Abstract

**Purpose:** Oncogenic gene fusions involving the 3′ region of **ROS1** kinase have been identified in various human cancers. In this study, we sought to characterize **ROS1** fusion genes in non–small cell lung cancer (NSCLC) and establish the fusion proteins as drug targets.

**Experimental Design:** An NSCLC tissue microarray (TMA) panel containing 447 samples was screened for **ROS1** rearrangement by FISH. This assay was also used to screen patients with NSCLC. In positive samples, the identity of the fusion partner was determined through inverse PCR and reverse transcriptase PCR. In addition, the clinical efficacy of **ROS1** inhibition was assessed by treating a **ROS1**-positive patient with crizotinib. The HCC78 cell line, which expresses the **SLC34A2–ROS1** fusion, was treated with kinase inhibitors that have activity against **ROS1**. The effects of **ROS1** inhibition on proliferation, cell-cycle progression, and cell signaling pathways were analyzed by MTS assay, flow cytometry, and Western blotting.

**Results:** In the TMA panel, 5 of 428 (1.2%) evaluable samples were found to be positive for **ROS1** rearrangement. In addition, 1 of 48 patients tested positive for rearrangement, and this patient showed tumor shrinkage upon treatment with crizotinib. The patient and one TMA sample displayed expression of the recently identified **SDC4–ROS1** fusion, whereas two TMA samples expressed the **CD74–ROS1** fusion and two others expressed the **SLC34A2–ROS1** fusion. In HCC78 cells, treatment with **ROS1** inhibitors was antiproliferative and downregulated signaling pathways that are critical for growth and survival.

**Conclusions:** **ROS1** inhibition may be an effective treatment strategy for the subset of patients with NSCLC whose tumors express **ROS1** fusion genes. *Clin Cancer Res;* 18(17); 1–10. ©2012 AACR.

Introduction

The identification of oncogenic drivers in tumor cells coupled with the targeting of these proteins by small-molecule inhibitors has become an increasingly successful treatment strategy for non–small cell lung cancer (NSCLC). This scenario is highlighted by the impressive clinical responses observed when EGFR receptor (**EGFR**) mutation–positive patients are treated with the EGFR inhibitors gefitinib and erlotinib and when **ALK** rearrangement–positive patients are treated with the kinase inhibitor crizotinib (1–3). However, for some patients with NSCLC, the identity of the oncogenic driver remains elusive. The characterization of the activated oncogenes in these "pan-negative" tumors is necessary so that more effective treatments for these patients can be developed.

**ROS1** is a receptor tyrosine kinase (RTK) that was initially discovered as the cellular homolog of the transforming **v-ros** sequence from the UR2 avian sarcoma virus (4, 5). The protein is composed of an intracellular C terminal portion containing the kinase domain, a single transmembrane domain, and a large N terminal extracellular domain that contains multiple fibronectin type III-like repeats (6). Unfortunately, very little is currently known about the roles of wild-type **ROS1** in the cell, and no ligand for this receptor has been identified (6, 7). Interestingly, aside from minor abnormalities in the reproductive tracts of males, mice lacking wild-type **ROS1** appear healthy (8).

Cancer-related genomic rearrangement involving **ROS1** was initially discovered in the human glioblastoma cell line U118MG (9, 10). In this line, an intrachromosomal deletion on chromosome 6 fused the 5′ region of a gene named **FIG** (a.k.a. **GOPC**) to the 3′ region of **ROS1** (10). **FIG–ROS1**...
Translational Relevance

Despite recent advances in our understanding of the genetics of non–small cell lung cancer (NSCLC), the oncogenic drivers(s) remain unidentified in many cases. Treatment options for these patients are limited. To improve the clinical outcomes in this population, the identification and characterization of novel driver oncogenes must be a priority. In this study, we identify a subpopulation of patients with NSCLC who express ROS1 gene fusions in their tumors. We show that these fusion genes function as oncogenic drivers by showing that fusion protein expression transforms noncancerous cells and that ROS1 inhibition downregulates growth factor–activated signaling pathways and inhibits proliferation in vitro. We also provide clinical evidence that crizotinib, a small-molecule tyrosine kinase inhibitor with activity against ROS1, has antitumor properties in a patient who expresses a ROS1 fusion gene. Therefore, ROS1 fusion-positive NSCLC represents a novel patient subset that may derive clinical benefit from ROS1 inhibition.

fusions have since been identified in samples from patients with cholangiocarcinoma and ovarian cancer at a frequency of 8.7% and 0.5%, respectively (11, 12). A phosphoproteomic screen of NSCLC cell lines and tumor samples identified one cell line and one tumor sample that expressed highly phosphorylated ROS1 (13). The cell line, HCC78, shows a chromosomal translocation that fused the 5' region of SLC34A2 to the 3' region of ROS1. A different translocation that fused the 5' region of CD74 to the 3' region of ROS1 was found in the tumor sample. Subsequent studies also observed SLC34A2–ROS1 and CD74–ROS1 gene fusions in samples from patients with NSCLC (14, 15). Recently, a screen of a large panel of NSCLC tumor samples identified 4 novel ROS1 fusion partners: TPM3, SDC4, EZR, and LRIG3, in addition to the SLC34A2 and CD74 fusions (16).

