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ORIGINAL ARTICLE

Role of the STAT3/survivin signaling pathway in the EML4-ALK-positive lung adenocarcinoma cell line H2228 before and after crizotinib-induced resistance*

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Abstract

Objective This study investigated the role of the STAT3/survivin signaling pathway in the EML4-ALK–positive lung adenocarcinoma cell line H2228 before and after crizotinib-induced resistance. The mechanism of resistance was studied.

Methods Cell viability was determined using the MTT assay. Crizotinib-induced apoptosis in H2228 and H2228 crizotinib-resistant cells treated with the indicated doses of crizotinib was measured at different times (24 h, 48 h, 72 h) using flow cytometry. The levels of p-ALK, ALK, p-STAT3, STAT3, and survivin after treatment of cells with 0, 0.3, and 1 μM crizotinib for 72 h were determined using Western blot analysis. DNA sequencing was used to identify mutations in H2228 crizotinib-resistant cells.

Results The crizotinib IC50 values in H2228 and H2228 crizotinib-resistant cells at 72 h were 334.5 nM and 3418 nM, respectively. The resistance index of H2228 crizotinib-resistant cells was 10.20. Crizotinib induced apoptosis in H2228 cells and reduced the levels of p-ALK, p-STAT3, and survivin. In contrast, no changes in the levels of p-ALK, p-STAT3, and survivin were observed in H2228 crizotinib-resistant cells. The mutations 2067G→A and 2182G→C in EML4-ALK were present in the H2228 crizotinib-resistant cells.

Conclusion Crizotinib decreased the viability of H2228 cells in a dose- and time-dependent manner. In the STAT3/survivin pathway, downregulation of p-ALK, p-STAT3, and survivin might contribute to crizotinib-induced apoptosis in H2228 cells. However, the STAT3/survivin pathway in H2228 crizotinib-resistant cells was unaffected by crizotinib treatment. Acquired resistance in H2228 cells might be related to ALK mutations.

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Lung cancer is the leading cause of cancer-related deaths, and nearly 80% of patients with non-small cell lung cancer (NSCLC) are diagnosed at an advanced stage. The efficacy of platinum-based doublet regimens is limited, with progression-free survival and median survival of only 4–5 months and 8–9 months, respectively.

A small inversion within chromosome arm 2p results in the fusion of the echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK) genes. Various break and fusion points within the EML4 locus in NSCLC cells give rise to different EML4-ALK isoforms, which appear to be present in 5% to 10% of NSCLC cases [1]. The most common EML4-ALK variants are 1 and 3, which together account for about 60% of EML4-ALK–positive lung cancer cases.

Crizotinib (PF-02341066), which inhibits the tyrosine kinase activity of ALK and the met proto-oncogene (MET), is effective in the treatment of EML4-ALK-positive NSCLC patients. However, as with other small molecule tyrosine kinase inhibitors, patients who are initially sensitive to crizotinib develop resistance in about a year [1–2].

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The signal transducer and activator of transcription 3 (STAT3)/survivin signaling pathway plays an important role in the growth, invasion, metastasis, and prognosis of lung cancer [3]. However, it is not clear whether this pathway is involved in crizotinib-induced acquired resistance. In this study, we assessed the effects of crizotinib on the EML4-ALK fusion gene-positive human lung adenocarcinoma cell line H2228 before and after inducing crizotinib resistance *in vitro*. In addition, we analyzed the key regulatory proteins in the STAT3/survivin signaling pathway, with the goal of elucidating the mechanisms of acquired crizotinib resistance.

Materials and methods

Materials

Cell line

The human NSCLC cell line H2228 was obtained from the American Type Culture Collection (ATCC). The cell line was cultured in Gibco RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and maintained under a humidified atmosphere of 5% CO_2 at 37 ℃.

