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Acquired Resistance to Dasatinib in Lung Cancer Cell Lines Conferred by *DDR2* Gatekeeper Mutation and *NF1* Loss

Ellen M. Beauchamp[1], Brittany A. Woods[1,7], Austin M. Dulak[1], Li Tan[3], Chunxiao Xu[1], Nathanael S. Gray[2], Adam J. Bass[1,6], Kwok-kin Wong[1,4], Matthew Meyerson[1,5,6], and Peter S. Hammerman[1,6]

Abstract

The treatment of non–small cell lung cancer has evolved dramatically over the past decade with the adoption of widespread use of effective targeted therapies in patients with distinct molecular alterations. In lung squamous cell carcinoma (lung SqCC), recent studies have suggested that *DDR2* mutations are a biomarker for therapeutic response to dasatinib and clinical trials are underway testing this hypothesis. Although targeted therapeutics are typically quite effective as initial therapy for patients with lung cancer, nearly all patients develop resistance with long-term exposure to targeted drugs. Here, we use *DDR2*-dependent lung cancer cell lines to model acquired resistance to dasatinib therapy. We perform targeted exome sequencing to identify two distinct mechanisms of acquired resistance: acquisition of the T654I gatekeeper mutation in *DDR2* and loss of *NF1*. We show that *NF1* loss activates a bypass pathway, which confers *ERK* dependency downstream of *RAS* activation. These results indicate that acquired resistance to dasatinib can occur via both second-site mutations in *DDR2* and by activation of bypass pathways. These data may help to anticipate mechanisms of resistance that may be identified in upcoming clinical trials of anti-DDR2 therapy in lung cancer and suggest strategies to overcome resistance. *Mol Cancer Ther; 13(2); 475–82. ©2013 AACR.*

Introduction

For patients with lung adenocarcinoma, the most common subtype of non–small cell lung cancer (NSCLC), genomic studies have identified several targetable molecular alterations. Alterations in *EGFR* (1–3) and *ALK* (4, 5), and more recently, *BRAF* (6, 7), *ROS1* (8, 9), and *RET* (10–12), have been associated with marked responses to small molecule kinase inhibitors in clinical trials and therapies targeting *EGFR* and *ALK* genomic alterations are U.S. Food and Drug Administration (FDA) approved. These targeted therapies are both more efficacious and less toxic than standard chemotherapy in selected populations and have led to a paradigm shift in the management of lung adenocarcinoma.

Lung squamous cell carcinoma (lung SqCC) is the second most common subtype of NSCLC and is a lethal disease, which is diagnosed in 40,000 to 50,000 patients per year in the United States (13). The majority of patients present with locally advanced or metastatic disease. Recent studies of lung SqCC have identified recurrent alterations in tyrosine kinases including amplifications, mutations, and translocations of fibroblast growth factor receptors (FGFR; refs. 14–18) and mutations in the discoidin domain receptor 2 gene (*DDR2*; refs. 19, 20).

FGFR1 amplification and *DDR2* mutation have been associated with response to targeted agents in preclinical models and in early-phase clinical trials (19, 21, 22). These studies have led to interest in the development of targeted therapeutic approaches specifically for lung SqCCs given that there are no targeted agents approved in this disease. Several ongoing clinical trials are investigating therapeutic biomarkers in lung SqCCs, including *FGFR* mutations and amplifications as well as *BRAF*, *PIK3CA*, and *DDR2* mutations (www.clinicaltrials.gov).

In lung adenocarcinoma, the success of targeted therapies has been limited by the observation that nearly all treated patients develop acquired resistance to the targeted agent over time. Studies of lung cancer cell lines with sensitizing mutations and patient-derived rebiopsy specimens have demonstrated that acquired resistance can occur as a result of secondary mutations in the original target, such as *EGFR* T790M (23, 24) and *ALK* L1196M (25, 26), or by activation of parallel pathways, which bypass the original target such as *MET* (27) or *ERBB2* amplification (28). Preclinical models of acquired resistance have been robust in predicting mechanisms of resistance identified in the clinic (29–35) and there are substantial efforts

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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underway to identify strategies to overcome acquired resistance to targeted agents for multiple lung cancer genotypes. These issues are not limited to lung cancer as acquired resistance to targeted agents is an increasing problem across many cancer cell types, and multiple mechanisms of acquired resistance have been reported to date.

