Molecular Pathways: ROS1 Fusion Proteins in Cancer

Kurtis D. Davies and Robert C. Doebele

Abstract

Genetic alterations that lead to constitutive activation of kinases are frequently observed in cancer. In many cases, the growth and survival of tumor cells rely upon an activated kinase such that inhibition of its activity is an effective anticancer therapy. *ROS1* is a receptor tyrosine kinase that has recently been shown to undergo genetic rearrangements in a variety of human cancers, including glioblastoma, non–small cell lung cancer (NSCLC), cholangiocarcinoma, ovarian cancer, gastric adenocarcinoma, colorectal cancer, inflammatory myofibroblastic tumor, angiosarcoma, and epithelioid hemangioendothelioma. These rearrangements create fusion proteins in which the kinase domain of ROS1 becomes constitutively active and drives cellular proliferation. Targeting ROS1 fusion proteins with the small-molecule inhibitor crizotinib is showing promise as an effective therapy in patients with NSCLC whose tumors are positive for these genetic abnormalities. This review discusses the recent preclinical and clinical findings on *ROS1* gene fusions in cancer. *Clin Cancer Res; 19(15); 4040–5. ©2013 AACR.*

Background

Wild-type ROS1

The human *ROS1* gene was initially discovered as the homolog of the chicken *c-ros*, which is the proto-oncogene for *v-ros*, the transforming sequence of the UR2 avian sarcoma virus (1, 2). *ROS1* encodes a receptor tyrosine kinase (RTK) that is closely related to the *ALK* and *LTK* human RTKs (3). It is arranged as a single-pass transmembrane protein with an intracellular C-terminal tyrosine kinase domain and a large extracellular N-terminal domain. Interestingly, the extracellular domain contains sequences that are analogous to sequences found in cell adhesion molecules and extracellular matrix proteins (4). ROS1 protein expression in adult humans is highest in the kidney but is also found in the cerebellum, peripheral neural tissue, stomach, small intestine, and colon, with lower expression in several other tissues (5). Notably, ROS1 protein expression has been reported to be absent in normal human lung tissue (5).

Very little is currently known about the function of wild-type (WT) ROS1 (4, 6). Mice that lack the receptor appear to be relatively healthy, although they display an abnormality in the epididymis of males that prevents normal reproduction (7). In addition, a ligand for the receptor has not been found, although the structure of the extracellular domain has led to speculation that cellular attachment may play a role in ROS1 activation (4). The lack of a known ligand has impeded examination of downstream signaling that is activated by WT ROS1. However, several studies have been conducted using chimeric receptors that contain the ROS1 kinase domain fused to the ligand-binding domain of EGF receptor (EGFR) or TRKA. When cells expressing these chimeras were stimulated with the cognate ligand, activation of various combinations of PLCγ, phosphoinositide 3-kinase (PI3K)/AKT, STAT3, VAV3, and mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) signaling components was observed (8–12).

Discovery of *ROS1* gene fusions

The first evidence that gene fusions involving the *ROS1* kinase domain have transforming capability came from a study in which DNA from MCF-7 human mammary carcinoma cells was transferred to NIH-3T3 cells, which were then implanted into mice (2). It was discovered that a DNA fragment containing the *ROS1* 3′ region (that encodes the kinase domain) fused to an unidentified sequence was able to promote tumorigenicity. However, it was determined that the rearrangement in the DNA occurred experimentally because a corresponding sequence was not found in the MCF-7 cell line. The first discovery of a naturally occurring rearrangement involving *ROS1* came from the human glioblastoma cell line U118MG, in which a deletion on chromosome 6 resulted in fusion of the 3′ region of *ROS1* to the 5′ region of the *FIG* (*GOPC*) gene (13, 14). The *FIG–ROS1* gene fusion has also been found in cholangiocarcinoma, ovarian cancer, and non–small cell lung cancer (NSCLC) patient samples (5, 15–17). In addition, *SLC34A2–*, *CD74–*, *TPM3–*, *SDC4–*, *EZR–*, *LRIG3–*, *KDELR2–*, and *CCDC6–ROS1* gene fusions have been found in NSCLC samples (18–21). The *SLC34A2–ROS1* fusion has also been observed in gastric adenocarcinoma samples and a colorectal cancer sample, *YWHAE–* and *TFG–ROS1* gene fusions have been found in inflammatory myofibroblastic tumors, and

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the *CEP85L–ROS1* fusion has been discovered in an angiosarcoma patient sample (22–25). Owing to the apparent promiscuity of *ROS1* rearrangement, the list of fusion partners is likely to grow as more samples are examined. In all of the known fusion genes, the *ROS1* kinase domain is fully retained. Furthermore, the junction point on *ROS1* at the mRNA level invariably occurs at the 5′ end of exons 32, 34, 35, or 36.

