Excessive mitochondrial fragmentation triggered by erlotinib promotes pancreatic cancer PANC-1 cell apoptosis via activating the mROS-HtrA2/Omi pathways

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Abstract

Background: Mitochondrial fragmentation drastically regulates the viability of pancreatic cancer through a poorly understood mechanism. The present study used erlotinib to activate mitochondrial fragmentation and then investigated the downstream events that occurred in response to mitochondrial fragmentation.

Methods: Cell viability and apoptosis were determined via MTT assay, TUNEL staining and ELISA. Mitochondrial fragmentation was measured via an immunofluorescence assay and qPCR. siRNA transfection and pathway blockers were used to perform the loss-of-function assays.

Results: The results of our study demonstrated that erlotinib treatment mediated cell apoptosis in the PANC-1 pancreatic cancer cell line via evoking mitochondrial fragmentation. Mechanistically, erlotinib application increased mitochondrial fission and reduced mitochondrial fusion, triggering mitochondrial fragmentation. Subsequently, mitochondrial fragmentation caused the overproduction of mitochondrial ROS (mROS). Interestingly, excessive mROS induced cardiolipin oxidation and mPTP opening, finally facilitating HtrA2/Omi liberation from the mitochondria into the cytoplasm, where HtrA2/Omi activated caspase-9-dependent cell apoptosis. Notably, neutralization of mROS or knockdown of HtrA2/Omi attenuated erlotinib-mediated mitochondrial fragmentation and favored cancer cell survival.

Conclusions: Together, our results identified the mROS-HtrA2/Omi axis as a novel signaling pathway that is activated by mitochondrial fragmentation and that promotes PANC-1 pancreatic cancer cell mitochondrial apoptosis in the presence of erlotinib.

Keywords: Erlotinib, Mitochondrial fragmentation, Mitochondrial apoptosis, mROS, HtrA2/Omi
The biological behavior of cancer is closely regulated by mitochondria [4, 5]. Sufficient ATP supply, intracellular calcium homeostasis, metabolic signaling transduction, and cell apoptosis management are affected by mitochondria [6–8]. In addition, mitochondria are also the key target of several chemotherapeutics and radiotherapies [9]. A recent study has reported that pancreatic cancer death, proliferation, and metastasis are modulated by mitochondrial homeostasis, especially mitochondrial fission [10]. Excessive mitochondrial fission induces cancer cell oxidative injury and subsequently mediates mitochondrial ATP depletion; this effect impairs PANC-1 cell proliferation and evokes mitochondrial apoptosis [10]. Notably, this conclusion is also supported by other studies. In colorectal cancer, the activation of mitochondrial fission is associated with SW837 cell apoptosis and migration inhibition [11]. In gastric cancer, abnormal mitochondrial fission contributes to cancer cell oxidative stress and energy undersupply [12]. In breast cancer, Drp1-mediated mitochondrial fission suppresses breast cancer cell invasion [13]. This information indicates that mitochondrial fission has a well-characterized role in the regulation of cancer viability. However, the downstream molecular events of mitochondrial fission activation remain to be discovered.

Based on a previous study in a mouse model of cardiac ischemia reperfusion injury, the activation of mitochondrial fission promotes the formation of mitochondrial fragmentation, and these mitochondrial deaths contain a decreased mitochondrial potential [14]. In addition, mitochondrial fragmentation can activate cell death via two mechanisms [15]; one mechanism is driven via HK2/VDAC1 disassociation-mediated mPTP opening, and the other involves mROS-induced cardiolipin oxidation. Notably, mitochondrial ROS (mROS) overloading, as a primary result of mitochondrial fragmentation [16], has been noted in different disease models such as those of gastric cancer [17], breast cancer [18], and leukemia [19]. Subsequently, excessive mitochondrial oxidative injury can activate the HtrA2/Omi-related apoptotic pathway in a manner that is dependent on caspase-9 activity [11]. This evidence indicates that the downstream effectors of mitochondrial fragmentation include mROS overproduction, HtrA2/Omi upregulation, caspase-9 activation, and mitochondrial apoptosis augmentation. Given these factors, we want to know whether mitochondrial fragmentation regulates pancreatic cancer viability via mROS-HtrA2/Omi-caspase-9 pathways.

