Mutations in the DDR2 Kinase Gene Identify a Novel Therapeutic Target in Squamous Cell Lung Cancer

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INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in the United States, with >157,000 deaths projected in 2010 (1). The more common type of lung cancer, non–small-cell lung cancer (NSCLC), accounts for 85% of cases and carries a grim prognosis; 70% of patients present with advanced and often incurable disease at the time of diagnosis (2).

Despite these statistics, a great deal of progress has been made in the targeted treatment of patients with NSCLC, largely owing to the development of small-molecule inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase (3–5). Patients who respond to EGFR kinase inhibitors are much more likely to have the adenocarcinoma subtype of NSCLC (6). Patients with the other principal subtype of NSCLC, lung squamous cell cancer (SCC), very rarely respond to these agents; few advances have been made in the treatment of this type of lung cancer, which accounts for 25% of NSCLC. In addition to EGFR, several other promising therapeutic targets have been identified in the laboratory, including EML4-ALK, KRAS, and MET; drugs directed against these proteins are being tested in clinical trials (7–10). However, it appears that these targets are likely limited to adenocarcinomas as well. A recent report has suggested that targeting FGFR1 amplifications in SCC of the lung may be a promising therapeutic strategy, although fibroblast growth factor receptor inhibitors are not currently in clinical use for the treatment of lung cancer (11). Given the burden of disease from lung SCC, we sought to identify new therapeutic targets by examining the tyrosine kinase of lung SCCs for novel mutated kinases.

In this article, we report the identification of novel somatic mutations in the discoidin domain receptor 2 (DDR2) tyrosine kinase gene at a frequency of 3.8% (n = 11) in a sample set of 290 lung SCC samples. DDR2, a receptor tyrosine kinase that binds collagen as its endogenous ligand, has been previously shown to promote cell migration, proliferation, and survival when activated by ligand binding and phosphorylation (12–18). DDR1 and DDR2 mutations have been noted in several cancer specimens, including 4 DDR1 mutations (W385C, A496S, F866Y, and F824W) and 2 DDR2 mutations in lung cancer (R105S and N456S); these reports, however, have not been confirmed...
in independent samples, and functional characterization of the mutations has not been reported (19–21). We show that DDR2 mutation status is associated with sensitivity to the tyrosine kinase inhibitor dasatinib or to short hairpin RNA (shRNA)–mediated depletion of DDR2. We also show that DDR2 mutations are oncogenic and that their ability to transform cells can be blocked by treatment with dasatinib or with a combination of tyrosine kinase inhibitors. Furthermore, we report a DDR2 kinase domain mutation in a clinical trial subject with SCC of the lung who had a radiographic response to combination therapy with erlotinib and dasatinib and who did not have an EGFR mutation. Together, these data suggest that DDR2 may be an important therapeutic target in SCCs.

**RESULTS**

**DDR2 Is Mutated in Lung SCC**

We performed Sanger sequencing of 201 genes, including the entire tyrosine kinase, in an initial set of 20 primary lung SCC samples and matched normal controls; we identified somatic missense mutations in 25 genes in our discovery sample set, including 6 in tyrosine kinase genes (Fig. 1A). Recurrent somatic mutations were identified in TP53 (n = 8) and in the tyrosine kinase genes: DDR2 (n = 2) and kinase insert domain receptor (KDR; n = 2; Fig. 1A). Subsequent sequencing of 6 of the mutated tyrosine kinase genes (DDR2, FGFR2, NTRK2, JAK2, FLT3, and CDK8), selected on the basis of being possible therapeutic targets, in a secondary screen of 48 lung SCC samples, including 13 cell lines, revealed 4 additional DDR2 mutations (Fig. 1A) as well as 3 FLT3 mutations, 2 NTRK2 and JAK2 mutations, and 1 mutation in each of FGFR2 and CDK8.

Given that DDR2 was the most frequently mutated gene in the primary and secondary screens, we sequenced DDR2 in a validation cohort of 222 primary lung SCC samples, which yielded an additional 5 samples with mutation, resulting in an overall frequency of 3.8% (n = 11) in 290 total samples and an overall frequency of 3.2% in primary lung SCC samples when cell lines were excluded (n = 9 of 277; Fig. 1A). Mutations were found both in the kinase domain and in other regions of the protein sequence, and 2 mutations were identified at G774 (Fig. 1B). The L239R and I638F mutations were identified in the HCC-366 and NCI-H2286 SCC cell lines, respectively, and the remainder of the mutations were found in primary SCC samples. The majority of the mutations resided in regions of high degrees of amino acid conservation, compared with the murine, zebrafish, and Caenorhabditis elegans homologs of DDR2 (Supplementary Fig. S1). Additional genomic analysis of previously reported copy number and gene expression datasets did not reveal any evidence of DDR2 overexpression in SCCs, compared with normal lung or lung adenocarcinoma, nor did we identify copy number alterations in DDR2 (data not shown; refs. 19, 22–24). A query of the limited clinical information accompanying the sequenced samples did not identify any significant correlation of DDR2 mutation status with age, sex, or smoking status of the patients.

