Activation of Src signaling mediates acquired resistance to ALK inhibition in lung cancer

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Abstract. Anaplastic lymphoma kinase (ALK) fusion oncogenes occur in approximately 3-5% of non-small cell lung cancer (NSCLC) cases. Various ALK inhibitors are in clinical use for the treatment of ALK-NSCLC, including the first generation ALK inhibitor, crizotinib, and recently the more highly potent alectinib and ceritinib. However, most tumors eventually become resistant to ALK specific inhibitors. To address the mechanisms underlying the development of ALK inhibitor resistance, we used iTRAQ quantitative mass spectrometry and phosphor-receptor tyrosine kinase arrays to investigate intracellular signaling alterations in ALK inhibitor resistant NSCLC cell lines. Src signaling was identified as an alectinib resistance mechanism, and combination treatment with ALK and Src inhibitors was highly effective for inhibiting the growth of ALK inhibitor resistant cells in vitro and in mouse xenograft models. Furthermore, phospho-receptor tyrosine kinase activation and downstream PI3K/AKT signaling was effectively blocked by inhibiting Src in alectinib resistant cells. Finally, we showed that the combined use of ALK and Src inhibitors inhibited the growth of other ALK-NSCLC cell lines, including those that were ceritinib or lorlatinib resistant. Our data suggest that targeting Src signaling may be an effective approach to the treatment of ALK-NSCLC with acquired resistance to ALK inhibitors.

Introduction

Lung cancer is the most common cause of cancer mortality, with 1.6 million deaths annually (1,2). Non-small cell lung cancers (NSCLCs) are currently sub-classified based on the presence of actionable driver oncogenes, and molecular targeted therapies have been developed to specifically treat cancers harboring these driver mutations (3). Anaplastic lymphoma kinase (ALK) inhibitors are clinically effective for the treatment of NSCLC patients whose tumors harbor ALK fusion genes, including the echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion (EML4-ALK) (4).

The first generation ALK inhibitor, crizotinib, can elicit dramatic and durable responses in ALK-NSCLCs (5). In the last few years, alectinib and ceritinib have been developed for the treatment of ALK-NSCLCs (6,7). Unfortunately, the development of resistance to ALK inhibitors is common and arises through a variety mechanisms (8,9). One of the most important of these mechanisms is the activation of alternative survival pathways, including EGFR, MET, cKIT and IGFR signaling (10). Although activation of Src tyrosine kinase signaling is linked to crizotinib resistance, this survival pathway was not linked to the development of resistance to other ALK inhibitors (11,12).

Heat shock protein (Hsp) 90 inhibitors are effective for the treatment of ALK-NSCLC (13). Hsp90 is a major intracellular molecular chaperone that interacts with a wide variety of intracellular proteins, and promotes correct protein folding and conformation. Hsp90 inhibitors promote ALK degradation, and are clinically effective for the treatment of patients with tumors carrying ALK rearrangements, regardless of resistance to ALK inhibitors (14,15).

The aims of the present study were to clarify the mechanisms underlying resistance to ALK specific inhibitors in NSCLC, and identify novel targets that may ultimately form the basis for the design of more effective therapies for overcoming acquired resistance.

Materials and methods

Cell culture and compounds. The H3122 (EML4-ALK variant 1) NSCLC cell line was kindly provided by Dr Pasi A. Jänne at Dana-Farber Cancer Institute (Boston, MA, USA). H2228 (EML4-ALK variant 3) cells were purchased from the...
American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained at 37°C in 5% CO₂ using RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin, supplemented with 10% fetal bovine serum (FBS) (R-10 medium). Alectinib was provided by Chugai Pharmaceutical Co., Ltd. (Camakura, Japan) and AUYY22 was provided by Novartis Pharma AG (Basel, Switzerland). Ceritinib, lorlatinib, saracatinib, dasatinib and bosutinib were purchased from Selleck Chemicals (Houston, TX, USA). Each compound was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for cell culture experiments.

Generation of H3122-AFR, H3122-LDKR and H3122-PFR cell lines. H3122 cells were treated with alectinib at a starting concentration of 1 nM and cells were maintained at ~70% confluence. After every passage at a given concentration of drug, the concentration of alectinib was increased until a final concentration of 100 nM was achieved. The resulting pool of resistant cells (designated H3122-AFR) was maintained in R-10 medium containing 100 nM alectinib. Other ALK inhibitor resistant cell lines were established in a similar manner. The H3122 derivatives, H3122-LDKR and H3122-PFR, were maintained in R-10 medium with 1 µM ceritinib or 100 nM lorlatinib, respectively.

