Acquired Resistance to Crizotinib from a Mutation in CD74–ROS1

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SUMMARY

Crizotinib, an inhibitor of anaplastic lymphoma kinase (ALK), has also recently shown efficacy in the treatment of lung cancers with ROS1 translocations. Resistance to crizotinib developed in a patient with metastatic lung adenocarcinoma harboring a CD74–ROS1 rearrangement who had initially shown a dramatic response to treatment. We performed a biopsy of a resistant tumor and identified an acquired mutation leading to a glycine-to-arginine substitution at codon 2032 in the ROS1 kinase domain. Although this mutation does not lie at the gatekeeper residue, it confers resistance to ROS1 kinase inhibition through steric interference with drug binding. The same resistance mutation was observed at all the metastatic sites that were examined at autopsy, suggesting that this mutation was an early event in the clonal evolution of resistance. (Funded by Pfizer and others; ClinicalTrials.gov number, NCT00585195.)
A 48-year-old woman with a distant history of light smoking presented with progressive dyspnea. Cytologic analysis of a malignant pleural effusion in the right lung was performed (Fig. 1A), and a diagnosis of metastatic lung adenocarcinoma was made. Genetic studies of the patient’s cancer cells revealed no mutations in KRAS or EGFR and no ALK translocation (not shown). She was started on first-
line chemotherapy with carboplatin and pemetrexed, but her condition deteriorated, as indicated by worsening discomfort in the right side of her chest, increased fluid output from a right pleural catheter (up to 1 liter per day), and progressive fatigue, weight loss, intermittent fevers, hypoxemia, and hypercalcemia. After three cycles of chemotherapy, repeat imaging of the chest confirmed marked progression of cancer throughout the right lung and pleura.

Additional molecular testing was performed. A ROS1 rearrangement within tumor cells was revealed on fluorescence in situ hybridization (FISH) (Fig. 1B). Reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assays that were performed on total RNA extracted from tumor cells showed that this rearrangement leads to expression of a fusion transcript joining exon 6 of CD74 to either exon 34 (a major splice form) or exon 35 (a minor splice form) of ROS1 (Fig. 1C). Since CD74 is a type II integral membrane protein with a cytoplasmic N-terminal, we would expect that only the major splice variant that contains an additional transmembrane domain (encoded by ROS1 exon 34) would be oncogenic because of the positioning of the ROS1 tyrosine kinase domain in the intracellular compartment (Fig. 1C).

The patient was enrolled in a clinical trial investigating the safety and efficacy of crizotinib in cancers with ROS1 translocations (ClinicalTrials.gov number, NCT00585195). She began taking 250 mg of crizotinib twice daily, and within a week she noted a substantial reduction in her dyspnea and fatigue and a substantial increase in appetite. As compared with a computed tomographic (CT) scan obtained before she began treatment with crizotinib, a repeat CT scan of the chest obtained after 2 months of treatment with crizotinib re-

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**Figure 2.** Identification of an Acquired ROS1 G2032R Mutation at the Time of Resistance to Crizotinib.

Axial CT scans of the chest (Panel A) show the patient’s disease burden before treatment, after a response to crizotinib, and at the time of crizotinib resistance. Sanger sequencing of RT-PCR products (Panel B) before and after treatment with crizotinib shows the acquired c.6094G→A mutation, which encodes for p.Gly2032Arg. (These coding and amino acid sequences are numbered in accordance with National Center for Biotechnology Information [NCBI] reference sequences CCDS5116 and NP_002935.2, respectively.) In all six malignant sites examined at autopsy (Panel C), the c.6094G→A ROS1 mutation was detected by means of Sanger sequencing of RT-PCR products. Genomic DNA sequencing of ROS1 exon 38 in the patient’s grossly and microscopically normal liver tissue shows the nonmutated ROS1 sequence; the CD74–ROS1 fusion transcript could not be detected on RT-PCR in the normal liver. In the summary of autopsy findings at right, the presence of the G2032R mutation is indicated by a plus sign, and the absence of the G2032R mutation by a minus sign.
revealed a dramatic response to treatment (Fig. 2A). However, 1 month later, the patient’s respiratory symptoms worsened while she was still taking crizotinib, and imaging of the chest showed disease progression (Fig. 2A).

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STUDY OVERSIGHT
The patient and her family provided informed consent for the genetic research studies, which were performed in accordance with protocols approved by the institutional review board at Massachusetts General Hospital. The first author wrote the manuscript in collaboration with the senior academic authors; no one other than the authors was involved in the writing of this report. All authors vouch for the accuracy and completeness of the report. The patient participated in a clinical trial funded by Pfizer.