Oncogenicity of ROS1 fusion proteins

The transforming potential of these cancer-related ROS1 fusion proteins is well established. Expression of the known fusion variants (with the exception of *KDELR2–*, *CCDC6–*, *YWHAE–*, *TFG–*, and *CEP85L–ROS1* fusions, which to date have not been tested) in fibroblasts was shown to result in anchorage-independent growth, foci formation, and tumorigenicity (15, 19, 26, 27). In addition, expression of the *FIG–ROS1* and *SDC4–ROS1* fusions in murine Ba/F3 cells has been shown to result in interleukin 3–independent proliferation, and this proliferation was sensitive to treatment with small-molecule ROS1 inhibitors (5, 15, 28). In mice, the *FIG–ROS1* fusion gene has been shown to promote the formation of astrocytomas when ectopically expressed in the basal ganglia, and the *EZR–ROS1* fusion gene has been shown to promote lung adenocarcinoma when ectopically expressed in lung epithelium (29, 30). Unfortunately, only three published cell lines have been found to naturally express *ROS1* fusion genes: the U118MG glioblastoma line described above, U138MG (which was derived from the same patient as U118MG and expresses the same *FIG–ROS1* fusion), and the HCC78 NSCLC line that expresses the *SLC34A2–ROS1* fusion (13, 14, 18). We have found the U118MG line to be relatively insensitive to ROS1 inhibition in proliferation assays, suggesting that the FIG–ROS1 fusion protein is not the sole oncogenic driver in these cells (unpublished observations). On the contrary, multiple studies have shown that proliferation of HCC78 cells is sensitive to pharmacologic ROS1 inhibition as well as RNA interference-mediated *ROS1* knockdown (18, 28, 31–34). Interestingly, treatment with the EGFR inhibitor gefitinib was shown to enhance sensitivity to ROS1 inhibition in HCC78 cells, suggesting that ROS1 cooperates with EGFR to promote proliferation in this line (28).

ROS1 fusion protein activation and signaling

The mechanism by which ROS1 fusion proteins become constitutively active is currently unknown. For other cancer-related RTK fusions, such as *ALK*, the fusion partner provides a dimerization domain that induces constitutive oligomerization and thus activation of the kinase (35). However, for ROS1, it is unclear whether dimerization is involved in activation of the WT receptor. Furthermore, the v-ros product of the UR2 avian sarcoma virus and the FIG–ROS1 fusion protein have been shown to exist only as monomers (26, 36). In agreement with this, many of the known ROS1 fusion partners to do not contain dimerization domains (19). Localization of the FIG–ROS1 fusion protein at the Golgi apparatus has been shown to be critical

for transforming ability (but not kinase activity; ref. 26). However, the localization of other ROS1 fusion variants seems to vary, with cytoplasmic, plasma membrane, and perinuclear patterns being reported (5, 15, 18, 27, 37).

Several studies have examined the signaling pathways that are activated by ROS1 fusion proteins. Expression of the FIG–ROS1, CD74–ROS1, or SDC4–ROS1 fusions in fibroblasts or Ba/F3 cells has been shown to result in autophosphorylation of ROS1 and phosphorylation of SHP-2, MAP–ERK kinase, ERK, STAT3, and AKT, and these effects were blocked by pharmacologic inhibition of ROS1 (15, 27–29). ROS1 inhibitors also led to reductions in phosphorylated ROS1, SHP-2, AKT, and ERK in HCC78 cells (28, 33). Interestingly, a recent report suggested that downstream signaling may differ depending on the fusion partner of ROS1 (27). It was observed that CD74–ROS1 but not FIG–ROS1 led to phosphorylation of E-Syt1, and that this differential signaling activity led to a more invasive phenotype of *CD74–ROS1*-transduced cells (27). Overall, the signaling activated by ROS1 fusions seems to mainly involve common growth and survival pathways that are also activated by other RTKs (Fig. 1).

