Knockdown of the DJ-1 (PARK7) gene sensitizes pancreatic cancer to erlotinib inhibition

Xiangyi He,1,2 Yunwei Sun,1,2 Rong Fan,1 Jing Sun,1 Douwu Zou,1 and Yaozong Yuan1

1Department of Gastroenterology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib, in combination with gemcitabine, has been shown to be a promising therapy in the treatment of pancreatic cancer. Our previous study showed that DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC/extracellular signal-regulated kinase (ERK)/uPA. The aim of this study was to evaluate whether knockdown of DJ-1 expression can sensitize pancreatic cancer cells to erlotinib treatment. Knockdown of DJ-1 expression accelerated erlotinib-induced cell apoptosis and improved the inhibitory effect of erlotinib on pancreatic cancer cell proliferation (for the BxPC-3, PANC-1, and MiaPACa-2 cell lines, regardless of KRAS mutation status) in vitro and in xenograft tumor growth in vivo. Knockdown of DJ-1 decreased K-RAS expression, membrane translocation, and activity in BxPC-3 cells. Knockdown of DJ-1 also decreased K-RAS, H-RAS, and N-RAS expression in PANC-1 and MiaPACa-2 cells. Knockdown of DJ-1 synergistically inhibited Akt and ERK1/2 phosphorylation with erlotinib in pancreatic cancer cells. These findings indicate that DJ-1 may activate the Ras pathway, reinforcing erlotinib drug resistance. Therefore, blocking DJ-1 in combination with the EGFR tyrosine kinase inhibitor erlotinib may be an attractive therapeutic target in pancreatic cancer.

INTRODUCTION

Pancreatic cancer is considered to be one of the most lethal forms of cancer. It is the 14th most commonly diagnosed cancer worldwide, but the 7th most common cause of cancer-related death. The only potentially curative treatment is surgical resection of the tumor; however, most patients are diagnosed at a late stage, systemic therapies have been intensively studied but have resulted in limited improvement. Numerous targeted agents have been evaluated alone or in combination therapy in metastatic pancreatic cancer. Unfortunately, most agents thus far have failed to improve patient survival.

Erlotinib is an orally administered quinazoline derivative that is a potent inhibitor of epidermal growth factor receptor (EGFR)-related tyrosine kinase. In combination with gemcitabine, it became the first combination therapy for non-resectable pancreatic ductal adenocarcinoma (PDAC) to show an improvement in overall survival compared with single-agent gemcitabine combination therapy, but it has a marginal therapeutic benefit, as it conferred a mean survival benefit of only 2 weeks. The minor effect of erlotinib may be due to the high percentage of activating K-RAS mutations, which occur in up to 90% of patients with PDAC, because constitutive activation of K-RAS makes cancer cells resistant to upstream EGFR inhibition. Therefore, overcoming erlotinib resistance is an important area of research.

DJ-1, also known as PARK7, exhibits multiple functions in both cancer and Parkinson’s disease. It was originally identified as an oncogene that can transform mouse NIH 3T3 cells in combination with H-RAS. In a previous study, we found that DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC/extracellular signal-regulated kinase (ERK)/uPA. It has also been reported that DJ-1 directly binds c-Raf and serves as a positive regulator of the EGF/Ras/ERK pathway. Given the role of DJ-1 in the EGF/Ras/ERK pathway, it will be interesting to investigate whether inhibition of DJ-1 could increase the anti-cancer effect of erlotinib.

