Dasatinib enhances cisplatin sensitivity in human esophageal squamous cell carcinoma (ESCC) cells via suppression of PI3K/AKT and Stat3 pathways

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Abstract
The clinical efficacy of cisplatin in esophageal squamous cell carcinoma (ESCC) treatment remains undesirable. Src, a non-receptor tyrosine kinase involved in multiple fields of tumorigenesis, recently has been indicated as a promising therapeutic target in the treatment of solid tumors including ESCC. However, whether inhibition of Src activity can increase cisplatin efficacy in ESCC cells remains unknown. The present study found that inhibition of Src by its inhibitor-dasatinib sensitized ESCC cells to cisplatin in vitro. Our data also suggest a likely mechanism for this synergy that dasatinib reduces expression of critical oncogenic members of the signaling pathways, such as AKT or Stat3, and cisplatin-resistant molecules, such as ERCC1 and BRCA1, under the control of Src. Furthermore, dasatinib could sensitize ESCC cells to another platin-based agent, carboplatin. Therefore, this study provides a potential target for improving cisplatin efficacy in ESCC therapy.

Introduction
Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide and occurs at a relatively high frequency in China [1]. Cisplatin is the most frequently used chemotherapeutic agent for ESCC treatment. Unfortunately, the clinical efficacy of cisplatin in ESCC treatment remains undesirable.

Src is a non-receptor tyrosine kinase involved in multiple fields of tumorigenesis including proliferation, migration, and angiogenesis, thus recently indicated as a promising therapeutic target in the treatment of solid tumors including ESCC [2–5]. Molecular mechanistic studies reveal that hyperactivation of Src is central mediator in multiple signaling pathways of tumors, such as interacting with tyrosine kinase receptors and subsequently activating the Raf/MEK/MAPK pathway and PI3K/AKT pathway, or regulating pro-tumorigenic gene expression via transcription factors, such as Stat3 [6–9]. Dasatinib, currently entering clinical trials for various malignancies to inhibit the Src activity, increases cisplatin cytotoxicity in non-small cell lung cancer (NSCLC) or breast cancer cells in vitro [10,11]. However, there are still lacks of direct evidences that dasatinib enhances the cisplatin efficacy in ESCC treatment.

In this study, we sought to explore the potential correlation between inhibition of Src activation and cisplatin efficacy in ESCC cells. The results demonstrate a striking synergistic cytotoxic effect between Src inhibitor-dasatinib and cisplatin in ESCC cells. Our data also suggest a likely mechanism for this synergy that dasatinib reduces expression of critical members of the growth-promoting and apoptosis-resistant molecules under the control of Src. This study provides a potential target for improving cisplatin efficacy in ESCC therapy.

Materials and methods

Antibodies and reagents

The sources of the antibodies were: anti-pAKT (Ser473), anti-pStat3 (Tyr705), anti-pERK1/2 (Thr202/Tyr204), anti-pSrc

Abbreviations used:
ESCC, esophageal squamous cell carcinoma; NSCLC, non-small cell lung cancer; ICEL, enhanced chemiluminescence detection kit; NEEC, normal esophageal epithelial cells.
of ESCC cell lines, confluent KYSE410 and KYSE150 cells (generously provided by Dr. Y. Shimada (Kyoto University)), were grown in PRM1640 medium supplemented with 10% Fetal bovine serum.

Cell lines

Primary cultures of normal esophageal epithelial cells were established from fresh specimens of the adjacent noncancerous esophageal tissue. The human ESCC cell lines-KYSE140, KYSE150, KYSE30, KYSE410, KYSE450, KYSE510 (generously provided by Dr. Y. Shimada (Kyoto University)), were grown in PRM1640 medium supplemented with 10% Fetal bovine serum.