Clinical–Translational Advances

Although several studies have shown aberrant/overexpression of WT ROS1 in various cancers, the clinical relevance of this expression has not been completely examined (4, 6, 37). Likewise, the clinical significance of *ROS1* rearrangement and fusion protein expression in most cancers where it has been found has been largely unexplored. What is clear from the minimal data available is that rearrangement of *ROS1* is a relatively uncommon event, occurring in cell lines and/or patient samples at the following rates: 8.7% (2/23) in cholangiocarcinomas, 0.5% (1/200) in ovarian cancers, 0.6% (3/495) in gastric adenocarcinomas, 0.8% (2/236) in colorectal cancers, 7.7% (2/26) in inflammatory myofibroblastic tumors, 2.9% (1/34) in angiosarcomas, and 5% (1/20) in epithelioid hemangioendotheliomas (15, 16, 22–25). Although *ROS1* rearrangement has been observed in two cell lines that were derived from a single glioblastoma patient, there have been no published reports of rearrangements being found in clinical specimens from this disease, and therefore no accurate estimation of prevalence can be given.

Prevalence of *ROS1* rearrangement in NSCLC

In contrast with the above cancers, a great deal of information has been published about *ROS1* rearrangement in NSCLC. Several studies have attempted to determine the prevalence of rearrangement by screening large (>100) numbers of patient samples. The first such study screened 202 lung adenocarcinoma samples from East Asian never-smoker patients by reverse transcriptase PCR (RT-PCR) using primers that would detect *CD74–ROS1* and *SLC34A2–ROS1* fusions (38). Two out of 202 samples were found to be positive for the *CD74–ROS1* fusion gene (no *SLC34A2–ROS1* fusions were found). However, because the screening method was limited to only two of the known

fusion partners, it is possible that other *ROS1* rearrangement–positive samples existed in this panel (38). Three subsequent studies used "break-apart" FISH to screen large sample panels. In this assay, the separation of differentially labeled FISH probes that span the common *ROS1* breakpoint is used to determine whether rearrangement has occurred, meaning that the assay should detect *ROS1* gene rearrangements regardless of the fusion partner. These unbiased studies found that 18 of 1,073 (1.7%), 13 of 1,476 (0.9%), and 5 of 428 (1.2%) patients with NSCLC had undergone *ROS1* rearrangement in their tumors (19, 28, 32). When histology was taken into account, it was found that 18 of 694 (2.6%), 13 of 1,116 (1.2%), and 3 of 244 (1.2%) of adenocarcinomas were positive, whereas 0 of 200 (0%), 0 of 233 (0%), and 2 of 138 (1.4%) squamous cell carcinomas were positive (19, 28, 32). Immunohistochemistry (IHC) with a ROS1-specific antibody has also been used to determine prevalence of rearrangement (5). In this study, 9 of 556 (1.6%) NSCLC samples [8/246 (3.3%) adenocarcinomas and 1/20 (5%) large cell carcinomas] were found to stain positive, and in all of these cases where

FISH analysis was possible, FISH confirmed *ROS1* rearrangement (5). Overall, the above studies strongly suggest that the prevalence of ROS1 rearrangement in NSCLC is 1% to 2%, with the majority of cases being of adenocarcinoma histology. Although this is a seemingly low number, it equates to potentially 20,000 or more patients per year worldwide who will present with this specific disease (39).