Importantly, the ROS1 kinase domain is retained in all of these fusion events, and the expressed fusion genes have been reported to be oncogenic. The FIG–ROS1 fusion promoted anchorage-independent growth and tumorigenicity when expressed in NIH3T3 and RAT1 cells and interleukin (IL)3-independent proliferation when expressed in Ba/F3 cells (11, 17). In support of these findings, ectopic expression of the FIG–ROS1 fusion in the basal ganglia of mice led to the formation of astrocytomas (18). Furthermore, expression of the SLC34A2, CD74, TPM3, SDC4, EZR, and LRIG3–ROS1 fusion genes in NIH3T3 cells resulted in transformation in vitro and tumorigenicity in vivo (11, 16). The mechanism of transformation by these constructs has been reported to involve upregulation of the phosphatase SHP-2, the PI3K/AKT/mTOR pathway, the JAK/STAT pathway, and the MAPK/ERK pathway (11, 18). Furthermore, in HCC78 cells, kinase inhibitors with activity against ROS1 have been shown to inhibit proliferation and siRNAs against ROS1 have been shown to induce apoptosis (13, 15, 19).

In this study, we screened a large NSCLC tissue microarray (TMA) panel to determine the prevalence of ROS1 rearrangement. The fusion partner in all positive TMA samples was determined. We also identified a patient with NSCLC whose tumor was found to express the recently discovered SDC4–ROS1 fusion gene. This patient exhibited tumor shrinkage upon treatment with crizotinib, a U.S. Food and Drug Administration (FDA)-approved ALK inhibitor that has activity against ROS1. Finally, we explored the cellular effects of ROS1 inhibition by treating HCC78 cells with ROS1 inhibitors.

Materials and Methods

Tissue microarray panel

Tissue from 447 surgically resected Caucasian patients with NSCLC who received a radical resection of a primary NSCLC during the period 2000 to 2004 at the Istituto Clinico Humanitas (Milan, Italy) was included in a TMA. Three cores (0.6-mm diameters) were available from each patient. Details of the TMA construction have been previously described (20). Institutional Review Board approval was obtained from the Istituto Clinico Humanitas.

Fluorescence in situ hybridization

A customized ROS1 break-apart probe set was designed using clones RP11-623N3 (117,654–117,833) and RP11-170O13 (117,830–117,971) telomeric (5') and clones RP11-59K17 (117,449–117,626) and CTD-2314K7 (117,338–117,438) centromeric (3') to the common breakpoint of ROS1. The clones are separated by a small distance (28,365 bp) and are overlapped or very close in the native copy of the gene. The FISH assays and analyses were conducted as previously described with minor modifications (20). Using the ROS1 break-apart probe set, 3' and 5' signals physically separated by >1 signal diameter were considered split. Specimens were considered positive for ROS1 rearrangement if more than 15% of the cells showed split signals or single 3' signals. The FISH analysis was conducted under blinded conditions without access to clinical, pathologic, or molecular features. FISH analysis of the TMA samples was conducted on the 0.6-mm formalin-fixed, paraffin-embedded (FFPE) cores. FISH analysis for the University of Colorado (Aurora, CO) was conducted on FFPE biopsy specimens.

Immunohistochemistry

Immunohistochemistry was conducted using standard techniques for TTF1 (Cell Marque Clone 8G7G3-1 at 1:100 dilution), p63 (Biocare Medical clone BC4A4 prediluted), and CK5/6 (DAKO Cytomation Clone D5/16 B4 at 1:15 dilution). Briefly, 4-μm slides were used and subjected to antigen retrieval for 30 minutes in High pH Cell Conditioner 1 (Ventana Medical Systems). Endogenous biotin blocker was used for p63 and TTF1 stains. p63 and CK5/6 were detected using the iView DAB Detection Kit (Ventana Medical Systems).
Medical Systems). TTF1 was detected using UltraView Polymer DAB Detection Kit (Ventana Medical Systems).