To this end, erlotinib is the first-line anti-tumor drug for the treatment of pancreatic cancer in the clinic [20]. Several human studies have verified the efficacy of erlotinib in improving the 5-year survival rate of patients with pancreatic cancer [21, 22]. Molecular investigations report that several biological processes are modulated by erlotinib, including mTOR inhibition [23], epidermal growth factor receptor downregulation [24], and epidermal interstitial transformation (EMT) suppression [25]. However, no study that explores the role of erlotinib in triggering mitochondrial stress has been conducted. In the present study, erlotinib was applied to activate mitochondrial fragmentation in a human PANC-1 pancreatic cancer cell line. Then, we explored the regulatory mechanism of mitochondrial fragmentation on cell viability in the presence of erlotinib.
Invitrogen, #459220), complex II (CII-30, 1:1000, Abcam, #ab110410), complex IV subunit II (CIV-II, 1:1000, Abcam, #ab110268). Next, the membranes were visualized using an enhanced chemiluminescence system (ECL; Pierce Company, USA) [28].

**MTT assay, caspase activity detection and LDH release assay**

MTT was used to analyze the cellular viability [29]. Cells (1 × 10⁶ cells/well) were cultured on a 96-well plate at 37 °C with 5% CO₂. Then, 40 μl of MTT solution (2 mg/ml; Sigma-Aldrich) was added to the medium for 4 h at 37 °C with 5% CO₂. Subsequently, the cell medium was discarded, and 80 μl of DMSO was added to the wells for 1 h at 37 °C with 5% CO₂ in the dark. The OD of each well was observed at A490 nm via a spectrophotometer (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA) [30]. In brief, to measure caspase-9 activity, 5 μl of LEHD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 1 h at 37 °C. Then, the absorbance at 400 nm was recorded via a microplate reader to reflect the caspase-3 and caspase-9 activities. To analyze caspase-3 activity, 5 μl of DEVD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 2 h at 37 °C [31].

**ELISA**

Glutathione (GSH, Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. G7570; Promega Corporation, Madison, WI, USA) and SOD (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. S0056) and glutathione peroxidase GPX, (Beyotime Institute of Biotechnology, China; Catalog No. C1158) were used according to the manufacturer’s protocol [32]. In brief, to measure caspase-9 activity, 5 μl of LEHD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 1 h at 37 °C. Then, the absorbance at 400 nm was recorded via a microplate reader to reflect the caspase-3 and caspase-9 activities. To analysis caspase-3 activity, 5 μl of DEVD-p-NA substrate (4 mM, 200 μM final concentration) was added to the samples for 2 h at 37 °C.

**Small interfering RNA transfection**

To inhibit HtrA2/Omi expression, two independent siRNAs against HtrA2/Omi were transfected into PANC-1 cells according to a previous study [27]. Briefly, the cells were seeded onto 6-well plates and then incubated with Opti-Mineral Essential Medium (Invitrogen; Thermo Fisher Scientific Inc., USA). The mPTP opening rate was calculated as a ratio to that of the control group. The relative mPTP opening was measured as a ratio to that of the control group.

**Detection of mitochondrial membrane potential and mPTP opening**

To observe the mitochondrial potential, JC-1 staining (Thermo Fisher Scientific Inc., Waltham, MA, USA; Catalog No. M34152) was used. Then, 10 mg/ml JC-1 was added to the medium for 10 min at 37 °C in the dark to label the mitochondria. Normal mitochondrial potential showed red fluorescence, and damaged mitochondrial potential showed green fluorescence [36]. The mPTP opening rate was detected using calcein-AM (Sigma, Cat. No. 17783) as described previously [36]. Briefly, cells were incubated with calcein-AM for 30 min at 37 °C in the dark. Next, PBS was used to wash the cells three times. Finally, the optical density (OD) at an absorbance of 579 nm was recorded using a multifunction microplate reader (Epoch 2; BioTek Instruments, Inc., USA). The mPTP opening rate was calculated as a ratio to that of the control group [14]. The relative mPTP opening was measured as a ratio to that of the control group.

