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

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Abstract

Background: 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/ERK/uPA. The aim of this study was to evaluate whether silencing DJ-1 expression can sensitize pancreatic cancer cells to erlotinib treatment. METHODS: Knockdown of DJ-1 expression, combined with erlotinib treatment, was performed in the pancreatic cancer cell lines BxPC-3, PANC-1 and MiaPACa-2. Cell proliferation was assessed with the CCK-8 assay and BrdU incorporation, while apoptosis was measured using terminal deoxynucleotidyl ransferase (TdT)-mediated dUTP nick-end labeling. RAS activity and activation of the downstream pathways involving AKT and ERK1/2 were explored to determine the underlying mechanism. RESULTS: Knockdown of DJ-1 expression accelerated erlotinib-induced cell apoptosis, and improved the inhibitory effect of erlotinib on pancreatic cancer cell proliferation 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. CONCLUSIONS: DJ-1 may activate the RAS pathway, reinforcing erlotinib drug resistance. Blocking DJ-1 in combination with the EGFR tyrosine kinase inhibitor erlotinib may be an attractive therapeutic target in pancreatic cancer.

Background

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 seventh most common cause of cancer-related death [1]. The only potentially curative treatment is surgical resection of the tumor; however, only 20% of patients at most are eligible for initial resection [2]. As the majority of 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 with chemotherapy in metastatic pancreatic cancer. Unfortunately, most agents thus far have failed to improve patient survival [2]. 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 has a marginal therapeutic benefit as it conferred a mean survival benefit of only 2 weeks [3]. 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 make 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 [4]. It was originally identified as an oncogene that can transform mouse NIH3T3 cells in combination with H-RAS [5]. In a previous study, we found that DJ-1 promotes invasion and metastasis of pancreatic cancer cells

by activating SRC/ERK/uPA [6]. It has also been reported that DJ-1 directly binds c-Raf and serves as a positive regulator of the EGF/Ras/ERK pathway [7]. 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.

Methods

PDAC cell lines, siRNA, and inhibitors

BxPC-3 , MiaPACa-2 and PANC-1 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). Erlotinib (Selleckchem, 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 short hairpin RNA (shRNA) and negative control (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 small interfering RNA (siRNA) was also purchased from Genepharma. The target sequences of DJ-1 shRNA and DJ-1 siRNA were 5'-GCTCTGTTGGCTCATGAAATA-3' and 5'-GAAUUUAUCUGAGUCUGCUUU-3', respectively. PANC-1 cells were transfected with the recommended concentration of siRNA using Lipofectamine 2000™ (ThermoFisher Scientific, Waltham, MA, USA), followed by one change of medium according to the manufacturer's instructions. Stable shRNA knockdown cells (BxPC-3/NC shRNA and BxPC-3/DJ-1 shRNA) were established as previously described [6].

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 Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay was used to determine cell viability in 96-well plates according to the manufacturer's instructions. IC_{50} (50% inhibitory concentration) values were calculated by nonlinear regression analysis from three independent replicate experiments. Following treatment, cells were incubated with 10 μM bromodeoxyuridine (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-APC (eBioscience, Carlsbad, CA, USA). Cell cycle distribution was assessed by FACSCalibur analysis (BD Biosciences, San Jose, CA, USA).

Apoptosis assay (immunofluorescence TUNEL assay)

Apoptosis was assessed using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit. The *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 [6]. Briefly, cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinyldifluoride 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, NRAS, HRAS, DJ-1, pERK, ERK, pAkt, 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 transcription-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 the 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'-

ACGCATCGTGTTATCTCTGG -3' (antisense). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primers were 5'- CACCCACTCCTCCACCTTTG -3' (sense) and 5'- CCACCACCCTGTTGCTGTAG -3' (antisense). All primers were synthesized by MWG Biotech AG (Shanghai, China). PCR was performed using the ABI Prism 7300 in a total volume of 50 μl using an FQ-PCR kit (Jrdun Biotechnology).

In vivo human xenograft tumors

Wild-type BALB/c mice (female ,six weeks) were purchased from Shanghai Sippe-Bk Lab Animal Co. Ltd. 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/d, 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 9d of treatment. 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 guidelines and following ethical review by the laboratory animal ethics committee of Ruijin Hospital, Shanghai Jiao-Tong University school of Medicine.

