Molecular Changes Associated with Acquired Resistance to Crizotinib in ROS1-Rearranged Non–Small Cell Lung Cancer

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Abstract

Purpose: Although ROS1-rearranged non–small cell lung cancer (NSCLC) is sensitive to crizotinib, development of resistance is inevitable. Here, we identified molecular alterations in crizotinib-resistant tumors from two NSCLC patients with the CD74–ROS1 rearrangement, and in HCC78 cells harboring SLC34A2–ROS1 that showed resistance to crizotinib (HCC78CR).

Experimental Design: ROS1 kinase domain mutations were examined in fresh tumor tissues from two NSCLC patients and HCC78CR1-3 cells by direct sequencing. Ba/F3 cells expressing ROS1 secondary mutations were constructed to evaluate resistance to crizotinib. An upregulated pathway was identified using phospho-receptor tyrosine kinase array, EGFR signaling antibody array, and RNA sequencing (RNA-seq). Cell proliferation and ROS1 downstream signaling pathways were compared between HCC78 and HCC78CR1-3 cells.

Results: The ROS1 G2032R mutation was identified in crizotinib-resistant tumors from one patient. Furthermore, HCC78CR1 and CR2 cells harbored a novel ROS1 L2155S mutation (73.3% and 76.2%, respectively). ROS1 G2032R and L2155S mutations conferred resistance to crizotinib in Ba/F3 cells. Evidence of epithelial-to-mesenchymal transition with downregulated E-cadherin and upregulated vimentin was observed in HCC78CR1-2 cells and in the other patient. RNA-seq and EGFR signaling antibody array revealed that the EGFR pathway was significantly upregulated in HCC78CR3 versus HCC78 cells. Cells with the ROS1 mutation and upregulated EGFR were sensitive to foretinib, an inhibitor of c-MET, VEGFR2, and ROS1 and irreversible EGFR kinase inhibitors plus crizotinib, respectively.

Conclusions: Molecular changes associated with acquired crizotinib resistance in ROS1-rearranged NSCLC are heterogeneous, including ROS1 tyrosine kinase mutations, EGFR activation, and epithelial-to-mesenchymal transition. Clin Cancer Res; 21(10); 2379–2387. ©2015 AACR.

Introduction

Oncogenic drivers have been discovered as druggable targets in patients with non–small cell lung cancer (NSCLC), and tyrosine kinase inhibitors (TKI) have emerged as a particularly successful treatment modality (1, 2). EGFR TKIs allow prolonged survival in NSCLC patients with activating EGFR mutations (3). However, acquired resistance to EGFR TKIs is inevitable, and various molecular mechanisms have been uncovered to possibly overcome resistance (4, 5). Likewise, an ALK inhibitor showed superior outcomes compared with standard chemotherapy in previously treated ALK-positive NSCLC patients (6). However, a fraction of tumors eventually became resistant to the ALK inhibitor by acquiring secondary ALK mutations or alternative pathway alterations (7–9).

Recently, the c-ros oncogene 1, which is a receptor tyrosine kinase (ROS1) rearrangement that is present in tumors of 0.7% to 1.7% of NSCLC patients, has been identified as a new target in NSCLC (10). Crizotinib showed in vitro and clinical activities against ROS1-rearranged NSCLC (11). In addition, crizotinib showed the objective response rate of 72% in patients with advanced ROS1-rearranged NSCLC (12). Despite the promising efficacy of crizotinib, ROS1-rearranged tumors developed acquired resistance to crizotinib after median progression-free survival of 19.2 months. The mechanism of acquired resistance to crizotinib was partly mediated by the ROS1 G2032R mutation in a patient with metastatic adenocarcinoma harboring CD74–ROS1 fusion (13) or EGFR pathway activation in a patient with NSCLC harboring SDC4–ROS1 fusion (14). However, due to the rarity of ROS1-rearranged NSCLC with acquired resistance to crizotinib, its mechanisms are not fully understood in ROS1-rearranged NSCLC.

