BRIEF REPORT

ROS1 Fusions Rarely Overlap with Other Oncogenic Drivers in Non–Small Cell Lung Cancer

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ABSTRACT

Introduction: Chromosomal rearrangements involving the gene *ROS1* define a distinct molecular subset of NSCLCs with sensitivity to ROS1 inhibitors. Recent reports have suggested a significant overlap between *ROS1* fusions and other oncogenic driver alterations, including mutations in *EGFR* and *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 receptor tyrosine kinase gene (*ALK*). Clinicopathologic features and genetic testing results were reviewed. We also examined a separate database of *ROS1*-rearranged NSCLCs identified through the commercial FoundationOne assay (Foundation Medicine, Cambridge, MA).

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 noncanonical *KRAS* I24N mutation of unknown biological significance. In a separate *ROS1* fluorescence in situ hybridization–positive case, targeted sequencing failed to confirm a *ROS1* fusion but instead identified a *KRAS* G13D mutation. No concurrent mutations in B-Raf proto-oncogene, serine/threonine kinase gene (*BRAF*), erb-b2 receptor tyrosine kinase 2 gene (*ERBB2*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene (*PIK3CA*), AKT/serine threonine kinase 1 gene (*AKT1*), or mitogen-activated protein kinase kinase 1 gene (*MAP2K1*) were detected. Analysis of an independent data set of 166 *ROS1*-rearranged NSCLCs identified by FoundationOne demonstrated rare cases with co-occurring driver mutations in *EGFR* (one of 166) and *KRAS* (three of 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.

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Keywords: ROS1; Non–small cell lung cancer; NSCLC; Concurrent mutations

Introduction

ROS1 is a validated therapeutic target in NSCLC. Chromosomal rearrangements involving the *ROS1* gene occur in 1% to 2% of NSCLCs[1–4] and are clinically associated with a history of never smoking, younger age,

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and the adenocarcinoma histologic type.[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. In an early-phase study of crizotinib therapy in advanced *ROS1*-rearranged NSCLC, the objective response rate was 72% and the median progression-free survival was 19.2 months.[6] Two additional studies since then have demonstrated similarly high response rates (range 71%–80%) to crizotinib in *ROS1*-rearranged NSCLC, although the median progression-free survival in these studies was shorter at 9 to 10 months.[7,8] On the basis of 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*, *EGFR*, and anaplastic lymphoma receptor tyrosine kinase gene (*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 that tested positive for *ROS1* rearrangement by immunohistochemistry (IHC), 36% were reported to harbor concomitant oncogenic driver mutations (including in *EGFR*, *KRAS*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene (*PIK3CA*), and B-Raf proto-oncogene, serine/threonine kinase gene (*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 patients with *ROS1*-rearranged NSCLC who underwent genotyping of other oncogenes, including *KRAS*, *EGFR*, and *ALK*, to determine the frequency of concurrent driver alterations in *ROS1*-rearranged NSCLC.

Methods

Study Population

Seventy patients with *ROS1*-rearranged NSCLC who were 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 patients with *ROS1*-rearranged NSCLC were identified through use of the FoundationOne next-generation sequencing (NGS) assay at Foundation Medicine (Cambridge, MA) (the FM cohort). A total of eight patients were included in both cohorts.

Molecular Testing

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

More comprehensive genotyping data (defined as sequencing for hotspot mutations in >10 genes) were available for 44 patients in the MGH cohort. The sequencing assay used for each patient is listed in Supplementary Table 1. Genes analyzed in each sequencing platform are listed in Supplementary Table 2. The FoundationOne, 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 by using FISH (n = 38), targeted sequencing or PCR (n = 13), or both FISH and sequencing (n = 11). Clinicopathologic features of these 62 patients with *ROS1*-rearranged NSCLC 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 molecule gene (*CD74*) (n = 16), syndecan 4 gene (*SDC4*) (n = 4), ezrin gene (*EZR*) (n = 2), and solute carrier family 34 (type II sodium/phosphate cotransporter), member 2 gene (*SLC34A2*) (n = 2).[1–4] Twelve patients underwent *ROS1* testing by both FISH and NGS, of whom 11 had concordant positive results

Table 1. Baseline Characteristics

Characteristic	n (N = 62)	%
Age, y		
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
White	45	72.6
African American	5	8.1
Hispanic	2	3.2
Unknown	1	1.6
Type of tumor		
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

(Fig. 1). In the patient with discordant results (patient 53), the results of FISH were 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 (see Fig. 1). The discordant *ROS1* case (the aforementioned patient 53) 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 (see Fig. 1). Patient 53, the patient with discordant *ROS1* testing results (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 7 months. The remaining 60 *ROS1*-positive cases had wild-type *KRAS*.