Statistical analysis

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

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 erlortinib treatment, a 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 shRNA or siRNA decreased the expression of DJ-1 by 51.19% or 61.36%, respectively, in BxPC-3 or PANC-1 cells (Fig. 1A–D). The IC_{50} (50% inhibitory concentration) value of erlotinib in BxPC-3/DJ-1 shRNA cells was lower than in BxPC-3/NC shRNA cells (20 μM vs. 67 μM; $p < 0.05$, Fig. 1c). Given that the IC_{50} value of erlotinib in PANC-1 cells was greater than 500 μM and could not be achieved, we treated PANC-1 cells with 100 and

200 μM erlotinib. Silencing DJ-1 expression in PANC-1 cells decreased the treatment/non-treatment cell number ratio with treatment with both 100 and 200 μM erlotinib (Fig. 1F). In order to rule out the possibility of cell-type specific effect, we investigated the effect of silencing DJ-1 expression on erlortinib sensitivity in another KRAS mutant cell line MiaPACa-2. It showed same effect as PANC-1. Silencing DJ-1 expression in MiaPACa-2 cells decreased the treatment/non-treatment cell number ratio with treatment with 10 to 200 μM erlotinib (Fig. 1G).

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. 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 Figure 2A and B, 5 μmol and 50 μmol 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 was decreased by 58.7% and 91.7% following treatment with 5 μm and 50 μmol erlotinib, respectively (both $p < 0.05$ vs. BxPC-3/NC shRNA, Fig. 2A, B). 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 (Fig. 2C, D).

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

The TUNEL 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% and 13.1%, respectively, compared with 3.0% in the untreated control. In BxPC-3/DJ-1 shRNA cells, apoptosis was increased to 10.5% and 21.7% following treatment with 5 and 50 μM erlotinib, respectively, compared with 2.8% in the untreated control (both $p < 0.05$ vs. BxPC-3/NC shRNA cells, Fig. 3A, B). PANC-1 cells were more resistance to erlotinib treatment. Following treatment with 100 μM erlotinib for 48 h, the proportion of apoptotic cells was increased by 49.0%. Following knockdown of DJ-1, 100 μM erlotinib increased apoptosis by 121.8% (Fig. 3C, D, $p < 0.05$ vs. PANC-1/NC siRNA cells). Inhibition of DJ-1 expression also dramatically increased erlotinib-induced apoptosis in MiaPACa-2 (Fig 3E,F $p < 0.05$ vs. NC siRNA cells or erlotinib alone).

Inhibition of DJ-1 expression sensitizes KRAS wide type 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*. Because we were unable to successfully generate a PANC-1 cell xenograft, BxPC-3 cells were used for the *in vivo* study. 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 4. 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 d reduced tumor growth of the BxPC-3/NC shRNA xenograft, while DJ-1 inhibition combined with erlotinib treatment showed the slowest tumor growth.

Silencing 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 $p < 0.05$ vs. BxPC-3/NC shRNA cells), while N-RAS expression was not reduced (Fig. 5A, B). Knockdown of DJ-1 expression in both PANC-1 and MiaPACa-2 cells significantly reduced K-RAS, H-RAS, and N-RAS expression (Fig. 5C, D, E,F all $p < 0.05$ vs. PANC-1/NC siRNA cells).

Given the important role played by K-RAS in the development of pancreatic cancer, we tested K-RAS activity after silencing DJ-1 expression in BxPC-3 cells. Knockdown of DJ-1 expression decreased K-RAS activity (Fig. 6A), membrane translocation (Fig. 6B), and mRNA production (Fig. 6C). Rasal1 negatively regulates K-RAS activation [8]. Expression of Rasal1 was increased by DJ-1 knockdown, but decreased by ectopic expression of DJ-1 (Fig. 6D, E), 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 were further decreased in BxPC-3/DJ-1 shRNA cells treated with 50 μM erlotinib (Fig. 7A). By 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 following treatment with 100 uM erlotinib upon knockdown of DJ-1 expression in PANC-1 cells (fig. 7B). The synergistic effect of DJ-1 knockdown and erlotinib on inhibition of ERK1/2 and AKT phosphorylation was also observed in MiaPACa-2 (Fig. 7C).

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 also increased the inhibitory effect of erlotinib on pancreatic cancer cell proliferation *in vitro* and *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 MEK [9, 10], AKT2 [11, 12], HDACs [13], and PI3K/mTOR [10, 12]. Jing *et al.* reported that erythropoietin-producing hepatocellular receptor 2 (EphA2), rescued by miR-124 downregulation, conferred erlotinib resistance in pancreatic cancer cells Capan-1, which contain a mutated copy of K-RAS [14]. However, none of these attempts have succeeded clinically thus far. Oncogenic K-RAS mutation is the signature genetic event in the progression and growth of PDAC [15]. It has been reported that nearly 90% of PDAC cases harbor mutationally activated K-RAS [15]. 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 [5]. Takahashi-Niki et al. [7] 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. Firstly, 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. Secondly, 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 [8]. Rasal1 has been identified as a tumor-suppressor gene in many cancers, including thyroid cancer [16], gastric cancer [17], bladder cancer [18], and colorectal cancer [8]. Ohta et al.[8] 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 [4], 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. Here, 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 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 [10].