The aim of this study was to discern the crizotinib resistance mechanisms in ROS1-rearranged NSCLC using tumor samples from 2 patients with acquired resistance to crizotinib and an in vitro crizotinib resistance induction model.
**Translational Relevance**

We used fresh tumor tissues from two patients with ROS1-rearranged non-small cell lung cancer (NSCLC) and HCC78CR1 cells to evaluate acquired resistance mechanisms to crizotinib. Our mechanistic findings associated with crizotinib resistance indicated that molecular changes associated with acquired crizotinib resistance in ROS1-rearranged NSCLC are heterogeneous: ROS1 tyrosine kinase mutations, EGFR activation, and epithelial-to-mesenchymal transition. Secondary ROS1-mutant NSCLC cells were sensitive to foretinib, an inhibitor of c-MET, VEGFR2, and ROS1. In addition, NSCLC cells with coactivated EGFR and ROS1 were inhibited by irreversible EGFR inhibitors alone or in combination with crizotinib. Future treatment strategies for ROS1-positive NSCLC patients with acquired crizotinib resistance should be personalized based on the specific molecular changes observed.

**Materials and Methods**

**Patients and tumor samples**

Patient 1, a 46-year-old female never-smoker, underwent right lower lobectomy with mediastinal lymph node dissection and received 4 cycles of adjuvant vinorelbine plus cisplatin chemotherapy for pathologic stage IIIA pulmonary adenocarcinoma. Multiple lung metastases developed 10 months after surgery and 4 cycles of pemetrexed plus cisplatin chemotherapy were administered. Patient 2, a 50-year-old female never-smoker, was diagnosed as stage IV pulmonary adenocarcinoma and failed first-line gemcitabine plus cisplatin and second-line pemetrexed chemotherapy. These patients were enrolled into a crizotinib phase I study (NCT00585195) after ROS1 rearrangement was detected by ROS1 fluorescence in situ hybridization (FISH) in tumor samples of the patients. ROS1 FISH was considered positive when split or isolated red signals were observed in more than 15% of 50 analyzed cells (13). ROS1-rearrangement probes were kindly provided by Professor Anthony John Iafrate (Massachusetts General Hospital, Boston, MA). Crizotinib at a dosage of 250 mg twice daily was administered to patients 1 and 2, who showed ROS1 FISH-positive cells of 86% and 70%, respectively. Response evaluation was based on the Response Evaluation Criteria in Solid Tumors version 1.0. Pretreatment and crizotinib-resistant tumor tissues were collected after informed consent as follows: paraffin-embedded surgical tissues before crizotinib and fresh tumor tissues after crizotinib for patient 1, and fresh tumor tissues before and after crizotinib for patient 2. This study was approved by the Institutional Review Board of Seoul National University Hospital (H-1404-072-572).

**Cell lines and reagents**

HCC78 and Ba/F3 cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. HCC78CR1, -2, and -3 cells were established as previously described (9). Briefly, HCC78 cells were maintained in RPMI-1640 medium that contained crizotinib at a starting concentration of 100 nmol/L until a final concentration of 1 μmol/L over 6 months. Then, the resulting resistant cells were maintained in RPMI-1640 medium with 0.5 μmol/L crizotinib and were designated as HCC78CR1, -2, and -3 cells. HCC78 and HCC78CR1-3 cells were authenticated by short tandem repeat analysis of DNA (Supplementary Table S1). HCC78, HCC78CR1, -2, -3, and retroviral vector-transduced Ba/F3 cells were grown in RPMI-1640 medium supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin (Gibco), and 2 mmol/L L-glutamine. Plat-E cells were purchased from Cell Biolabs, Inc. and maintained in Dulbecco’s Modified Eagle Medium supplemented with 1 μg/mL puromycin, 10 μg/mL blasticidin, 1% penicillin/streptomycin, and 10% FBS. Crizotinib, NVP-TAE684, afatinib, dacotimib, foretinib, and gefitinib were purchased from Selleck Chemicals and cetuximab from Merck Serono. Antibodies to total ROS1 (#3266), phosphorylated (p)-ROS1 Tyr 2274 (#3078), total AKT (#4685), p-AKT (#4060S), total ERK p42/44 (#9102), p-ERK (#9106), total Stat3 (#1393), p-Stat3 (#9145S), total EGFR (#4267S), p-EGFR (#3777S), p-SHP2 (#3751), total S6 (#2217S), p-S6 (#4858S), mTOR (#2988S), p-mTOR (#5536), GAPDH (#5174), p-PLCγ1 (#8713S), PARP (#9542), and E-cadherin (#3195) were purchased from Cell Signaling Technology. Antibodies to total SHP2 (#610621), anti-actin, α-smooth muscle, anti-fibronectin, and vimentin were purchased from BD Bioscience, Sigma-Aldrich, abcam, and Santa Cruz Biotechnology, respectively. These antibodies were used for immunoblotting, and the blots were subsequently washed, transferred, and developed using prepared Enhanced Lumi-Light Western Blotting Substrate (Roche).