GENETIC STUDIES
A break-apart CD74–ROS1 FISH assay was used to identify ROS1 rearrangements and gene copy number in specimens of malignant tissue. Complementary DNA was reverse-transcribed from total RNA that was extracted from samples obtained from the patient and then amplified by means of PCR with primers to CD74 and ROS1. Sanger sequencing was performed on RT-PCR products. Genomic DNA extracted from patient samples was used both for deep sequencing of ROS1 exons 34 to 42 and for exon sequencing of a panel of 409 cancer-related genes. Detailed information on the isolation and sequencing of nucleic acid and descriptions of other laboratory techniques are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

ANALYSIS OF THE ROS1 G2032R MUTANT
Expression plasmids were made containing the nonmutated or mutated CD74–ROS1 coding sequence and were used for transient transfection assays in 293T cells. For soft-agar assays, NIH-3T3 cells were infected with lentivirus encoding the nonmutated or mutated CD74–ROS1. The ROS1 kinase domain (residues 1934 to 2232) was expressed in Sf21 cells and purified for use in the in vitro enzymatic assays and for the purpose of determining the crystal structure of the nonmutant ROS1–crizotinib complex (structure deposited in the Worldwide Protein Data Bank, entry 3zbf).

RESULTS

GENETIC ANALYSIS OF RESISTANT TUMORS
To identify the mechanisms of resistance to crizotinib, we obtained a biopsy specimen of a lesion in the right lung that was enlarging while the patient was receiving crizotinib and compared this specimen with one obtained before treatment with crizotinib began. The persistence of the ROS1 rearrangement in the resistant lesion was confirmed by means of FISH, which also showed no ROS1 gene amplification (Fig. S1 in the Supplementary Appendix). Total RNA was extracted from tumor tissue, and the sequencing of an RT-PCR product spanning the CD74–ROS1 fusion junction revealed a c.6094G→A, p.Gly2032Arg (G2032R), mutation that had not been detected in the pretreatment specimen (Fig. 2B, and Fig. S2 in the Supplementary Appendix).

After the patient died, an autopsy was performed. All the sites of disease that were examined had the G2032R mutation (Fig. 2C), suggesting that its occurrence was an early event in the clonal expansion of crizotinib-resistant tumor cells. Deep sequencing (>10,000 reads) of the 3’ portion of the ROS1 gene involved in the rearrangement (exons 34 to 42) was performed on all autopsy specimens; no somatic mutations other than G2032R were revealed within ROS1, and the G2032R mutation was not detected in the sample of malignant cells assessed before treatment with crizotinib (Fig. S2 in the Supplementary Appendix). Exon sequencing of a panel of 409 genes implicated in carcinogenesis independently confirmed the presence of the G2032R mutation in tumors that were resistant to treatment and also identified mutations in three other genes — TP53, ataxia–telangiectasia mutated (ATM), and NOTCH2 — in both the pretreatment and the crizotinib-resistant tumor specimens but not in normal tissue (Fig. S2 in the Supplementary Appendix). No mutations in EGFR or KRAS were detected on exon sequencing.

MOLECULAR BASIS OF CRIZOTINIB RESISTANCE
The glycine at position 2032 is conserved in all human ROS1 paralogs and in several other, more distantly related tyrosine and serine–threonine kinases (Fig. 3A). To determine whether an arginine substitution at this highly conserved residue conferred resistance to ROS1 inhibitors, we transfected 293T cells with an expression plasmid encoding ei-
ROS1 in a dose-dependent manner, with IC\textsubscript{50} values of approximately 30 nM and 50 nM, respectively (half-maximal inhibitory concentration) values of nonmutant or G2032R CD74–ROS1. The transfected cells were subsequently treated with increasing doses of tyrosine kinase inhibitors. Although crizotinib and TAE684\textsuperscript{14} (Fig. 3B) inhibited the phosphorylation of nonmutant CD74–ROS1 in a dose-dependent manner, with IC\textsubscript{50} values of approximately 30 nM and 50 nM, respectively (Fig. S3 in the Supplementary Appendix), they were ineffective against the G2032R mutant, for which IC\textsubscript{50} values for both compounds were greater than 1000 nM. We did not observe activity of the selective ALK inhibitor CH5424802\textsuperscript{15} against either the nonmutant or mutant forms of CD74–ROS1.