Dasatinib is an FDA-approved oral tyrosine kinase inhibitor, which has been studied in several trials of patients with lung cancer (36–38). Although the response rate to dasatinib as a single agent has been low in general, several responders have been reported, including 2 individuals with *DDR2* S768R mutations (19, 22) and an individual with a *BRAF* mutation (39), suggesting that specific biomarkers may be predictive of response to dasatinib. An international clinical trial is now accruing patients with these genotypes to address the efficacy of dasatinib in genomically selected patient cohorts with advanced NSCLC (NCT01514864). There has been no study reported to date of mechanisms of acquired resistance to dasatinib in the lung cancer population.

DDR2 is a receptor tyrosine kinase, which functions as a cellular collagen receptor (40, 41). Activation of DDR2 has been associated with a number of cellular phenotypes including transformation, migration, and differentiation (40, 42). *DDR2* mutations are present in 3% to 4% of patients with lung SqCC and have been reported in other cancer types at comparable frequencies including lung adenocarcinoma, uterine cancer, stomach cancer, bladder cancer, melanoma, colorectal cancer, and head and neck cancer (www.cbioportal.org). DDR2 engages a number of downstream signaling pathways including PI3K/AKT, Src, MEK/ERK, and NF-κB to drive cell survival, proliferation, migration, and transformation (43–47). DDR2 is potently inhibited by several FDA approved tyrosine kinase inhibitors including dasatinib, nilotinib, imatinib, and ponatinib (48). Although all of these kinase inhibitors are active against *DDR2*-mutated lung cancer cell lines, dasatinib is the most potent *in vitro* and *in vivo*. The potency of dasatinib against DDR2 has led to the design of clinical trials testing its efficacy in patients with lung cancer with *DDR2* mutations.

Here we report the generation of 2 cell line models of acquired resistance to dasatinib in *DDR2*-mutated lung cancer cell lines (HCC-366 and NCI-H2286), which have been previously shown to be dasatinib sensitive and *DDR2* dependent (19). Targeted exome-sequencing analysis of these lines demonstrated acquisition of a second-site mutation (T654I) in *DDR2* in HCC-366 as the only novel mutation as compared with the parental cell line. In contrast, 3 alterations were identified in NCI-H2286, none of which was a second-site mutation in *DDR2*. In NCI-H2286, loss of *NF1* was the most potent alteration associated with acquired resistance and activated a bypass pathway characterized by increased RAS and ERK activity.

Materials and Methods

Cell culture

Lung cancer cell lines (NCI-H2286, HCC-366, and NCI-H1703) were obtained from the ATCC and maintained in RPMI 1640 (Invitrogen) plus 10% fetal calf serum (Gemini). Experiments were performed within 6 months of receipt of cell lines from ATCC and no further authentication was performed. Dasatinib was obtained from LC labs. NCI-H2286 and HCC-366 cells were grown in increasing concentrations of dasatinib beginning at 100 nmol/L and increasing to 1.5 μmol/L over a period of 3 to 4 months.

DNA sequencing

Genomic DNA was prepared from HCC-366, NCI-H2286, and 2 independent clones of NCI-H2286-DR and HCC-366-DR, using the Qiagen DNeasy Kit. DNA was quantified by Pico-Green (Invitrogen) and 200 ng used for solution-based hybrid capture using the Oncopanel Capture Kit (49). This was followed by massively parallel sequencing on the Illumina Hiseq 2000. FASTQ files were aligned using the Picard pipeline and mutations called using Mutect and annotated using Oncotator using the parental cell line as the reference sequence (20). Analysis methods are also described at www.broadinstitute.org/cancer/cga.

Immunoblotting

Cellular lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. Immunoblotting was performed using the Nupage system (Invitrogen) with 100 μg of lysate. Primary antibodies used were: NF1 (Santa Cruz Biotechnology), DDR2 (Bethyl Laboratories), p-TYR clone 4G10 (Millipore), Erk (Cell Signaling Technologies), p-Erk1/2 (Thr202/Tyr204; Cell Signaling Technologies), Src (Cell Signaling Technologies), p-Src (Tyr416; Cell Signaling Technologies), Mek (Cell Signaling Technologies), p-Mek (Ser217/221; Cell Signaling Technologies), Akt (Cell Signaling Technologies), p-Akt (Ser473; Cell Signaling Technologies), β-actin (Sigma), and vinculin (Sigma). Immunoprecipitation was performed by incubating 2 mg of lysate with 10 μL antibody for 1 hour at 4° with shaking. Thirty microliters of protein A agarose (Invitrogen) was then added, followed by a 2 hour incubation at 4° with shaking. The agarose was washed 3 times with RIPA buffer and resuspended in SDS sample buffer (Boston BioProducts). Immunoblotting was then performed using the Nupage system (Invitrogen).