**TUNEL assay and cardiolipin staining**

Apoptotic cells were detected with an In Situ Cell Death Detection Kit (Thermo Fisher Scientific Inc., USA; Catalog No. C1024) according to the manufacturer’s protocol. Briefly, cells were fixed with 4% paraformaldehyde at 37 °C for 15 min. Blocking buffer (3% H₂O₂ in CH₃OH) was added to the wells, and then cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were incubated with TUNEL reaction mixture for 1 h at 37 °C.
DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to counterstain the nuclei, and the numbers of TUNEL-positive cells were recorded [37]. Cardiolipin oxidation was stained with 10-N-nonylacridine orange (NAO; 2 mmol/l; Molecular Probes, Eugene, OR, USA). Under normal conditions, NAO interacts with nonoxidized cardiolipin and generates a characteristic green fluorescence. However, upon cardiolipin oxidation, NAO cannot interact with cardiolipin, and this result is accompanied by a drop in green fluorescence. Accordingly, the green fluorescence intensity of NAO was used to quantify the cardiolipin oxidation with the help of Image-Pro Plus 6.0; Media Cybernetics, Rockville, MD, USA) [16].

RNA extraction and qPCR analysis

For mRNA expression analysis, total RNA was isolated using Trizol (Invitrogen, Carlsbad, California, USA) according to a previous study. Then, cDNA was synthesized using 1 mg RNA and the First-Strand Synthesis Kit (Fermentas, Flamborough, Ontario, Canada) according to a previous study [38]. The cycling conditions were as follows: 92 °C for 7 min, 40 cycles of 95 °C for 20 s and 70 °C for 45 s. β-actin was amplified as an internal standard. All the primer sequences are listed below: Drp1 forward 5′-CGT GGT ATG TCTG-3′, reverse prime 5′-AACGT-3′; OPA1 (forward prime 5′-GCT ACT TGT GCC GTA TAC-3′, reverse prime 5′-CGT TAC CTT GAG-3′), OPA1 (forward prime 5′-TGT CCA GTC CGT AAC TGA-3′, reverse prime 5′-TTC GAT ACC TGA CTTAC-3′), Mfn2 (forward prime 5′-TTGCGATTCCAATCCCTGA C-3′, reverse prime 5′-TTGAGTCCCTTTCGAGGTTTCTA-3′), Mfn2 (forward prime 5′-CCTCTTGGCTCAAGTCTCTTT-3′, reverse prime 5′-ATCCGTGTAATGATGCTGT-3′), Mff (forward prime 5′-GAACTTGCCTCTTTTGTTTCTAC-3′, reverse prime 5′-ATCCGTGTAATGATGCTGT-3′), Mff (forward prime 5′-CTTCCAGTCTCCCTAATGGGA TGA C-3′, reverse prime 5′-TGGACAGCTGGGCTTTCG-3′), reverse prime 5′-ATCCGTGTAATGATGCTGT-3′). Statistical analysis

Data are expressed as the mean±SE of triplicate samples. Statistical analysis for multiple comparisons was analyzed by a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. p values below 0.05 were considered statistically significant.

Results

Erlotinib dose-dependently promotes PANC-1 pancreatic cancer cell apoptosis

First, erlotinib was incubated with PANC-1 pancreatic cancer cells. Then, cell viability was observed using the MTT assay and LDH-cytotoxicity assay. Compared to the control group, erlotinib treatment reduced the viability of PANC-1 cells (Fig. 1a, b), and this effect was achieved in a dose-dependent manner. This finding was also found in erlotinib-treated PaCa-2 pancreatic cancer cells (Fig. 1c, d). To explore whether the reduction in cell viability was attributable to excessive cell apoptosis, the TUNEL assay was used. The number of TUNEL-positive cells was decreased in a dose-dependent manner. This finding was also found in erlotinib-treated PaCa-2 pancreatic cancer cells (Fig. 1c, d). To explore whether the reduction in cell viability was attributable to excessive cell apoptosis, the TUNEL assay was used. The number of TUNEL-positive cells was decreased in a dose-dependent manner.