Cell proliferation/viability assay

Proliferation/viability of cells was determined using MTS method as previously described with minor modifications [12]. 1×10^4 cells in 100 µl of RPM1640 medium were seeded in 96-well plates. Once confluent, cells were treated with different doses of dasatinib (10–500 nM), or 50 nM dasatinib, 5, 10, or 25 µM cisplatin, 5 µM carboplatin alone or 50 nM dasatinib combined with indicated doses of cisplatin, or 5 µM carboplatin, 5 µM etoposide, 0.5 µM SN38, or 1 µM amrubicin for 72 h before analysis. Then the medium was aspirated, and incubated with MTS solution (Promega) for 1 h. The viable cell number was directly proportional to the formazan product, which could be measured spectrophotometrically at 490 nm.

Colony formation assay

5×10^3 cells were seeded into 60 mm dishes in PRM1640 plus 10% FBS and cultured both in the absence and presence of agents as indicated. After 10 days, the cells were washed with PBS, fixed with methanol and 0.1% crystal violet. The colonies were counted and then photographed.

Flow cytometry analysis of apoptosis and caspase activity assay

The protocols and reagents used for flow cytometry analysis of apoptosis and caspase 3/7 activity assays were all strictly according to our previous studies [13]. For evaluating the apoptotic rate of ESCC cell lines, confluent KYSE410 and KYSE150 cells were diluted to 8×10^3/ml and incubated for 24 h at 37 °C in 6-well plates. Then, cells were treated with indicated agents for 48 h. The cells were collected, washed in cold PBS, and resuspended in 100 µl 1× binding buffer with 3×10^5 cells containing 5 µl of Annexin V-FITC (Pharmingen, San Diego, CA) and 5 µl of PI (Pharmingen, San Diego, CA). Samples were mixed gently and incubated at room temperature in the dark for 20 min. A minimum of 10,000 cells within the gated region were analyzed. Four hundred µl of 1× binding buffer was added to each sample tube, and the samples were analyzed by FACS (Beckman Coulter, CA). Bivariate flow cytometry was used to simultaneously measure log green fluorescence (Annexin-V-staining) versus log red fluorescence (PI staining). The following controls were used: unstained cells; cells stained with Annexin-V-FITC only (early apoptosis); Cells that were Annexin-V-FITC+/PI+ were considered positive for late apoptosis or necrosis.

Cell invasion assay

The effects of dasatinib (50 nM), cisplatin (5 µM) or their combination on the invasion of KYSE410 and KYSE150 cells was determined using modified Boyden chambers supplemented with Transwell with 8 µm pore size polycarbonate membrane filters precoated with 50 µl of Matrigel (1.25 mg/ml). During MTS assay, equal KYSE410 and KYSE150 cells (5×10^4 cells) of the second group suspended in the serum-free DMEM of 100 µl in the presence or absence of cisplatin were seeded onto the upper chamber of Matrigel-coated filter inserts. Serum-containing DMEM (700 µl) was added to the lower chamber. After 16-h incubation, filter inserts were removed from the wells. The cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 15 min, and then the invaded cells were determined as eight high-power fields of cells were counted in each well under an inverted microscope at 100× magnification. Invasion was calculated as the relative invasive score of treated group (invaded cell number/total cell number assayed by MTS represented by OD490 divided by that of control.

HUVEC tube formation assay

Matrigel (50 µl) was pipetted into each well of a 96-well plate and polymerized for 30 min at 37 °C. HUVEC (1×10^5) in 150 µl of conditioned medium from KYSE410 or KYSE 150 cells treated with indicated agent were added to each well and incubated at 37 °C in 5% CO_2 for 16 h. Pictures were taken under a 100× bright-field microscope, and the capillary tubes were quantified by counting length.

Immunoblotting analysis

Cells were lysed and clarified and protein extracts (50 µg) were separated via 8–12% SDS-PAGE and then transferred to NC membranes. The membranes were subsequently incubated with anti-Src antibody, anti-pSrc antibody, anti-AKT antibody, anti-pAKT antibody, anti-Stat3 antibody, anti-pStat3 antibody, anti-c-myc antibody, anti-MMP-9 antibody, anti-VEGF antibody, and anti-GAPDH antibody. All of the first antibodies were diluted at 1:1000 except for GAPDH at 1:5000. Antibody binding was detected by enhanced chemiluminescence detection kit (ECL) (UK Amersham International plc).