RNAi

shRNA vectors targeting *NF1* were obtained from The RNAi Consortium (TRC) at the Broad Institute. Hairpin shNF1a corresponds to TRC clone TRCN0000039714, shNF1b corresponds to clone TRCN0000039716, shNF1c corresponds to clone TRCN0000039715, and shNF1e corresponds to clone TRCN0000039713. All hairpins were provided in the PLKO.1 vector. Hairpins targeting GFP, LacZ29, or LacZ1650 were also obtained from TRC and

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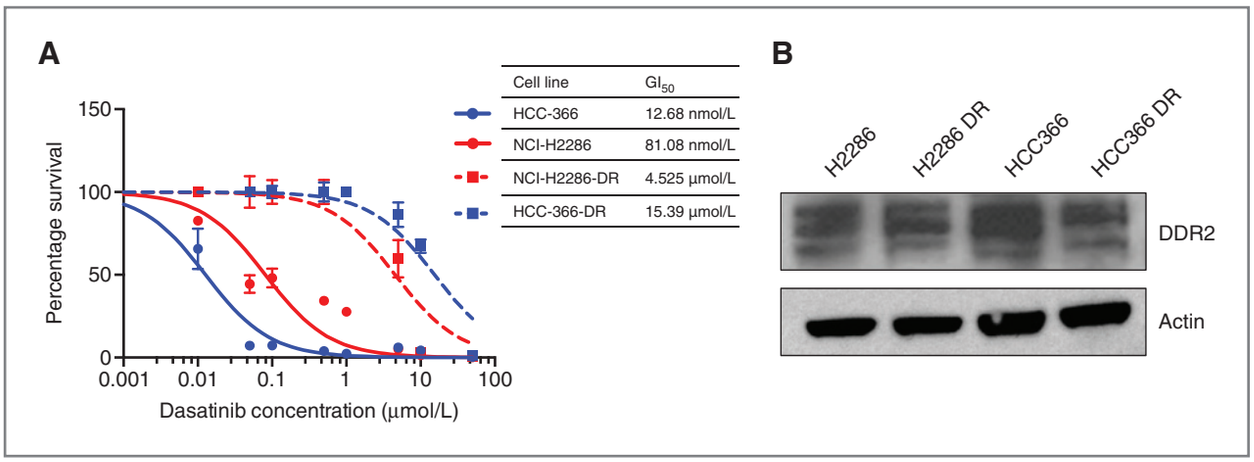

Figure 1. Dasatinib-resistant lines generated from *DDR2*-dependent lung cancer cell lines exhibit decreased sensitivity to dasatinib and have acquired alterations not present in the parental cell lines. A, proliferation of NCI-H2286, HCC-366, and generated dasatinib-resistant lines NCI-H2286-DR and HCC-366-DR grown for 6 days in the presence of dasatinib. Calculated GI_{50}s for each line are shown in the table. B, immunoblot showing levels of DDR2 in NCI-H2286 and HCC-366 parental and dasatinib-resistant lines.

used as controls. Lentivirus expressing the shRNAs was generated using a previously described triple transfection system with 293T cells as the packaging cell line. NCI-H2286, HCC-366, and NCI-H1703 were infected with a 1:8 titer of virus for 6 hours in the presence of 8 μg/mL polybrene. Cells were selected for stable expression of the shRNA construct by puromycin selection at 2 μg/mL for NCI-H2286 and 4 μg/mL for NCI-H1703 and HCC-366. Knockdown was confirmed by both real-time PCR and immunoblot. ERK1/2 knockdown was performed using siRNAs purchased from Cell Signaling Technologies (SignalSilence). Cells were transfected with a mixture of the siRNA and Lipofectamine 2000 (Invitrogen) and were assayed at 24, 48, and 72 hours.

RAS activity assay

The activation of RAS was detected using the Ras Activation Kit (Millipore) according to the manufacturer's instructions. In short, 300 μg of cell lysate was incubated with Raf1 Ras-binding domain conjugated to glutathione S-transferase for 30 minutes at 4°C. Precipitates were washed 3 times, resuspended in 2× Laemmli sample buffer, and resolved by SDS-PAGE using 12% gels. Proteins were transferred to Immobilon-FL membranes and subjected to immunoblotting with the RAS10 antibody (Millipore).

Cell proliferation and viability assays

Cell proliferation was measured with the Cell-Titer-Glo reagent (Promega) per the manufacturer's instructions. Cells were plated at a density of 1,500 cells/well in clear-bottomed 96-well plates. Drug was added the following day and cell proliferation was measured 6 days later using a standard 96-well plate luminometer. Percent survival at a given drug concentration was determined by comparing the luminescence at that concentration to that of untreated cells of the same cell type. For the *ERK* knockdown experiment, cells were seeded at a density of 250,000 cells/plate in 60 mm dishes. siRNA was added the following day, and cell viability was measured 24, 48, and 72 hours after addition of siRNA with a Vi-CELL reader, which stained cells with trypan blue and generated 50 independent images for each sample.