Patient characteristics

Although the number of published *ROS1* rearrangement–positive clinical cases remains fairly low, patterns are beginning to emerge in regard to patient characteristics. In general, *ROS1* rearrangement occurs in the absence of other known oncogenic drivers (*EGFR* mutations, *KRAS*, mutations, *ALK* rearrangements). However, exceptions to this rule have been reported, specifically with the finding of patients who are positive for *ROS1* rearrangement concurrent with an *EGFR* mutation (5). In addition, *ROS1* rearrangement–positive patients tend to be younger and never-smokers (19, 32). Interestingly, this pattern of patient characteristics mirrors that of
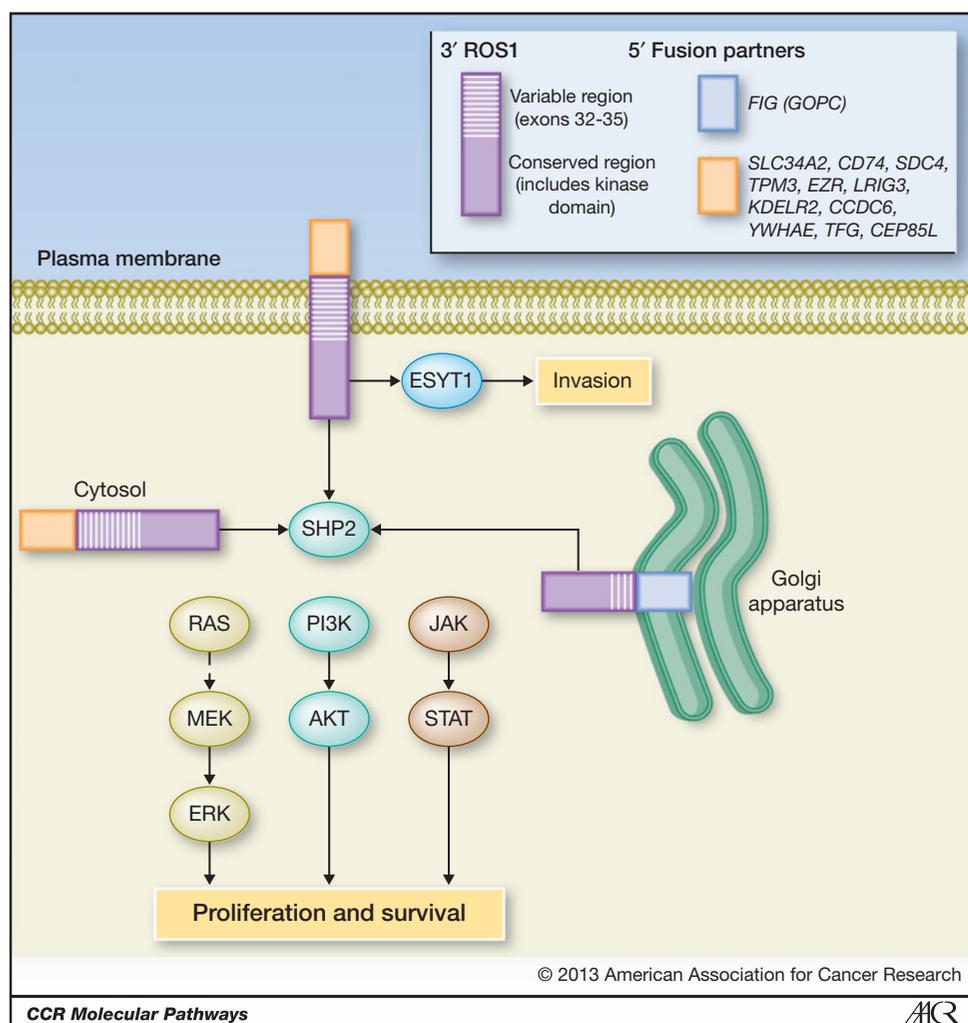

Figure 1. Schematic of ROS1 fusion proteins and activation of downstream signaling pathways. FIG–ROS1 fusions have been found to be localized to the Golgi apparatus, whereas other ROS1 fusion variants have been reported to be plasma membrane associated or cytosolic. ROS1 fusion proteins activate growth and survival signaling pathways common to other RTKs. ESYT1 activation may be a fusion variant–specific downstream signaling component that influences invasion and metastasis.

ALK rearrangement–positive NSCLC (40). Despite these trends, it is important to take into account that many patients who are positive for rearrangements do not fit this pattern, so these characteristics should not be used for patient screening.