**DDR2 Mutant Cell Lines Are Selectively Sensitive to Tyrosine Kinase Inhibitors**

To assess whether targeting DDR2 might be a promising therapeutic strategy in lung SCC, we analyzed several tyrosine kinase inhibitors reported to inhibit DDR2, including imatinib and dasatinib, drugs that are FDA approved for clinical use in targeting BCR-Abl in chronic myelogenous leukemia and acute lymphoblastic leukemia, c-KIT in gastrointestinal stromal tumors, and platelet-derived growth factor receptor in chronic myelomonocytic leukemia (21, 25–28). Fluorescence resonance energy transfer (FRET) measurements provided in vitro dissociation rate constant (Kd) values of dasatinib (5.4 nM) and imatinib (71.6 nM) for recombinant DDR2 (Supplementary Table S1).

Dasatinib showed particular efficacy against SCC cell lines bearing DDR2 mutations because it inhibited proliferation of the DDR2 mutant NCI-H2286 and HCC-366 cells with calculated 50% inhibitory concentrations (IC50) of 139 and 140 nM, respectively (Fig. 2A). Of note, a recent pharmacokinetic analysis of dasatinib in lung cancer patients revealed that peak concentrations of dasatinib were in the range of 300 ng/mL (615 nM) at the maximum tolerated dose of 140 mg daily, a dose approved for use in leukemias (29). Imatinib was less potent when tested in the same cell lines with respective IC50s of 1.2 and 1.0 μM for the DDR2 mutant NCI-H2286 and HCC-366 cell lines (Supplementary Fig. S2A). Dasatinib and imatinib were less effective against the A549 cell line, which is known to harbor a KRAS mutation and does not have any DDR2 mutations (calculated IC50 of 7.4 μM for dasatinib and 2.3 μM for imatinib). Consistent with previous reports, the NCI-H1703 SCC cell line, which contains a PDGFRα amplification, was sensitive to both drugs, serving as a positive control for our assay (30, 31). Notably, no other somatic mutations have been reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) database for NCI-H2286 or HCC-366 lines to suggest alternative dasatinib targets; a previous report examining the drug sensitivities of 83 NSCLC cell lines identified HCC-366 as the lung SCC line most sensitive to dasatinib, although NCI-H2286 and NCI-H1703 were not assayed (32). Treatment of the DDR2 mutant cell lines with dasatinib appeared to lead to cell death instead of cell-cycle arrest, as measured by trypan blue exclusion (Supplementary Fig. S2B). Dasatinib treatment was associated with an increase in cellular annexin V staining, suggesting that the treated cells died by apoptosis (data not shown).

To validate DDR2 as a relevant target of dasatinib in SCCs, we ectopically expressed a DDR2 transgene with a threonine-to-methionine mutation at amino acid 654, a mutation site shown previously to render DDR2 insensitive to dasatinib in a manner similar to the ability of the T790M mutation in EGFR to confer acquired resistance to the tyrosine kinase inhibitors erlotinib and gefitinib (33). We introduced the dasatinib-insensitive DDR2 “gatekeeper” mutant in cis with the observed L239R and I638F mutations in the HCC-366 and NCI-H2286 cell lines, respectively, as well as alone in NCI-H1703. Expression of the gatekeeper mutation led to a decrease in dasatinib sensitivity in both DDR2 mutant cell lines and had a modest effect on NCI-H1703 (Fig. 2B;
Figure 1. Sequencing of squamous lung cancer samples identifies recurrent mutations in DDR2. A, primary, secondary, and validation screens for DDR2 mutations in lung SCC samples. B, amino acid sequence of DDR2, with positions of identified mutations shown in the context of the known domain structure of DDR2.

Transgene expression is shown in Supplementary Fig. S2E). Whereas the calculated IC₅₀ for NCI-H1703 did not change with ectopic expression of the gatekeeper, the IC₅₀ increased by 35-fold for NCI-H2286 and 209-fold for HCC-366. Of interest, a parallel sequencing project in our laboratory identified a T654I mutation in DDR2 in a primary endometrial carcinoma sample (data not shown).