RESULTS

Inhibition of DJ-1 expression sensitizes pancreatic cancer cells to erlotinib treatment in vitro

To investigate whether inhibition of DJ-1 can sensitize pancreatic cancer cells to erlotinib treatment, a Cell Counting Kit-8 (CCK-8) assay was conducted to determine the difference in cellular viability of two pancreatic cancer cell lines with and without combination treatment of DJ-1 inhibition and erlotinib. BxPC-3 cells contain wild-type K-RAS, while K-RAS is mutated in PANC-1 and MiaPACa-2 cells. Transfection of DJ-1 short hairpin RNA (shRNA) or small interfering RNA (siRNA) decreased the expression of DJ-1 by 51.19% or 61.36%, respectively, in BxPC-3 or PANC-1 cells (Figures 1A and 1D). The IC50 (50% inhibitory concentration) value of erlotinib in BxPC-3/DJ-1 shRNA cells was lower than in BxPC-3/negative control (NC) shRNA cells (20 μM versus 67 μM; p < 0.05, Figure 1C). Knockdown of DJ-1 expression in PANC-1 cells also decreased DJ-1 expression by 62% (Figure 2E) and IC50 of erlotinib from 433.33 to 51.19 μM; p < 0.05, Figure 2F).
In order to rule out the possibility of a cell type-specific effect, we investigated the effect of knockdown DJ-1 expression on erlotinib sensitivity in another KRAS mutant cell line, MiaPACa-2, which showed same effect as PANC-1 cells. Knockdown of DJ-1 expression in MiaPACa-2 cells decreased the IC50 of erlotinib from 244.0 to 57.6 μM (Figure 1G, p < 0.01).

Inhibition of DJ-1 expression acts in synergy with the inhibitory effect of erlotinib on proliferation of pancreatic cancer cells

We next studied the synergistic effect of DJ-1 inhibition and erlotinib on cell proliferation. 5-Bromo-2′-deoxyuridine (BrdU) incorporation was used to detect the proliferation rate in pancreatic cancer cells. Erlotinib decreased the proportion of BrdU-positive cells more extensively in BxPC-3/DJ-1 shRNA cells than in BxPC-3/NC shRNA cells. As shown in Figures 2A and 2B, 5 and 50 μM erlotinib treatment decreased the number of BrdU-positive cells by 37.7% and 81.5%, respectively, in BxPC-3/NC shRNA cells compared with the control. However, in BxPC-3/DJ-1 shRNA cells, the number of BrdU-positive cells decreased by 58.7% and 91.7% following treatment with 5 and 50 μM erlotinib, respectively (both p < 0.05 versus BxPC-3/NC shRNA, Figures 2A and 2B). The same phenomenon was observed in PANC-1 cells, where erlotinib (100 μM) decreased BrdU incorporation in PANC-1 cells transfected with DJ-1 siRNA but not in PANC-1 cells transfected with NC siRNA (Figures 2C and 2D).

Inhibition of DJ-1 expression increased erlotinib-induced apoptosis in pancreatic cancer cells

The TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay was used to detect apoptosis. Treatment with 5 or 50 μM erlotinib for 48 h significantly increased the proportion of BxPC-3/NC shRNA TUNEL-positive cells to 7.3% (2.3-fold to control) and 13.1% (4.1-fold to control), respectively, compared with 3.0% in the untreated control. In BxPC-3/DJ-1 shRNA cells, apoptosis was increased to 10.5% (3.7-fold, p < 0.05 versus BxPC-3/NC shRNA cells, Figures 3A and 3B) and 21.7% (9.7-fold, p < 0.05 versus BxPC-3/NC shRNA cells, Figures 3A and 3B) following treatment with 5 and 50 μM erlotinib, respectively, compared with 2.8% in the untreated control. Following treatment with 100 μM erlotinib for 48 h in PANC-1, the proportion of apoptotic cells was increased to 1.47-fold (compared to control). Following knockdown of DJ-1, 100 μM erlotinib increased apoptosis to 2.9-fold (Figures 3C and 3D, p < 0.05 versus PANC-1/NC siRNA cells). Inhibition of DJ-1 expression dramatically increased erlotinib-induced apoptosis in MiaPACa-2 cells compared with control (Figures 3E and 3F, 2.96 versus 1.73, p < 0.05 versus NC siRNA cells or erlotinib alone).