The failures of clinical trials thus far suggest that there are challenges associated with developing effective RAS therapeutics in pancreatic cancer [15]. 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 inhibitor. This may yield new and interesting targets for the development of future therapies.

Conclusion

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.

Abbreviations

EGFR epidermal growth factor receptor; PDAC pancreatic ductal adenocarcinoma; ATCC American Type Culture Collection; NC negative control; TUNEL terminal deoxynucleotidyl transferase dUTP nick-end labeling; RT-PCR reverse transcription-polymerase chain reaction.

Declarations

Ethical Approval and Consent to participate

Patient studies – not applicable, no human subjects involved. Animals studies –All experiments were carried out according to ARRIVE guidelines and following ethical review by the laboratory animal ethics committee of Ruijin Hospital, Shanghai Jiao-Tong University school of Medicine.

Consent for publication

All of the authors are aware of and agree to the content of the paper and their being listed as a co-author of the paper.

Availability of data and materials

All of the data and material in this paper are available when requested.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XY HE YW SUN DW ZOU and YZ YUAN conceived and designed the study. XY HE YW SUN performed the experiments. R FAN and J SUN performed the data analysis. XY HE and YW SUN wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

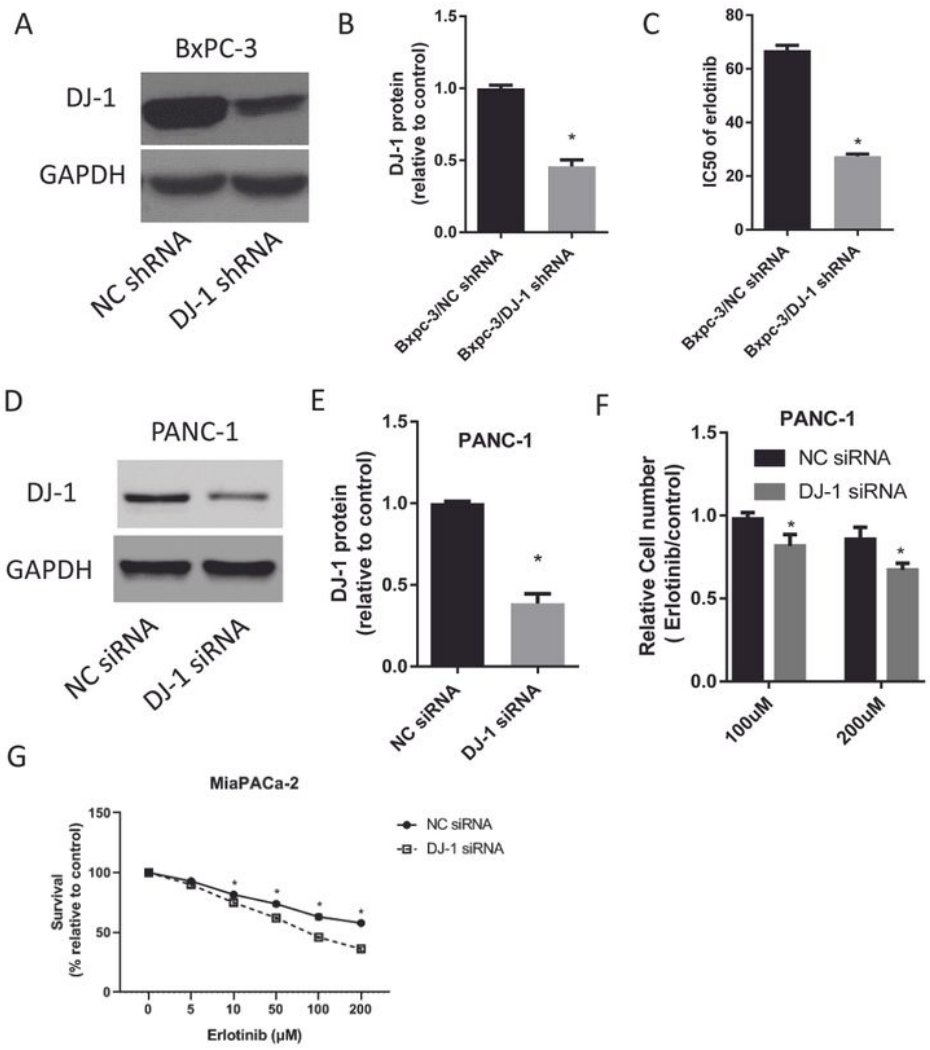

Figure 1

Figure 1

Silencing of DJ-1 expression sensitizes pancreatic cancer cells to erlotinib treatment in vitro. A. Western blot analysis of DJ-1 expression in BxPC-3/DJ-1 shRNA and BxPC-3/negative control (NC) shRNA cells. B. Densitometry quantification of (A). C. BxPC-3/DJ-1 shRNA and BxPC-3/NC shRNA cells were treated with various doses of erlotinib for 72 h followed by CCK-8 analysis of the IC50 value of erlotinib. D. Western blot analysis of DJ-1 expression in PANC-1 cells transfected with DJ-1 or NC siRNA for 72 h. E.