Cell growth assays. For MTS assays, 3-4x10³ cells were plated into 96-well plates. After 24 h, cells were treated with drugs, and incubated for a further 72 h. CellTiter 96® AQour solution (Promega, Madison, WI, USA) was then added and plates were incubated for an additional 4 h. Optical density was measured at 490 nm and the data were graphically displayed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

For colony formation assays, 5.0x10⁴ cells were plated into 6-well plates and treated with drugs 24 h later. After 6 days, the media containing drugs were refreshed. One week after media replacement, plates were washed with phosphate-buffered saline (PBS) once, and stained with 500 µl of 0.5% crystal violet with 25% methanol for 1 h and photographed.

Immunoblot analysis. Cells grown under the previously specified conditions were lysed in cell lysis buffer (Wako Pure Chemical Industries) with PhosSTOP Phosphatase inhibitor cocktail (Roche, Basel, Switzerland) and Complete EASYPack (Roche). After cell lysis, lysates were centrifuged at 14,000 x g for 10 min at 4°C and the supernatant was used for subsequent procedures. Western blot analyses were conducted after protein separation by SDS-PAGE electrophoresis and transfer to Immobilon-P membrane (Merck KGaA, Darmstadt, Germany). Immunoblotting was performed according to the antibody manufacturer’s recommendations. Antibodies against phospho-ALK (#4144), ALK (#3633), phospho-EGFR (#3777), EGFR (#4267), phospho-Src (#6943), Src (#42109), phospho-AKT (#4060), AKT (#9272), phospho-P130Cas (#4011), P130Cas (#13846), phospho-Crk II (#3491), Crk II (#3492), phospho-CrKL (#3181), CrKL (#3182), phospho-Paxillin (#2541), phosphor-FAK (#3283), FAK (#3285), pan-phospho-Tyrosine (P-Tyr-100) (#9411) and β-actin (#4967) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Immunofluorescence/immunocytochemistry. A total 5x10⁴ of H3122-AFR cells were plated in 8-well chamber slides 1 day before the drug treatment. The cells were fixed with 2% paraformaldehyde at room temperature 8 h after drug treatment. The fixed cells were incubated with primary P-Tyr-100 and Src antibodies diluted in PBS containing 0.5% Block Ace (DS Pharma Biomedical, Osaka, Japan) for 1 day. After washing with PBS, the fixed cells were incubated with appropriate secondary antibodies conjugated to Alexa dye 488 or 594 (Thermo Fisher Scientific) for 1 h before mounting. Nuclei were counterstained with DAPI. The images were taken using an Olympus Fluoview FV1000 confocal laser scanning microscope, and analyzed with Olympus Fluoview ver4.2a Viewer software.

Phospho-receptor tyrosine kinase (RTK) array analysis. A PathScan RTK Signaling Antibody Array kit (Cell Signaling Technology) was used to profile relative tyrosine phosphorylation levels. A total of 2x10⁵ cells was seeded in 10-cm dishes 1 day before drug treatment with alectinib and saracatinib, and the cells were harvested 1 day after drug treatment. Phospho-RTK array analysis was performed according to the manufacturer’s protocol. The average pixel densities of duplicate spots were determined using ImageJ software (http://imagej.nih.gov/ij/) and phospho-RTK percentages were calculated relative to the average control intensity.

Next-generation sequencing. Genomic DNA from H3122-AFR cells was isolated using the DNaseasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol. Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific), a previously validated panel for targeted amplicon sequencing was utilized (Thermo Fisher Scientific). Briefly, 10 ng of gDNA was amplified by PCR using Ion AmpliSeq Library Kit (Thermo Fisher Scientific) and the sequencing was performed on an Ion PGM System according to the manufacturer's protocol. Sequencing reads were multiplexed, quality-filtered and aligned to the human reference genome (GRCh37) using the Torrent Suite software (ver. 5.0.4; Thermo Fisher Scientific). Variants were identified with the Variant Caller software (ver. 5.0.4.0; Thermo Fisher Scientific). The quality of all variants called was manually confirmed by Integrative Genomics Viewer software (IGV; ver. 2.3.59).

