Original Article

Dasatinib suppresses invasion and induces apoptosis in nasopharyngeal carcinoma

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Abstract: Dasatinib, an orally available tyrosine kinases inhibitor (TKI), potently inhibits SRC which was found to activate RTKs that induce trastuzumab de novo and acquired resistance. To evaluate the potential of Dasatinib in the treatment of Nasopharyngeal Carcinoma, we used a variety of assays to measure its effects on cell proliferation, apoptosis, and migration. This work aimed to test the antitumor effects of the inhibitor in vitro to determine whether in vivo analyses were warranted. Cell growth rate and 50% inhibitory concentration was calculated by MTT assay. Dasatinib-induced apoptotic cells were investigated by Annexin V/PI staining. Proteins from cell extracts were analyzed by Western blot. Cell motility was investigated by Transwell. Our study showed that Dasatinib significantly inhibited CNE2 proliferation and induced apoptosis in vitro. Phospho-AKT, phospho-MEK, phospho-ERK expression was significantly reduced when treated with dasatinib which means the downregulated RAS/RAF/MEK/ERK and PI3K/AKT pathway activity. Dasatinib significantly inhibited the motility of CNE2 as well as Phospho-FAK expression. Dasatinib exhibit antitumor effects of nasopharyngeal carcinoma by downregulating MAPK and PI3K/AKT pathways activity and FAK phosphorylation. This suggests that dasatinib would have therapeutic activity against NPC.

Keywords: Nasopharyngeal carcinoma, dasatinib, CNE2, PI3K/AKT, RAS/RAF/MEK/ERK, FAK

Introduction

Nasopharyngeal carcinoma (NPC) is a highly prevalent and invasive head and neck cancer in China. EGFR, AKT, and ERK were found constitutively activated in nasopharyngeal carcinoma [1]. EGFR inhibitors Lapatinib and Cetuximab are considered promising anti-cancer agents for NPC with anti-invasion and anoikis-sensitization activities, but with poor clinical results [2]. So we speculate some supplementary mechanisms were involved. In the research of EGFR inhibitor resistant mechanism, SRC was found to activate RTKs that induce trastuzumab de novo and acquired resistance [3]. However, SRC family kinases are potently inhibited by Dasatinib. Dasatinib is an orally available tyrosine kinases inhibitor (TKI). It potently inhibits BCR-ABL and SRC family kinases (SRC, LCK, YES, FYN), but also inhibited P-mitogen-activated protein kinase (MAPK), P-Akt levels as well as FAK activity [4, 5]. To evaluate the potential of Dasatinib in the treatment of Nasopharyngeal Carcinoma, we used a variety of assays to measure its effects on cell proliferation, apoptosis, and migration. Our studies suggest that this inhibitor potently suppresses c-Src activation and induces multiple antitumor effects, making it a candidate for in vivo tests of its effectiveness in nasopharyngeal carcinoma.

Materials and methods

Cell culture and reagents

The immortalized human nasopharyngeal carcinoma cells (CNE2) are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37°C. Dasatinib was purchased from Selleck chemicals. GAPDH antibody was obtained from Boster biology (Wuhan, China). MTT was purchased from Sigma-Aldrich Co. (St. Louis, Missouri). ERK, Phospho-ERK (tyr204) antibodies were from Santa Cruz (Indian Gulch, California). SRC, Phospho-SRC (tyr416), AKT, Phospho-AKT (ser473), MEK, Phospho-MEK (ser217/221), FAK, Phospho-FAK (tyr397) antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts). Annexin V-FITC Apoptosis Detection Kit was from Invitrogen Inc. (Carlsbad, California).
Western blot analysis

Cells were collected on ice and washed with PBS, whole cell extracts were generated by direct lysis with 1 × Cell Lysis Buffer (Cell Signaling Technology) adding 1 ml PMSF immediately before use. Cell lysate was collected after centrifugation (12,000 rpm, 15 minutes). Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Samples were boiled by addition 6 × SDS sample buffer for 10 min at 100°C and resolved by SDS-PAGE and transferred to PVDF membranes (Roche). After membranes were blocked with 5% skim milk in TBST containing 0.05% Tween 20 (PBST) for 1~2 hours, it was probed with various primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies for at least 2 hours at room temperature, then washed in TBST and visualized with enhanced chemiluminescence’s reagent following the manufacturer’s instructions (Thermo Fisher Scientific Inc). Immunoreactivity was detected using the Amersham ECL Prime Western Blot detection reagent (GE Healthcare, Fairfield, CT, USA) according to the manufacturer’s instructions.