**Phospho-receptor tyrosine kinase and EGFR signaling antibody arrays**

Phospho-receptor tyrosine kinase (RTK) were detected with the Human Phospho-RTK Array Kit (R&D Systems) according to the manufacturer’s protocols. Total EGFR, p-EGFRs (Thr669, Tyr845, Tyr998, and Tyr1068), EGFR L858 mutant, EGFR with deletion at E746–A750, and EGFR downstream molecules were detected using the PathScan EGFR Signaling Antibody Array Kit (Cell Signaling). Image analysis was performed using a LAS-3000 imaging system (Fuji Photo Film Co.).

**RT-PCR, PCR, and sequencing**

Total RNA was isolated from the HCC78, HCC78CR1, -2, and -3 cell lines and tumor tissue samples using a RNA Mini kit (Invitrogen). Each sample was reversely transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). PCR amplification was performed with specific primers according to protocols. PCR primers for RT-PCR are listed in Supplementary Table S2. The images were captured using the Gel Logic 200 imaging system (Kodak). The PCR products were purified with a PCR purification kit (Invitrogen). Sequencing of PCR products was performed as described below. Genomic DNA was isolated from cell pellets or tissues from patients using an ALL-prep DNA/RNA micro kit (Qiagen). Exons of EGFR and KRAS were amplified from genomic DNA using the High Fidelity plus PCR System (Roche) and sequenced bidirectionally by Sanger sequencing with the primers specific for EGFR exons 18 to 21 and KRAS exons 1 and 2 (Supplementary Table S2).

**Cloning, site-directed mutagenesis, and construction of retroviral vector-transduced Ba/F3 cell lines**

SLC34A2–ROS1 variant cDNAs from HCC78, HCC78CR1, -2, and -3 cells and CD74–ROS1 variant cDNAs from fresh tumor tissues from patients 1 and 2 were amplified using specific primers (Supplementary Table S2). The PCR products were cloned into the
TOPO-TA vector (Invitrogen) and transformed into DH5α E. coli bacteria (Life Technologies); the ROS1 kinase domain was sequenced in individual clones. To generate CD74–ROS1 mutants, each mutation identified from cell lines and patients was introduced using site-directed mutagenesis (Agilent Technologies) with mutant-specific primers according to the manufacturer’s instructions. Primers used for site-directed mutagenesis are listed in Supplementary Table S2. The sequencing products were analyzed using an electropherogram. The Basic Local Alignment and Search Tool (BLAST database, NCBI) was used to identify the query sequences. Each CD74–ROS1 variant gene was subcloned into the retrovector pMx-puro (Cell Biologs), which was transduced into Ba/F3 cells according to the manufacturer’s instructions. Retrovector-transduced Ba/F3 cells were selected by puromycin treatment and subsequently cultured in the absence of IL3 for 4 weeks.

**Quantitative real-time PCR**

Complementary cDNA was prepared as described above. Quantification of gene expression levels and an internal reference gene (GAPDH) was performed using the SYBR green I detection method. The Power SYBR Green PCR Master Mix (Life Technologies) was used according to the manufacturer’s instructions. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The Applied Biosystems StepOnePlus Real-Time PCR System (Life Technologies) was used for cDNA amplification and analysis. Primers used for the amplification of EGFR, heparin-binding (HB)-EGF, and GAPDH are listed in Supplementary Table S2.

**Cell proliferation assays**

NSCLC cells and retrovector-transduced Ba/F3 cells were cultured in 96-well plates in the presence of drugs or vehicle for 72 hours. Ba/F3 cell proliferation was analyzed using the CCK-8 colorimetric assay (Dojindo), and the absorbance was measured at 450 nm in an Eon Microplate Spectrophotometer (BioTek). NSCLC cell proliferation was analyzed using the CellTitre-Glo Luminescent cell viability assay (Promega) according to the manufacturer’s instructions, and the luminescent signal was measured in a PerkinElmer Victor Light 1420 Luminescence Counter (PerkinElmer).