Real-time RT PCR

For RNA isolation and real-time RT PCR analysis of ERCC1 and BRCA1, RNA was isolated from KYSE410 and KYSE150 cells using Trizol reagent according to the manufacturer’s protocol (Invitrogen). cDNA was synthesized using iScript reverse transcriptase reagent from 2 µg of total RNA. RT PCR analysis was performed on an Mx3000P QPCR system (stratagene) using Sybr-green and ∆∆Ct method was used to normalize transcripts to that of GAPDH and to calculate fold induction relative to control group. Primer sequences for real-time RT PCR analysis of ERCC1 and BRCA1 were: ERCC1, 5’- CCTTATCCGATCTACAAGAC -3’ and 5’- TATGCGGCG TAGGCTGAGGG -3’; BRCA1, 5’- GAACCGTGGCAGAACCTC -3’ and 5’- CCAAGGTAGAGAATTGGAACAC -3’.

Statistical analysis

All statistical analyses were evaluated by Student’s test for simple comparisons between two groups and one-way ANOVA for comparisons among multiple groups using GraphPad Prism version 5.0 (GraphPad Software Inc, La Jolla, CA) and JMP7.0 software (SAS) (Tyr416), anti-Src, anti-AKT, anti-Stat3, anti-MMP-9, and anti-GAPDH (Cell Signaling Technology). Antibodies against c-myc and VEGF were from Santa Cruz and Abcam, respectively. Dasatinib, LY294002 (PI3K/AKT inhibitor), U0126 (MEK inhibitor), cisplatin, carboplatin, etoposide, and SN38 were purchased from Selleck chemicals. Amrubicin was from Santa Cruz. S3I-201(Stat3 inhibitor) was purchased from Millipore.
Institute Inc, Cary, US). In MTS assay, the half-maximal inhibitory concentration (IC_{50}) was calculated using SPSS 12.0 software (SPSS Inc, US). All tests were two-sided and expressed as mean ± S.D. A P value of less than 0.05 was considered significant.

Results

Inhibition of Src activity sensitizes ESCC cells to cisplatin

We first evaluated the levels of phospho/total Src in ESCC cells and normal esophageal epithelial cells (NEEC). As shown in Fig. 1A, the levels of phospho/total Src was markedly higher in ESCC cell lines than that in NEEC. To explore the anti-proliferative effect of Src inhibitor-dasatinib in ESCC cells, we performed the MTS assay in human ESCC cell lines—KYSE410 and KYSE150. Dose–response curve in these cells showed that IC_{50} value of dasatinib in KYSE410 or KYSE150 cells was 91 or 115 nM, respectively (Fig. 1B). Then, the relatively low dose of dasatinib-50 nM was selected for the subsequent experiments. Treatment of the KYSE410 cells with 50 nM dasatinib reduced cell growth by about 25%, and cisplatin of 5 μM even had less cytotoxicity. Combination treatment of dasatinib (50 nM) and cisplatin (5 μM) significantly reduced cell growth and produced more effectively growth-inhibitory effect than cisplatin at higher doses (10 μM and 25 μM). Enhanced growth inhibition by dasatinib was also observed using 10 μM and 25 μM cisplatin. For example, while 10 μM cisplatin alone reduced growth to about 13%, the combination of dasatinib and 10 μM cisplatin reduced growth to 68%, effective reduction in cell growth versus cisplatin alone (Fig. 1C). The similar results were also obtained in KYSE150 cells (Fig. 1D).

Anchorage-dependent colony formation assay was then used to specifically evaluate the synergistic effect of dasatinib and cisplatin on the growth of ESCC cells in vitro. As shown in Fig. 1E, combining...
50 nM dasatinib and 5 μM cisplatin could produce more effective anchorage-dependent growth-inhibitory effect than each agent alone in KYSE410 cells. The similar results were also obtained in KYSE150 cells (Fig. 1E).