Reagents and instruments

Crizotinib was obtained from Selleck Chemicals. RPMI-1640 medium, FBS, and trypsin were purchased from Gibco (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and dimethyl sulfoxide were purchased from Amresco (USA). The Annexin V-FITC Apoptosis Detection kit and the bicinchoninic acid (BCA) kit were purchased from BD Biosciences and Merck, respectively. Antibodies against human ALK, phospho-ALK (Tyr1604), STAT3, phospho-STAT3, survivin, and β-actin were obtained from Cell Signaling Technology.

Methods

Cell culture

Cells were cultured in RPMI-l640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37 ℃ in a 5% CO_2 atmosphere. The solution was removed every 2–3 days, and 1–2 mL of trypsin-EDTA solution was added. The flask was incubated at room temperature until the cells detached. Fresh culture medium was added, and the solution was aspirated and dispensed into clean culture flasks.

Establishment of crizotinib-resistant H2228 (H2228 CR) cells

H2228 cells were seeded at –70% confluence in 25 cm^2 dishes in RPMI 1640 medium with 10% FBS at an initial crizotinib concentration of 50 nM. The cells were maintained in medium containing fresh crizotinib, which was changed every 72 h. Cells were passaged once they reached confluence. After every two passages at a given concentration of crizotinib, the concentration of drug was increased in half-log intervals until a final concentration of 1 μM was reached. The resulting resistant cells (H2228 CR cells) were maintained in RPMI medium with 10% FBS containing 1 μM crizotinib. The MTT assay was used to assess the viability of H2228 CR cells, and the resistance index (RI) was calculated from the formula: RI = resistant cells IC50 / parental cells IC50. H2228 CR cells were cultured in crizotinib-free medium for >1 month to eliminate the effects of crizotinib.

MTT assay

H2228 cells and H2228 CR cells were digested with 0.25% trypsin until the cells detached. The cells were centrifuged, the supernatant was discarded, and the cells were resuspended in complete growth medium. After the cell density was adjusted to 8000 cells/well, the cells were seeded in 96-well plates at 100 μL/well, and incubated overnight at 37 °C in a 5% CO_2 atmosphere. H2228 cells and H2228 CR cells were then exposed to the indicated concentration of crizotinib for 72 h. Each assay, consisting of six replicate wells, was repeated at least three times. The absorbance (Abs) at 490 nm was measured with a Multiskan Spectrum spectrophotometer. Cell viability was calculated as: [(Abs experimental group – Abs blank group) / (Abs control group – OD blank group)] × 100%.

Analysis of the effect of crizotinib on apoptosis using flow cytometry

H2228 cells or H2228 CR cells were digested with 0.25% trypsin and centrifuged. The supernatant was discarded, and the cells were resuspended in complete growth medium. The cell were seeded in 6-well plates at 2 mL/well. The indicated dose of crizotinib was added to H2228 cells and H2228 CR cells. Cells were cultured for 24 h, 48 h, and 72 h. Cells were harvested by exposure to 0.25% trypsin without EDTA, washed with phosphate-buffered saline solution (PBS), and centrifuged at 300 × g for 5 min. The cell pellets were resuspended in 100 mL of Annexin-V-FLUOS labeling solution and incubated for 15 min at room temperature in the dark. After the addition of propidium iodide and fluorescein isothiocyanate, the cells were resuspended in 400 mL of Annexin-V-FLUOS labeling solution, and fluorescence was analyzed with a flow cytometer.

Western blot analysis

Cells were washed twice with ice-cold PBS and then lysed in a solution containing phosphatase inhibitors, protease inhibitors, and phenylmethanesulfonyl fluoride. The protein concentration of the cell lysates was measured using a BCA kit, and equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8% or 10% gel. The separated proteins were transferred to polyvinylidene fluoride membranes, which were then exposed to 5% bovine serum albumin in PBS for 2 h at room temperature before overnight incu-

bation at 4 ℃ with primary antibodies. The membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat antibodies to rabbit or horse antibodies to mouse IgG. All membranes were developed using Superstar ECL Plus.