**Whole-exome sequencing and RNA sequencing**

Whole-exome sequencing (WES) was performed on normal blood and on pretreatment and crizotinib-resistant fresh tumors from patient 2 according to the Illumina Nextera Turbo protocol. FastQC was used for sequence quality control. Alignment against human genome hg19 was performed using the Burroughs Wheeler Aligner. The GATK, VarScan2, and UnifiedGenotyper algorithms were used for variant calling. Functional annotation of genetic variants was performed using ANNOVAR. RNA was extracted from HCC78 and HCC78CR3 cells using the miNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions and analyzed by Illumina RNA sequencing (RNA-seq). SNIPR and Funseq algorithms were used for variant calling, and SNVs and Indels were identified and compared between the cell lines. Quantification of gene expression was performed, and differentially expressed genes were identified using the Tophat–cufflink–cuffdiff axis. The existence of an enriched pathway was compared (Supplementary Table S2). PCR amplicons from HCC78 cells represented truncations of SLC34A2 at exon 5 and ROS1 at exon 22, and HCC78CR3 cells showed resistance to ALK inhibitors (Fig. 1A). In addition, cDNA from HCC78CR3 cells was analyzed using an electropherogram. The Basic Local Alignment and Search Tool (BLAST database, NCBI) was used to identify the query sequences. Each CD74–ROS1 variant gene was subcloned into the retrovector pMx-puro (Cell Biologs), which was transduced into Ba/F3 cells according to the manufacturer’s instructions. Retrovector-transduced Ba/F3 cells were selected by puromycin treatment and subsequently cultured in the absence of IL3 for 4 weeks.

**Results**

Patient outcomes and the identification of the ROS1 fusion transcript

Patient 1 achieved a partial response to crizotinib and experienced disease progression at 12 months (Fig. 1A). A new cystic and solid mass on the right ovary was resected revealing a metastatic adenocarcinoma from the lung with thyroid transcription factor-1 expression. The patient continued crizotinib treatment beyond progression due to ongoing clinical benefits. Patient 2 underwent bronchoscopy-guided tumor removal before crizotinib and after disease progression due to total collapse of the left lung. Although partial response was achieved after the initiation of crizotinib, tumors progressed to the brain at 5 months. Expression of SLC34A2–ROS1 and CD74–ROS1 fusion transcripts was confirmed by RT-PCR with specific primers SLC34A2–E4F/ROS1-E34R and CD74–E5F/ROS1-E34R, respectively (Supplementary Table S2). PCR amplicons from HCC78 cells represented truncations of SLC34A2 at exon 5 and ROS1 at exon 32 or 34, and fresh tumor tissues from the 2 patients exhibited truncations of CD74 at exon 6 and ROS1 at exon 34 (Supplementary Fig. S1A and S1B).

**ROSI kinase mutations in crizotinib-resistant tumors**

The c.6094G→A mutation that encoded for Gly2032Arg (G2032R) was identified at a frequency of 100% in crizotinib-resistant tumors from patient 1 (Fig. 1B). In addition, c.6464T→C substitution encoding Leu2155Ser (L2155S) was observed in HCC78CR1–2 cells (Fig. 2B) and showed an oncogenic potential (Supplementary Fig. S2). cDNA clones from HCC78CR1 and HCC78CR2 cells harbored the L2155S mutation at a frequency of 73.3% and 76.2%, respectively. The crystal structure revealed that Pseudo DELTA DELTA G value is 5.76 and ROS1 L2155S mutation is predicted to be highly destabilizing and cause protein structural modification (Fig. 2C). This might interfere with crizotinib binding. However, none of the cDNA clones from normal and pretreatment tumor tissues of patient 1 or HCC78 cells harbored any mutation within the ROS1 kinase domain (Fig. 1B, 1B, and 1B). In addition, no secondary ROS1 mutation was found in normal, pretreatment, and crizotinib-resistant tumor tissue from patient 2 using direct sequencing as well as WES analysis (Supplementary Table S3).