Dasatinib enhanced cisplatin-induced apoptosis of ESCC cells in vitro

We then investigated the synergistic effect of dasatinib and cisplatin on apoptosis of ESCC cells. FCM data indicated that there were a low percentage of KYSE410 cells undergoing apoptosis in both control and 5 μM cisplatin-treated cells. 50 nM dasatinib alone increased the apoptotic rates in KYSE410 cells. However, the combination of 50 nM dasatinib and 5 μM cisplatin produced much higher apoptotic rate than each agent alone (Fig. 2A, left panel). Importantly, this increase in apoptotic rate was also reflected in the caspase3/7 activity of these cells (Fig. 2B, left panel). A similar increase in caspase activity and apoptosis was also observed with KYSE150 cells (Fig. 2A, right panel, and Fig. 2B, right panel). Together, these data show that the combination of dasatinib with cisplatin significantly enhances the cisplatin-mediated apoptosis in ESCC cells.

Dasatinib enhances the anti-invasive and anti-angiogenic ability of cisplatin in ESCC cells in vitro

To investigate the effect of dasatinib, cisplatin or dasatinib/cisplatin combination on invasion of ESCC cells, KYSE410 cells were treated with these agents alone or their combination for 16 h and

![Fig. 2. Effects of dasatinib and cisplatin alone or in combination on the apoptosis of ESCC cells.](image-url)

(A) KYSE410 (left panel) and KYSE150 (right panel) cells were treated with 5 μM cisplatin, or 50 nM dasatinib, and their combinations for 48 h, cell apoptosis was evaluated by FCM assay, respectively. (B) KYSE410 (left panel) and KYSE150 (right panel) cells were treated with 5 μM cisplatin, or 50 nM dasatinib, and their combinations for 48 h, cell apoptosis was evaluated by caspase 3/7 activity, respectively. Bars are mean ± SD from at least three independent experiments. **P < 0.01; ***P < 0.001.
then invasion was assessed by Matrigel invasion assay. As shown in Fig. 3A, 5 μM cisplatin could not inhibit the invasion of KYSE410 cells. 50 nM dasatinib alone downregulated the invasive ability of KYSE410 cells. However, the combination of 50 nM dasatinib/5 μM cisplatin produced lower invasive index than each agent alone. Similar results were also obtained in KYSE150 cells (Fig. 3B).

Furthermore, HUVEC tube formation assay indicated that 5 μM cisplatin could not inhibit the conditioned medium of KYSE410 cells-induced tube formation of HUVECs. 50 nM dasatinib alone downregulated the conditioned medium of KYSE410 cells-induced tube formation of HUVECs. However, the combination of 50 nM dasatinib/5 μM cisplatin produced lower tube formation index than each agent alone (Fig. 3C). Similar results were also obtained in KYSE150 cells (Fig. 3D).

**Dasatinib sensitizes ESCC cells to carboplatin and other anti-tumor agents**

Another cisplatin parent compound, carboplatin, could also be used for the treatment of ESCC [14]. To determine whether the synergistic effect of dasatinib with cisplatin was a class effect or specific for cisplatin, KYSE410 cells were treated with carboplatin alone and in combination with dasatinib. Treatment of KYSE410 cells with 50 nM dasatinib alone reduced tumor growth of 14% or 6%, respectively. The combination of 50 nM dasatinib/5 μM cisplatin produced lower invasive index than each agent alone (Fig. 3B). Similar results were also obtained in KYSE150 cells (Fig. 3D).

We further evaluated the synergistic effect of dasatinib with other clinical anti-tumor agents, such as etoposide, SN38, and amrubicin. As shown in Fig. 4E–G, treatment of KYSE410 and KYSE150 with 5 μM etoposide (Fig. 4E), 0.5 μM SN38 (Fig. 4F), or 1 μM amrubicin (Fig. 4G) had little effect on growth of ESCC cell lines. As expected, the combination of 50 nM dasatinib with etoposide, SN38, or amrubicin could effectively reduce cell growth compared with etoposide, SN38, or amrubicin treatment alone.