DNA sequencing

The primers were designed. Total RNA was extracted from cells and subjected to PCR. DNA was sequenced by HuaDa Genomics Company (China).

Statistical analysis

Quantitative data are shown as the mean ± standard deviation. Two sets of data were analyzed using the unpaired two-tailed Student's *t*-test, and multiple sets of data were analyzed using one-way ANOVA. The data were analyzed using the SPSS Statistics version 16.0 software, and $P < 0.05$ was considered statistically significant. Probit regression analysis was used to calculate the IC50 with a 95% confidence interval.

Results

Effects of crizotinib on the viability of H2228 parental cells and H2228 CR cells

Crizotinib decreased the viability of H2228 cells in a concentration-dependent manner. The differences in viability at all concentrations were statistically significant ($P < 0.05$). The IC50 values of H2228 and H2228 CR cells were 334.5 nM and 3418 nM, respectively. The resistance index of H2228 CR cells was 10.2 (Fig. 1).

Effects of crizotinib on apoptosis in H2228 parental cells and H2228 CR cells

Crizotinib induced apoptosis in H2228 parental cells, but not in H2228 CR cells ($P < 0.05$). However, the differences in the apoptotic fractions of H2228 CR cells treated with 0.3 μM and 1 μM crizotinib were not statistically significant ($P > 0.05$; Fig. 2). At 0.3 μM crizotinib, the apoptotic fraction of H2228 cells at 24, 48, and 72 h was 19.2% ± 0.6%, 25.3% ± 1.6%, and 43.5% ± 3.2%, respectively, while that of H2228 CR cells was 3.5% ± 0.7%, 7.0% ± 0.6%, and 11.7% ± 1.4%, respectively. At 1 μM, the apoptotic fraction of H2228 CR cells at 24, 48, and 72 h was 3.6% ± 1.0%, 7.9% ± 0.2%, and 13.1% ± 0.7%, respectively.

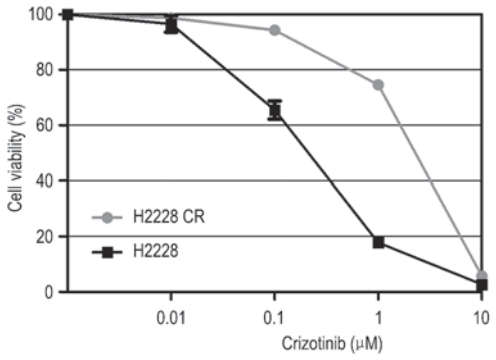

Fig. 1 Effects of crizotinib on the viability of H2228 parental cells and H2228 CR cells

Effects of crizotinib on the levels of apoptosis-related proteins in the STAT3/survivin signaling pathway

Crizotinib downregulated the expression of survivin by inhibiting the STAT3 signaling pathway in H2228 cells. The levels of p-STAT3 and p-ALK were also downregulated. Immunoblot analysis showed that crizotinib did not inhibit p-ALK, p-STAT3, or survivin in H2228 CR cells (Fig. 3).

Results of DNA sequencing

Two substitution mutations at 2067 and 2182 in ALK part V3b were found, an G to A mutation and a G to C mutation. The 2067G→A and 2182G→C substitutions resulted in arginine→lysine (Arg→Lys) and glutamic acid→aspartic acid (Glu→Asp) changes, respectively (Fig. 4). No mutations were found in EML4.

Discussion

The STAT3/survivin signaling pathway plays an important role in tumorigenesis and cell development [5]. STAT3 participates in proliferation and differentiation, apoptosis, immune evasion, angiogenesis, invasion and metastasis in human cancers [4]. Activated STAT3 inhibits tumor cell apoptosis by upregulating the expression of survivin, the smallest of the inhibitors of apoptosis proteins.