RNA isolation
Isolation of RNA from FFPE samples was accomplished using the RecoverAll Total Nucleic Acid Isolation Kit from Ambion according to the manufacturer’s protocol. For total RNA isolation from the frozen tumor sample from the University of Colorado patient, tissue was homogenized and then resuspended in Tri-Reagent from Ambion. Homogenized samples were incubated at room temperature for 7 minutes to dissociate nucleoproteins and then subjected to organic extraction by the addition of chloroform followed by centrifugation. The aqueous layer was isolated and RNA precipitated by isopropanol incubation followed by 2 washes in 70% ethanol. The RNA pellet was resuspended in nuclease-free water.

Patients and treatment
Patients at the University of Colorado (CU) were screened for the presence of a ROS1 gene fusion after Institutional Review Board-approved consent was obtained. One patient (of 48 consented) was identified with evidence of a ROS1 gene fusion, and this patient was enrolled on an expanded cohort of the Pfizer phase I trial of PF-02341066 (crizotinib, Xalkori; http://clinicaltrials.gov/ct2/show/NCT00585195).

Reverse transcriptase-PCR
To identify the fusion partner of ROS1 in the TMA samples, reverse transcriptase (RT)-PCR was carried out using the SuperScript III First-Strand Synthesis System (Invitrogen) with a previously published ROS1 primer located in exon 34 (ROS1 E34R; ref. 14). First-strand synthesis was carried out as above followed by a 20-minute RNaseH digestion at 37°C. Individual PCR reactions were carried out to amplify either SLC34A2–ROS1, CD74–ROS1, or SDC4–ROS1 using the previously published primers (SLC34A2-E4F, CD74-E5F, ROS1 E34F) along with a primer to SDC4 of our design (SDC4-E2F; ref. 14). PCR conditions for detecting the ROS1 fusion partners included an initial denaturation at 95°C for 5 minutes followed by 10 cycles of touchdown PCR (annealing temperature ranging from 60°C to 55°C with a 0.5 decrease per cycle and a 1-minute extension at 72°C) and 30 cycles of PCR (annealing temperature at 55°C and 1-minute extension at 72°C). PCR products were resolved on a 2% agarose gel. Positive PCR products for the ROS1 fusions were excised from agarose gel, purified (Wizard SV Gel and PCR Clean Up Kit; Promega), and sequenced. All primer sequences are listed in Supplementary Table S1.

Lentiviral constructs and transduction
To create the SDC4–ROS1 (exon 32) construct, RT-PCR was carried out on the RNA sample from the CU patient. This was done as above using primers to the 5’ end of SDC4 (SDC4 E1F and 3’ end of ROS1 (ROS1 E43F). Restriction sites (XhoI and NotI) were added to the PCR product for subsequent cloning into a lentiviral expression plasmid (pCDH-MCS1-EF1-Puro; System Biosciences). Production of lentivirus was achieved by co-transfecting this plasmid (or empty pCDH-MCS1-EF1-Puro as a control), pCMV-VSV-G, and pCMVΔR8.2 into 293T cells using TransIT-293 transfection reagent (Mirus Bio) as previously described (21). Viral supernatants were collected 72 hours after transfection and added to Ba/F3 or NIH3T3 cells in the presence of 8 µg/mL polybrene (Millipore). Media were replaced after 24 hours of incubation. Infected cells were selected through puromycin treatment (2 µg/mL).

Cell lines and reagents
HCC78 was a kind gift from Dr. John D. Minna (The University of Texas Southwestern Medical Center, Dallas, TX). Ba/F3 cells were a kind gift from Dr. Dan Theodorescu (University of Colorado Comprehensive Cancer Center, Aurora, CO). 293T and NIH3T3 cells were purchased from American Type Culture Collection. Crizotinib (PF-02341066) and PF-04217903 were obtained from Pfizer Inc. NVP-TAE684 and gefitinib were purchased from Selleck Chemicals. Antibodies used were as follows: ROS1 pY2274 (3078, Cell Signaling), total ROS1 (sc-6347, Santa Cruz Biotechnology), SHP-2 pY542 (3751, Cell Signaling), total SHP-2 (610621,

www.aacrjournals.org
Clin Cancer Res; 18(17) September 1, 2012
OF3

Published OnlineFirst August 23, 2012; DOI: 10.1158/1078-0432.CCR-12-0550

Downloaded from clincancerres.aacrjournals.org on April 15, 2017. © 2012 American Association for Cancer Research.
BD Biosciences), AKT pS437 (4058, Cell Signaling), total AKT (2920, Cell Signaling), STAT3 pY705 (9145, Cell Signaling), total STAT3 (9139, Cell Signaling), extracellular signal–regulated kinase (ERK) pT202/Y204 (9101, Cell Signaling), total ERK (9107, Cell Signaling), α-tubulin (sc-8035, Santa Cruz Biotechnology), and GAPDH (MAB274, Millipore).