Densitometry quantification of (D). F. PANC-1 cells were transfected with DJ-1 or NC siRNA for 48 h followed by treatment with the indicated doses of erlotinib for 72 h. A CCK-8 assay was then performed to assess cell viability. G. MiaPACa-2 cells were transfected with DJ-1 or NC siRNA for 48 h followed by treatment with the indicated doses of erlotinib for 72 h. A CCK-8 assay was then performed to assess cell viability. *p < 0.05 vs. NC shRNA or NC siRNA; n = 3.

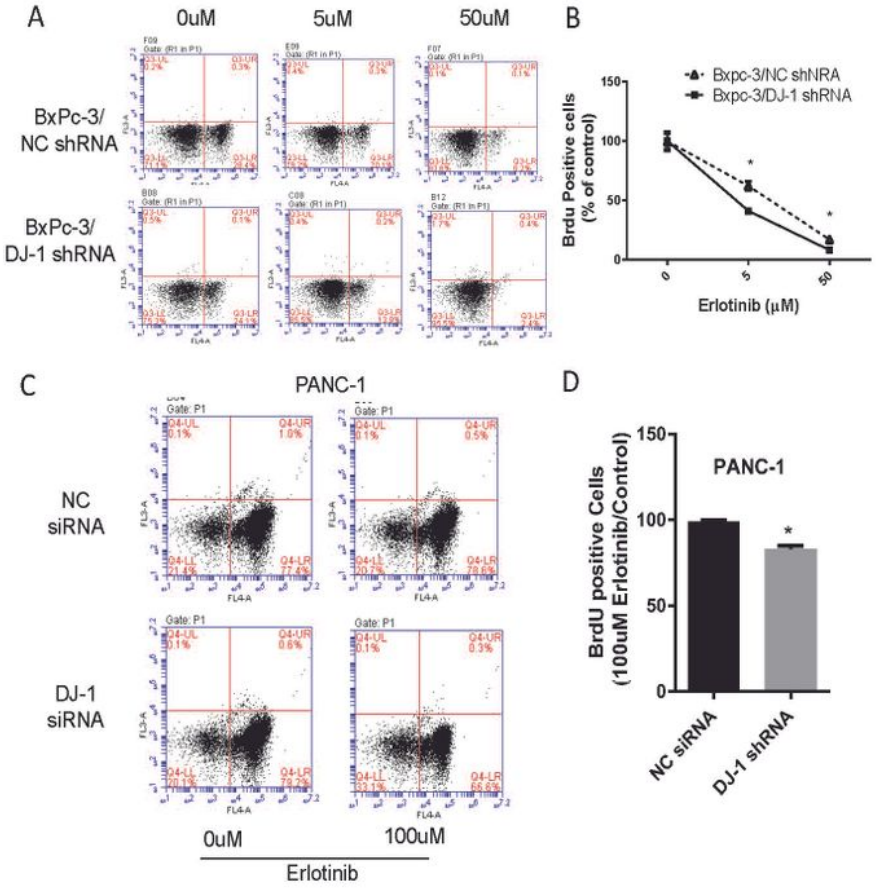

Figure 2

Figure 2

Inhibition of DJ-1 expression acts in synergy with the inhibitory effect of erlotinib on proliferation of pancreatic cancer cells. A. BxPC-3/DJ-1 shRNA and BxPC-3/NC shRNA cells were treated with the indicated doses of erlotinib for 72 h. BrdU incorporation combined with flow cytometry analysis was used to assess cell proliferation. B. Quantification of (A). *p < 0.05 vs. NC shRNA; n = 3. C. Cells were treated as described in Figure 1D and BrdU incorporation and flow cytometry were performed to assess cell proliferation. D. Quantification of (C). *p < 0.05 vs. NC siRNA; n = 3.