Results

Generation and analysis of dasatinib-resistant cell lines

We cultured 2 cell lines, HCC-366 and NCI-H2286, previously shown to be *DDR2*-mutated, *DDR2* dependent and dasatinib sensitive (19) in increasing concentrations of dasatinib to generate resistant cell populations. The previously reported GI_{50} for these lines with dasatinib treatment is 140 nmol/L and we increased dasatinib doses to a target of at least 10 times the baseline GI_{50}. Two independent clones were generated for each cell line and calculated GI_{50s} for the resistant pooled lines were 15.4 μmol/L for HCC-366-DR and 4.5 μmol/L for NCI-H2286-DR (Fig. 1A). Levels of DDR2 protein did not change as a result of the acquisition of dasatinib resistance (Fig. 1B).

To define mechanisms of resistance to dasatinib in these cell lines, we extracted genomic DNA from resistant clones and parental lines. Targeted exome sequencing was performed using the previously described Oncopanel hybrid-capture system (49) followed by massively parallel sequencing using the Illumina Hiseq 2000. A total of 645 genes were targeted and a mean sequencing depth of 320× was achieved across covered regions. Variant calling using Mutect (ref. 20; www.broadinstitute.org/cancer/cga) to annotate variants present in the resistant and not parental lines identified only one variant in one of the HCC-366 dasatinib-resistant lines (*DDR2* T654I), a mutation previously reported by our group and others (Table 1).

DDR2 T654I is a gatekeeper mutation site, which confers dasatinib resistance in a manner analogous to *EGFR* T790M (50). We have shown previously that expression of

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Table 1. List of nucleotide variants present in the dasatinib-resistant lines and not in the parental lines, identified by targeted exome sequencing

Cell line	Nucleotide variant	Amino acid change
HCC-366-DR-1	DDR2 1961C>T	DDR2 T654I
HCC-366-DR-2	None	
NCI-H2286-DR-1	NF1 1392 splice G>A	NF1 P464 splice (exon 12)
NCI-H2286-DR-1	EPHA7 1678G>T	EPHA7 V560L
NCI-H2286-DR-2	IL6ST 1990G>T	IL6ST A664S

DDR2 T654I mutants in HCC-366 and NCI-H2286 confers dasatinib resistance, with an increase in GI_{50} of 209- and 35-fold, respectively, for dasatinib in HCC-366 and NCI-H2286 (19). Although dasatinib treatment led to decreased phosphorylation of mutated DDR2 in parental NCI-H2286 and HCC-366 cells, expression of the *DDR2* T654I acquired mutation blocked this effect (Supplementary Fig. S1). Similarly, dasatinib treatment led to decreased phosphorylation of DDR2 in NCI-H2286-DR but not HCC366-DR (Supplementary Fig. S1). In NCI-H2286-DR clones, we did not identify any second-site mutations in *DDR2*, but we did identify variants including a splice-site mutation in *NF1* and missense mutation in *EPHA7* (V560L) in one clone and *IL6ST* (A664S) in a second clone (Table 1).

Functional assessment of mutations associated with acquired resistance

Given that the *DDR2* T654I has been extensively characterized in the past by our group, we did not pursue additional experiments to further characterize the gatekeeper mutation (19, 49). Our observation that DDR2 phosphorylation was maintained in HCC-366-DR with dasatinib suggests a similar gatekeeper mechanism in our study (Supplementary Fig. S1).

We focused our analysis on the NCI-H2286 dasatinib-resistant clones by determining protein expression for the 3 genes identified by targeted sequencing (IL6ST, EPHA7, and NF1). No difference in protein level was noted in EPHA7 or IL6ST in the resistant clones as compared with the parental lines (data not shown), but we observed a decrease in NF1 protein in the dasatinib-resistant clone harboring the *NF1* splice site mutation (Fig. 2A, lane 2). Ectopic expression of *IL6ST* A664S in parental NCI-H2286 cells resulted in no effect on dasatinib sensitivity and was not pursued further (Supplementary Fig. S2). Given recent reports that *EPHA7* is a tumor suppressor (51), we also evaluated whether knockdown of *EPHA7* might drive dasatinib resistance but this was not observed (Supplementary Fig. S2).