Dasatinib was originally designed as an inhibitor of Src and is a multitargeted tyrosine kinase inhibitor (34). Dasatinib treatment is associated with toxicity in patients, including myelosuppression and the development of pleural and pericardial effusions (35, 36). In an attempt to identify additional agents that could potently inhibit DDR2 with less associated toxicity, we screened a panel of 20 tyrosine kinase inhibitors that, on the basis of their respective structures, were predicted to have the potential to inhibit DDR2. We found that nilotinib, a second-generation BCR-ABL inhibitor, as well as AP24534, a third-generation BCR-ABL inhibitor that displays activity against BCR-ABL and imatinib-resistant BCR-ABL (36), inhibited the proliferation of SCC lines harboring DDR2 mutations (Supplementary Fig. S2C and D). We observed that AP24534 treatment resulted in a greater degree of inhibition than did nilotinib treatment, a finding in agreement with calculated in vitro Kᵦ values of 35.4 nM for nilotinib and 9.0 nM for AP24534, compared with 5.4 nM for dasatinib (Supplementary Table S1).

shRNAs Targeting DDR2 Kill DDR2 Mutant SCC Cell Lines

As an independent measure of DDR2 dependency, we expressed shRNAs targeting DDR2, using lentiviral vectors in the NCI-H2286, HCC-366, and NCI-H1703 cell lines. We screened a set of shRNA-expressing plasmids for the ability to knock down DDR2 mRNA expression by real-time PCR in NCI-H2286 cells and selected 2 hairpins for further analysis because of their ability to reduce DDR2 mRNA levels by approximately 50% (data not shown). We observed that knockdown of DDR2 by these 2 sh-RNAs led to a reduction in proliferation of the 2 DDR2 mutant cell lines but not of PDGFRα-amplified NCI-H1703 cells, which had been sensitive to imatinib and dasatinib in our proliferation assays (Fig. 2C). The reduction in proliferation appeared to correlate with the degree of knockdown because the observed phenotype was greater with shRNA-2 than shRNA-5 (Fig. 2C) and seemed to result from cell death, not cell-cycle arrest (data not shown).

To assess the specificity of the observed knockdown phenotype, we performed a similar experiment in NCI-H2286 and HCC-366 cells ectopically expressing their described...
mutated forms of DDR2 (I638F and L239R, respectively) and then knocked down endogenous DDR2 with shRNA-2, which targets the 3’ untranslated region (UTR) of DDR2 and would not be expected to interfere with ectopic expression of DDR2. We observed for both NCI-H2286 and HCC-366 that ectopic expression of DDR2 attenuated the antiproliferative effect of endogenous DDR2 knockdown and that the effect was of greater magnitude in NCI-H2286, perhaps owing to a greater degree of off-target effects in HCC-366 (Fig. 2D).

**DDR2 Mutations Are Associated with Dasatinib Sensitivity In vivo**

To analyze the effects of dasatinib treatment in a somewhat more physiologic setting, we performed xenograft studies in athymic nude mice in which we injected cohorts of mice with NCI-H2286, HCC-366, NCI-H1703, and A549 cells. HCC-366 cells did not form tumors in the mice and could not be analyzed further. Following tumor formation of the 3 tested lines, mice were treated with dasatinib at 50 mg/kg by oral gavage for 2 weeks or with vehicle control. Dasatinib treatment led to a decrease in tumor size in the NCI-H1703 and NCI-H2286 lines, but not in A549, consistent with our *in vitro* results (Fig. 3).

**DDR2 Mutations Are Oncogenic, and DDR2-Driven Transformation Is Dasatinib Sensitive**

We examined whether DDR2 mutations could confer an oncogenic gain-of-function phenotype. Ectopic expression of a subset of the DDR2 mutants identified in our primary and secondary screens (*n* = 2 of 6) promoted the formation of colonies in soft agar of NIH-3T3 cells (Supplementary Fig. S3A). Colony formation was greatest in the L63V and I638F mutants at a level comparable to that driven by expression of the gain-of-function L858R mutation in *EGFR* and
Fig. S3C). The third-generation BCR-Abl inhibitor AP24534 was also effective in killing the IL-3–independent Ba/F3 cells expressing mutant forms of DDR2, suggesting that this class of drugs may be effective against DDR2-driven neoplasms, whereas the second-generation BCR-Abl inhibitor nilotinib demonstrated modest activity against the DDR2-transformed Ba/F3 cells (Supplementary Figs. S3D and S4A). Survival of Ba/F3 cells in the absence of IL-3 was associated with maintenance of STAT5 phosphorylation, as has been previously shown (Supplementary Fig. S4B; ref. 38).