Crizotinib, an ALK/c-MET dual tyrosine kinase inhibitor, inhibits proliferation and induces apoptosis in EML4-ALK-positive NSCLC cells, but the mechanism has not been fully explained [6]. Our findings showed that crizotinib induced apoptosis in H2228 cells in a concentration- and time-dependent manner. However, crizotinib did not inhibit the expression of ALK or STAT3. Instead, crizotinib markedly inhibited ALK and STAT3 phosphorylation and downregulated survivin in H2228 cells. Survivin prevents cell apoptosis by directly or indirectly inhibiting the activation of cysteine proteases [6]. Presumably, in H2228 cells harboring the type 3 variant of EML4-ALK, phosphorylation of ALK or a related protein activates the downstream STAT3/survivin signaling pathway. Crizotinib suppressed this process by inhibiting ALK phosphorylation and consequently downregulating survivin, thereby decreasing proliferation and increasing apoptosis in H2228 cells. However, crizotinib had no pro-apoptotic effects in H2228 CR cells and failed to reduce the level of

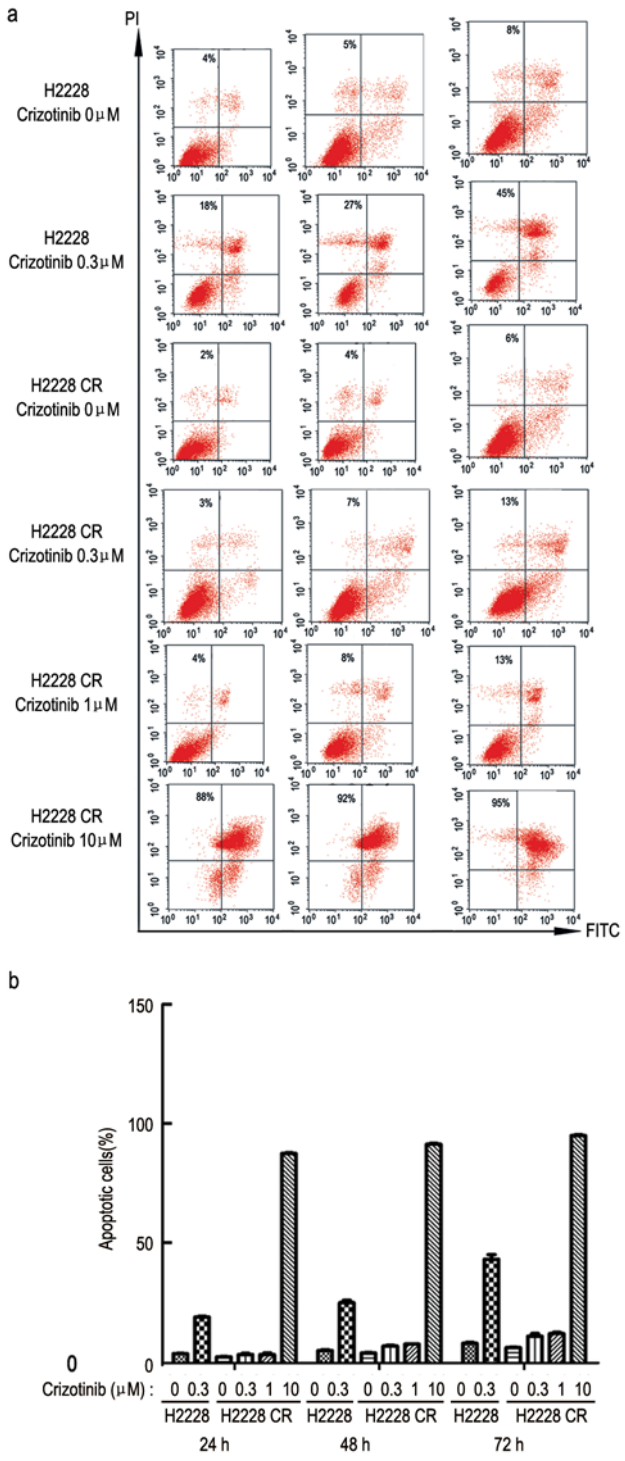

Fig. 2 Apoptosis in H2228 and H2228 CR cells at different time points after treatment with crizotinib at the indicated doses

p-ALK, the activation of STAT3, or the expression of survivin. The main mechanisms for acquired-resistance to crizotinib include mutations in the ALK kinase domain, copy number gain of the ALK fusion gene, and activation