**Inhibition of Src activity-induced sensitivity of ESCC cells to cisplatin is mainly dependent on suppression of PI3K/AKT and Stat3 pathways**

To more specifically evaluate the Src-mediated oncogenic pathways, such as PI3K/AKT, Stat3, or ERK pathway in regulating dasatinib-mediated cisplatin sensitization, we treated ESCC cells with cisplatin in the presence of Stat3 inhibitor, AKT inhibitor, or ERK inhibitor, respectively. As shown in Fig. 5A and B, inhibition of PI3K/AKT or Stat3 pathway but not ERK pathway could synergize with 5 μM cisplatin to inhibit growth of KYSE410 and KYSE150 cells. Then, immunoblot assay was performed to analyze the activities of Src, AKT, or Stat3 after dasatinib incubation. The activities of these proteins were represented as the levels of phosphorylated/total forms. As shown in Fig. 5C and D, 10 nM dasatinib had little or no effect on Akt and Stat3 activities. When the dose of dasatinib was used up to 50 nM, it could sufficiently suppress both Akt and Stat3 activities in KYSE410 and KYSE150 cells. Taken together, these results indicate that inhibition of Src activity enhances cisplatin efficacy in ESCC cells probably dependent on suppression of the PI3K/AKT and Stat3 pathways.

We further evaluated whether downstream effectors of the PI3K/AKT and Stat3 pathways—c-myc and MMP-9, are involved in inhibition of Src-enhanced cisplatin cytotoxicity, we extracted total proteins from KYSE410 cells after treatment with control solvent, 5 μM cisplatin, 50 nM dasatinib or their combination. As shown in Fig. 5D, 5 μM cisplatin alone could not downregulate the levels of c-myc and MMP-9. 50 nM dasatinib could inhibit the expression of these two proteins. Furthermore, combining...
cisplatin and dasatinib produced more inhibitory rate on c-myc and MMP-9 expression than each agent alone in KYSE410 cells. Similar results were also obtained in KYSE150 cells (Fig. 5D). Taken together, these observations suggest that inhibition of Src activity enhances cisplatin efficacy in ESCC cells mainly through suppression of the PI3K/AKT and Stat3 pathways-induced c-myc and MMP-9 expression.

Dasatinib inhibits cisplatin-induced mRNA transcription of DNA repair and synthesis genes

The effect of dasatinib alone or in combination with cisplatin on the expression of genes, such as ERCC1 and BRCA1, potentially involved in the cisplatin resistance was then investigated using quantitative real-time PCR. As Fig. 6A showed, these two genes were upregulated after 5 μM cisplatin treatment. Conversely, 50 nM dasatinib effectively suppressed the cisplatin-induced mRNA transcription. The similar results were also obtained in KYSE150 cells (Fig. 6B).

Discussion

This study investigates the influence of inhibition of Src activity by dasatinib on cisplatin sensitivity in ESCC cells. The results indicate that dasatinib dramatically increases the growth-inhibitory effect of cisplatin in ESCC cell lines. Notably, we observe that the underlying mechanism of dasatinib-enhanced cisplatin cytotoxicity mainly via suppressing PI3K/AKT and Stat3 pathways, as well as inhibiting cisplatin-induced mRNA transcription of DNA repair and synthesis genes.

Our study shows that combinations of the low concentration of dasatinib and cisplatin had a stronger inhibitory effect on the growth of KYSE410 cells than either agent alone at the same concentration. Moreover, a combination of dasatinib and cisplatin at both low doses had an even more potent inhibitory effect on the growth of KYSE410 cells than cisplatin used individually at a high concentration. A combination of dasatinib (50 nM) and cisplatin (5 μM) produced more significantly apoptotic rate than each agent alone. The combination of dasatinib and cisplatin can also increase caspase activity to a level similar to the apoptotic rate, suggesting combination of dasatinib and cisplatin significantly enhances the efficacy of cisplatin for reaching the threshold of caspase activation and promoting of the apoptosis in KYSE410 cells. We then confirms this combinatorial effect of dasatinib and cisplatin in another ESCC cell line-KYSE150 cells, suggesting that the combination of dasatinib and cisplatin is general applicable for treating ESCC cells. Taken together, the present study comprehensively provided the cytotoxic profiles of dasatinib and cisplatin in ESCC treatment and suggested that the combination of dasatinib and cisplatin produced a more-than-additive or possibly synergistic effect in ESCC cells. Furthermore, previous studies have confirmed the role of...
Dasatinib on cisplatin cytotoxicity in various cancer cell lines, such as non small cell lung cancer, small cell lung cancer, and laryngeal squamous cell carcinoma [11,15–17]. Consistent with these studies, our finding that the combination of dasatinib and cisplatin produce effective synergistic effect on the ESCC cells, indicating that the synergistic effect of dasatinib and cisplatin is not specific to certain cancer cells.