Other Co-occurring Genetic Alterations

Of the 62 patients, 44 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, which are summarized in Table 2 and Supplementary Table 3. Recurrent co-occurring genetic alterations included tumor protein p53 gene (*TP53*) mutations (11 of 43 tested cases [25.6%]), catenin beta 1 gene (*CTNNB1*) mutations (three of 43 tested cases [7.0%]), and cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) or cyclin-dependent kinase inhibitor 2B gene (*CDKN2B*) loss (three of 22 tested cases [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 the 39 cases tested for erb-b2 receptor tyrosine kinase 2 gene (*ERBB2*) exon 20 insertions, none harbored these mutations. Similarly,

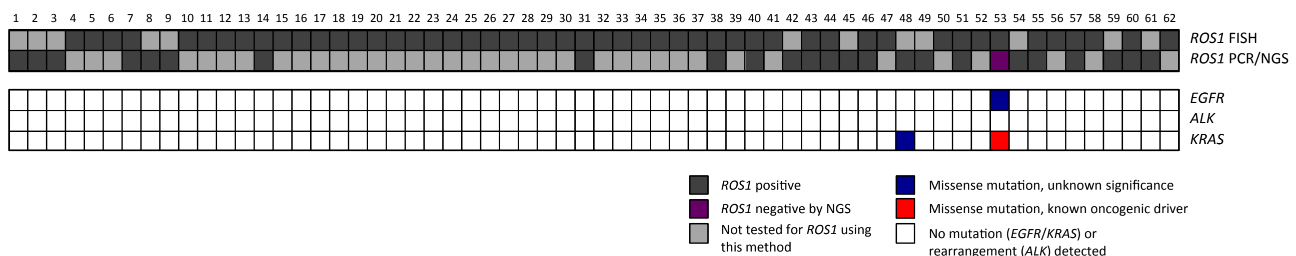

Figure 1. *ROS1* rearrangements are generally mutually exclusive with oncogenic driver alterations in *EGFR*, *KRAS*, and anaplastic lymphoma receptor tyrosine kinase gene (*ALK*). One case (patient 53) in the Massachusetts General Hospital cohort had *ROS1* testing results that were positive by fluorescence in situ hybridization (FISH) (*dark gray*) but negative by next-generation sequencing (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 Massachusetts General Hospital cohort tested negative (*white*) for concurrent *EGFR* and *KRAS* mutations and *ALK* rearrangements.

Table 2. Concomitant Genetic Alterations in *ROS1*-Rearranged NSCLC

Patient	Genes with Alterations	Prior Systemic Therapy
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[a]
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[b]	*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

Note: Prior systemic therapy includes chemotherapy and/or crizotinib.
[a]This tumor sample was derived after crizotinib therapy. The pre-crizotinib therapy tumor sample did not harbor the *ROS1* G2032R mutation, which is a known crizotinib-resistant mutation.[17]
[b]This tumor sample tested positive for *ROS1* rearrangement by fluorescence in situ hybridization, but targeted RNA sequencing did not detect a *ROS1* fusion transcript.
ATM, ATM serine/threonine kinase gene; *CTNNB1*, catenin beta 1 gene; *TP53*, tumor protein p53 gene; *DNMT3A*, DNA methyltransferase 3 alpha gene; *SMAD4*, SMAD family member 4 gene; *APC*, APC, WNT signaling pathway regulator gene; *MAP2K4*, mitogen-activated protein kinase kinase 4 gene; *SF3B1*, splicing factor 3b subunit 1 gene; *TSC2*, tuberous sclerosis 2 gene; *BAP1*, BRCA1 associated protein 1 gene; *CHEK2*, checkpoint kinase 2 gene; *KIT*, KIT proto-oncogene receptor tyrosine kinase gene; *IDH1*, isocitrate dehydrogenase (NADP(+)), 1 cytosolic gene; *CDKN2A*, cyclin-dependent kinase inhibitor 2A gene; *FLT1*, fms related tyrosine kinase 1 gene; *PRKDC*, protein kinase, DNA-activated, catalytic polypeptide gene; *RUNX1*, runt-related transcription factor 1 gene; *CCND1*, cyclin D1 gene; *ARID1A*, AT-rich interaction domain 1 gene; *CDKN2B*, cyclin-dependent kinase inhibitor 2B gene; *FGF19*, fibroblast growth factor 19 gene; *FGF4*, fibroblast growth factor 4 gene; *FGF3*, fibroblast growth factor 3 gene; *MSH6*, mutS homolog 6 gene.

oncogenic mutations in *PIK3CA*, mitogen-activated protein kinase kinase 1 gene (*MAP2K1*), AKT/serine threonine kinase 1 gene (*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 et al.,[12] nine of the 25 of *ROS1*-positive cases (36%) 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 two of 62 (3.2%)—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

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

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, and G12C). In addition, five cases (3.0%) had a concurrent *PIK3CA* mutation (E453Q, E453K, E545K, E726K, and E970K), whereas 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 data set; therefore, whether the co-occurring mutations arose de novo or after 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 programmed cell death 1 inhibitor pembrolizumab.[18] Thus, establishing the correct tumor genotype is critical for patient management.

In this study, we examined two separate cohorts of patients with *ROS1*-rearranged NSCLC. Taking into account the eight patients included in both cohorts, the total number of patients with *ROS1*-rearranged NSCLC in this study was 220. This represents the largest series of patients with *ROS1*-positive NSCLC 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 results and

was likely *ROS1* negative. Although 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 they are in contrast to the findings of other recent reports.[11,12,19–22] One explanation for the discrepancy may be the difference in *ROS1* testing techniques. At present, options for *ROS1* detection include IHC, FISH, reverse transcriptase 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 definitive standard. However, FISH can be technically challenging, and its interpretation can vary depending on the laboratory, leading to false-positive and false-negative results. ROS1 IHC is not a validated screening assay for *ROS1* rearrangement and is more complicated than ALK IHC given background expression of ROS1.[23] 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-positive results. 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, it carries the theoretical risk for 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 tyrosine kinase inhibitor despite a positive testing result), as illustrated by patient 53 in the MGH cohort.

Although 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 intermetastatic tumor heterogeneity. Although 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 been present at sites other than the one at which biopsy was performed. 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. Patients with advanced NSCLC 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.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at http://dx.doi.org/10.1016/j.jtho.2017.01.004.

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