DDR2-transformed cell lines Maintain src Phosphorylation and are especially sensitive to Dual inhibition of DDR2 and src

Given that the type I kinase inhibitor dasatinib was more potent in DDR2-transformed Ba/F3 cells than the more target-specific type II inhibitors nilotinib and imatinib, we sought to test whether the potency of dasatinib in this system might be due to the effects of dasatinib on kinases in addition to DDR2. Dasatinib treatment also inhibited the colony formation of NIH-3T3 cells expressing the L858R mutation in EGFR, consistent with previous reports, and did so to a lesser extent in NIH-3T3 cells stably expressing the activating G12V KRAS mutation (Fig. 4A; refs. 32, 37).

Because the observed gain-of-function phenotype was modest for many of the DDR2 mutants in NIH-3T3 cells, we evaluated the transforming potential of DDR2 in the interleukin-3 (IL-3)–dependent hematopoietic cell line Ba/F3. We observed that ectopic expression of all 6 DDR2 mutants identified in our primary and secondary screens led to IL-3–independent growth of Ba/F3 cells, as did high expression levels of wild-type DDR2; no differences were observed in the time to transformation or the rate of IL-3–independent proliferation (Supplementary Fig. S3B). A kinase-dead DDR2 transgene (K608E) did not support the IL-3–independent growth of Ba/F3 cells (Supplementary Fig. S3B). Although culture with the less potent DDR2 inhibitor imatinib did not lead to significant killing of Ba/F3 cells expressing DDR2 mutations, compared with cells grown in the presence of IL-3, culture with dasatinib led to cell death in all cell lines expressing DDR2 mutants, with a mean calculated IC50 of 680 nM for the mutants and 30 μM for the control (Fig. 4B; Supplementary Fig. S3C). The third-generation BCR-Abl inhibitor AP24534 was also effective in killing the IL-3–independent Ba/F3 cells expressing mutant forms of DDR2, suggesting that this class of drugs may be effective against DDR2-driven neoplasms, whereas the second-generation BCR-Abl inhibitor nilotinib demonstrated modest activity against the DDR2-transformed Ba/F3 cells (Supplementary Figs. S3D and S4A). Survival of Ba/F3 cells in the absence of IL-3 was associated with maintenance of STAT5 phosphorylation, as has been previously shown (Supplementary Fig. S4B; ref. 38).

DDR2-Mutated Cell Lines Maintain Src Phosphorylation and Are Especially Sensitive to Dual Inhibition of DDR2 and Src

Given that the type I kinase inhibitor dasatinib was more potent in DDR2-transformed Ba/F3 cells than the more target-specific type II inhibitors nilotinib and imatinib, we sought to test whether the potency of dasatinib in this system might be due to the effects of dasatinib on kinases in addition to DDR2. Dasatinib has previously been shown to require Src for maximal kinase activity (16), and we observed that levels of phosphorylated Src were maintained in Ba/F3 cells expressing DDR2 mutants in the absence of IL-3 (Supplementary Fig. S4B). To determine whether the ability of DDR2 mutations to confer IL-3–independent proliferation in Ba/F3 cells might depend on both DDR2 and Src activity, we treated Ba/F3 cells expressing DDR2 with AZD0530, a highly...
selective Src-family kinase inhibitor that displays minimal activity against DDR2, in comparison with the other inhibitors described in this article (in vitro \(K_{\text{d}}\), 291 nM; Supplementary Table S1; ref. 39). As with nilotinib treatment, AZD0530 had a modest effect on the proliferation of the IL-3–independent DDR2-expressing Ba/F3 cells (Supplementary Fig. S4A and C). However, when Ba/F3 cells expressing the L63V DDR2 mutation were grown in 50-nM nilotinib, a concentration associated with little effect on proliferation of wild-type Ba/F3 cells or Ba/F3 cells expressing DDR2 mutations (Supplementary Fig. S4A), the addition of AZD0530 led to a marked reduction in proliferation of Ba/F3 cells expressing DDR2 L63V; this finding suggested that coordinated activity of DDR2 and Src-family kinases may be required for the ability of DDR2-mutated Ba/F3 cells to grow in the absence of IL-3, thereby providing a possible explanation for the potency of dasatinib in this system (Fig. 4C; Supplementary S5A). A similar additive effect of AZD0530 was observed when the Ba/F3 cells were cotreated with AZD0530 and 50 nM of either AP24534 or dasatinib (see Fig. 4C for L63V; for a more detailed version of this experiment, including additional DDR2 mutants, see Supplementary Fig. S5). AZD0530 reduced Src and STAT5 phosphorylation in a dose-dependent fashion in the DDR2 L63V-expressing Ba/F3 cells when used as a single agent or in combination with nilotinib, AP24534, or dasatinib (Fig. 4D; Supplementary S4D).