Xenograft studies. All mouse xenograft experiments were performed using age matched 8-12 week-old female Nu/Nu mice. After subcutaneous injection of 5.0x10⁶ H3122-AFR cells, mice were randomized into four groups, and treated with either vehicle, alectinib alone, saracatinib alone, or a combination of the two, for the indicated duration of treatment. Tumor size was measured by calipers twice a week. Tumor volume was calculated as length x width² x 0.51. Treatment was initiated when tumors exceeded 100 mm³. Nude mice bearing H3122-AFR xenografts were treated with vehicle only (Control, n=11), alectinib (20 mg/kg, n=10), saracatinib (50 mg/kg, n=11), combination (alectinib 20 mg/kg + saracatinib 50 mg/kg, n=9) for the indicated number of days.
Each drug was prepared in the following solvents: alectinib (0.02 N HCl, 10% DMSO, 10% CremophorEL, 15% PEG400, 15% HPCD), saracatinib (0.5% HPMC and 0.1% Tween-80). Alectinib was administered by oral gavage and saracatinib was administered by intraperitoneal injection.

**iTRAQ sample preparation and analysis.** Protein samples were diafiltered, reduced, alkylated and trypsin digested according to the iTRAQ protocol (Thermo Fisher Scientific). The samples were then labeled using the iTRAQ reagents. Peptides were desalted on a Strata-X 33 µm polymeric reversed phase column (Phenomenex, Inc., Torrance, CA, USA) and dissolved in a buffer containing 10 mM KH2PO4 in 10% acetonitrile before separation by strong cation exchange liquid chromatography on an Agilent 1100 HPLC system. Peptides were eluted with a linear gradient of 0-400 mM KCl. Eight fractions containing the peptides were collected and desalted on Strata-X columns. The fractions were analyzed by electrospray ionization mass spectrometry using the Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1260 Chip Cube nanospray interface (Agilent Technologies) on an Agilent 6540 mass spectrometer (Agilent Technologies).

Data analysis was performed according to a previous report (16). Spectral data for each sample were compared against the Swiss-Prot database with taxonomy set to *Homo sapiens* using ProteinPilot™ 4.5 software (AB Sciex, Redwood City, CA, USA). Average ratios were calculated by comparing the intensities of the peptide signals before and after drug treatment. A P≤0.05 indicates statistically significant differential expression.

**Statistical analysis.** The measurements are presented as means ± SEM. Results were analyzed by the Student’s t-test using GraphPad Prism version 6.0 (GraphPad Software). P<0.05 were considered to be statistically significant.

**Results**

**Generation of ALK lung cancer cell line resistance models.** We generated ALK inhibitor resistant lung cancer cell lines by exposing H3122 cells to increasing doses of the alectinib, ceritinib and lorlatinib. The alectinib resistant H3122 cell line derivative was designated as H3122-AFR. The gDNA extracted from H3122-AFR cells was sequenced using Next-generation sequencing but secondary ALK mutation was not found. Ceritinib and lorlatinib resistant H3122 cell line derivatives were designated as H3122-LDKR and H3122-PFR, respectively.

**Sensitivity of HSP90 inhibitor in H3122 and H3122-AFR.** H3122 and H3122-AFR cell lines had the same sensitivity to AUY922, and ALK protein expression in H3122-AFR cells was inhibited 4 h after the addition of AUY922 (Fig. 1A and B). Immunoblotting analysis of H3122 and H3122-AFR before and after AUY922 treatment for 4 h revealed that ALK proteins were degraded in both cell lines. However, the expression of Hsp70 proteins was increased in both H3122 and H3122-AFR after AUY922 treatment, consistent with a compensatory activation of co-chaperones in response to Hsp90 inhibition (Fig. 1C).

**Chaperone peptides/protein screening in ALK resistant cell lines.** As we previously reported (17), the treatment of H3122 cells with Hsp90 inhibitors resulted in the degradation of ALK proteins and the induction of apoptosis, which suggested that growth/survival signaling in these cells was dependent on chaperone activity. To address this hypothesis, we screened for Hsp90 client proteins in H3122 cells before and after the addition of the Hsp90 inhibitor, AUY922. Similarly, proteins/peptides extracted from H3122-AFR cells were analyzed before and after treatment with AUY922 to study the changes of client proteins after acquired resistance.

