HHS Public Access

Author manuscript

J Thorac Oncol. Author manuscript; available in PMC 2018 May 01.

Published in final edited form as:

J Thorac Oncol. 2017 May ; 12(5): 872–877. doi:10.1016/j.jtho.2017.01.004.

ROS1 fusions rarely overlap with other oncogenic drivers in non-small cell lung cancer

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Abstract

Introduction—Chromosomal rearrangements involving the ROS proto-oncogene 1 receptor tyrosine kinase gene (*ROS1*) define a distinct molecular subset of non-small cell lung cancer (NSCLC) with sensitivity to ROS1 inhibitors. Recent reports have suggested a significant overlap between *ROS1* fusions and other oncogenic driver alterations, including mutations in epidermal growth factor receptor (*EGFR*) and KRAS proto-oncogene (*KRAS*).

Methods—We identified patients at our institution with *ROS1*-rearranged NSCLC who had undergone testing for genetic alterations in additional oncogenes, including *EGFR, KRAS*, and anaplastic lymphoma kinase (*ALK*). Clinicopathologic features and genetic testing results were reviewed. We also examined a separate database of *ROS1*-rearranged NSCLCs identified through a commercial FoundationOne assay.

Results—Among 62 patients with *ROS1*-rearranged NSCLC evaluated at our institution, none harbored concurrent *ALK* fusions (0%) or *EGFR* activating mutations (0%). *KRAS* mutations were detected in two cases (3.2%), one of which harbored a concurrent non-canonical KRAS I24N mutation of unknown biological significance. In a separate *ROS1* FISH-positive case, targeted

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Disclosures:

The remaining authors (**JJL, LLR, LAF, JKL**) have no other disclosures to declare.

LIST OF SUPPLEMENTAL DIGITAL CONTENT

Supplemental Table 1. Genotyping platforms.

Supplemental Table 2. Genes analyzed in the genotyping platforms.

Supplemental Table 3. Concomitant genetic alterations in *ROS1*-rearranged NSCLC: List of mutations.

sequencing failed to confirm a *ROS1* fusion, but instead identified a KRAS G13D mutation. No concurrent mutations in *BRAF, ERBB2, PIK3CA, AKT1*, or *MAP2K1* were detected. Analysis of an independent dataset of 166 *ROS1*-rearranged NSCLCs identified by FoundationOne demonstrated rare cases with co-occurring driver mutations in *EGFR* (1/166) and *KRAS* (3/166), and no cases with co-occurring *ROS1* and *ALK* rearrangements.

Conclusions—*ROS1* rearrangements rarely overlap with alterations in *EGFR, KRAS, ALK*, or other targetable oncogenes in NSCLC.

Keywords

ROS1; non-small cell lung cancer; NSCLC

INTRODUCTION

ROS proto-oncogene 1 receptor tyrosine kinase (ROS1) is a validated therapeutic target in non-small cell lung cancer (NSCLC). Chromosomal rearrangements involving the *ROS1* gene occur in 1–2% of NSCLCs,[1–4] and are clinically associated with never smoking history, younger age, and adenocarcinoma histology.[2] NSCLC cells harboring oncogenic *ROS1* fusions are dependent on ROS1 signaling for their viability.[1,5]

In the clinic, identification of patients with NSCLC harboring *ROS1* fusions is crucial, as these patients can have marked responses to ROS1-targeted tyrosine kinase inhibitors (TKIs). In an early-phase study of crizotinib in advanced *ROS1*-rearranged NSCLC, the objective response rate was 72% and median progression-free survival was 19.2 months.[6] Two additional studies since then have demonstrated similarly high response rates (ranging 71–80%) to crizotinib in *ROS1*-rearranged NSCLC, although the median progression-free survival in these studies was shorter at 9–10 months.[7,8] Based on its safety and efficacy, crizotinib was granted approval by the United States Food and Drug Administration and the European Medicines Agency for treatment of advanced *ROS1*-positive NSCLC. Several additional inhibitors with ROS1 activity are now being developed, including lorlatinib (NCT01970865), cabozantinib (NCT01639508), entrectinib (NCT02568267), ceritinib (NCT02186821), and DS-6051b (NCT02279433).