**Observation of a DDR2 Kinase Domain Mutation in a Clinical Trial Subject with a Radiographic Response to Combination Therapy with Dasatinib and Erlotinib**

Two recent early-phase clinical trials of dasatinib have been reported in which subjects with advanced-stage lung cancer were treated with either dasatinib or a combination of dasatinib and erlotinib (29, 40). One of 7 subjects with a lung SCC exhibited significant shrinkage in tumor size while undergoing therapy with a combination of dasatinib and erlotinib; unlike the other subject in the study who exhibited...
a response to treatment, and who had adenocarcinoma, no evidence of \textit{EGFR} mutation was found in the subject with SCC. The patient was a 59-year-old white woman with a one-third pack-per-day smoking history for 38 years who quit 1 year before her diagnosis of lung cancer. She was found to have a left lower lobe stage I (tumor, 2; node, 0; metastasis, 0) SCC and received primary treatment with weekly carboplatin and paclitaxel, with concomitant 70 Gy of radiation, resulting in a complete response. However, approximately 1 year later, the disease progressed within the radiation field, and treatment was initiated with a standard dose of carboplatin and paclitaxel, without response. She then began combination dasatinib and erlotinib therapy on protocol. A restaging computed tomographic (CT) scan after nearly 2 months indicated tumor shrinkage, and the patient experienced improved symptoms (resolved dyspnea and cough; Fig. 5A and B). She remained on the treatment regimen for 14 months until it had to be discontinued due to treatment-induced airspace disease and pleural effusions.

We performed directed sequencing of \textit{DDR2} in a pretreatment tumor specimen derived from this individual and identified a novel \textit{DDR2} kinase domain mutation, S768R, which was present in 844 of 3020 (28\%) reads obtained by 454 sequencing (data not shown) and independently verified by Sanger sequencing (Supplementary Fig. S6). The mutation could not be verified as somatic because no normal DNA was available for this individual, who is deceased. This correlation could not be further explored because no other SCC subjects responded to therapy in this study or a subsequent one of dasatinib alone (\(n = 13\) total). We performed 3-dimensional modeling of the S768R mutation in the context of the DDR2 kinase domain, which suggested that the S768R substitution is likely to alter the kinase activity of DDR2 (Supplementary Fig. S7).

**DISCUSSION**

We report the identification of 11 novel mutations in \textit{DDR2} in a screen of 290 SCC samples, yielding an overall mutation rate of 3.8\% in all samples and 3.2\% in primary SCCs, a rate comparable to the fraction of lung adenocarcinoma patients bearing \textit{ALK} fusions, a genomic event associated with...
dramatic responses to the ALK inhibitor crizotinib (10, 41). At this time, it remains an important question whether DDR2 mutations will be primarily found in SCCs of the lung or whether they may be present in SCCs originating from other tissues, such as those of the head and neck, the skin, or tumors of different histologic types. Furthermore, the rate of DDR2 mutations was lower in our validation screen than in our initial mutation screens, and it will likely take additional sequencing efforts, such as The Cancer Genome Atlas, which is upcoming, to further define the prevalence of DDR2 mutations. Of note, we have found 2 additional DDR2 mutations in a sample set of endometrial carcinomas (T654I and T685S), as well as a mutation in a colorectal cancer patient (T692N), supporting the possibility that DDR2 mutations may be present in multiple cancer types. A search of the COSMIC database is notable for DDR2 mutations in renal cell carcinoma, glioblastoma multiforme, and the previously mentioned lung adenocarcinoma samples. In addition, our initial and secondary screens consisted largely of samples from the United States whereas the validation screen consisted of more samples from European patients, suggesting that demographics may also affect the rate of observed DDR2 mutations. A recent report in which >1500 genes were sequenced in a cohort of 63 squamous cell lung cancers, using mismatch repair technology, did not identify any DDR2 mutations (42); however, we calculate that the sample size was not large enough to detect a statistically significant difference in the rates of DDR2 mutations, when compared with our study, in which a power of 0.8 and alpha of 0.05 were assumed (43).

We evaluated the effects of ectopic expression of 6 mutant forms of DDR2 in NIH-3T3 cells and Ba/F3 cells and showed that mutated DDR2 could function as an oncogene in either context, although with differing potency. We did not complete an assessment of all identified DDR2 mutants, nor did we evaluate the effects of expression of mutated DDR2 in the more appropriate context of primary squamous lung cells in a mouse or other model organism. The creation of these models is currently in progress and will be critical to more fully characterize the function of mutated DDR2.