For chaperone peptides/protein screening, we analyzed protein expression in H3122 and H3122-AFR cells in response to AUY922 treatment by iTRAQ quantitative mass spectrometry (16,18). iTRAQ detected 1154 proteins/peptides in each cell line, including 72 candidate proteins in H3122, and 63 candidate proteins in H3122-AFR that had P≤0.05. There were 14 candidate proteins that were common to both H3122 and H3122-AFR (Fig. 2A). The candidate proteins in the iTRAQ data were converted to gene lists in DAVID (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov). KEGG (Kyoto Encyclopedia of Genes and
Genomes, http://www.genome.jp/kegg/) intracellular pathway analysis of the DAVID gene lists revealed two deregulated signaling cascades, including the focal adhesion pathway and the insulin signaling pathway (Fig. 2B and Table I). Activation of the focal adhesion pathway, involving paxillin and crkII, could be a mechanism for resistance. Immunoblotting revealed that H3122-AFR cells had higher phosphorylation levels of paxillin, crkII, and Src compared with H3122 cells (Fig. 2C). Taken together, these data suggested that these pathways could be associated with resistance mechanisms.

**Activation of Src signaling mediates acquired resistance to alectinib in H3122-AFR cells.** Src was reported to confer resistance to crizotinib, but not alectinib in a cell line-derived...
We hypothesized that the Src protein could be a therapeutic target in H3122-AFR. To test this hypothesis, Src phosphorylation was inhibited with the Src inhibitor, saracatinib. In colony formation assays, the growth of H3122-AFR cells was inhibited more potently by combined treatment with alectinib (500 nM) and saracatinib (1 µM) alone or in combination on H3122 and H3122-AFR cells.

The H2228 cell line is resistant to ALK TKIs compared with the H3122 cell line due to pre-existing activation of EGFR signaling (data not shown). Colony formation assays showed that combined treatment with each ALK inhibitor alone, or saracatinib alone (Fig. 4A and B).

The H2228 cell line is resistant to ALK TKIs compared with the H3122 cell line due to pre-existing activation of EGFR signaling (data not shown). Colony formation assays showed that combined treatment with each ALK inhibitor alone, or saracatinib alone (Fig. 4A and B).

**Dual blockade of ALK and phospho-Src in other ALK resistant cells.** To study whether activation of Src signaling is a mechanism of acquired resistance to ceritinib or lorlatinib, we employed colony formation assays to confirm the combined effects of each ALK inhibitor and saracatinib. In the H3122-LDKR and H3122-PFR cell lines, dual blockade of ALK and phosphor-Src inhibited tumor growth more effectively compared with each ALK inhibitor alone, or saracatinib alone (Fig. 4A and B).

The H2228 cell line is resistant to ALK TKIs compared with the H3122 cell line due to pre-existing activation of EGFR signaling (data not shown). Colony formation assays showed that combined treatment with each ALK inhibitor and saracatinib effectively inhibited H2228 cell growth (Fig. 4C).
This result showed that the combined use of ALK and Src inhibitors could be effective in different contexts of ALK inhibitor resistance.

Inhibition of Src overcomes resistance by inhibition of alternative RTKs in H3122-AFR cells. Previous reports showed that Src is a common node downstream of multiple resistance pathways (19,20). We confirmed that H3122-AFR cells had higher phosphorylation levels of Src, paxillin and crk II, as well as crkL, P130Cas and FAK compared with H3122 by western blotting (data not shown). we assessed the roles of these Src pathway related proteins in the development of ALK resistance.
inhibitor resistance by studying the combined effect of alectinib and saracatinib on Src signaling in H3122-AFR cells by western blotting. When tumors were treated with saracatinib alone, the phosphorylation levels of Src pathway proteins decreased (Fig. 5A). We also found that EGFR phosphorylation was decreased following treatment with saracatinib alone. On the other hand, ALK phosphorylation levels decreased in response to alectinib alone. Combined treatment with alectinib and saracatinib induced a reduction in AKT phosphorylation (Fig. 5B). In addition, we confirmed that phospho-RTK activity was higher in untreated H3122-AFR cells compared with H3122 cells, and RTK activity decreased in H3122-AFR cells in response to saracatinib alone (Fig. 5D).