Inhibition of DJ-1 expression sensitizes pancreatic cancer cells to erlotinib treatment in vivo

Next, we tested whether inhibition of DJ-1 would sensitize pancreatic cancer cells to erlotinib treatment in vivo. BxPC-3/DJ-1 shRNA and
BxPC-3/NC shRNA (subcutaneous) xenografts were treated with 50 mg/kg/day erlotinib (intraperitoneal) for 9 d. Tumor growth of all groups is shown in Figure 4A. There was no difference in tumor growth between the BxPC-3/DJ-1 shRNA and BxPC-3/NC shRNA xenografts. Treatment with erlotinib for 9 days reduced tumor growth of the BxPC-3/NC shRNA xenograft, while DJ-1 inhibition combined with erlotinib treatment showed the slowest tumor growth. In Mia-PACa-2 xenografts, knockdown of DJ-1 expression or erlotinib...
treatment alone slightly decreased tumor growth. However, the combination of DJ-1 inhibition and erlotinib treatment dramatically retarded tumor growth of MiaPACa-2 xenografts compared with control and any treatment alone (Figure 4B).

Knockdown of DJ-1 expression decreased RAS expression and activation

To investigate the mechanism underlying how inhibition of DJ-1 sensitizes PDAC to erlotinib treatment, we assessed RAS expression and activation of the downstream AKT/ERK pathway. K-RAS and H-RAS protein concentrations were significantly decreased in BxPC-3/DJ-1 shRNA cells compared with BxPC-3/NC shRNA cells (both p < 0.05 versus BxPC-3/NC shRNA cells), while N-RAS expression was not reduced (Figures 5A and 5B). Knockdown of DJ-1 expression in both PANC-1 and MiaPACa-2 cells significantly reduced K-RAS, H-RAS, and N-RAS expression (Figures 5C–5F, all p < 0.05 versus NC siRNA cells).

Given the important role played by K-RAS in the development of pancreatic cancer, we tested K-RAS activity after knockdown of DJ-1 expression in BxPC-3 cells. Knockdown of DJ-1 expression decreased K-RAS activity (Figure 6A), membrane translocation (Figure 6B), and mRNA production (Figure 6C). RASAL1 negatively regulates K-RAS activation. Expression of RASAL1 was increased by DJ-1 knockdown (Figure 6D) but decreased by ectopic expression of DJ-1 (Figure 6E), suggesting that DJ-1 may inhibit RASAL1 expression, which then inhibits K-RAS activation.

Synergistic effect of DJ-1 knockdown and erlotinib on inhibition of ERK1/2 and AKT phosphorylation

The ERK1/2 and AKT pathways are two important downstream pathways of RAS and DJ-1. We determined the phosphorylation levels of ERK1/2 and AKT following knockdown of DJ-1 and treatment with erlotinib. As shown in Figure 7, the levels of both phosphorylated AKT (p-AKT) and phosphorylated ERK1/2 (p-ERK1/2) decreased following treatment with 50 μM erlotinib in BxPC-3/NC shRNA cells. As shown previously, knockdown of DJ-1 expression decreased p-ERK1/2, and the levels of p-ERK1/2 and p-AKT were further decreased in BxPC-3/DJ-1 shRNA cells treated with 50 μM erlotinib (Figure 7A; see also Figure S1). In contrast, the levels of p-AKT and p-ERK1/2 were resistant to 100 μM erlotinib treatment alone in PANC-1 cells, which contain a mutated copy of K-RAS. However, levels of both p-AKT and p-ERK1/2 were decreased further following treatment with 100 μM erlotinib upon knockdown of
DISCUSSION

DJ-1 is a versatile protein. We previously showed that DJ-1 promotes metastasis of pancreatic cancer.\(^6\) In the present study, we uncover a new function for DJ-1 that sensitizes pancreatic cancer cells to erlotinib treatment. Inhibition of DJ-1 not only accelerated erlotinib-induced apoptosis, but it also increased the inhibitory effect of erlotinib on pancreatic cancer cell proliferation \textit{in vitro} and \textit{in vivo}. Furthermore, these effects are independent of the K-RAS mutation status, as both wild-type and mutant cell lines were shown to be sensitive to the combination treatment.