The precise mechanism by which mutated DDR2 promotes cellular transformation is unclear. Whereas ectopic expression of DDR2 correlated with STAT5 and Src phosphorylation in transformed Ba/F3 cells and chemical inhibition of Src and DDR2 appeared to exhibit an additive, if not synergistic, effect in DDR2-transformed Ba/F3 cells, the mechanism by which mutations in DDR2 activate downstream signaling is not known. It is possible that the kinase domain mutations, in a manner similar to the modeled mutation at S768, alter the kinase activity of DDR2. The observation that ectopic expression of wild-type DDR2 was sufficient to transform Ba/F3 cells suggests that increased DDR2 signaling activity is a potential mechanism of transformation. It is also possible that mutations in the discoidin domain or unclassified regions of DDR2 could impact upon the ligand binding or localization of DDR2; prior reports have shown that DDR2 mutations in familial spondylometaphyseal dysplasia alter the ligand binding and membrane trafficking of DDR2 (44, 45).

In addition to discussing identification of recurrent somatic mutations in the DDR2 kinase gene, we show that dasatinib can efficiently inhibit the proliferation of DDR2-mutated SCC cell lines in vitro and in vivo, as well as cells ectopically expressing mutant DDR2. Together, these data identify a potential first therapeutic target in lung SCC for which clinically approved drugs already exist, thereby providing a rationale for clinical trials of tyrosine kinase inhibitors. We also report a DDR2 kinase domain mutation in a patient with lung SCC who exhibited a radiographic response to the combination of dasatinib and erlotinib and who did not harbor an EGFR mutation. Although this is an interesting result, we think that caution should be exercised in its interpretation, given that only 1 response was reported in a SCC patient in the trial, thus precluding a thorough assessment of the correlation. Further, the subject discontinued therapy in the context of unacceptable toxicity after 14 months of treatment, suggesting that investigation of tyrosine kinase inhibitors other than dasatinib is warranted. Although it is not possible to definitively conclude that this patient responded specifically to dasatinib treatment due to a DDR2 mutation, we believe it is unlikely that the response was due to erlotinib, in light of the absence of an EGFR kinase domain mutation and the previously noted GI_{50} of 990 nM for erlotinib in the DDR2 mutant HCC-366 cell line (32). A search of the literature did not uncover any earlier reports of erlotinib as a potent inhibitor of DDR2, and our initial experiments in the DDR2 mutant cell lines HCC-366 and NCI-H2286 indicate a sensitivity to erlotinib that is at least 10-fold less than sensitivity to dasatinib (data not shown).

In conclusion, we hope our data may stimulate the initiation of larger clinical trials of dasatinib or other tyrosine kinase inhibitors in patients with lung SCC and testing of these patients for DDR2 mutations, potentially leading to a less toxic and more effective treatment for this deadly disease.

METHODS

Ethics Statement

All animal experiments were performed under an Institutional Animal Care and Use Committee–approved animal protocol at the Dana-Farber Cancer Institute (Boston, MA) and all experiments involving human DNA were performed in accordance with Institutional Review Board (IRB)—approved protocols as described below.

Collection of Lung SCC Samples

For the primary and secondary screens, tumor samples were obtained from the Dana-Farber Cancer Institute/Brigham and Women’s Hospital/Harvard Cancer Center (Boston, MA) under Institutional Protocol 02-180, approved by the Dana-Farber/Harvard Cancer Center IRB in September 2002 and renewed yearly thereafter. This is a general tissue collection protocol for patients with lung cancer who have consented to tissue collection for research, including DNA sequencing, prior to surgery. All patients with resectable, biopsy-proven lung SCCs (as diagnosed by a board-certified anatomic pathologist) were eligible. These eligible subjects participated in a detailed informed consent procedure prior to enrollment on the protocol, which included a discussion of the use of tissue samples for DNA sequencing studies and written documentation of consent. In addition, DNA samples from de-identified patients with lung SCCs were obtained from the Ontario Cancer Institute for the primary and secondary screens as part of a Dana-Farber/Harvard Cancer.
Center IRB-approved collection of de-identified tumor samples for DNA sequencing studies. IRB approval for collection of de-identified samples was subject to a review of the local IRB-approved protocols for all external sites to ensure that an adequate informed consent process had taken place.