Genetic alterations in oncogenic drivers in NSCLC, including KRAS proto-oncogene (*KRAS*), epidermal growth factor receptor (*EGFR*), and anaplastic lymphoma kinase (*ALK*), are generally deemed mutually exclusive.[9] Initial studies suggested that *ROS1* rearrangements do not overlap with *EGFR* mutations or *ALK* rearrangements.[2,4,6,10] However, conflicting findings have since been reported.[11,12] For example, in a recent analysis of 25 NSCLCs positive for *ROS1* rearrangement by immunohistochemistry (IHC), 36% were reported to harbor concomitant oncogenic driver mutations (including in *EGFR, KRAS, PIK3CA*, and *BRAF*).[12] Five of the six patients with tumors harboring concurrent *EGFR* mutations in this cohort derived significant clinical benefit from an EGFR inhibitor and did not receive a ROS1-targeted therapy,[12] raising the question of whether *ROS1* rearrangements truly define a distinct molecular subset of NSCLC.

Herein, we examined *ROS1*-rearranged NSCLC patients who underwent genotyping of other oncogenes including *KRAS, EGFR*, and *ALK*, in order to determine the frequency of concurrent driver alterations in *ROS1*-rearranged NSCLC.

METHODS

Study population

Seventy patients with *ROS1*-rearranged NSCLC seen at Massachusetts General Hospital (MGH) between 2007 and October 2016 were identified. Of these, 62 had known mutational status of *KRAS* (exon 2), *EGFR* (exons 18–21) and *ALK*, and these patients (the MGH cohort) were selected for an institutional review board-approved retrospective analysis. Records were reviewed to extract data on clinicopathologic characteristics and tumor genotyping. An independent group of 166 *ROS1*-rearranged NSCLC patients were identified using the FoundationOne next-generation sequencing (NGS) assay at Foundation Medicine (the FM cohort). A total of eight patients were included in both cohorts.

Molecular testing

In the MGH cohort, *ROS1* testing was performed using fluorescence in situ hybridization (FISH), targeted RNA sequencing using anchored multiplex polymerase chain reaction as previously described,[13] commercially available FoundationOne NGS (Foundation Medicine, Cambridge, MA), or a commercial real-time polymerase chain reaction (PCR) assay (Clarient/NeoGenomics Laboratories, Fort Myers, FL). FISH was performed on formalin-fixed paraffin-embedded (FFPE) tumor tissue using a break-apart assay as previously described,[2] and determined to be positive if >15% of tumor cells demonstrated split signals.

More comprehensive genotyping data (defined as sequencing for hotspot mutations in >10 genes) was available for 44 patients in the MGH cohort. The sequencing assay used for each patient is listed in Supplemental Table 1. Genes analyzed in each sequencing platform are listed in Supplemental Table 2. The FoundationOne (Foundation Medicine, Cambridge, MA), Smart Genomics (PathGroup, Brentwood, TN), and LUNGSEQ (Medfusion, Lewisville, TX) panels are commercially available. The MGH SNaPshot[13] and Dana-Farber Cancer Institute (DFCI) OncoPanel[14] assays have been previously described.

RESULTS

Identification of *ROS1* rearrangements

ROS1 fusions were identified in 62 patients in the MGH cohort using FISH (n = 38), targeted sequencing or PCR (n = 13), or both FISH and sequencing (n = 11). Clinicopathologic features of these 62 *ROS1*-rearranged NSCLC patients are summarized in Table 1. In the 24 cases of *ROS1* fusions detected by NGS or PCR, four previously reported *ROS1* fusion partners were identified: *CD74* (n = 16), *SDC4* (n = 4), *EZR* (n = 2), and *SLC34A2* (n = 2).[1–4] Twelve patients underwent *ROS1* testing by both FISH and NGS, of whom 11 had concordant positive results (Figure 1). In the patient with discordant results (patient 53), FISH was positive with split signals in 44 of 50 tumor cell nuclei, but RNA sequencing on the same tumor did not detect a *ROS1* fusion.

Genetic alterations of *ALK, EGFR*, and *KRAS*

All 62 cases were tested for *ALK* rearrangements, *EGFR* mutations, and *KRAS* mutations. None had a concurrent *ALK* fusion. A concurrent *EGFR* activating mutation was also not detected (Figure 1). The discordant *ROS1* case (patient 53, mentioned above) was found to harbor an EGFR C781F mutation. This variant, which lies within the kinase domain, has not been previously reported, and its biological consequence is unknown.[15,16]

Two cases (3.2%; patients 53 and 48) had a *KRAS* mutation (Figure 1). Patient 53, the patient with discordant *ROS1* testing (FISH-positive/NGS-negative) and EGFR C781F, was also found to harbor a KRAS G13D activating mutation. This patient, a 25-pack-year former smoker, was treated with crizotinib with no documented response, but experienced a sustained response to nivolumab. Patient 48 had a KRAS I24N mutation, which does not lie within a functional KRAS domain and is not a known oncogenic driver mutation. This patient responded to crizotinib for over seven months. The remaining 60 *ROS1*-positive cases had wild-type *KRAS*.