Next we analyzed phospho-RTK signaling in H3122 and H3122-AFR cells using human phospho-RTK arrays. These data showed a decrease in ALK phosphorylation in H3122-AFR cells, and an increase in the phosphorylation of multiple RTKs in H3122-AFR cells compared with H3122 cells (data not shown). In addition, there was a decrease in the phosphorylation of multiple RTKs, especially the members of EGFR/HER, insulin receptor (IR) and HGFR families in H3122-AFR cells after combined treatment with alectinib and saracatinib (Fig. 5D).

We also carried out two-color immunofluorescence staining to study the effects of combined ALK and Src inhibition on H3122-AFR cells. These data addressed the expression of phospho-tyrosine proteins and total Src in H3122-AFR cells, and also revealed the co-localization of phospho-tyrosine proteins and Src. Double immunofluorescence staining showed markedly diminished phospho-RTK and Src signals in H3122-AFR cells after combined treatment with alectinib and saracatinib (Fig. 5E). Taken together, these data indicate that Src inhibition overcomes alectinib resistance in H3122-AFR cells in vitro.

**Combined effects of alectinib and saracatinib in H3122-AFR xenograft models.** To evaluate the combined effect of alectinib and saracatinib in vivo, H3122-AFR xenograft tumors were established in nude mice. The combined treatment with alectinib and saracatinib resulted in significant tumor regression compared with treatment with each compound alone (Fig. 6A). During the treatment, body weight or individual appearance were not changed. At 22 days after treatment, when the average size of tumors in the control group exceeded 500 mm³, the percent change in tumor volume was significantly decreased by combined treatment, while the volume of some tumors increased in response to monotherapy (Fig. 6B).

**Discussion**

We identified the activation of Src signaling as a mechanism of acquired resistance to ALK inhibitors in NSCLC cells. Our data also showed that RTK activation and downstream PI3K/AKT signaling can be effectively blocked by inhibiting Src.

The activation of alternative survival pathways is a common mechanism driving the development of drug resistance in cancer cells. Indeed, the activation of Src is involved in the resistance to the crizotinib used for the treatment of NSCLC and other cancers (11).

iTRAQ-based quantitative proteomic analysis has contributed to the discovery of novel therapeutic targets for cancer (21,22) and recently An et al (12) identified CrkL activation as an NSCLC resistance mechanism by proteomics analysis. However, the role of Src activation in acquired resistance to alectinib, ceritinib and lorlatinib has not been previously reported. We used iTRAQ proteomics and Hsp90-inhibitors to identify chaperone peptides/proteins in ALK resistant cell lines. The screening results revealed that the focal adhesion pathway, involving paxillin and crkII activation, could be involved in ALK resistance. Previous reports identified interactions between Src and proteins in the focal adhesion pathway (23,24) and in the present study, ALK resistant cells had higher phosphorylation levels of Src-related proteins suggesting that this pathway could be a therapeutic target in ALK resistant cell lines.

Previous studies demonstrated that Src interacts with multiple RTKs and facilitates activation of downstream pathways (25-28). In this study, combined treatment with ALK and Src inhibitors effectively reduced PI3K/AKT mediated survival and proliferation signaling. We showed that dual blockade of ALK and Src inhibited alternative RTKs which were involved in ALK resistance in H3122-AFR cells. Activation of EGFR, IR and HGFR signaling are known mechanisms of resistance in ALK resistance in H3122-AFR cells. Activation of these alternative survival pathways is likely to be primarily responsible for mediating ALK resistance in H3122-AFR cells because secondary ALK mutations and ALK amplification were not identified in this cell line.

Phospho-RTK arrays also showed that combined treatment with ALK and Src inhibitors led to decreased phosphorylation of multiple RTKs. Although the levels of phospho-RTK inhibition were not dramatic, the efficacy of combined treatment was greater than the efficacy of ALK or Src inhibitors alone in vitro and in vivo. These results suggest that Src is a common node downstream of multiple resistance pathways in ALK-NSCLCs, which is consistent with previous investigations of RET rearranged NSCLCs and breast cancer (19,20).

Although it is important to validate our findings using clinical specimens, our data suggest that targeting Src signaling may be an effective approach for the treatment of ALK-NSCLCs that are resistant to ALK inhibitors.

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