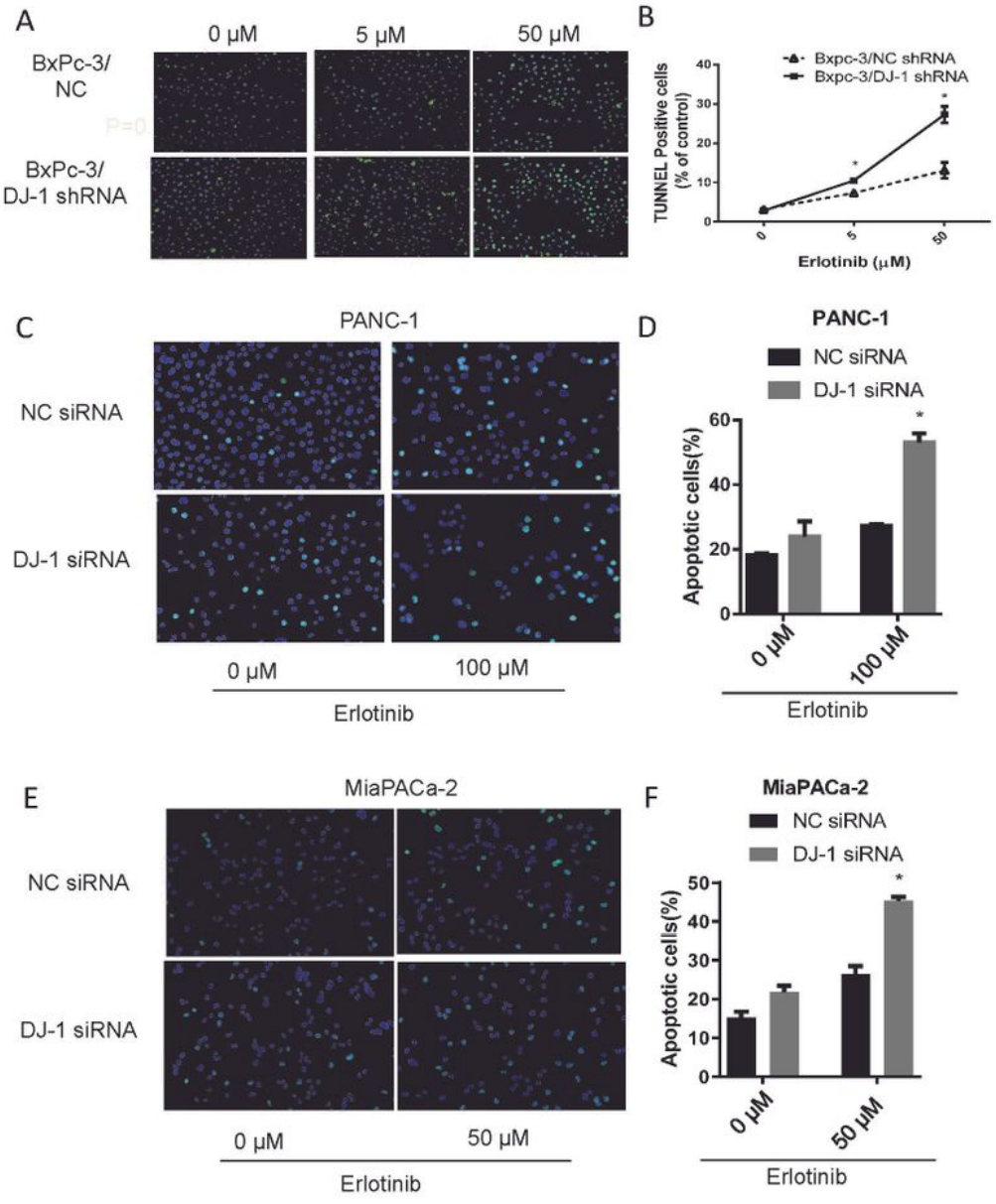

Figure 3

Figure 3

Inhibition of DJ-1 expression accelerated erlotinib-induced apoptosis in pancreatic cancer cells. A. Cells were treated as described in Figure 2A. TUNEL assay was used to quantify the number of apoptotic cells. B. Quantification of (A). *p < 0.05 vs. NC shRNA; n = 3. C. PANC-1 Cells were treated as described in Figure 1D. TUNEL assay was performed to quantify the number of apoptotic cells. D. Quantification of (C). E. MiaPACa-2 Cells were transfected with DJ-1 or NC siRNA for 48 h followed by treatment with the indicated doses of erlotinib for 72 h. TUNEL assay was performed to quantify the number of apoptotic cells. F. Quantification of (E). *p < 0.05 vs. NC siRNA; n = 3.