Other co-occurring genetic alterations

Forty-four of the 62 patients underwent more comprehensive tumor genotyping (*i.e.*, sequencing of >10 genes). Among these 44 cases, 24 did not have additional genetic changes other than a *ROS1* fusion detected. Twenty cases were found to have additional alterations, summarized in Table 2 and Supplemental Table 3. Recurrent co-occurring genetic alterations included *TP53* mutations (11 of 43 tested cases, or 25.6%), *CTNNB1* mutations (3 of 43 tested cases, or 7.0%), and *CDKN2A*/B loss (3 of 22 tested cases, or 13.6%).

Notably, all 44 cases with additional genotyping were found to be wild-type for *BRAF* V600. Thirty-seven of the 44 cases were tested for *BRAF* non-V600 mutations, and all were wild-type. Among 39 cases tested for *ERBB2* exon 20 insertions, none harbored these mutations. Similarly, oncogenic mutations in *PIK3CA, MAP2K1, AKT1*, and *NRAS* were not detected in the tested cases (n = 44, 37, 39, and 44, respectively), indicating that *ROS1* fusions are generally mutually exclusive with other driver mutations in NSCLC.

In the recently published report by Wiesweg and colleagues,[12] 36% (9 of 25) of the *ROS1*-positive cases were found to harbor overlapping oncogenic mutations in *EGFR, KRAS, PIK3CA*, or *BRAF*. If this were the true frequency of overlap, then we would expect approximately 22 cases in the MGH cohort to have a concurrent driver mutation. However, only 3.2% (2 of 62)—a statistically significantly lower proportion ($P < 0.001$)—of the *ROS1*-rearranged cases in this cohort had a mutation detected in these oncogenes.

Independent analysis of 166 *ROS*-rearranged NSCLCs

In order to validate our findings regarding the frequency of driver mutations that co-occur with *ROS1* rearrangements, a separate dataset of NSCLCs sequenced at Foundation Medicine was queried. Among a total of 17,538 NSCLC cases that underwent sequencing, 166 cases (0.95%) harbored a *ROS1* fusion. Of note, eight of these cases were included in the MGH cohort described above.

Among the 166 *ROS1*-rearranged NSCLCs in the FM cohort, no concomitant *ALK* fusions (0%) were detected. One case (0.6%) had a concurrent *EGFR* activating mutation (L858R), and three (1.8%) had a concurrent *KRAS* driver mutation (Q61R, G12R, G12C). In addition, five cases (3.0%) had a concurrent *PIK3CA* mutation (E453Q, E453K, E545K, E726K, and E970K), while none (0%) had a *BRAF* V600E or a mutation in *ERBB2, MAP2K1*, or *AKT1*, again highlighting the significantly low prevalence of concurrent driver mutations. Of note, clinical information was not available for patients in this dataset; therefore, whether the co-occurring mutations arose *de novo* or post-treatment is unknown.

DISCUSSION

Current guidelines recommend upfront molecular testing for all patients with advanced lung adenocarcinoma. Identification of an actionable driver mutation directs patients to first- and often second-line targeted therapy, which typically results in durable clinical responses.[3] Importantly, at this time, detection of *EGFR, ALK*, or *ROS1* also directs patients away from first-line treatment with the PD-1 inhibitor pembrolizumab.[17] Thus, establishing the correct tumor genotype is critical for patient management.

In this study, we examined two separate cohorts of *ROS1*-rearranged NSCLC patients. Taking into account the eight patients included in both cohorts, the total number of *ROS1*-rearranged NSCLC patients in this study is 220. This represents the largest series of *ROS1*-positive NSCLC patients with additional molecular assessments published to date. Among the 220 patients, there were no cases of *ROS1* fusions co-occurring with *ALK* fusions and only one case with co-occurring *ROS1* fusion and *EGFR* activating mutation. Interestingly, a total of four cases of the 220 harbored a *KRAS* activating mutation. However, one of these cases had discordant *ROS1* FISH and NGS testing, and was likely *ROS1*-negative. While the FM cohort demonstrated co-occurrence of *ROS1* rearrangement and *PIK3CA* mutations in five cases, no overlap with other oncogenic drivers, including *BRAF* (V600E), *ERBB2, NRAS, AKT1*, and *MAP2K1* were identified. Altogether, these findings indicate that *ROS1* rearrangements rarely overlap with other driver mutations in NSCLC.