Cell migration assay

Migration was detected by the Transwell chamber assay. For Transwell chamber migration assay, CNE2 cells were detached by trypsin and resuspended in serum-free medium. Then the cells were seeded on the upper chamber in a 24-well Transwell plate with 8mm pore polycarbonate filters (Corning) at a density of 1 × 10⁵ cells/well in 200 μL serum free RPMI 1640 medium. While 750 μl RPMI 1640 medium containing 10% FBS was applied to the lower chamber as a chemoattractant. After incubation, the cells on the top surface of the filter were scraped using a cotton swab while the invading cells on the lower surface of the membrane filter were fixed with 4% paraformaldehyde and stained with 5% Giemsa solution, then counted with a light microscope.

MTT assay

CNE2 cells were seeded in 96-well plates at a concentration of 8 × 10⁴ cells/well. The cells were then incubated in a 37°C CO₂ incubator
overnight then treated with different concentrations of Dasatinib for indicated time. MTT solution (5 mg/ml) was added at a volume of 10 µl in each well and was incubated for 4~6 hours. Absorbance values were measured at the wavelength of 570 nm. Inhibitory rates were calculated by Microsoft Excel and IC50 values were calculated using the Calcusyn software (Biosoft Corporation, Cambridge, UK).

**Results**

Dasatinib inhibited the proliferation of nasopharyngeal carcinoma cells in vitro

To further investigate the growth inhibition of nasopharyngeal carcinoma treated by Dasatinib, the Annexin V-PI assay was carried out using the Annexin V-FITC Apoptosis Detection Kit. CNE2 cells were seeded in 6 well plates at a concentration of 2 × 10^5 cells/well and was incubated for 48 hours. The seeded cells were treated with the desired concentrations of Dasatinib for indicated time. Treated cells were collected and harvested. The resulting pellets were immediately resuspended in the provided binding buffer and subsequently stained with 5 µl of FITC Annexin V and 5 µl of PI. The mixture was left to incubate at room temperature for 15 minutes and then analyzed by flow cytometry using an FC 500 MPL instrument (Beckman Coulter, Miami, FL, USA).

**Apoptosis assay**

The Annexin/V-PI assay was carried out using the Annexin V-FITC Apoptosis Detection Kit. CNE2 cells were seeded in 6 well plates at a concentration of 2 × 10^5 cells/well overnight. The seeded cells were treated with the desired concentrations of Dasatinib for indicated time. Treated cells were collected and harvested. The resulting pellets were immediately resuspended in the provided binding buffer and subsequently stained with 5 µl of FITC Annexin V and 5 µl of PI. The mixture was left to incubate at room temperature for 15 minutes and then analyzed by flow cytometry using an FC 500 MPL instrument (Beckman Coulter, Miami, FL, USA).

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Dasatinib, MTT assay was used to calculate the IC50 value in CNE2 cell line. After treated by Dasatinib at indicated concentration for 3 days, the proliferation was inhibited gradually. And when at 9.85 μM, Dasatinib induced 50% apoptotic cells (Figure 1). The data indicates that Dasatinib has antitumor effects in nasopharyngeal carcinoma in vitro.

**Dasatinib inhibited phosphorylation of SRC in nasopharyngeal carcinoma**

To exam whether SRC inhibition is involved in growth inhibition induced by Dasatinib, we detected SRC activity after Dasatinib treatment. Dasatinib caused complete or near-complete inhibition of Src activity, as measured by phosphorylation at Y416 by Western blotting after treatment for 24 hr with concentrations of 2.5, 5, 10 μmol/L in CNE2 cell line (Figure 2). The data suggests that Dasatinib inhibited proliferation of nasopharyngeal carcinoma cells in a SRC-dependent manner.

**Dasatinib inhibits Src downstream signaling**

As we found that SRC activity was significantly inhibited by Dasatinib in nasopharyngeal carcinoma lines, but total SRC levels were not affected. Therefore, the effects of Dasatinib on Src and known downstream targets were examined by Western blotting in cell treated for 24 hours. Two main survival signaling pathways, MAPK and PI3K/AKT pathway are well understood downregulated by antitumor inhibitors [14, 15]. As expected, the phosphorylation of AKT, MEK and ERK were transiently decreased by Dasatinib in CNE2 cells (Figure 3). Since MAPK and PI3K/AKT are investigated as downstream of SRC, a more striking correlation was found between the antiproliferative effects of Dasatinib and the modulation of MAPK and PI3K/AKT pathways activity.