The validation screen was performed at the University of Cologne and Max Planck Institute for Neurological Research (Cologne, Germany). Samples were taken in accordance with a tissue collection protocol approved by the University of Cologne Ethics Committee; this protocol included a detailed informed consent process with discussion of genetic testing prior to the subject’s surgery and written documentation of consent. Again, all patients with biopsy-proven lung SCCs were eligible regardless of disease stage, as long as their tumors were considered resectable. De-identified samples were also collected from additional European sites, including Haukeland University Hospital (Bergen, Norway), University Hospital Zurich (Zurich, Switzerland), Université Joseph Fourier (Grenoble, France), Oslo University Hospital (Oslo, Norway), Jena University Hospital (Jena, Germany), and University Medical Center Groningen (Groningen, The Netherlands). At all sites, samples were obtained in accordance with an IRB-approved tissue collection protocol, and the collection of de-identified samples from these sites was approved by the University of Cologne Ethics Committee after a review of local collection protocols.

For the single patient sample obtained from the recent clinical trial of combination therapy with dasatinib and erlotinib in advanced lung cancer, DNA was provided to the University of Cologne by the H. Lee Moffitt Cancer Center (Tampa, FL). DNA was obtained under an IRB-approved protocol at the H. Lee Moffitt Cancer Center and the sequencing of the de-identified sample by both the 454 and the Sanger method was performed with the approval of the University of Cologne Ethics Committee after a review of the collection protocol.

In all instances, specimens were continuously selected at the site of surgery, to avoid sampling bias, and all samples were de-identified prior to processing for DDR2 sequencing. When available, de-identified correlative clinical data were provided with the de-identified tumor DNA.

In the discovery set, 20 patient samples and matched normal DNA were used for sequencing 201 genes, including 90 kinases. All mutations were confirmed as somatic. Mutations were identified using an automated mutation caller and then verified manually with comparison made to the matched normal sequence in the case of all primary tumor samples. In the secondary screening set, 35 additional patient samples and 13 SCC cell lines were used for sequencing the 6 mutated tyrosine kinases identified in the primary screening (DDR2, FGFR2, NTRK2, JAK2, CDK8, and FLT3). In the validation screen, 222 total samples underwent sequencing of the DDR2 gene. In all cases except D125Y, matched normal DNA was available to verify the mutation as somatic.

Cell Culture
A549, NCI-H2286, HCC-366, and NCI-H1703 cells were obtained from the core collection at the Dana-Farber Cancer Institute, having previously been purchased from the American Type Culture Collection and used to establish a collection of early-passage lung cancer cell lines that were analyzed by fingerprinting and single-nucleotide polymorphism arrays (32). All cells used for the experiments described in this article were obtained from freezers made at that time, and no further validation was performed. Lung cancer cell lines were grown in RPMI 1640 (Invitrogen) with 10% fetal calf serum, NIH-3T3 cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech) with 10% bovine serum, and Ba/F3 cells were grown in RPMI supplemented with 10% bovine serum and IL-3 (BD Biosciences) at 10 ng/mL. For IL-3 withdrawal experiments, Ba/F3 cells were collected via centrifugation, washed once in sterile PBS, and then resuspended in media without IL-3. Colony formation assays in NIH-3T3 cells were performed in 6-well plates in which 25,000 NIH-3T3 cells were plated in triplicate in 1 mL of 0.33% top agar over 2 mL of 0.5% bottom agar. After 3 weeks, colonies were counted using NIH ImageJ software.

Vectors
The full-length DDR2 cDNA was obtained from Origene and cloned into the EcoRI site of the retroviral vector pWzIBlast and pbabe-puro following the addition of a C-terminal FLAG tag by PCR. Mutants were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Strategene). All mutations were verified by sequencing. shRNA lentiviral vectors for DDR2 were obtained from The RNAi Consortium at the Broad Institute (Cambridge, MA; 46, 47). DDR2 shRNA-2 corresponds to TRC clone TRCN0000121117, with hairpin sequence 5′-CCGG-CCCTGGAGGTTCCATCATTTA-3′ targeting the 3′ UTR of DDR2. DDR2 shRNA-5 corresponds to TRC clone TRCN0000121121, with hairpin sequence 5′-CCGG-CCTGGAGGTTCCATCATTTA-3′ targeting the coding sequence of DDR2. Both hairpins were provided in the pLKO vector. A hairpin targeting GFP (shGFP) was obtained from TRC and used as a control.

Viral Infections
The DDR2 transgene was expressed in the lung cancer cell lines NIH-3T3 and Ba/F3, using retroviral transduction with the pWz vector, as has been previously described. Briefly, 293T cells were used to generate the virus and were cotransfected with the appropriate pWzl or pbabe vector and a packaging vector using Fugene (Roche). Cells were subjected to 2 rounds of overnight infection in the presence of polybrene. Stable cells were generated using blastidin selection at 10 μg/mL for 3T3, Ba/F3, and A549; 2 μg/mL for NCI-H2286 and NCI-H1703; and 1 μg/mL for HCC-366. Lentiviral infections were performed per the online TRC protocol (48), with 293T cells transfected with the suggested 3-vector combination of pLKO, VSVG, and delta 8.9. Virus was collected and used to infect the lung cancer cell lines for 6 hours in the presence of polybrene. Stable cell lines were generated using puromycin selection at a concentration of 2 μg/mL for NCI-H2286 and 4 μg/mL for NCI-H1703, A549, and HCC-366.