These findings are in line with early studies suggesting minimal overlap between *ROS1* fusions and *ALK* fusions or *EGFR* mutations,[2,4,6,10] but are in contrast to other recent reports.[11,12,18–21] One explanation for the discrepancy may be the difference in *ROS1* testing techniques. At present, options for *ROS1* detection include IHC, FISH, RT-PCR, and DNA or RNA sequencing. Each detection method is associated with distinct advantages and challenges. *ROS1* break-apart FISH was used as the diagnostic assay in the global crizotinib study[6] and is widely regarded as the gold standard. However, FISH can be technically challenging, and interpretation can vary depending on the laboratory, leading to false positives and false negatives. ROS1 IHC is not a validated screening assay for *ROS1* rearrangement and is more complicated than ALK IHC given background expression of ROS1.[22] In one recent study reporting a high prevalence of concurrent driver mutations with *ROS1*, 25 ROS1 IHC-positive cases were examined.[12] Of these, only roughly half (n = 13) were positive for *ROS1* rearrangement by FISH. Several of the cases found to harbor concomitant mutations in *EGFR, KRAS, BRAF*, and *PIK3CA* were, in fact, *ROS1* FISH-negative,[12] suggesting that the IHC result for these cases may have represented false

positives. Lastly, NGS offers an alternative diagnostic option with the advantage that it can identify the fusion partner, detect novel fusions, and allow for multiplex testing. On the other hand, NGS requires significantly more tissue and time for data analysis than FISH or IHC, and additionally carries the theoretical risk of identifying novel fusion variants that may not be functionally relevant. Given the distinct characteristics of each diagnostic modality, *ROS1* testing using orthogonal methods may be informative in the face of inconclusive initial screening results or inconsistent clinical behavior (*e.g.*, lack of response to a TKI despite a positive testing result), as illustrated by patient 53 in the MGH cohort.

While concomitant mutations in currently targetable oncogenes were rare, a number of additional genetic aberrations were detected by NGS in our *ROS1*-rearranged NSCLC cohort, including *TP53* mutations (in 25.2%), *CDKN2A*/B loss (in 13.6%), and *CTNNB1* mutations (in 7%). Future investigations in larger patient cohorts are needed to define the true frequencies of co-occurring genomic alterations, and to understand whether the genetic changes that co-occur with *ROS1* fusions may be biologically and therapeutically relevant.

There are several potential limitations of this study. First, concomitant genetic alterations may have been missed if they were present at very low allelic frequencies below the analytic sensitivity threshold of the targeted NGS platforms (< 5%), and if they occurred outside the hotspot regions covered by the specific assays. Second, tumor biopsy specimens carry the inherent limitation that they do not capture inter-metastatic tumor heterogeneity. While driver mutations are generally thought to be truncal events present at all sites of disease, other co-occurring genetic alterations could have evolved later and have been present at sites other than the one biopsied. Liquid biopsies (*i.e.*, circulating tumor DNA analysis) and deeper sequencing technologies could help overcome these limitations.

In summary, we have found that *ROS1* rearrangements rarely co-occur with other oncogenic drivers. These findings establish *ROS1*-rearranged NSCLC as a distinct molecular subset of lung cancer. Advanced NSCLC patients found to harbor *ROS1* fusions should be treated with a ROS1 inhibitor. If concurrent driver mutations are identified, an orthogonal testing methodology should be considered to confirm the molecular diagnosis before proceeding with targeted therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

JFG has served as a compensated consultant or received honoraria from Novartis, Bristol-Myers Squibb, Merck, Ariad, Loxo, Genentech, Boehringer-Ingelheim, Jounce Therapeutics, Incyte, Clovis, and Kyowa Hakko Kirin. **MMK** has served as a compensated consultant for Merrimack Pharmaceuticals, ACD, and H3 Biomedicine. **AJI** has received personal fees from ArcherDx, Roche, and Chugai, and has a patent issued to ArcherDx. **ATS** has served as a compensated consultant or received honoraria from Pfizer, Novartis, Genentech/ Roche, Ariad, Ignyta, Daiichi-Sankyo, Taiho, Blueprint Medicines, Loxo, EMD Serono, and Foundation Medicine. **SMA, MB, ABS**, and **VAM** are employees of, and have equity interest, in Foundation Medicine, Inc.