**Src inhibition by dasatinib leads to apoptosis in nasopharyngeal carcinoma**

CNE2 were treated with 5, 10, 20, 40 μmol/L Dasatinib in complete medium for 48 hours. Cells were stained with both propidium iodide and Annexin V and analyzed by fluorescence-activated cell sorting analysis. Dasatinib induced apoptosis in a dose-dependent manner. Cells treated with 40 μmol/L Dasatinib,
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apoptosis rate was increased to 13.5% (Figure 4). The result is consistent with the MTT assay.

Src inhibition by Dasatinib causes decreased migration.

FAK was well known as downstream of SRC, which functions in invasive migration [6-9]. We want to know whether Dasatinib inhibit tumor migration by decrease FAK activity. FAK phosphorylation level was detected in CNE2 after Dasatinib treatment (Figure 5). As predicted, phosphorylation of FAK at Y397 decreased in a dose-dependent manner. CNE2 cells were treated with 0, 50, 100 nmol/L Dasatinib and cell migration was measured using Transwell assay. The number of cells that had migrated into the scratch after 6 hours was calculated. Cell migration was significantly inhibited by Dasatinib (Figure 6). These data indicate that decreased migration by Dasatinib treatment correlated with the inhibition of FAK phosphorylation.

Discussion

Two major categories of EGFR inhibitors resistance have been proposed, de novo resistance and acquired resistance. The former due to genetic alterations of receptor tyrosine kinases and the later due to the acquisition of alternative RTK signaling activation. However, in a Trastuzumab resistance study, SRC is demonstrated activated in both de novo and acquisition resistance with ERBB2-overexpressing breast cancer, and targeting SRC by Dasatinib overcomes Trastuzumab resistance both in vitro and in vivo [3]. While EGFR and SRC are found overexpressed in nasopharyngeal carcinoma, targeting EGFR by Lapatinib and Cetuximab shows no benefit in clinical. So we guess SRC activation is to blame to induce EGFR inhibitors de novo resistance in nasopharyngeal carcinoma. Dasatinib has been report- ed to be an effective therapeutic molecule for the treatment of EBV-associated malignancies by inhibiting B-cell colony formation by bone marrow cells transgenic for LMP2A gene of Epstein-Barr virus [10]. Solid tumor nasopharyngeal carcinoma tumorigenesis is associated with Epstein-Barr virus (EBV) infection and latency, but may have different antitumor mechanism from lymphoma. In this study, we investigated the therapeutic efficacy of the protein tyrosine kinase inhibitor Dasatinib in Nasopharyngeal Carcinoma. Our data demonstrated that treatment with Dasatinib results in anti-proliferation of CNE2 cell line in vitro and inducing apoptosis. This effect of Dasatinib appears to be mediated by inhibition of SRC phosphorylation. However, changes in the phosphorylation of specific downstream signaling proteins by Dasatinib did seem to correlate with proliferation and survival. We also examined the Ras-MAPK pathway and PI3K/AKT pathway because this two are affected by SRC, possibly via their interactions with SHC [11, 12]. These results showed that inhibition of SRC have sustained effects on the MAPK cascade and PI3K/AKT in nasopharyngeal carcinoma.

Src is part of the focal adhesion complex that links integrins to the cytoskeleton [12, 13]. Src inactivation is associated with stabilization of this complex and reducing cell motility and invasion. Previous study shows that low dose of Dasatinib inhibited cell migration and invasion independent of any effects on cell proliferation and survival. In this study, we investigated cell motility was inhibited by Dasatinib. And we found that FAK overexpression and hyperphosphorylation have been reported in a variety of human cancers, including sarcomas and carcinomas of the breast, colon, thyroid, prostate, oral cavity, liver, stomach and ovary [15]. So we suggest Dasatinib inhibits migration by down-regulating FAK activity.

This drug is currently being examined in patients with AML and CLL [16, 17], and also in several solid tumors including breast cancer, colorectal cancer, prostate cancer and head and neck cancer [18-21]. The early results provided here suggest that this agent may also have antitumor activity in nasopharyngeal carcinoma. Testing for Dasatinib sensitivity prior to study entry might be a way to select a patient population benefitting from Dasatinib.

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Disclosure of conflict of interest

None.

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