Cellular proliferation

Cells were seeded in 96-well plates at a density found to result in exponential growth throughout the course of the assay. Immediately after seeding (Ba/F3) or on the day following seeding (HCC78), the indicated doses of the inhibitors were added. Three days following drug addition, 20 μL of MTS reagent (Promega) was added to each well. Following a 1-hour incubation, absorbance at 490 nm was measured using a microplate reader (Molecular Devices). IC_{50} values were calculated using Prism software from GraphPad.

Cell-cycle analysis

Cells were seeded in 6-well plates and then treated with drug the next day. Following treatment, cells were washed and then permeabilized with 70% ethanol. Cells were then stained with propidium iodide (BD Pharmingen) and analyzed on a Gallios flow cytometer (Beckman Coulter). Cell-cycle distribution analysis was conducted using ModFit software (Verity Software House).

Phospho-array and immunoblotting

Phosphorylated RTKs were measured with the Human Phospho-RTK Array Kit (ARY001) from R&D Systems per the manufacturer’s instructions. Immunoblotting was conducted as previously described (22).

Results

In light of recent studies that observed ROS1 gene fusions in various human cancers, we sought to determine the prevalence of ROS1 rearrangement in NSCLC. To this end, we screened 447 NSCLC samples in a TMA using a break-apart FISH assay. The clinical and pathologic features of the patients in this TMA are summarized in Supplementary Table S2. In this assay, tumor tissue is stained with 2 fluorescently labeled probes; one specific for the 5’ region of ROS1 and one specific for the 3’ region. Similar to ALK FISH, separation of the probes (observed by fluorescent microscopy) is indicative of a genetic rearrangement involving the gene (23). We found 5 positives of 428 evaluable samples, suggesting that approximately 1.2% of NSCLC tumors have undergone rearrangements involving ROS1 (Fig. 1A). In the FISH analysis, both split signals and single 3’ signals were observed (Supplementary Table S3). The number of cells positive for rearrangement ranged from 25% to 84% per sample (Supplementary Table S3). Of the positive patients, the age ranged from 41 to 71 years (Table 1). Three of the positive patients were female and 2 were male and 3 were former or current smokers and 2 were never-smokers. Histologically, 3 of the patients presented with adenocarcinomas and 2 with squamous cell carcinomas. The histology of the 2 squamous specimens was confirmed by independent concordance among 3 separate pathologists and lack of TTF-1 staining and presence of p63 staining by immunohistochemistry (Supplementary Fig. S2).

Genetic rearrangement does not necessarily prove expression of a fusion gene, so we analyzed the positive samples by RT-PCR. At the time these studies were conducted, only the SLC34A2–ROS1 and CD74–ROS1 fusions had been identified in NSCLC. Therefore, we screened the positive TMA samples with primers designed to recognize these fusions (Fig. 1B). Amplified DNA was then gel isolated and sequenced to verify identity (data not shown). We found that 2 of the positive samples expressed the SLC34A2–ROS1 fusion and 2 expressed the CD74–ROS1 fusion. Similar to HCC78 cells, the 2 samples positive for the SLC34A2–ROS1 fusion expressed equivalent levels of the long and short transcripts (SLC34A2 exon 4 fused to ROS1 exons 32 and 34, respectively). The 2 samples positive for the CD74 fusion expressed only the short transcript (CD74 exon 6 fused to ROS1 exon 34).

