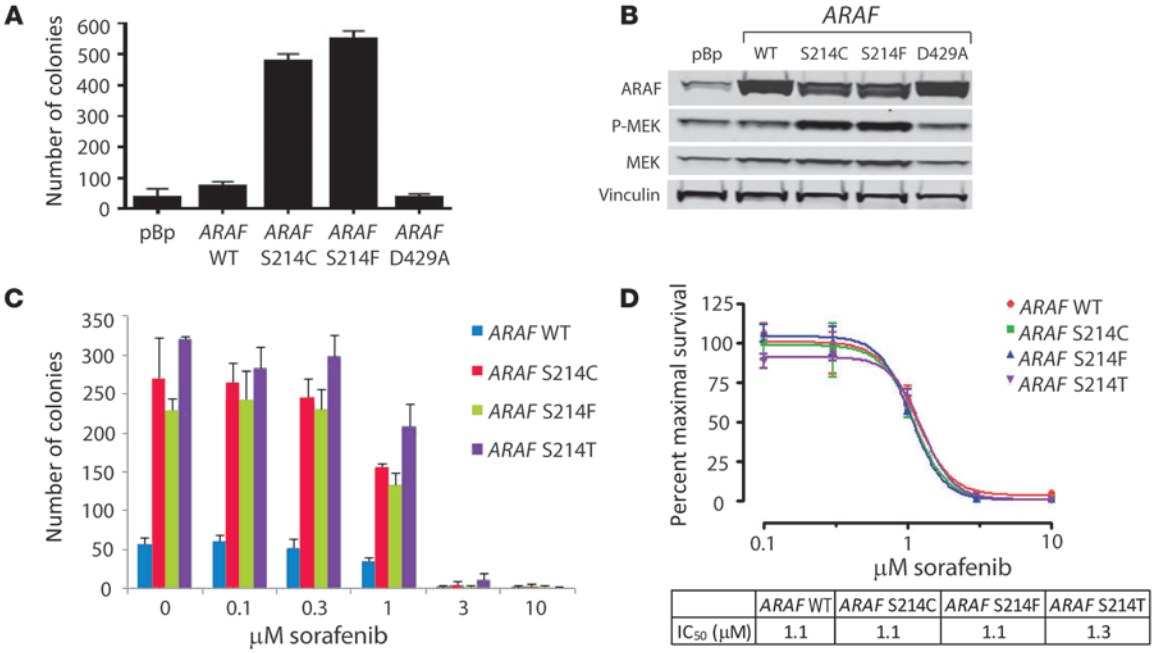
Figure 2

ARAF mutation found in a sorafenib-responsive lung adenocarcinoma defines a novel Raf family somatic mutation hot spot. (**A**) Read alignments in tumor DNA (TD, pink) and normal DNA (ND, green) and tumor RNA (TR, aqua), supporting *ARAF* S214C somatic mutation call (variant bases: brown, G; green, A; blue, C; red, T) in sorafenib-responder case. (**B**) Aligned ARAF, RAF1, and BRAF protein domain models overlaid with publicly available (http://cancergenome.nih.gov/), published (3), and sorafenib-responder somatic mutation data (SR-12), colored by tumor of origin. Red labels denote the putative *RAF1*/*ARAF* hot spot. *BRAF* p.V600E mutations are excluded for simplicity. luad, lung adenocarcinoma; skcm, cutaneous melanoma; coad, colorectal adenocarcinoma; stad, gastric adenocarcinoma; hnsc, head and neck squamous cell carcinoma; kirp, kidney renal papillary cell carcinoma; blca, bladder carcinoma; read, rectal adenocarcinoma; lgg, lower-grade glioma; thca, thyroid carcinoma; ucec, uterine corpus endometrial carcinoma; lusc, lung squamous cell carcinoma; gbm, glioblastoma multiforme; brca, breast adenocarcinoma; prad, prostate adenocarcinoma; cesc, cervical squamous cell carcinoma; kirc:kirp, kidney renal clear cell carcinoma; ov, ovarian carcinoma.

serine-threonine kinase in the Raf protein family, which also includes *RAF1* (also known as *CRAF*) and *BRAF*, a lung adenocarcinoma and melanoma oncogene. Like other Raf family proteins, ARAF transduces MAP kinase pathway signals from Ras to MEK and ERK and is a sorafenib target; however, unlike its paralogs, *ARAF* has never been implicated in tumorigenesis (6, 13).