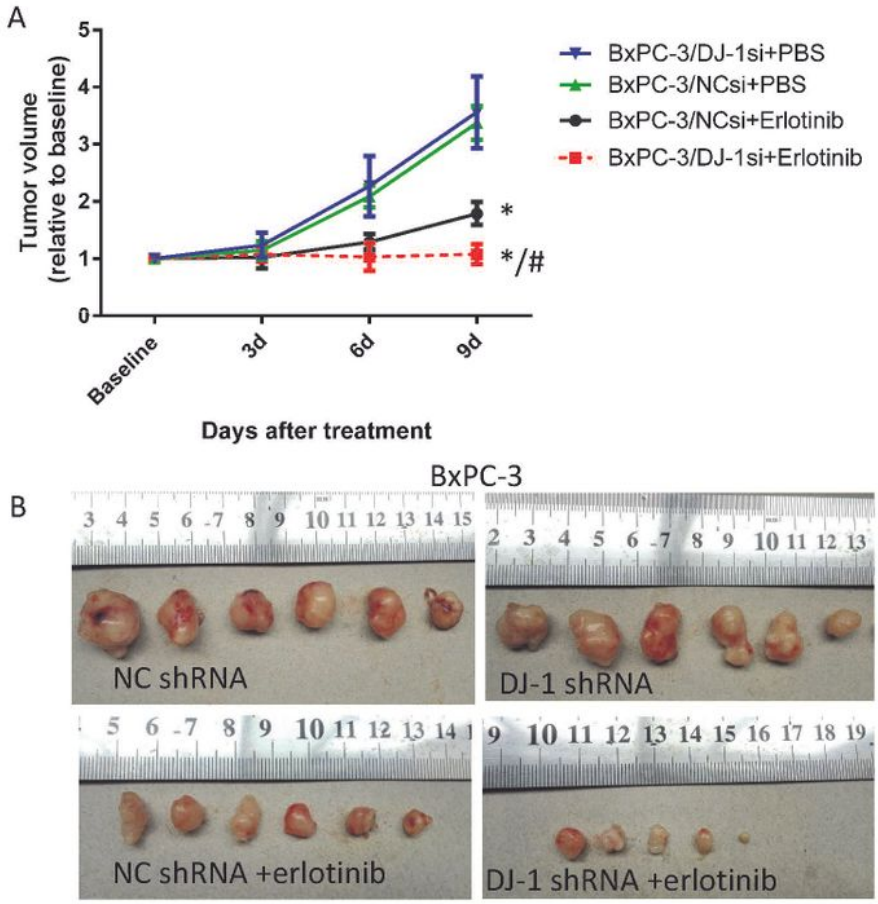

Figure 4

Figure 4

Inhibition of DJ-1 expression sensitizes pancreatic cancer cells (BxPC-3) to erlotinib treatment in vivo. BxPC-3/NC shRNA or BxPC-3/DJ-1 shRNA subcutaneous xenografts were treated with phosphate-buffered saline or erlotinib (50 mg/kg/d, intraperitoneal) for 9 d. Tumor growth is shown in (A) and an image of the tumor following removal after treatment is shown in (B).