The finding that a subset of patients with NSCLC are positive for ROS1 rearrangement prompted us to begin testing patients with NSCLC at the University of Colorado Anschutz Medical Campus by FISH (as described above). We found 1 patient positive for ROS1 rearrangement of the 48 tested (Fig. 2A). The clinical and pathologic features of the patients tested are summarized in Supplementary Table S4. The positive patient is a 65-year-old male never-smoker with adenocarcinoma that was found to be wild-type for EGFR and KRAS and negative for rearrangement of ALK. To confirm expression of a ROS1 fusion gene and to identify the fusion partner in this patient’s tumor, we conducted the same RT-PCR assays as for the TMA samples above. However, we found this patient’s sample to be negative for both the SLC34A2–ROS1 and CD74–ROS1 fusions (Fig. 1B). We then used inverse PCR to determine the identity of the fusion partner. This technique is commonly used to amplify unknown regions of DNA that are adjacent to known regions (24). Sequencing of the inverse PCR product revealed that this patient’s tumor has undergone a chromosomal translocation between chromosomes 6 and 20 that fused the 3’ region of ROS1 to the 5’ region of SDC4. We then carried out RT-PCR on the CDNA using primers specific for SDC4 and ROS1 and observed 2 different species: a predominant long form and a minor short form (Fig. 1B). This same assay conducted on the remaining positive TMA sample revealed similar products, although in the TMA sample the short form was the predominant band (Fig. 1B). Sequencing of these products revealed a fusion of SDC4 exon 2 to ROS1 exon 32 in the long form and a fusion of SDC4 exon 2 to ROS1 exon 34 in the short form, suggestive of alternative splicing of a single fusion gene (Fig. 2B). Interestingly, these are the same ROS1 breakpoints observed in samples with SLC34A2–ROS1, CD74–ROS1, and EZR–ROS1 fusions (13, 14, 16). When a construct containing the long form of this fusion gene was introduced into Ba/F3 cells, the ROS1 fusion protein was expressed and...
this led to IL3-independent growth, a hallmark of an activated oncogene (Fig. 2C).

Crizotinib is a kinase inhibitor that has recently been approved by the FDA for the treatment of patients with NSCLC who test positive for \textit{ALK} gene rearrangements. However, this molecule also has activity against ROS1 (25). Therefore, it was hypothesized that treatment of a ROS1 fusion–positive patient with crizotinib may be an effective treatment strategy. The patient identified with the SDC4–ROS1 gene fusion was treated with 250 mg crizotinib orally twice daily in 28-day cycles within a molecularly defined cohort in the first-in-man study of crizotinib. After 2 cycles (56 days) of continuous crizotinib therapy, there was a 57% tumor shrinkage using Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 with an associated decrease in standard uptake value from 10.8 to 3.7. This response was confirmed on subsequent imaging and therefore consistent with a partial response to crizotinib (Fig. 2D). Recently, another case of a ROS1 fusion gene–positive patient with NSCLC who responded to crizotinib was reported, although the fusion partner was not identified (15).

To examine the cellular effects of ROS1 inhibition, we treated SDC4–ROS1 expressing Ba/F3 cells with crizotinib and NVP-TAE684, another small-molecule ALK inhibitor with activity against ROS1 (19). We found that, similar to Ba/F3 cells expressing the EML4-ALK fusion protein, both

![Figure 1. ROS1 FISH and RT-PCR analysis of NSCLC TMA panel. A, ROS1 rearrangement–positive samples analyzed by break-apart FISH assay. Red probes are hybridized to the 5‘ region of ROS1 and green probes to the 3‘ region. Yellow arrows denote fused signals (nonrearranged ROS1 allele), whereas green and red arrows denote 3‘ and 5‘ regions of ROS1, respectively, that have been separated by rearrangement. Left image shows split signal pattern (patient #1), whereas right image shows single green signal pattern (patient #4). B, agarose gel electrophoresis of RT-PCR products from ROS1 rearrangement–positive TMA samples (along with samples from HCC78 cells and CU patient for comparison). The approximate expected positions of long transcripts (fusion to ROS1 exon 32), short transcripts (fusion to ROS1 exon 34), and primer dimers (PD) are shown. In all cases, amplified DNA was gel isolated and sequenced (data not shown).](image-url)
Table 1. ROS1 fusion gene–positive patient characteristics

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Sex/age</th>
<th>Stage</th>
<th>Smoking status</th>
<th>Histology</th>
<th>ROS1 fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/51</td>
<td>III</td>
<td>Former</td>
<td>Squam</td>
<td>SLC34A2–ROS1 (4:32 + 34)</td>
</tr>
<tr>
<td>2</td>
<td>M/61</td>
<td>I</td>
<td>Former</td>
<td>Adeno</td>
<td>SLC34A2–ROS1 (4:32 + 34)</td>
</tr>
<tr>
<td>3</td>
<td>F/68</td>
<td>III</td>
<td>Never</td>
<td>Adeno</td>
<td>SDC4–ROS1 (2:32 + 34)</td>
</tr>
<tr>
<td>4</td>
<td>M/71</td>
<td>I</td>
<td>Current</td>
<td>Squam</td>
<td>CD74–ROS1 (8:34)</td>
</tr>
<tr>
<td>5</td>
<td>F/41</td>
<td>III</td>
<td>Never</td>
<td>Adeno</td>
<td>CD74–ROS1 (8:34)</td>
</tr>
</tbody>
</table>

NOTE: For ROS1 fusion, numbers represent fusion point [exon of partner; exon(s) of ROS1].