In contrast, we screened four shRNAs to evaluate knockdown of *NF1* in NCI-H2286 by immunoblot and real-time PCR (Fig. 2A and B). All four shRNA vectors decreased *NF1* mRNA and 3 of 4 had an appreciable effect on protein level (Fig. 2A and B). We then assessed whether *NF1* knockdown in parental NCI-H2286 cells impacted the sensitivity to dasatinib and observed a decrease in dasatinib sensitivity using all of the shRNA vectors (Fig. 2C). In an average of 3 independent experiments, the GI_{50} was 65.9 nmol/L for the parental line expressing control shRNA targeting the *lacZ* gene (shLACZ) and 1.9 μmol/L in the dasatinib-resistant line. Parental NCI-H2286 cells expressing shRNA vectors targeting *NF1* displayed GI_{50}s ranging from 122 to 544 nmol/L, a 1.85- to 8.3-fold shift in GI_{50} compared with the parental cell line expressing shLACZ.

Correlation of dasatinib resistance with maintained ERK activation

We next aimed to elucidate downstream signaling alterations in NCI-H2286-DR through comparison of effector molecule phosphorylation relative to parental NCI-H2286 by Western blotting. DDR2 has been previously shown to engage the PI3K/AKT, Src, and ERK/MAPK signaling cascades (43–47). In addition, ERK1/2 activity is essential for DDR2-driven cellular migration and differentiation (40–42). Although we did not observe any difference in the levels of p-AKT, p-MEK, or p-Src when comparing the response of resistant and parental NCI-H2286 lines to dasatinib, we observed that p-ERK levels were maintained in dasatinib-treated NCI-H2286-DR cells and not in the parental cell lines (Fig. 3). Interestingly, both p-Src and p-MEK were decreased to a comparable fashion in parental NCI-H2286 cells and NCI-H2286-DR suggesting that effects of dasatinib on at least one of its cellular targets may be independent of the changes in signaling observed in the context of dasatinib resistance. To this effect, we observed no difference in sensitivity of NCI-H2286 and NCI-H2286-DR cells to the MEK inhibitor GSK1120212 (Supplementary Fig. S3).

NF1 is a negative regulator of RAS activity. Given that we observed increased p-ERK1/2 in NCI-H2286-DR, we measured RAS activity by RAS GTPase assay. We observed RAS activity was elevated in NCI-H2286-DR as compared with NCI-H2286 by RAS GTPase assay (Fig. 4A). Because we observed that ERK1/2 but not MEK phosphorylation was elevated in NCI-H2286-DR as compared with parental NCI-H2286 in the setting of dasatinib, we hypothesized that ERK1/2 may be required for cell viability or proliferation in the dasatinib-resistant cells. Previous work has shown that ERK1/2 is the most critical effector of the MAPK pathway in the setting of resistance to vemurafenib in melanoma cell lines (52) with MEK activity less critical. To address whether analogous

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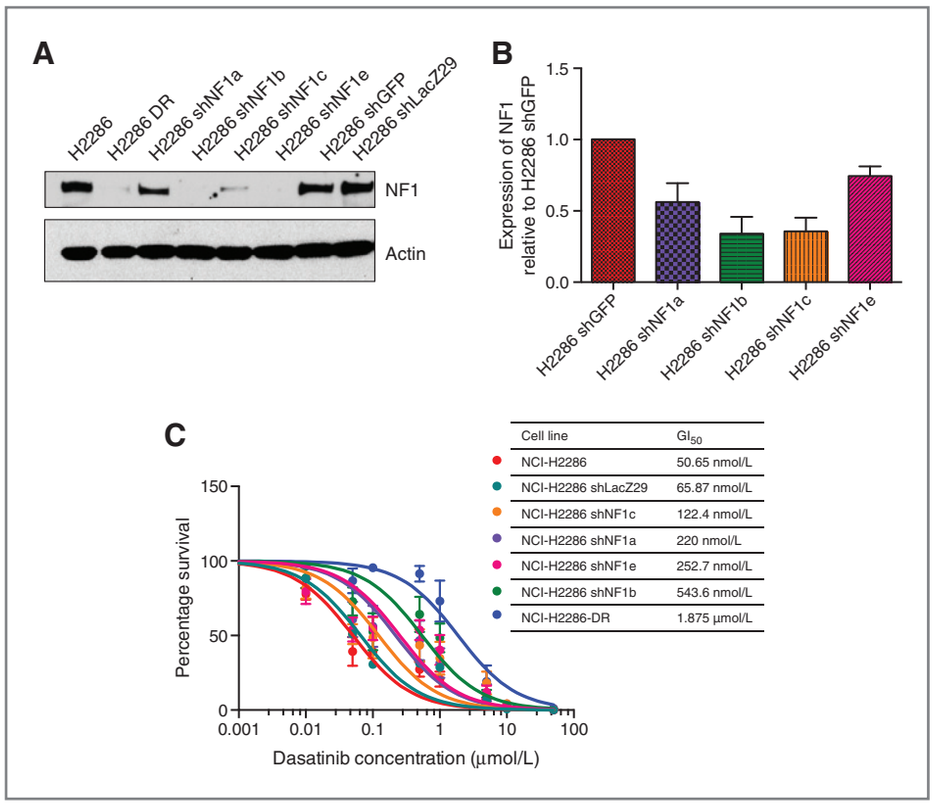