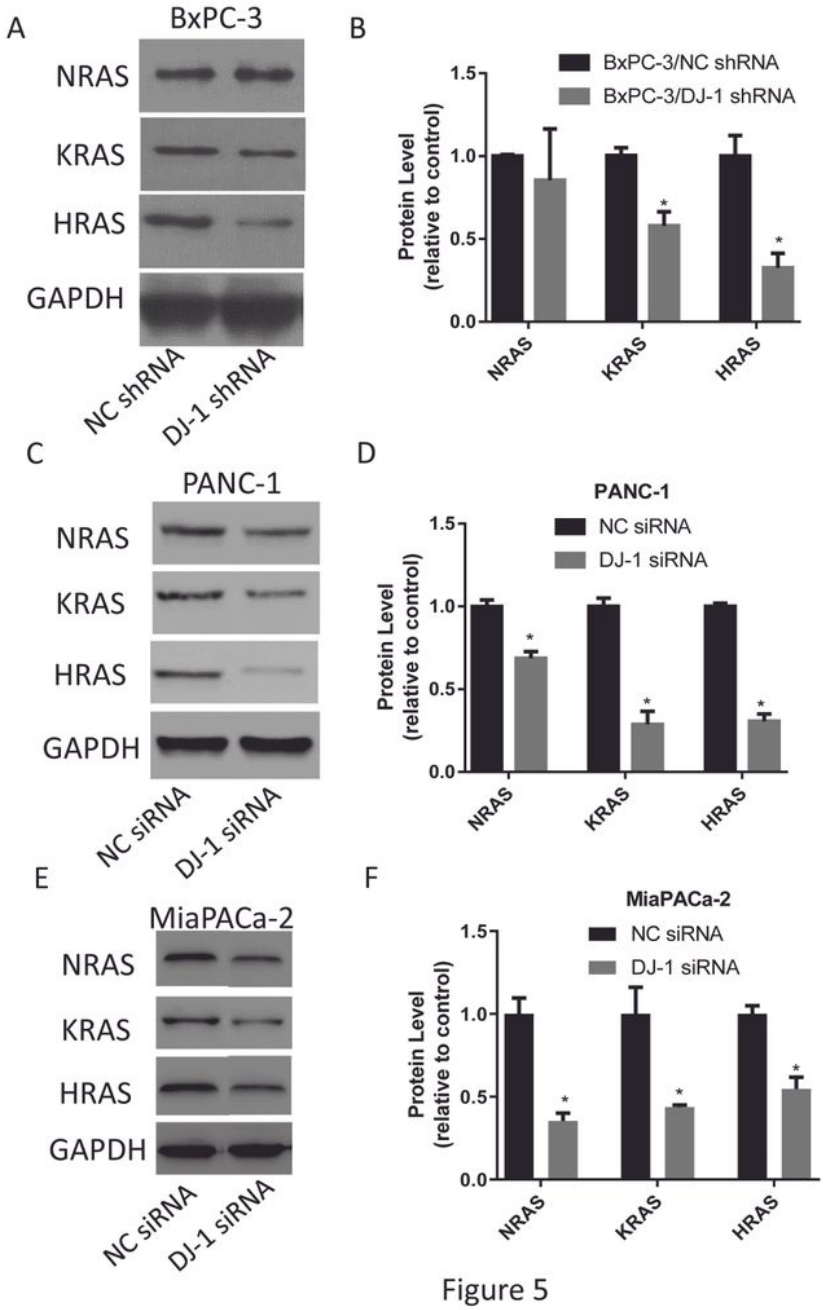

Figure 5

Figure 5

Silencing of DJ-1 expression decreased RAS expression. A. Western blot analysis of H-RAS, K-RAS, and N-RAS expression in BxPC-3/NC shRNA and BxPC-3/DJ-1 shRNA cells. B. Quantification of (A). *p < 0.05 vs. NC shRNA; n = 3. C. Western blot analysis of H-RAS, K-RAS, and N-RAS expression in PANC-1 cells transfected with DJ-1 or NC siRNA for 72 h. D. Quantification of (C). E. Western blot analysis of H-RAS, K-RAS, and N-RAS expression in MiaPACa-2 cells transfected with DJ-1 or NC siRNA for 72 h. F. Quantification of (E). *p < 0.05 vs. NC siRNA; n = 3.