Abbreviations: Adeno, adenocarcinoma; F, female; M, male; Squam, squamous cell carcinoma.

Davies et al.

Discussion

Genetic rearrangements that fuse the kinase-domain containing 3′ regions of tyrosine kinases to the 5′ regions of unrelated genes are found in multiple tumor types. Because these fusion genes are often the oncogenic drivers of the tumor cells, they represent ideal targets for therapeutic intervention. The potential for clinical success using this approach has been well established by the use of imatinib in BCR–ABL fusion–positive chronic myelogenous leukemia and crizotinib in ALK rearrangement–positive NSCLC (3, 26). In this study, we characterized ROS1 genetic rearrangement in NSCLC and established ROS1 fusion proteins as attractive drug targets.

SDC4 is a heparan sulfate proteoglycan that plays a role as a coreceptor in focal adhesion signaling complexes (27). Through an inverse PCR technique, we identified an SDC4–ROS1 gene fusion in a ROS1 FISH–positive NSCLC patient (Fig. 2). Another group has also identified SDC4–ROS1 fusions in NSCLC samples (16). Our characterization of this fusion gene in 2 patient-derived tumor samples revealed the existence of 2 species: a long form in which SDC4 is fused to ROS1 exon 32 and a short form in which SDC4 is fused to ROS1 exon 34. SDC4 fusions to ROS1 exons 32 and 34 were also found in the study by Takeuchi and colleagues (16). Interestingly, this is the exact same pattern that is observed for the SLC34A2–ROS1 and CD74–ROS1 fusions (13, 14). Together, these findings are highly suggestive of a common break point 3′ of ROS1 exon 32 and subsequent alternative splicing of the transcript that removes ROS1 exons 32 and 33. However, there does not seem to be a conserved pattern for this alternative splicing. In HCC78 cells, 2 of our TMA samples, and a recently published patient sample, both long and short forms of the SLC34A2–ROS1 fusion were shown to be expressed (Fig. 1B; refs. 13, 16). In contrast, another report found only the long form of SLC34A2–ROS1 in a patient sample (15). We observed only the short form of the CD74–ROS1 fusion in TMA samples, as did the study from Berghofer and colleagues, however the studies from Li and colleagues and Takeuchi and colleagues found patients who expressed both forms (in addition to ones that expressed only the short form; refs. 14–16). Finally, we observed different ratios of long to short forms of the SDC4–ROS1 fusion in our CU patient sample and TMA sample (Fig. 1B), and the study from Takeuchi and colleagues, found patients who only expressed the long form (16). Clearly, future studies are needed to address the importance of this splicing event.
We used FISH analysis to determine the prevalence of ROS1 rearrangement and found 5 positives of 428 evaluable samples in a TMA panel. The FISH patterns observed in this study did not seem to correlate with a specific fusion gene partner, similar to ALK FISH where multiple FISH patterns are observed for EML4-ALK (23). Our study is the first to identify ROS1 gene fusions in NSCLC cases with squamous cell carcinoma histology. It should be noted that the number of ROS1-positive cases are small in this series and therefore the proportion of squamous cell cases observed here may not reflect that of the general population of patients with NSCLC. Other activated oncogenes (including BRAF, PIK3CA, ALK, and others) in NSCLC are found in both adenocarcinoma and squamous cell carcinoma histologies (28). Our study also showed a significant proportion of current and former smokers and a similar caution should be used when trying to extrapolate from small numbers. Data suggest for both EGFR mutation–positive and ALK gene rearrangement–positive patients that a large proportion...
expressed the other known SLC34A2 fusions by RT-PCR using only primers specific for SLC34A2. E6; A20 (2.4 nmol/L), crizotinib for 3 days and then analyzed by MTS assay. Values represent the mean ± SEM (n = 4). Calculated IC50 values were as follows: NVP-TAE684–SD2/R32 (12.3 nmol/L), E6; A20 (2.4 nmol/L), crizotinib–SD2; R32 (31 nmol/L), E6; A20 (83 nmol/L). B, HCC78 cells were treated with dose ranges of NVP-TAE684 or crizotinib for 3 days and then analyzed by MTS assay. Values represent the mean ± SEM (n = 5). Calculated IC50 values were 105 nmol/L for NVP-TAE684 and 775 nmol/L for crizotinib. C, HCC78 cells were treated with dose ranges of NVP-TAE684 (left) or crizotinib (right) for 24 hours and then stained with propidium iodide. Cell-cycle distribution was then assessed by flow cytometry. Results are representative of 2 independent experiments. D, HCC78 cell lysates were analyzed by phospho-RTK array. The positions of phospho-EGFR and phospho-MET spots are indicated. Unlabeled spots at the 4 corners of the array are the positive control. E, left, HCC78 cells were treated with dose ranges of gefitinib (EGFR kinase inhibitor) or PF-04217903 (MET kinase inhibitor) for 3 days and then analyzed by MTS assay. Values represent averages of 2 independent experiments. Right, HCC78 cells were treated with a dose range of NVP-TAE684 alone or in the presence of 1 μmol/L gefitinib or 1 μmol/L PF-04217903 for 3 days and then analyzed by MTS assay. Values represent the mean ± SEM (n = 4). Calculated IC50 values were 157 nmol/L for vehicle, 43 nmol/L for +1 μmol/L gefitinib, and 141 nmol/L for +1 μmol/L PF-04217903. +1 μmol/L gefitinib was less than + vehicle and +1 μmol/L PF-04217903 as determined by one-way ANOVA analysis followed by Bonferroni multiple comparison test (P < 0.05).