Funding source:

This work was supported by the National Institutes of Health (5R01CA164273, to ATS), Be a Piece of the Solution, and LungStrong.

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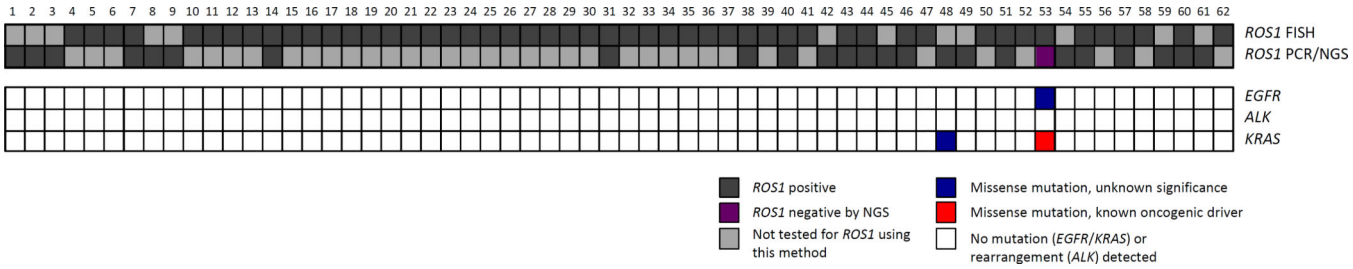

Figure 1.

ROS1 rearrangements are generally mutually exclusive with oncogenic driver alterations in *EGFR, KRAS*, and *ALK*. One case (patient 53) in the MGH cohort had *ROS1* testing that was positive by FISH (dark grey) but negative by NGS (purple). This case was found to harbor a KRAS G13D mutation (red) and an EGFR C781F mutation of unknown significance (blue). Another case (patient 48) had a KRAS I24N mutation of unknown significance (blue). All other *ROS1*-rearranged NSCLC cases in the MGH cohort tested negative (white) for concurrent *EGFR* and *KRAS* mutations and *ALK* rearrangements.

Table 1

Baseline Characteristics.

Characteristic	No (*N* = 62)	%
Age, years		
Median	52	
Range	22–84	
Sex		
Male	23	37.1
Female	39	62.9
Smoking history		
Never smoker	48	77.4
Light smoker (<10 pack-years)	5	8.1
Smoker (≥10 pack-years)	9	14.5
Ethnicity		
Asian	9	14.5
Caucasian	45	72.6
African-American	5	8.1
Hispanic	2	3.2
Unknown	1	1.6
Pathology		
Adenocarcinoma	62	100
Squamous	0	0
Stage at diagnosis		
IA	3	4.8
IB	2	3.2
IIA	1	1.6
IIB	1	1.6
IIIA	7	11.3
IIIB	2	3.2
IV	46	74.2

Table 2

Concomitant Genetic Alterations in *ROS1*-Rearranged NSCLC.

Patient	Genes with Alterations	Prior Systemic Therapy[a]
1	*ATM, CTNNB1, TP53, DNMT3A*	Yes
2	*TP53*	No
6	*TP53*	No
10	*TP53*	No
14	*TP53, SMAD4, APC*	No
24	*TP53, ROS1* (p.G2032R)	Yes[b]
42	*MAP2K4, SF3B1*	No
43	*TP53*	Yes
45	*TSC2*	No
46	*TP53*	No
48	*KRAS* (p.I24N), *CTNNB1, TP53*	No
49	*BAP1, CHEK2*	No
50	*CTNNB1*	Yes
53[c]	*KRAS* (p.G13D), *EGFR* (p.C781F), *KIT, IDH1*	No
54	*CDKN2A, TP53*	No
55	*FLT1, PRKDC, RUNX1*	No
57	*TP53*	No
59	*CCND1, ARID1A, CDKN2A*/B, *FGF19, FGF4, FGF3*	No
60	*MSH6, CDKN2A*	No
61	*CDKN2A*/B	No

[a] Prior systemic therapy includes chemotherapy and/or crizotinib.

[b] This tumor sample was derived post-crizotinib. The pre-crizotinib tumor sample did not harbor the ROS1 G2032R mutation, which is a known crizotinib-resistant mutation.[23]

[c] This tumor sample tested positive for *ROS1* rearrangement by FISH, but targeted RNA sequencing did not detect a *ROS1* fusion transcript.