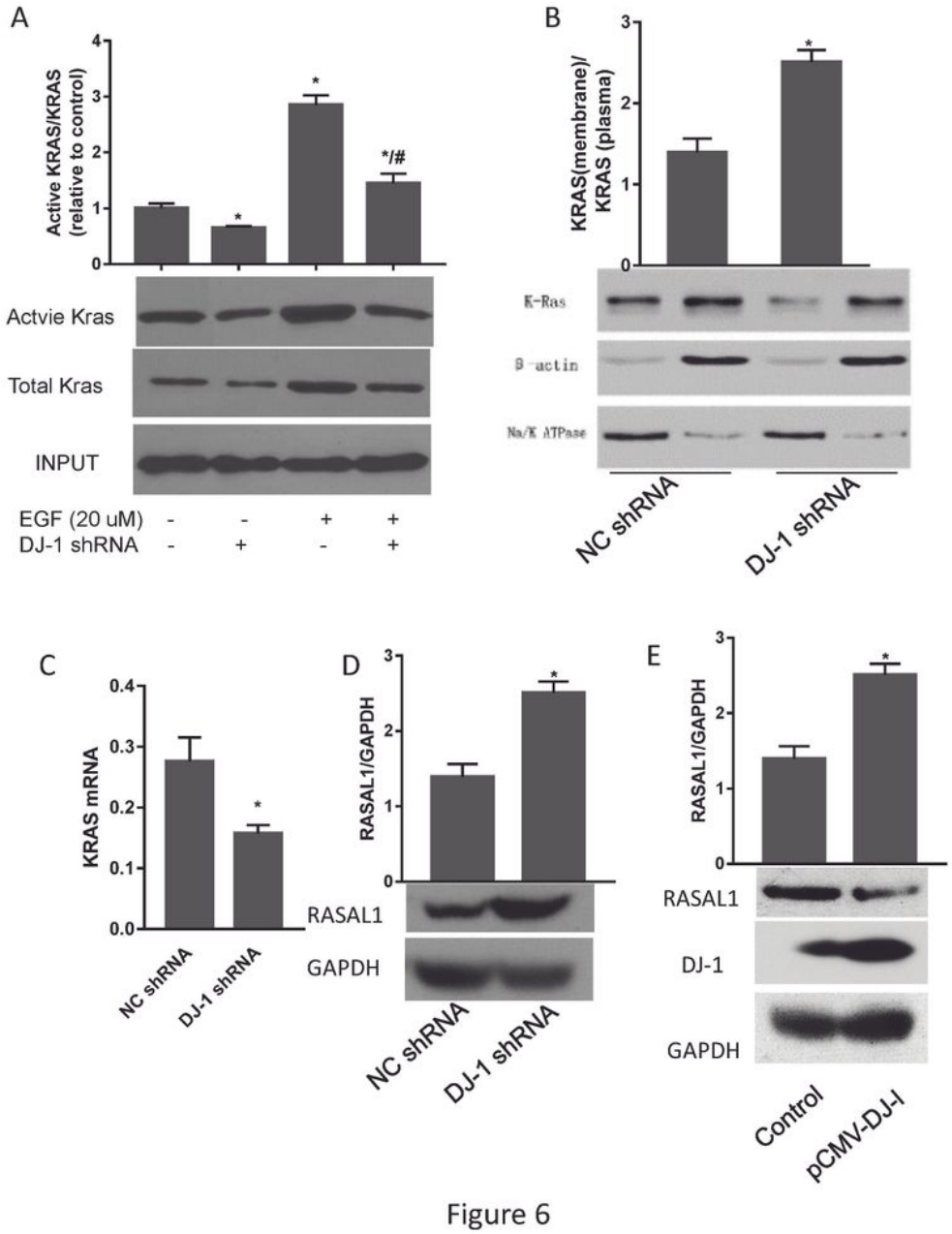

Figure 6

Figure 6

Silencing 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 and cytoplasmic K-RAS. 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. *p < 0.05 vs. control; n = 3.

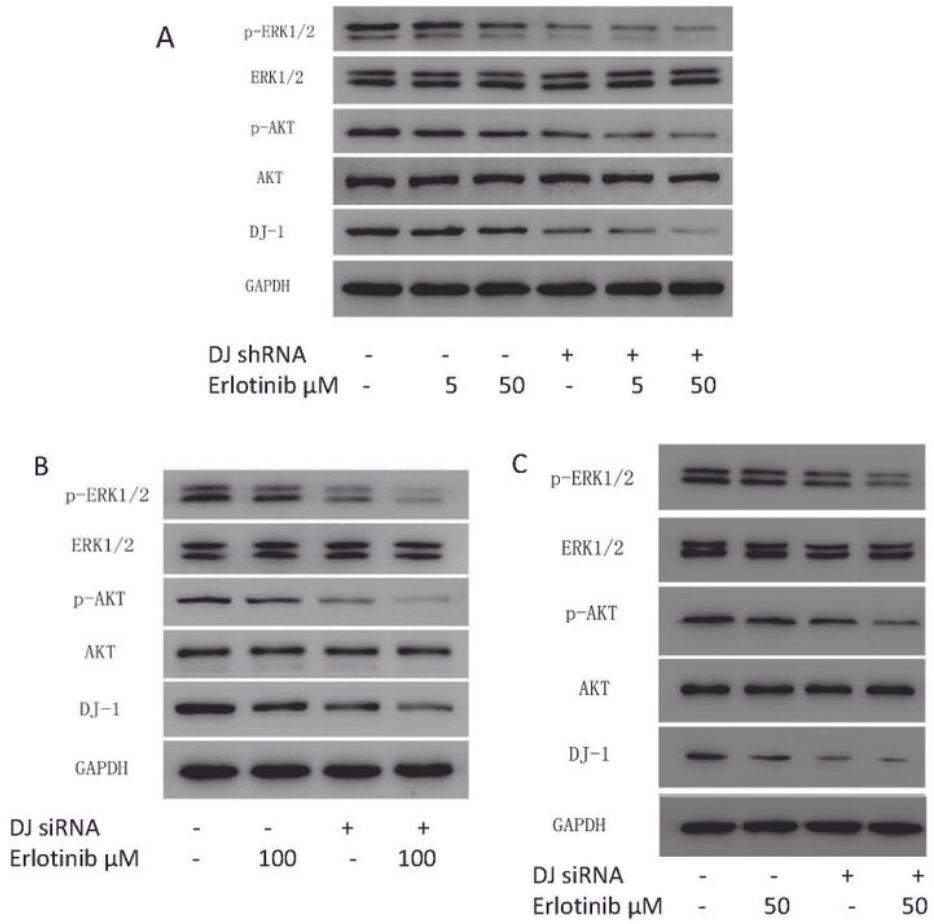

Figure 7

Figure 7

Synergistic effect of DJ-1 knockdown and erlotinib on inhibition of ERK1/2 and AKT phosphorylation. A. AKT and ERK1/2 phosphorylation in BxPC-3/NC shRNA and BxPC-3/DJ-1 shRNA cells exposed to the indicated doses of erlotinib for 72 h. B. AKT and ERK1/2 phosphorylation of PANC-1 cells transfected with DJ-1 or NC siRNA for 48 h followed by treatment with 100 μM erlotinib for 72 h. C. AKT and ERK1/2 phosphorylation of MiaPACa-2 cells transfected with DJ-1 or NC siRNA for 48 h followed by treatment with 50 μM erlotinib for 72 h.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklist2014.docx