Figure 3. ROS1 inhibition is antiproliferative in cells expressing ROS1 gene fusions. A, Ba/F3 cells transduced with an SDC4–ROS1 (exon 32) construct (SD2; R32) or an EML4–ALK (exon 20) construct (E6; A20) were treated with dose ranges of NVP-TAE684 (left) or crizotinib (right) for 3 days in the absence of IL3 and then analyzed by MTS assay. Values represent the mean ± SEM (n = 4). Calculated IC50 values were as follows: NVP-TAE684–SD2/R32 (12.3 nmol/L), E6; A20 (2.4 nmol/L), crizotinib–SD2; R32 (31 nmol/L), E6; A20 (83 nmol/L). B, HCC78 cells were treated with dose ranges of NVP-TAE684 or crizotinib for 3 days and then analyzed by MTS assay. Values represent the mean ± SEM (n = 5). Calculated IC50 values were 105 nmol/L for NVP-TAE684 and 775 nmol/L for crizotinib. C, HCC78 cells were treated with dose ranges of NVP-TAE684 (left) or crizotinib (right) for 24 hours and then stained with propidium iodide. Cell-cycle distribution was then assessed by flow cytometry. Results are representative of 2 independent experiments. D, HCC78 cell lysates were analyzed by phospho-RTK array. The positions of phospho-EGFR and phospho-MET spots are indicated. Unlabeled spots at the 4 corners of the array are the positive control. E, left, HCC78 cells were treated with dose ranges of gefitinib (EGFR kinase inhibitor) or PF-04217903 (MET kinase inhibitor) for 3 days and then analyzed by MTS assay. Values represent averages of 2 independent experiments. Right, HCC78 cells were treated with a dose range of NVP-TAE684 alone or in the presence of 1 μmol/L gefitinib or 1 μmol/L PF-04217903 for 3 days and then analyzed by MTS assay. Values represent the mean ± SEM (n = 4). Calculated IC50 values were 157 nmol/L for vehicle, 43 nmol/L for +1 μmol/L gefitinib, and 141 nmol/L for +1 μmol/L PF-04217903. +1 μmol/L gefitinib was less than + vehicle and +1 μmol/L PF-04217903 as determined by one-way ANOVA analysis followed by Bonferroni multiple comparison test (P < 0.05).

of approximately 40% in both molecular subtypes are current or former smokers (29, 30).

Three recent studies also sought to determine the frequency of ROS1 rearrangement in NSCLC (14–16). In the study by Li and colleagues, 2 of 202 (~1%) East Asian never-smoker patients with adenocarcinoma were found to express the CD74–ROS1 fusion, whereas no SLC34A2–ROS1 fusions were found (14). Because this study screened samples by RT-PCR using only primers specific for CD74 and SLC34A2, it is possible that additional patients in this cohort expressed the other known ROS1 fusions. In the study by Bergethon and colleagues, a similar FISH assay to that used in our study found that 18 of 1,073 (1.7%) patients with NSCLC had undergone ROS1 rearrangement (15). Of these 18 positive samples, 5 expressed the CD74 fusion and 1 expressed the SLC34A2 fusion. In the study by Takeuchi and colleagues, FISH and RT-PCR analysis found 13 of 1,476 (0.9%) NSCLC samples to express ROS1 fusions (2 to 3TMM, 3 to SDC4, 1 to SLC34A2, 3 to CD74, 2 to EKR, 1 to LRIG3, and 1 unknown; ref. 16). These studies, together with our study, suggest that the prevalence of ROS1 rearrangement is 1% to 2% of all NSCLC cases. As it is estimated
that there are approximately 1.6 million new patients with lung cancer diagnosed each year worldwide, and 80% to 85% of these cases are of NSCLC histology, it can be predicted that there are 12,000 to 27,000 new patients per year who harbor tumors that express ROS1 fusion genes (31).