**HCC78CR cells showed resistance to ALK inhibitors**

HCC78CR1, 2, and 3 cells showed resistance to crizotinib at least 8-fold higher IC50 compared with parental HCC78 cells (Fig. 2A). In addition, these cell lines showed resistance to foretinib, a potent ROS1 inhibitor that is effective against ROS1 G2032R-mutant cells (16), and TAE684, a potent ALK inhibitor (Supplementary Fig. S2). Whereas crizotinib suppressed p-ROS1, p-SHP2, p-S6, and p-ERK expression in HCC78 cells, it ineffectively suppressed expression of p-SHP2 and p-ERK in HCC78CR cells (Supplementary Fig. S3).

**ROS1 secondary mutations conferred resistance to crizotinib**

Ba/F3 cells expressing cDNA encoding wild-type (WT) CD74–ROS1, CD74–ROS1 G2032R, CD74–ROS1 L2155S, CD74–ROS1 L2026M (i.e., a gatekeeper mutation), and CD74–ROS1 G2101A (an analogue of the ALK G1269A mutation) were constructed to
evaluate the resistant profiles. Ba/F3 cells expressing ROS1-mutant CD74–ROS1 transcripts showed resistance to crizotinib, compared with those expressing WT CD74–ROS1 after exposure to crizotinib, in a dose-dependent manner (Fig. 3A). Although ROS1 G2101A, L2026M, and G2032R-expressing Ba/F3 cells were sensitive to foretinib, ROS1 L2155S-mutant Ba/F3 cells were resistant to foretinib (Fig. 3B and Supplementary Fig. S4). ROS1 G2032R and L2155S clones were resistant to TAE684 (Fig. 3C and Supplementary Fig. S4). p-ROS1 was downregulated to a lesser degree in ROS1-mutant Ba/F3 cells than in WT ROS1 Ba/F3 cells after exposure to crizotinib (Fig. 3D).

EGFR pathway activation in HCC78CR3 cells

There were no EGFR and KRAS mutations in HCC78 and HCC78CR cells according to the results of Sanger sequencing. The p-EGFR at Thr669 and Tyr1068, p-MEK1/2 at Ser221, and p-PLCγ1 at Ser1248, and p-ERK1/2 at Thr202/Tyr204 were more highly expressed in HCC78CR3 cells than in HCC78 cells. In contrast, expression of p-PARP at Asp214 was high in HCC78 cells (Fig. 4A). After exposure to 1 μmol/L crizotinib, p-ERK1/2 at Thr202/Tyr204 was upregulated in HCC78CR3 cells compared with HCC78 cells. RNA-seq revealed that EGFR expression was upregulated by 2.6-fold in HCC78CR3 cells compared with HCC78 cells (Supplementary Table S4). Among EGFR ligands, only HB-EGF was 2.4-fold higher in HCC78CR3 cells (Supplementary Table S4). Quantitative RT-PCR analysis showed high expression levels of EGFR and HB-EGF in HCC78CR3 cells (Supplementary Fig. S5). In addition, HB-EGF and EGFR ligands induced resistance to crizotinib in HCC78 cells (Supplementary Fig. S5). The EGFR pathway was activated, but epithelial-to-mesenchymal transition (EMT) was not observed in HCC78CR3 cells by pathway enrichment analysis (Supplementary Table S5). The upregulation of p-EGFR was not observed in crizotinib-resistant tumors of patient 2 (data not shown).

Inhibition of EGFR in HCC78CR cells

HCC78 and HCC78CR cells were resistant to gefitinib as well as cetuximab (Supplementary Fig. S6). Dacomitinib and afatinib were active against HCC78CR3 cells, with lower IC50 values observed for HCC78 and HCC78CR1-2 cells (Fig. 4B). Crizotinib in combination with dacomitinib or afatinib showed synergistic inhibition in HCC78CR3 cells (Fig. 4C) as well as in HCC78 and HCC78CR1-2 cells (Supplementary Figs. S7 and S8). The expression levels of p-ERK, p-AKT, and p-S6 were significantly decreased in HCC78CR3 cells treated with dacomitinib or afatinib versus cells treated with crizotinib alone (Fig. 4D). HCC78CR3 cells also showed upregulation of p-EGFR (Tyr1068) and total EGFR as compared with HCC78 cells, irrespective of crizotinib treatment or drug exposure time (Supplementary Fig. S9).