Clinical detection

Several methods are available for detecting the presence of *ROS1* rearrangements in patient tumor samples. As mentioned above, RT-PCR has been used to successfully identify positive cases (30, 38, 41). However, as the list of fusion partners for *ROS1* is relatively large and still growing, using a fusion partner-specific assay like RT-PCR is likely to miss some positive cases. The FISH assay, also described above, will detect rearrangement regardless of fusion partner, and this is the diagnostic that has been used in the phase I clinical trial of crizotinib to identify positive patients (42). However, FISH can be more technically demanding compared with other clinical diagnostics and this limits its use. IHC is a common clinical assay that can be used to detect aberrant ROS1 expression in tumor samples (5). Importantly, the ability of this assay to specifically identify *ROS1* rearrangement–positive cases relies upon the absence of WT ROS1 expression in rearrangement-negative NSCLC tumors. This issue remains controversial, with some studies reporting WT ROS1 expression in normal lung and nonrearranged NSCLC samples and others not (4, 5, 37). A different expression-based approach to identify *ROS1* rearrangement uses the fact that the 3′ region of the gene (that is included in the fusion gene) should have higher mRNA expression levels than the 5′ region. This principle was applied in a recent study that used "NanoString" probes to show rearrangement in a patient with NSCLC who was subsequently found to be positive for the *FIG–ROS1* fusion (17). Finally, next-generation sequencing has emerged as a powerful approach to look for genetic abnormalities in tumor samples in an unbiased fashion, and this technique has identified five novel *ROS1* fusion variants (20, 21, 24, 25). In this method, genetic rearrangements that create fusion genes can be identified either using the aforementioned "unbalanced" expression concept (if the transcriptome is sequenced) or by sequencing of fragments that span the fusion point (25). Importantly, next-generation sequencing can be used to simultaneously test for multiple oncogenic drivers (including activating mutations and rearrangements), making it a very attractive option for streamlined diagnostic analysis.

Treatment of tumors with *ROS1* rearrangements

The strategy of oncogene-directed therapy in lung cancer has led to significant clinical benefit in *ALK* rearrangement–positive and *EGFR* mutation–positive patients treated with crizotinib or EGFR kinase inhibitors, respectively, with remarkable overall response rates, progression-free survival times, and superiority compared with standard chemotherapy (40, 43). Therefore, it is of great interest to determine whether *ROS1* rearrangement–positive NSCLC patients benefit from ROS1-targeted therapy. Fortuitously, crizoti-

nib, which has now been approved by the U.S. Food and Drug Administration for use in *ALK* rearrangement–positive NSCLC, is also a potent ROS1 inhibitor (34). Initial results from a phase I trial of crizotinib, 250 mg twice daily, in *ROS1* FISH-positive NSCLC patients have been very encouraging, with a reported objective response rate of 57% and a disease control rate at 8 weeks of 79%, results that are very similar to those observed for crizotinib in *ALK* FISH-positive lung cancer (42, 44). Other kinase inhibitor drugs, such as AP26113, are reported as ROS1 inhibitors, and clinical trials have planned to enroll *ROS1* rearrangement–positive patients (ClinicalTrials.gov NCT01449461; ref. 45). In addition, similar to ALK fusion-positive cells, preclinical data suggest that HSP90 inhibition is antiproliferative in ROS1 fusion–positive cells (46). Consequently, a clinical trial of the HSP90 inhibitor AT13387 is recruiting patients who are positive for *ROS1* rearrangement, and results are eagerly awaited (ClinicalTrials.gov NCT01712217).

Future directions

It is now clear that *ROS1* rearrangement–positive NSCLC is a clinically relevant subclass of this disease. Given the apparent success of treating these patients with crizotinib, it will be interesting to see whether ROS1 inhibition will be examined in other cancer types in which rearrangement has been observed. Furthermore, because this genetic event is not confined to a specific cancer type, large-scale studies that screen patient samples should be conducted in all major cancer indications that thus far have not been examined.

Unfortunately, kinase inhibitor drugs are rarely (if ever) curative due to the emergence of acquired resistance. It should be fully expected that *ROS1* rearrangement–positive patients who respond to crizotinib will eventually experience disease progression despite continued treatment. Much has been learned recently in regard to resistance mechanisms to targeted therapy in *EGFR*-mutant and *ALK* rearrangement–positive NSCLC (35, 47). It will be imperative to use these studies as a guide to understand acquired resistance to ROS1 inhibition so that rational therapeutic strategies will someday be available for these patients.

Authors' Contributions
Conception and design: K.D. Davies, R.C. Doebele
Writing, review, and/or revision of the manuscript: K.D. Davies, R.C. Doebele
Study supervision: R.C. Doebele

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