Figure 2. NCI-H2286 cells treated with RNAi targeting *NF1* have decreased *NF1* mRNA and protein levels and decreased sensitivity to dasatinib. A, immunoblot showing NF1 protein levels in NCI-H2286, NCI-H2286-DR, and NCI-H2286 cells expressing shRNA vectors targeting *NF1* (shNF1a-e), *GFP* (shGFP), or *lacZ* (shLacZ29). B, relative levels of *NF1* mRNA in NCI-H2286 cells expressing shRNA vectors to knockdown *GFP* (shGFP) or *NF1* (shNF1a-e), measured by real-time PCR. C, proliferation of NCI-H2286, NCI-H2286-DR, and NCI-H2286 cells expressing shRNA vectors targeting *lacZ* or *NF1*, grown for 6 days in the presence of dasatinib. Calculated GI_{50}s are shown in the table.

activation of ERK1/2 downstream of NF1-mediated RAS activation was required for the resistance phenotype, we used RNAi to knockdown *ERK1/2* in NCI-H2286 and NCI-H2286-DR (Fig. 4B). We observed that knockdown of *ERK1/2* led to a dramatic decrease in cell viability and proliferation in both the parental and dasatinib-resistant cell line (Fig. 4B), suggesting that ongoing ERK1/2 activity is required in the setting of dasatinib resistance in NCI-H2286-DR as well as in the parental cell line. This may be because of the existence of a DDR2-ERK signaling axis in the parental line, which is bypassed by a NF1-ERK axis in the resistant line; in both cases, signaling through ERK is required for cell proliferation.

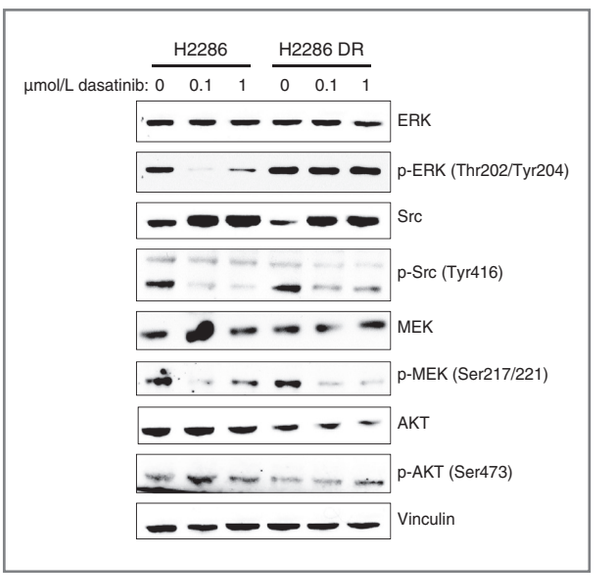

Figure 3. p-ERK levels are maintained in dasatinib-resistant, but not parental, NCI-H2286 following dasatinib treatment. Immunoblots showing relative levels of the proteins ERK, Src, MEK, and AKT and phosphoproteins p-ERK (Thr202/Tyr204), p-Src (Tyr416), p-MEK (Ser217/221), and p-AKT (Ser473) in NCI-H2286 and NCI-H2286-DR treated with various concentrations of dasatinib.

Generalizability of NF1 loss as a mechanism of acquired resistance to dasatinib

NF1 loss has been nominated as a mechanism of acquired resistance to both *BRAF* and *EGFR*-directed therapy (35). Activation of other signaling molecules in the RAS/RAF/MEK/ERK pathway has been described in the setting of acquired resistance to *BRAF* inhibitors (34). Given that *NF1* loss seemed to drive acquired resistance in the setting of NCI-H2286-DR, we also probed 2 additional dasatinib-sensitive lung cancer cell lines to examine if *NF1* loss may be a general mechanism of resistance to dasatinib in the lung cancer context.