EGFR-tyrosine kinase inhibitors are the standard treatment for advanced non-small cell lung cancer harboring EGFR mutations. However, in pancreatic cancer, erlotinib is not widely used. Numerous studies have been performed in an effort to overcome erlotinib resistance in pancreatic cancer, investigating the use of inhibitors of...
mitogen-activated protein kinase (MAPK) kinase (MEK), AKT, histone deacetylase inhibitors (HDACis), and phosphatidylinositol 3-kinase (PI3K)/mTOR. Du et al. reported that erythropoietin-producing hepatocellular receptor 2 (EphA2), rescued by miR-124 downregulation, conferred erlotinib resistance in pancreatic cancer cells (Capan-1 cell line), which contain a mutated copy of K-RAS. However, none of these attempts has succeeded clinically thus far. Oncogenic K-RAS mutation is the signature genetic event in the progression and growth of PDAC. It has been reported that nearly 90% of PDAC cases harbor mutationally activated K-RAS. Constitutively activated K-RAS leads to activation of downstream pathways independent of EGFR activation. Therefore, K-RAS activation may be one of the reasons erlotinib has only a minor effect on pancreatic cancer.

DJ-1 was initially identified as a RAS cooperator. Takahashi-Niki et al. reported that DJ-1 binds to c-Raf, positively regulating the EGF/Ras/ERK pathway. In the present study, we showed a new function of DJ-1, activating RAS via several routes. First, knockdown of DJ-1 expression decreased K-RAS and H-RAS mRNA expression in both PANC-1 and BxPC-3 cells. Thus, this effect was independent of the mutational status of K-RAS. Second, knockdown of DJ-1 expression decreased K-RAS membrane translocation and increased RASAL1 expression in wild-type K-RAS BxPC-3 cells. RASAL1 functions as a negative modulator of the RAS signaling pathway by acting as a RasGAP that catalyzes RAS inactivation. RASAL1 has been identified as a tumor-suppressor gene in many cancers, including thyroid cancer, gastric cancer, bladder cancer, and colorectal cancer. Ohta et al. reported that RASAL1 expression was reduced in most colorectal cancer cells with a wild-type K-RAS gene but rarely in those with a mutant K-RAS gene. Our results also suggest that in wild-type K-RAS pancreatic cancer cells, knockdown of DJ-1 decreased RASAL1 expression. Therefore, inactivation of RAS by inhibition of DJ-1 may explain its role in augmenting the anti-cancer effects.

This study, as well as others, has shown that DJ-1 plays a major role in various pathways. It mediates cell survival and proliferation by activating the ERK1/2 and PI3K/Akt pathways. Herein, we show that treatment with erlotinib can inactivate both AKT and ERK when used in combination with DJ-1 inhibition; this may partly explain the synergistic effect of inhibition of DJ-1 and erlotinib treatment. Another study has also shown that combined blockage of the MAPK and PI3K/Akt/mTOR pathways with MEK and PI3K/ mTOR inhibitors resulted in anti-cancer effects in cell lines with acquired resistance to erlotinib. The failures of clinical trials thus far suggest that there are challenges associated with developing effective RAS therapeutics in pancreatic cancer. Our study has demonstrated that the combination of inhibition of DJ-1 and treatment with erlotinib inhibits downstream signaling independent of the K-RAS status by directly inhibiting RAS expression or by upregulating a RAS

Figure 6. Knockdown of DJ-1 expression decreased K-RAS expression, membrane translocation, and activity, but increased RASAL1 expression in BxPC-3 cells.