Cell Proliferation and Viability Assays
Cell proliferation was measured with the Cell-Titer-Glo Reagent (Promega) per the manufacturer’s instructions. For experiments with the SCC cell lines, cells were plated in clear-bottomed 96-well plates at a density of 1,500 cells per well. The following day the drug was added, and cell proliferation for the SCC lines was measured 6 days later. For Ba/F3, cells were plated at 5,000 cells per well and the drug was added the same day. Proliferation was measured 4 days later. Proliferation measurements were made using a standard 96-well plate luminometer/plate reader. Data are shown as relative values in which the luminescence at a given drug concentration is compared with that of untreated cells of the same type. Kinase inhibitors were purchased from LC Laboratories or were synthesized in the laboratory of N.S. Gray at Harvard Medical School. In vitro IC50s for DDR2 were determined for all compounds by Lanthascreen time-resolved–FRET kinase activity assays performed by Invitrogen. Cell viability was determined by CellTiter-Glo or CellTiter-Blue reagents (Promega) per the manufacturer’s instructions.
was measured using a Vi-CELL reader to stain cells with trypan blue and to generate 50 independent images for each measured sample. Annexin V (BD Biosciences) analysis was performed on dasatinib-treated cells 48 hours after the addition of the drug per the manufacturer’s protocol. For shRNA experiments, cells were plated at a density of 1,500 cells per well in 96-well plates following puromycin selection. Proliferation was measured 4 days later, in comparison with cells expressing a hairpin targeting GFP.

**Immunoblot**

Immunoblots were performed using the Nupage System (Invitrogen) per the manufacturer’s protocol. Cells were lysed in 1% Nonidet P-40 with protease (Roche) and phosphatase inhibitors (Calbiochem), and protein concentration was assayed with the Bradford reagent (Bio-Rad). Primary antibodies used were Flag-M2 (Sigma), phospho-Y417-Src (Cell Signaling Technologies), phospho-Y694-STAT5 (Cell Signaling), and Actin (Santa Cruz Biotechnology). A DDR2 antibody was generated for this project by Bethyl Laboratories. Secondary horseradish peroxidase–conjugated antibodies were all obtained from Pierce, and proteins were detected by pico-ECL (Thermo Scientific). Images were imported into Adobe Illustrator, using an Epson 4490 scanner. In some cases, brightness and contrast of the scanned images were adjusted for clarity and blots were cropped to display the area of interest in the images. In all cases, adjustment of brightness or contrast was applied uniformly to the image as a whole.

**Xenografts**

All animal experiments were performed according to institutional guidelines regarding animal safety. Nude mice were injected with the lung cancer cell lines at a density of 2.5 (A549), 3.0 (NCI-H1703), or 5.0 (NCI-H2226 and HCC-366) million cells per injection, in an attempt to control for the variable rates of tumor growth in the animals. Cohorts of 10 mice were injected at 3 sites for each cell type, and the mice were observed until the tumor volume approached 150 mL. for A549 and NCI-H1703 or 100 mL for NCI-H1703. At that time, mice were treated with dasatinib at 50 mg/kg or vehicle control daily for 2 weeks, and tumor size was measured during the treatment period.

**Statistics**

For proliferation and colony formation assays, mean values from a minimum of triplicate samples are reported, as well as SEs calculated by Microsoft Excel. IC50 values were obtained using GraphPad Prism software. Power and sample size calculations were performed using the Interactive Statistics Web Resource (43).

**Disclosure of Potential Conflicts of Interest**

M. Meyerson is a consultant to Novartis; receives research support from Novartis and Genentech; is a founding advisor and consultant to, and an equity holder in, Foundation Medicine; and is a patent holder for EGFR mutation testing, licensed to Genzyme Genetics. N.S. Gray receives research support from Novartis. E.B. Haura was the principal investigator of an industry-sponsored clinical trial of dasatinib and erlotinib in lung cancer, funded in part by Bristol-Myers Squibb and the American Society for Clinical Oncology. R.K. Thomas reports consulting and lecture fees (Sequenom, Sanofi-Aventis, Merck, Roche, Infinity, Boehringer, Astra-Zeneca, Johnson & Johnson, and Atlas-Biolabs) and research support (Novartis, AstraZeneca).

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