We selected 2 cell lines for this analysis: HCC-366, which harbors the *DDR2* mutation L239R, and NCI-H1703, which has no *DDR2* mutations but is dasatinib-sensitive because of dependence on *PDGFRA* amplification (53). We knocked-down *NF1* in both lines using the shRNA construct in Fig. 2, which led to the greatest degree of NF1

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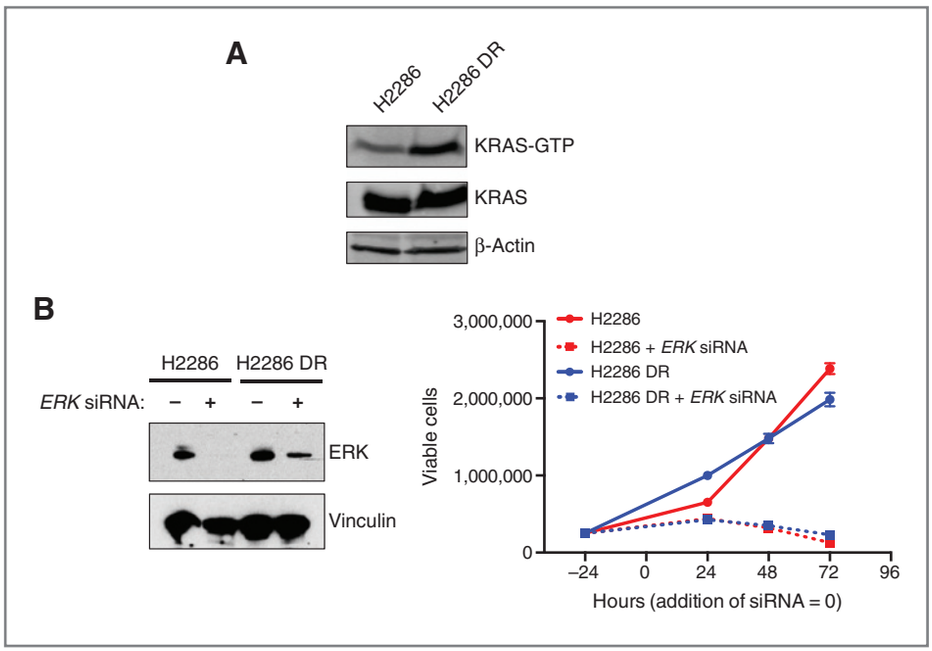

Figure 4. NCI-H2286-DR demonstrates increased RAS activity compared with parental NCI-H2286 and demonstrates ERK dependence. A, immunoblot showing RAS-GTP in NCI-H2286 and NCI-H2286-DR, measured by RAS GTP pull-down. B, cell viability of NCI-H2286 and NCI-H2286-DR 24, 48, and 72 hours after addition of siRNA targeting *ERK1/2*. The immunoblot shows the decrease in ERK protein levels in NCI-H2286 and NCI-H2286-DR upon knockdown of *ERK1/2* by RNAi.

depletion (Fig. 5A). We observed that *NF1* knockdown in both HCC-366 and NCI-H1703 led to a decrease in dasatinib sensitivity (Fig. 5B). The calculated GI_{50} shifts were 11.1-fold for NCI-H1703 (4.8–53.5 nmol/L) and 3.0-fold for HCC-366 (10.6–32 nmol/L), when comparing lines expressing the shRNA targeting *NF1* as compared with an shRNA targeting *GFP*.

Discussion

Here we have generated cellular models of acquired resistance to dasatinib using 2 independent *DDR2*-mutated lung cancer cell lines, both previously shown to be sensitive to dasatinib at comparable concentrations. Targeted exome sequencing of resistant lines identified 2 different modes or acquired resistance to dasatinib, second site (gatekeeper) mutations in *DDR2* and loss of *NF1*. These results are analogous to the cases of *EGFR* and *ALK* resistance in lung cancer in which both *cis* events such as gatekeeper mutations as well as *trans* events involving activation of other genes and bypass pathways have been shown to drive acquired resistance to *EGFR*- or *ALK*-directed therapy.

The development of pharmaceutical agents to selectively target gatekeeper mutations has been successful in the case of *EGFR* T790M with compounds such as WZ-4002 and CO-1686 (54) and for BCR-ABL with second- and third-generation ABL inhibitors. Our data suggest that the development of DDR2 inhibitors with activity against gatekeeper mutations is warranted given the likelihood that patients on clinical trials of dasatinib or other DDR-directed therapies will develop *DDR2* gatekeeper mutations associated with failure of initial therapy.