The overall 5-year survival rate of ESCC patients remains poor, which is largely attributable to the high rates of extensive invasion and metastasis. Thus, we further evaluate the anti-invasive and anti-angiogenic ability of this combination. In this study, cisplatin alone could not effectively inhibit the invasion and promoting-angiogenic ability of ESCC cells. However, when dasatinib was added, the anti-invasive and anti-angiogenic ability of cisplatin treatment was significantly extended, suggesting that administration of a combination of dasatinib and cisplatin had a synergistic effect in suppressing ESCC cell invasion and angiogenesis.

The present study implies that the molecular mechanism underlying Src inhibition-enhanced cisplatin effectiveness can be majorly classified into two categories. One involves oncogenic signaling proteins and the other involves the response to and repair of platinum-adducted DNA. Aberrant signaling pathways, such as the PI3K/AKT, MEK/ERK and Stat3 pathways, contribute to chemoresistance in various tumors [18–22]. In the present study, combination treatment of PI3K inhibitor-LY294002, or Stat3 inhibitor-S3I-201, and cisplatin resulted in a substantial decrease in cell growth as compared with each agent alone. However, inhibition of MEK/ERK pathway produced little effect on cisplatin efficacy in ESCC cells. Furthermore, dasatinib dose-dependently inhibited the AKT and Stat3 phosphorylation in ESCC cells. The results also show that the downstream proteins of PI3K/AKT and Stat3 pathways, such as c-myc, MMP-9, or VEGF can be effectively regulated by dasatinib treatment. Thus, our data strongly suggest that inhibition of Src increases cisplatin efficacy mainly
Fig. 6. Combination of dasatinib and cisplatin inhibits the activation of cisplatin-resistant genes. (A and B) KYSE410 (A) and KYSE150 (B) cells were treated with 5 μM cisplatin, or 50 nM dasatinib, and their combinations for 24-h. Real-time PCR analysis of ERCC1 and BRCA1 genes. Bars are mean ± SD from at least three independent experiments. ***P < 0.001.

via regulation of the PI3K/AKT and Stat3 pathways-activated protein signatures. Taken together, these data indicate that dasatinib enhances cisplatin efficacy in ESCC cells mainly dependent on inhibition of the PI3K/AKT and Stat3 pathways.

Many studies have shown that ERCC1 and BRCA1 play a critical role in the resistance of certain cancers to platinum-based agents [23–27]. This is highlighted by the observation that the higher expression of ERCC1 and BRCA1 are negatively correlated with the effect of chemotherapy in ESCC treatment. Our data demonstrate that dasatinib suppresses the expression of several cisplatin-resistant genes, such as ERCC1 and BRCA1, in ESCC cells. These results strongly suggest that suppression of ERCC1 and BRCA1 expression by dasatinib partially explains the synergy observed with cisplatin in ESCC cells.

It will be important to determine the molecular basis underlying the Src protein-mediated PI3K/AKT and Stat3 pathway activation. In vivo study is needed to observe the combinatorial effect of dasatinib and cisplatin in ESCC treatment. Notwithstanding these drawbacks, our study does indicate that dasatinib can enhance cisplatin or even other anti-tumor agents’ sensitivity in ESCC cells. Therefore, dasatinib may be a promising alternative for adjuvant chemotherapy in ESCC treatment.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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