ARAF S214C was detected in 11 of 23 tumor and 0 of 18 normal DNA reads and 48 of 56 tumor RNA reads (Table 1 and Figure 2A). *ARAF* was the second most highly expressed sorafenib target gene in our analysis (after *KIT*) and the only one harboring somatic sequence alterations (Supplemental Table 4). We identified 3 *ARAF* codon 214 somatic mutant cases across 564 lung adenocarcinomas profiled by The Cancer Genome Atlas (TCGA) and Imielinski et al. (3) and 3 additional cases with somatic mutations in a paralogous (p.S259) or nearly paralogous residue (p.S257) in the related gene *RAF1* (Figure 2B). Of these 6 lung adenocarcinoma cases, 4 lacked somatic mutations in any known lung adenocarcinoma oncogenes; 1 *RAF1* p.S257W mutant tumor harbored a *KRAS* G12V mutation, and a 1 *RAF1* p.S259F mutant tumor harbored a *BRAF* p.G469V somatic mutation (Supplemental Figure 3). Additional mutations in this putative hot spot were discovered among 21 tumor types (4,608 cases) profiled by TCGA, including colorectal adenocarcinoma (4 of 217 cases), gastric adenocarcinoma (2 of 264 cases), and cutaneous melanoma (3 of 269 cases) (Figure 2B and Supplemental Table 5). RAF1 p.S257 and p.S259 mutations have also been reported in ovarian cancer via the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and Noonan syndrome, a congenital disorder associated with constitutive Ras pathway signaling (14).

To examine the functional impact of the *ARAF* and *RAF1* mutations observed in lung adenocarcinoma, we ectopically expressed mutant alleles in immortalized tracheobronchial epithelial (AALE) cell lines. Ectopic expression of all 3 *ARAF* p.S214 lung adenocarcinoma variants substantially enhanced soft agar colony formation (Figure 3, A and C) and phospho-MEK levels (Figure 3B) relative to vector control and a kinase-dead (D429A) variant. Sorafenib treatment inhibited *ARAF*-induced AALE soft agar colony formation (Figure 3, C and D) and MEK phosphorylation (Supplemental Figure 3A) at concentrations (IC_{50} 1.0–1.3 μM, 95% CI 0.7–1.6) lower than those achievable in serum (6 μM) using standard oral



	ARAF WT	ARAF S214C	ARAF S214F	ARAF S214T
IC_{50} (μM)	1.1	1.1	1.1	1.3

Figure 3

ARAF p.S214 mutations found in lung adenocarcinoma are oncogenic and sensitive to sorafenib. (**A**) Soft agar colony formation by AALE cells expressing variants of *ARAF*. pBp, empty vector; S214C and S214F, *ARAF* p.S214 variants; D429A, kinase-dead *ARAF*. (**B**) Immunoblot indicating protein levels and phosphorylation status of MEK. (**C**) Response of ARAF mutant AALE soft agar colony formation to the indicated concentrations of sorafenib. (**D**) Dose-response curves for data shown in **C**. Data and error bars in **A**, **C**, and **D** represent mean ± SEM, respectively, obtained from triplicate experiments.

sorafenib doses (ref. 15 and Supplemental Table 6). Because MEK1 and MEK2 are downstream substrates of ARAF in the MAP kinase signaling pathway (13), we additionally tested trametinib, a MEK1 and MEK2 inhibitor (16). Trametinib also inhibited colony formation and decreased ERK phosphorylation in cells expressing the oncogenic *ARAF* mutants (IC_{50} 1–2 nM) (Supplemental Figure 4, B–D, and Supplemental Table 6). Similar to the *ARAF* variants, *RAF1* p.S257L and p.S259A induced anchorage-independent growth and increased MEK/ERK phosphorylation in AALE and NIH-3T3 cells (Supplemental Figure 5) in a sorafenib- and trametinib-sensitive manner (Supplemental Figure 6).

In summary, our genomic and functional results suggest the transforming *ARAF* codon 214 substitution as the most likely driver of this patient's tumor and determinant of its sorafenib response. The absence of known recurrent oncogenic alterations or alterations in other sorafenib targets (Supplemental Table 4) in our comprehensive profiling data supports this conclusion. Moreover, the discovery of additional patients with transforming *ARAF* codon 214 and *RAF1* codon 257 and 259 mutations in independent lung adenocarcinoma and pan-cancer data sets suggests somatic selection for a novel oncogenic hot spot in *ARAF* and *RAF1*, both which encode sorafenib targets (6). Inhibition of colony formation by sorafenib or trametinib in cells overexpressing wild-type *ARAF* or *RAF1* suggests that sorafenib and trametinib do not exhibit increased activity toward the mutant gene products, but rather that *ARAF* and *RAF1* mutations may confer an inhibitor-sensitive oncogene dependency. This is consistent with the current understanding of the mechanisms underlying the specificity of established targeted therapies (e.g., imatinib) (17). To the best of our knowledge, the *ARAF* and *RAF1* mutations profiled in this study have not been characterized previously as oncogenic somatic mutation hot spots in clinical cancer samples. Interestingly, one group has recently associated derived *RAF1* p.S257P mutations with in vitro PLX-4720 resistance in *BRAF* p.V600E mutant melanoma cells (18); however, all of the TCGA samples from patients with lung cancer analyzed in the current study were treatment naive.