EMT-like changes in tumor tissue from 1 patient and HCC78CR1-2 cells

WES of patient 2 tumor tissue did not reveal any genetic alteration in ROS1, EGFR, ALK, KRAS, or MET tyrosine kinases.
Instead, E-cadherin expression had disappeared in crizotinib-resistant tumor tissue (Fig. 5A, top right) as compared with pretreatment tumor tissue (Fig. 5A, top left). Vimentin expression was maintained or slightly increased in crizotinib-resistant tumor tissue (Fig. 5A, bottom right). The expression of vimentin and fibronectin was increased in HCC78CR1-2 cells (Fig. 5B), concomitant with a spindle-shaped morphology (Fig. 5C, top right and bottom left). Morphologic evidence of EMT was not observed in HCC78 (Fig. 5C, top left) or HCC78CR3 cells (Fig. 5C, bottom right).

**Discussion**

Our study demonstrated that acquired resistance mechanisms are heterogeneous in ROS1-rearranged NSCLC treated with crizotinib. Secondary ROS1 G2032R and L2155S mutations were identified in 1 patient (patient 1) with CD74–ROS1 rearranged NSCLC and in HCC78CR1-2 cells, respectively. EGFR was activated, and HB-EGF was overexpressed in HCC78CR3 cells, which was inhibited by irreversible EGFR TKIs. In addition, EMT-like features were observed in HCC78CR1-2 cells with the ROS1 L2155S mutation and in cells from the other ROS1-rearranged tumor (patient 2) without secondary ROS1 mutation.

The ROS1 G2032R mutation in the solvent front of the kinase domain was identified in one ROS1-rearranged NSCLC patient who showed acquired resistance to crizotinib. In addition, this mutation conferred resistance to crizotinib and TAE684 (13). Similarly, the ROS1 G2032R mutation was detected at a frequency of 100% in one of our patients (patient 1). In addition, Ba/F3-G2032R cells that were sensitive to foretinib were resistant to crizotinib and TAE684, like previous studies (13, 16). We identified a novel L2155S mutation in the ROS1 kinase domain in crizotinib-resistant HCC78CR1-2 cells, and L2155S-mutant clones were resistant to crizotinib, TAE684, and foretinib, suggestive of the highly resistant ROS1 mutation.
Resistance to crizotinib in ROS1-mutant Ba/F3 cells. Ba/F3 cells expressing cDNA that encoded WT CD74–ROS1, CD74–ROS1 G2032R, CD74–ROS1 L2155S, CD74–ROS1 L2026M, and CD74–ROS1 G2101A were constructed and exposed to crizotinib (A), foretinib (B), and TAE684 (C). IC50 was provided as mean ± SD. D, p-ROS1 expression was less suppressed after treatment with crizotinib for 6 hours in CD74–ROS1 G2032R, L2155S, L2026M, and G2101A-expressing Ba/F3 cells compared with that of CD74–ROS1-expressing Ba/F3 cells.

**Figure 3.**
Figure 4.

EGFR activation in HCC78CR3 cells. A, EGFR signals were more highly activated in HCC78CR3 cells than in HCC78 cells. EGFR and its downstream signaling molecules were compared between HCC78CR3 and HCC78 cells after vehicle, crizotinib for 24 hours, and crizotinib for 48 hours. Target map of subarray A (top) and subarray B (bottom) was expressed using the PathScan EGFR Signaling Antibody Array Kit (left). Activations of EGFR and its downstream signaling molecules were confirmed by Western blotting (right). B, cell viability assays were performed in HCC78 and HCC78CR1-3 cells treated with dacomitinib (left) and afatinib (right) for 72 hours. C, cell viability assays were plotted in HCC78CR3 cells treated with dacomitinib or afatinib with or without crizotinib. D, the expression levels of signaling molecules were shown after 4-hour incubation at 1 μmol/L of each drug concentration.
In addition, a structural model for ROS1 L2155S mutation suggests that it confers resistance to crizotinib through protein malfunction rather than steric inference with crizotinib binding. However, its clinical significance is unknown in CD74-ROS1-rearranged NSCLC tumors because its analogue, the ALK F1323 mutation, has not been found through mutagenesis screening (17) and in ALK-rearranged NSCLC from patients who showed acquired resistance to crizotinib (7–9). Interestingly, the ROS1 L2026M and G2101A mutations that were analogous to the ALK L1196M and G1269A mutations were moderately resistant to crizotinib by 38-fold and 10-fold, respectively, compared with ROS1 WT CD74–ROS1 clones. Foretinib, a multitargeted inhibitor of c-MET, VEGFR2, and ROS1 (16), was toxic to ROS1-mutant clones, with the exception of clones with the ROS1 L2155S mutation in our study. Similarly, ROS1-mutant cells that were discovered from accelerated mutagenesis screening were more sensitive to foretinib than crizotinib (16). Recently, PF-06463922, a potent ALK and ROS1 inhibitor, showed promising efficacy against ROS1 G2032R-mutant Ba/F3 cells (18), and an early clinical trial was initiated for patients with ALK- or ROS1-rearranged NSCLC (NCT01979865).