In vitro enzymatic assays also showed that the crizotinib concentration needed to achieve 50% enzyme inhibition (K\textsubscript{\text{app}}) was increased by a fac-
tor of 270 for the G2032R mutant kinase as compared with nonmutant ROS1 (K_{m}^{app} 570±29 nM vs. K_{m}^{app} 2.1±0.1 nM) (Fig. 3C). Furthermore, the ATP concentration needed to achieve half-maximal enzyme velocity (K_{m}^{app}) for the G2032R mutant was reduced by a factor of 3 as compared with that for nonmutant ROS1 (K_{m}^{app} 22 μM vs. K_{m}^{app} 65 μM) (Fig. S5 in the Supplementary Appendix) — perhaps contributing to the increased activity of the mutant kinase. However, in soft-agar assays, the transforming capacities of nonmutant and mutant CD74–ROS1 were similar (Fig. S6 in the Supplementary Appendix).

To elucidate the molecular basis of G2032R-mediated resistance, we determined the crystal structure of the phosphorylated nonmutant ROS1 kinase domain bound to crizotinib (Fig. 3D, left). Like ALK and c-MET (also known as the hepatocyte growth-factor receptor), ROS1 binds crizotinib at the ATP-binding site in the cleft between the N-terminal and C-terminal domains of the kinase. The G2032 residue sits at the solvent front in the distal end of the kinase hinge and creates a turn, putting the G2032 alpha carbon in position to engage in a van der Waals interaction with the pyrazole ring of crizotinib. An arginine at position 2032 has been modeled into an empty ROS1 ATP–inhibitor binding site (Fig. 3D) and is believed to sterically clash with the piperidine ring of crizotinib while still allowing for ATP binding (Fig. S7 in the Supplementary Appendix). These data provide a biochemical and structural basis for the resistance to crizotinib conferred by the G2032R mutation.

**DISCUSSION**

In this study, we report an acquired mutation for crizotinib resistance in a cancer driven by an oncogenic ROS1 fusion. We also present the crystal structure of crizotinib bound to the ROS1 kinase domain. Unlike the classic gatekeeper mutations for drug resistance that have been identified in ABL, EGF, and ALK, the G2032R ROS1 mutation is located in the solvent front of the kinase domain and is analogous to the G1202R ALK mutation identified in crizotinib-resistant ALK-rearranged lung cancers. The autopsy performed in this case revealed the presence of the CD74–ROS1 gene translocation at all sites of disease. Despite the genetic heterogeneity that has been identified at various metastatic sites in an individual patient, our findings support the notion that some oncogenic drivers are present in founder clones and therefore may be present at all sites of metastasis. It is also noteworthy that the same mechanism of acquired resistance — the G2032R mutation — was identified at all the sites of disease that were examined, and no other ROS1 kinase mutations were identified by deep sequencing. Thus, it appears that this mutation occurred early in the development of resistance and suggests that a potent inhibitor of this mutant kinase may have been clinically effective after the failure of crizotinib.

At the time of disease progression, when the patient was taking crizotinib, a repeat biopsy was performed to identify the mechanism of drug resistance. The biopsy was performed in accordance with a clinical protocol that was approved by the institutional review board at our institution specifically for patients in whom resistance has developed to targeted therapies such as crizotinib and erlotinib. Repeat biopsies have emerged as an invaluable tool for discovering clinically relevant resistance mechanisms, including secondary mutations within the target, activation of alternative signaling pathways, and in the case of EGFR-mutant lung cancer, small-cell transformation. Elucidating these mechanisms has helped to guide the development of new treatment strategies designed to overcome resistance.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

**Abbreviations**

ABL, Abelson tyrosine kinase; ALK, anaplastic lymphoma kinase; APL, acute promyelocytic leukemia; c-MET, hepatocyte growth factor receptor; CD74–ROS1, nonclassical ROS1 at the ATP-binding site in the cleft between the N-terminal and C-terminal domains of the kinase; c-MET, hepatocyte growth factor receptor; CD74–ROS1, nonclassical ROS1 fusion. We also present the crystal structure of crizotinib bound to the ROS1 kinase domain. Unlike the classic gatekeeper mutations for drug resistance that have been identified in ABL, EGF, and ALK, the G2032R ROS1 mutation is located in the solvent front of the kinase domain and is analogous to the G1202R ALK mutation identified in crizotinib-resistant ALK-rearranged lung cancers. The autopsy performed in this case revealed the presence of the CD74–ROS1 gene translocation at all sites of disease. Despite the genetic heterogeneity that has been identified at various metastatic sites in an individual patient, our findings support the notion that some oncogenic drivers are present in founder clones and therefore may be present at all sites of metastasis. It is also noteworthy that the same mechanism of acquired resistance — the G2032R mutation — was identified at all the sites of disease that were examined, and no other ROS1 kinase mutations were identified by deep sequencing. Thus, it appears that this mutation occurred early in the development of resistance and suggests that a potent inhibitor of this mutant kinase may have been clinically effective after the failure of crizotinib.

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