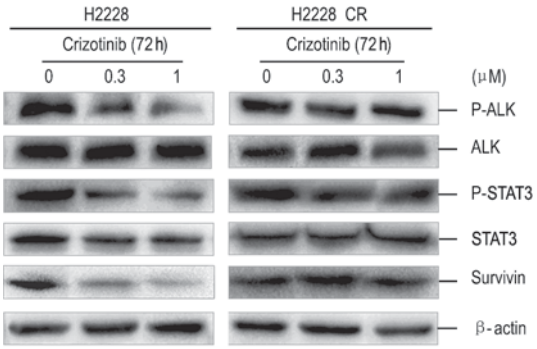

Fig. 3 H2228 parental and H2228 CR cells were treated with crizotinib at the indicated concentrations for 72 h. Cell lysates were immunoblotted to detect the indicated proteins

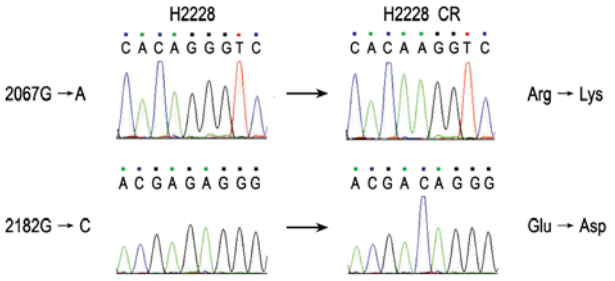

Fig. 4 DNA sequencing peaks and amino acids changes in H2228 parental cells and H2228 CR cells

of other signaling pathways [2, 7–8, 10].

The levels of p-STAT3 and survivin did not decrease in H2228 CR cells as they did in H2228 cells. This might be attributable to secondary mutations in the EML4-ALK fusion gene [2]. DNA sequencing of H2228 cells and H2228 CR cells identified 2067G→A and 2182G→C mutations in H2228 CR cells, which resulted in amino acids changes that might underlie the acquired resistance of H2228 cells. In this regard, Katayama *et al* [2] identified an L1196M mutation in the fusion gene of H3122 crizotinib-resistant cells. In addition, Choi *et al* [10] detected two mutations, L1196M and C1156Y, in a 28-year-old non-smoking male patient with EML4-ALK-positive adenocarcinoma who was treated with crizotinib for five months.

Point mutations in the region of the ALK kinase domain might change the structure of ALK and thus interfere with the binding of tyrosine kinase inhibitors [8, 10]. Whether the 2067G→A and 2182G→C mutations found in H2228 CR cells changes the structure of the protein has yet to be established. In our study, the protein levels of EML4-ALK were similar in H2228 CR cells and the parental cells, a finding that is inconsistent with the results of Katayama *et al* [2], who compared H3122 CR cells with the parental cell line. Yamaguchi *et al* [11] have shown that the mechanism of resistance to crizotinib in H3122 cells harboring EML4-ALK fusion variant 1 is activation

of EGFR, instead of fusion gene mutation or copy number gain of the ALK fusion gene. Furthermore, Kim *et al* [12] have shown that the epithelial-mesenchymal transition is a key factor for acquired resistance in H2228 cells. Whether H2228 CR cells acquire resistance through the activation of other signaling pathways requires further investigation.

Conclusion

The STAT3/survivin signaling pathway is a key modulator of the transforming activity of EML4-ALK in the EML4-ALK-positive lung adenocarcinoma cell line H2228 before and after crizotinib-induced resistance. These results provide a basis for the further development of ALK-targeted therapy for EML4-ALK–positive lung cancer patients.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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