(A) Glutathione-S-transferase (GST) pull-down analysis of K-RAS activity. (B) Western blot analysis of membrane-bound (M) and cytoplasmic K-RAS (P). (C) RT-PCR analysis of K-RAS mRNA levels. (D) Western blot analysis of RASAL1 in BxPC-3/NC shRNA and BxPC-3/DJ-1 shRNA cells. (E) Western blot analysis of RASAL1 in BxPC-3 cells transfected with DJ-1 expression vector or empty vector for 72 h. The data are shown as the mean ± SD (n = 3 per group). *p < 0.05 versus control (n = 3); Student’s t test.
inhibitor. This may yield new and interesting targets for the development of future therapies.

Our studies and those of others have supported the development of chemotherapeutic approaches targeting DJ-1. Several interventions were suggested to be used targeting DJ-1, such as using RNA interference (RNAi), inhibiting DJ-1 protein complex formation, and interfering with protein function. Recently, several compounds were identified as potent inhibitors of DJ-1 to inactivate the critical Cys106 residue by using biophysical methodologies and X-ray crystallography. All of these RNAi and small molecular inhibitors may facilitate clinical application of targeting DJ-1 in the future.

In summary, this study found that DJ-1 activates RAS through several mechanisms in pancreatic cancer and showed that erlotinib drug resistance may be overcome by inhibition of DJ-1. Dual EGFR and DJ-1 blockade may be an attractive therapeutic target in pancreatic cancer.

MATERIALS AND METHODS

PDAC cell lines, siRNA, and inhibitors

BxPC-3 (CRL-1687), MiaPACA-2 (CRL-1420), and PANC-1 (CRL-1469) cells were purchased from American Type Culture Collection (ATCC). Cell lines were typed by short tandem repeat profiling and were shown to conform to the ATCC reference standards (CellBank, Westmead, NSW, Australia). All of the cell lines were routinely tested for mycoplasma contamination. Erlotinib (Selleck Chemicals, Houston, TX, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) as 20 mM stock solutions and stored at −80°C.

Plasmids and transfection

DJ-1 shRNA and NC shRNA, a scrambled sequence of the shRNA target sequence containing the green fluorescent protein expression sequence, were purchased from GenePharma (Shanghai, China). DJ-1-specific siRNA was also purchased from GenePharma. The target sequences of DJ-1 shRNA and DJ-1 siRNA were 5'-GCTCTGTGGCTCAGTAAAT-3' and 5'-GAAUUUAUCUGAGUCGUUU-3', respectively. PANC-1 cells were transfected with the recommended concentration of siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA), followed by one change of medium according to the manufacturer’s instructions. After 48–72 h of siRNA transfection, cells were harvested for the following assay. Stable shRNA knockdown cells (BxPC-3/NC shRNA and BxPC-3/ DJ-1 shRNA) were established as previously described.

Cell proliferation assays (CCK-8 and BrdU incorporation)

Pancreatic cancer cells were treated with varying concentrations of erlotinib in medium supplemented with 10% fetal bovine serum for 48 h. The CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability in 96-well plates according to the manufacturer’s instructions. IC50 values were calculated by nonlinear regression analysis from three independent replicate experiments. Following treatment, cells were incubated with 10 μM BrdU (Sigma-Aldrich, Dallas, TX, USA) for 45 min at 37°C. Cells were then centrifuged and resuspended in 300 mL of phosphate-buffered saline. After fixation, cells were stained with anti-BrdU-allophycocyanin (APC) (eBioscience, Carlsbad, CA, USA). Cell cycle distribution was assessed by FACS analysis (BD Biosciences, San Jose, CA, USA).
Apoptosis assay (immunofluorescence TUNEL assay) 

Apoptosis was assessed using a TUNEL assay kit. An in situ cell death detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer’s instructions. Cells were plated on coverslips overnight and treated with various doses of erlotinib. Following treatment, cells were fixed and permeabilized with solutions. TUNEL reaction mixture (50 μL) was added to the sample. Slides were then incubated in a humid atmosphere for 60 min at 37°C in the dark and viewed using the Leica confocal microscopy system (Leica TCS SP5; Leica, Buffalo Grove, IL, USA) with 633-nm (red) and 488-nm (green) lasers.