The *ARAF/RAF1* mutations characterized in this study lie in a Raf CR2-domain phosphorylation site that negatively regulates Ras binding and RAF1 activation via binding of 14-3-3 (13, 19). This region is distinct from the kinase domain hot spots of BRAF (near p.G469 and p.V600), which are mutated in approximately 5% of patients with lung adenocarcinoma (3), suggesting that *ARAF* p.S214/*RAF1* p.S259 may function by a distinct biochemical mechanism. Our preliminary biochemical data suggests that *ARAF* also bound 14-3-3, and this binding was markedly attenuated in the setting of p.S214C mutation (Supplemental Figure 7). These results suggest that ARAF p.S214 mutations may potentiate Ras/Raf signaling by abrogating 14-3-3 and ARAF binding.

Clinical trials of sorafenib in patients with advanced non–small-cell lung cancer have demonstrated modest activity, with no survival advantage (7, 20). Though it is attractive to consider *ARAF/RAF1* mutations as possible biomarkers of sorafenib response in lung adenocarcinoma, additional profiling and functional characterization studies will be required to establish this link. This includes mutation data from additional sorafenib responders and/or sorafenib-response data from *ARAF* p.S214/*RAF1* p.S259 mutated cases. Our initial sequencing of 3 transient responders (<6-month progression-free survival) from the ECOG 2501 trial did not reveal additional Raf family mutations. Identification of additional *ARAF* p.S214/*RAF1* p.S257 mutant cases in lung ade-

nocarcinoma will likely require screening of large sets of patients, given the low frequency of these variants (1%). Though we believe that the cell line model used in this study should adequately recapitulate the observed overexpression of mutant *ARAF* in this patient's tumor (Table 1 and Supplemental Table 4), knockdown and inhibitor studies of cell lines with naturally occurring *ARAF/RAF1* mutations will be useful in definitively establishing essentiality and oncogene addiction in an endogenous context. Our preliminary analysis of the Cancer Cell Line Encyclopedia sequencing data (http://www.broadinstitute.org/ccle/home) did not reveal any *ARAF* p.S214/*RAF1* p.S257 mutant cell lines.

If recurrent but rare mutations underlie the oncogenicity and responsiveness of "driver-negative" lung adenocarcinomas, they are not likely to be nominated by statistical analysis of several hundred (or even thousands) of genome-sequenced cases. Our study suggests that a powerful, alternate approach to driver mutation discovery may be through the analysis of outlier patient responses and the identification of driver mutations through the preponderance of genomic, biochemical, and functional evidence.

Methods

Sequencing and bioinformatics. WGS of fresh tumor and peripheral blood DNA was performed using standard Broad Institute Illumina-based sequencing and analysis protocols (see Supplemental Methods). RNA-seq of the primary tumor specimen was performed using poly-A enrichment and standard Illumina protocols (see Supplemental Methods). Bioinformatics analyses and visualizations were generated using R Bioconductor packages (http://www.bioconductor.org) and CIRCOS (http://www.circos.ca).

Retroviral transduction and soft agar assays. Ectopic expression of mutant constructs and assessment of anchorage-independent proliferation were performed as described previously (21). See the Supplemental Methods for experimental details.

Immunoblotting. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, and 0.25% IGEPAL CA630. Protease inhibitors (Roche) and phosphatase inhibitors (Calbiochem) were added prior to use. Immunoblotting was performed as described previously (21) using antibodies specified in the Supplemental Methods.

Statistics. IC_{50} estimates and 95% CIs were obtained from sorafenib and trametinib inhibition dose-response data using least-squares nonlinear regression on a standard 4-parameter logistic model (see Supplemental Methods for additional details).

Study approval. The collection and genomic analysis of this patient sample was carried out in accordance with protocols approved by Institutional Review Boards at the Broad Institute of Harvard and MIT (Broad COUHES 1103004402) and Vanderbilt University (VICC THO 0547). Informed consent for genomic analysis was obtained from the patient at the time of sample collection.

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