Our finding that crizotinib-induced tumor regression in an SDC4–ROS1 fusion–positive patient together with a similar finding in a patient with an unknown fusion partner that was recently published indicate that inhibition of ROS1 may be an effective treatment strategy for this subpopulation of NSCLC (15). Crizotinib is a slightly more potent inhibitor of ROS1 fusion protein activity than ALK fusion protein activity when expressed in Ba/F3 cells (Fig. 3A). These data are consistent with cell-free in vitro kinase data where the IC50 of crizotinib for ROS1 is 0.11 nmol/L compared with an IC50 of 0.6 nmol/L for ALK (J. Chris tensen, personal communication). Given the success of crizotinib in ALK rearrangement–positive patients with NSCLC and the enhanced potency of crizotinib on ROS1 compared with ALK, this drug is likely to be an effective therapy for patients who display ROS1 gene rearrangements. Interestingly, crizotinib and NVP-TAE684 showed reduced potency in HCC78 cells [the only published NSCLC cell line that expresses a ROS1 fusion gene (SCL34A2–ROS1)] compared with Ba/F3 cells expressing the SDC4–ROS1 fusion (Fig. 3A, B). This relatively non-potent inhibition of HCC78 proliferation has also been observed in previous studies (15, 19). While it is possible that fusion partner identity influences sensitivity to ROS1 inhibition, we showed that the reduced sensitivity of HCC78 cells is at least partially due to EGFR activation (Fig. 3D, E). Indeed, a recent study observed that EGFR activation induced resistance to ALK inhibition in EML4–ALK expressing NSCLC cell lines (32).

In conclusion, approximately 1% to 2% of patients with NSCLC harbor tumors that are driven by ROS1 fusion proteins and several different 5' fusion partners for ROS1 exist. Inhibition of ROS1 kinase activity by small molecules is a promising treatment strategy for these patients.

**Disclosure of Potential Conflicts of Interest**

D.R. Camidge is a consultant/advisory board member for Pfizer. R.C. Doebele received research grants from Pfizer, Eli Lilly & Co., and ImClone Systems; honoraria from Speakers Bureau of Abbott Laboratories; is a consultant/advisory board member for Pfizer and Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: K.D. Davies, A.T. Le, D.R. Camidge, M. Varella-Garcia, R.C. Doebele


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.D. Davies, M.F. Theodoro, M.C. Skokan, D.L. Aisner, M. Incarbone, M. Roncalli, F. Cappuzzo, D.R. Camidge, M. Varella-Garcia, R.C. Doebele

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.D. Davies, A.T. Le, M.F. Theodoro, M.C. Skokan, D.R. Camidge, M. Varella-Garcia, R.C. Doebele

Writing, review, and/or revision of the manuscript: K.D. Davies, A.T. Le, D.L. Aisner, E.M. Berge, F. Cappuzzo, D.R. Camidge, M. Varella-Garcia, R.C. Doebele

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.D. Davies, A.T. Le, M.F. Theodoro, M.C. Skokan, E.M. Berge, M. Varella-Garcia, R.C. Doebele

Study supervision: A.T. Le, D.R. Camidge, M. Varella-Garcia, R.C. Doebele
Acknowledgments

The authors thank Blair Murphy, Aria Vaishnani, and Barbara A. Helfrich for technical assistance.

Grant Support

This research was supported by the University of Colorado Lung Cancer SPORE grant (P50CA058187) to M.arella-Garcia and R.C. Doebele and by funds from the Boettcher Foundation’s Webb-Waring Biomedical Research Program to R.C. Doebele. This work was also supported by the Italian Association for Cancer Research (AIRC) and Associazione Oncologia Translazionale (AOT). Pfizer provided financial support of ROS1 FISH testing at the University of Colorado.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 15, 2012; revised June 11, 2012; accepted July 2, 2012; published OnlineFirst August 23, 2012.

References


Clinical Cancer Research

Identifying and Targeting ROS1 Gene Fusions in Non–Small Cell Lung Cancer

Kurtis D. Davies, Anh T. Le, Mariana F. Theodoro, et al.

Clin Cancer Res  Published OnlineFirst August 23, 2012.

Updated version   Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-12-0550
Supplementary Material Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/08/22/1078-0432.CCR-12-0550.DC1

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.