Western blotting

Western blot analysis was performed as previously described. Briefly, cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore, Temecula, CA, USA). After blocking of non-specific binding sites with 5% fat-free milk, the blot was probed with primary antibodies against K-RAS, N-RAS, H-RAS, DJ-1, p-ERK, ERK, p-Akt, Akt, and β-actin (Cell Signaling Technology, Danvers, MA, USA). The bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized using an enhanced chemiluminescence system (Millipore, Billerica, MA, USA). The levels of each protein were standardized to the loading control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and quantified using ImageJ 1.41 (National Institutes of Health, Bethesda, MD, USA). For membrane-bound and cytoplasmic K-RAS expression, a Mem-PER plus membrane protein extraction kit (Thermo Scientific, Rockford, IL, USA) was used according to the manufacturer’s instructions.

K-RAS pull-down activation assay

A Ras pull-down activation assay kit (Cytoskeleton, Denver, CO, USA) was used to pull down activated RAS according to the manufacturer’s instructions. Activated K-RAS was then detected by western blotting as described above using anti-KRAS as the primary antibody.

Reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of K-RAS expression

RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with a first-strand cDNA synthesis kit for RT-PCR (Jrdun Biotechnology, Shanghai, China) with oligo(dt) primers according to the manufacturer’s instructions. Primers used for the amplification of human K-RAS were 5'-ACAAGGCACTGGGTATATGG-3' (sense) and 5'-ACGGATCGTGTATCTCTGG-3' (antisense). For GAPDH, primers were 5'-ACCCACCTCCTCACCCTTG-3' (sense) and 5'-CCACCACTCGTGTGCTTAG-3' (antisense). All primers were synthesized by MWG Biotech (Shanghai, China). PCR was performed using the ABI Prism 7300 in a total volume of 50 μL using a fluorescence quantitative (FQ)-PCR kit (Jrdun Biotechnology).

In vivo human xenograft tumors

Wild-type BALB/c mice (female, 6 weeks of age) were purchased from Shanghai Sippe-Bk Lab Animal Co. BxPC-3/NC shRNA or BxPC-3/DJ-1 shRNA cells (2 × 10^6) were implanted subcutaneously in the right flanks of 6-week-old female BALB/c nude mice. Mice were randomized to the control or erlotinib (50 mg/kg/day, intraperitoneal) groups when the tumor diameter reached 3 cm (n = 6 mice/group). Tumor growth was assessed every other day by caliper measurement of tumor diameter in the longest dimension (L) and at right angles to that axis (W). Tumor volume was estimated by the following formula: (L × W × W)/2. These mice were then euthanized by cervical dislocation after 9 days of treatment. For Mia-PACa-2, after transfection with NC siRNA and DJ-1 siRNA for 24 h, cells were collected and implanted subcutaneously (5 × 10^6 for each mouse) in the right flanks of 6-week-old BALB/c nude mice. The erlotinib treatment and tumor observation were performed as described above. The protocol was approved by the Institutional Animal Care and Use Committee (Ruijin Hospital, Shanghai Jiao-Tong University School of Medicine, Shanghai, China). All experiments were carried out according to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and following ethical review by the Laboratory Animal Ethics Committee of Ruijin Hospital, Shanghai Jiao-Tong University School of Medicine.

Statistical analysis

Data are represented as mean ± standard deviation from at least three independents. Data were analyzed by a Student’s t test, chi-square test, or analysis of variance as appropriate using the SPSS v20.0 statistical software (IBM, Armonk, NY, USA). A two-tailed p value ≤ 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.01.013.

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AUTHOR CONTRIBUTIONS

X.H., Y.S., D.Z., and Y.Y. conceived and designed the study. X.H. and Y.S. performed the experiments. X.H., R.F., and J.S. performed the data analysis. X.H. and Y.S. wrote and reviewed the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare on competing interests.
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