EGFR and its ligand HB-EGF were overexpressed in HCC78CR3, which showed the highest level of resistance to crizotinib, but did not harbor any ROS1 mutation. These results were confirmed by EGFR RTK array, Western blot, quantitative RT-PCR, and RNA-seq, and were also compared between HCC78 and HCC78CR3 cells. In addition, the EGFR cascade was upregulated in HCC78CR3 cells compared with HCC78 cells, whereas the EMT signature was less evident in HCC78CR3 cells when performing pathway enrichment analysis using differentially expressed genes. However, considering that HCC78CR3 cells that overexpressed EGFR signals were ineffective inhibited by cetuximab or gefitinib, an EGFR ligand, HB-EGF alone might not contribute to crizotinib resistance. Because irreversible EGFR TKIs alone or in combination with crizotinib effectively inhibited HCC78CR3 cell proliferation, these strategies might overcome crizotinib resistance associated with EGFR coactivation. EGFR phosphorylation at Thr669 or Tyr1068 was highlighted before crizotinib and enhanced after crizotinib in HCC78CR3 cells as compared with HCC78 cells. Although EGFR phosphorylation at Thr669 negatively modulates EGFR (19), this might be upregulated in HCC78CR3 because the basal level of EGFR phosphorylation is higher in HCC78CR3 cells than in HCC78 cells. These findings suggest that combined EGFR and ROS1 blockade might be a feasible strategy to overcome crizotinib resistance mediated by EGFR activation in ROS1-rearranged NSCLC. However, due to lack of EGFR upregulation in resistant tumors of patient 2, EGFR pathway activation might partly contribute to resistance to ROS1 inhibition, as observed in a previous study (14).

Morphologic and molecular evidence of EMT was observed in our HCC78CR1-2 cells that harbored the ROS1 L2155S mutation. Therefore, both EMT and the ROS1 kinase mutation (L2155S) in HCC78CR1-2 cells conferred crizotinib resistance; thus, these cells were relatively resistant to dual ROS1 and EGFR inhibition or gefitinib. An EMT-like feature, the loss of E-cadherin, was also observed in crizotinib-resistant tumors (patient 2) that did not harbor any ROS1 or alternative pathway mutation as determined by Nextera WES. Although TAE684-resistant HCC78 cells did not
revel EMT [14], crizotinib-resistant, ALK-rearranged H3122 cells showed an EMT-like, fibroblastic morphology similar to the HCC78/CR1-2 cells in our study [20]. This discrepancy might be due to the dual inhibition of ALK or ROS1 and c-MET.

In conclusion, we demonstrated that molecular changes associated with acquired resistance to crizotinib are heterogeneous in ROS1-rearranged NSCLC. ROS1 secondary mutations such as G2032R and L2155S, EGFR activation, and EMT were associated with acquired crizotinib resistance in ROS1-rearranged NSCLC. Secondary ROS1-mutant NSCLC cells (except those with the L2155S mutation) were sensitive to foretinib. In addition, NSCLC cells with coactivated EGFR and ROS1 rearrangement were inhibited by irreversible EGFR inhibitors alone or in combination with crizotinib. These strategies should be investigated in patients with ROS1-rearranged NSCLC whose tumors initially responded but subsequently showed resistance to crizotinib.

Disclosure of Potential Conflicts of Interest

D.-W. Kim is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: A. Song, T.M. Kim, D.-W. Kim, B. Keam, D.S. Heo


References


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