Loss of *NF1* has recently been reported to drive resistance to tyrosine kinase inhibitors in a number of settings including *BRAF*-driven melanoma and *EGFR*-driven lung cancer (35). *NF1* loss is also increasingly thought to play an

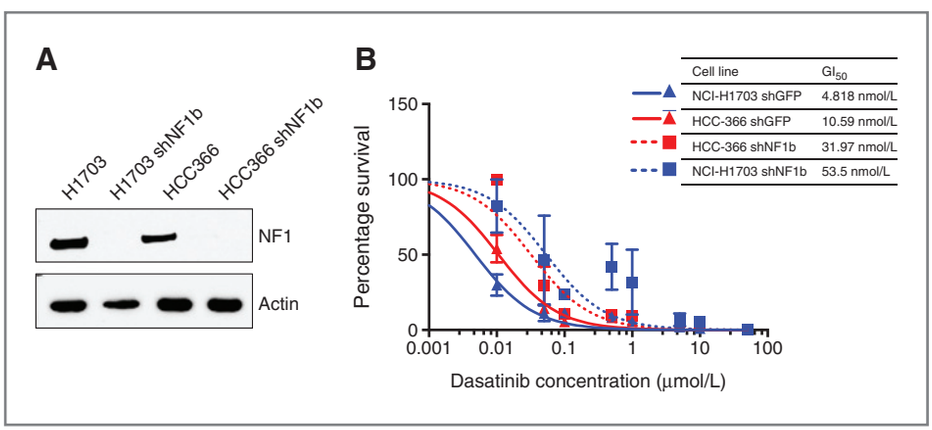

Figure 5. Knockdown of *NF1* in 2 additional dasatinib-sensitive cell lines leads to a decrease in dasatinib sensitivity. A, NF1 protein levels in NCI-H1703 and HCC-366 cells expressing an shRNA vector targeting *NF1* (shNF1b) are shown relative to parental NCI-H1703 and HCC-366 lines in the immunoblot. B, proliferation of NCI-H1703 and HCC-366 cells expressing an shRNA vector targeting either *NF1* or *GFP* was measured after 6 days in the presence of dasatinib. Calculated GI_{50}s are shown in the table.

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important role in oncogenesis in a wide spectrum of tumor types and has been reported as a recurrently mutated gene by a number of cancer genome studies (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Our data indicate another setting in which *NF1* loss contributes to tyrosine kinase inhibitor resistance and suggests that it can drive resistance to dasatinib in sensitive cell lines with different mechanisms of sensitivity to dasatinib. Given that *NF1* loss leads to RAS pathway activation, it is likely that its loss has the potential to drive resistance to tyrosine kinase inhibitors in a variety of settings given the central role of RAS signaling in cancer. Although knockdown of ERK1/2 was sufficient to kill NCI-H2286 dasatinib-resistant cells in our assays, there remains a need to develop improved inhibitors of RAS pathway effectors given the central role of this pathway in cancer development, progression, and therapeutic resistance. It is interesting to note that, we observed a dominant effect of ERK and not MEK downstream of *NF1* loss in our dasatinib-resistant cell lines. Although the mechanism of this observation remains unclear, a recent study has reported a similar observation in melanoma cell lines rendered resistant to the RAF inhibitor vemurafenib (52). It may be the case that MEK is subject to feedback inhibition in the setting of ERK activation downstream of *NF1* loss or a parallel MEK-independent pathway could be driving ERK activation.

Our data are unlikely to represent a full accounting of mechanisms of acquired resistance to dasatinib in lung cancer and it is also likely that some patients with *DDR2* mutations in ongoing clinical trials will display primary resistance to dasatinib given that many *DDR2* mutations have been described and some of these may not confer sensitivity to dasatinib. As we only profiled 645 genes in our sequencing assay and because knockdown of *NF1* did not fully recapitulate the degree of dasatinib resistance observed in NCI-H2286-DR, we expect that additional mechanisms will likely be found as has been the case for acquired resistance to *EGFR*- and *BRAF*-directed therapies. Furthermore, it is possible that more than one mechanism may drive resistance in a particular context and that some mechanisms may involve alterations other than DNA mutations, such as changes in gene expression or the epigenetic state of the cell. It remains to be seen what the diversity of mechanisms of acquired resistance to dasatinib will be. Given the ongoing clinical trials of dasatinib in genotypically selected patients with NSCLC, we think that the establishment of additional models of resistance, including serial biopsies of patients who respond to treatment on study, will be critical to allow for the best design of strategies to overcome acquired resistance to dasatinib.

Disclosure of Potential Conflicts of Interest

M. Meyerson has ownership interest (including patents) in and is a consultant/advisory board member for Foundation Medicine. P.S. Hammerman is a consultant/advisory board member for ARIAD, ImClone, and Molecular MD. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: B.A. Woods, K.-K. Wong, Peter S. Hammerman
Development of methodology: B.A. Woods, K.-K. Wong, Peter S. Hammerman
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