Flow cytometry assay

Flow cytometry was applied as a quantitative method for evaluating mitochondrial ROS levels according to a previous study [4]. In brief, PANC-1 cells were seeded onto 6-well plates and then treated with erlotinib. Subsequently, the cells were isolated using 0.25% trypsin and then incubated with MitoSOX red mitochondrial superoxide indicator (Molecular Probes, USA) for 30 min in the dark at 37 °C. Subsequently, PBS was used to wash cell two times, and then the cells were analyzed with a FACS Calibur Flow Cytometer. Data were analyzed by FACS Diva software. The experiment was repeated three times to improve the accuracy [39]. The number of apoptotic cells was analyzed quantitatively using the Annexin V–FITC/PI Apoptosis Detection Kit (BD Biosciences, USA). After treatment, the cells were harvested, resuspended in 100 µl of binding buffer, and then incubated with 5 µl of Annexin V–FITC/binding buffer mixture (30 min, 37 °C) in the dark. Subsequently, the cells were incubated with 10 µl of propidium iodide for 5 min and immediately analyzed by bivariate flow cytometry using a BD FACSCalibur cytometer [36].

Statistical analysis

Data are expressed as the mean±SE of triplicate samples. Statistical analysis for multiple comparisons was analyzed by a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. p values below 0.05 were considered statistically significant.
**Figure 1:**

**Panel a:** MTT assay (PANC1 cells) showing cell viability with various concentrations of erlotinib.

**Panel b:** LDH release (folds) (PANC1 cells) illustrating cellular damage.

**Panel c:** MTT assay (PaCa-2 cells) demonstrating cell viability with erlotinib.

**Panel d:** LDH release (folds) (PaCa-2 cells) indicating cellular damage.

**Panel e:** TUNEL analysis of PANC1 cells and PaCa-2 cells treated with different concentrations of erlotinib.

**Panel f:** Number of TUNEL-positive PANC1 cells (%) showing cellular apoptosis.

**Panel g:** Number of TUNEL-positive PaCa-2 cells (%) indicating cell death.

**Panel h:** Caspase-3 activity (folds) (PANC1 cells) reflecting apoptosis.

**Panel i:** Caspase-3 activity (folds) (PaCa-2 cells) illustrating cell death.
counted as the apoptotic index. As shown in Fig. 1e, f, erlotinib dose-dependently increased the apoptotic index in PANC-1 cells. Similarly, the number of TUNEL-positive cells was also elevated in PaCa-2 cells upon exposure to erlotinib (Fig. 1e, g). Furthermore, since cell apoptosis is primarily executed via caspase-3 activation, caspase-3 activity was determined via ELISA. Compared to the control group, caspase-3 activity was relatively increased in response to erlotinib treatment (Fig. 1h), which is suggestive of caspase-3 activation by erlotinib. This alteration was also noted in PaCa-2 cells (Fig. 1I). These data were further supported via quantitative analysis of cell apoptosis with the help of flow cytometry (Additional file 1: Figure S1). Together, our results indicated that erlotinib dose-dependently promoted PANC-1 and PaCa-2 cell apoptosis. Notably, no phenotypic difference was noted in erlotinib-mediated apoptosis in PANC-1 cells or PaCa-2 cells, and thus PANC-1 cells were used in the following study. In addition, we have found that the minimum concentration of erlotinib that induces cell death was 10 μM, and thus, 10 μM erlotinib was used to conduct the molecular investigations.

**Erlotinib induces mitochondrial fragmentation in PANC-1 pancreatic cancer cells via elevating mitochondrial fission and repressing mitochondrial fusion**

Subsequently, the mitochondrial morphology was observed via an immunofluorescence assay using a Tom-20 antibody [40]. Compared to the control group, we found that erlotinib treatment mediated the formation of mitochondrial fragmentation (Fig. 2a). Then, the average length of the mitochondria was measured after erlotinib treatment and was used to quantify mitochondrial fragmentation. As shown in Fig. 2b, the mean length of the mitochondria was ~9.1 μm at baseline. However, after treatment with erlotinib, the mean length of mitochondria was reduced to ~2.3 μm (Fig. 2b). In addition, the fluorescence intensity of Mff, an activator of mitochondrial fragmentation, was obviously increased in response to erlotinib treatment, compared to that in control group (Fig. 2c). Subsequently, to further confirm the promotive effect of erlotinib on mitochondrial fragmentation, Mdivi-1, an antagonist of mitochondrial fragmentation, was added into the medium of erlotinib-treated cells. Meanwhile, FCCP, an agonist of mitochondrial fragmentation, was used to incubate with normal cells, which was used as the positive control group. Then, mitochondrial fission, mitochondrial length and Mff expression were evaluated again. Compared to the control group, FCCP triggered mitochondrial fragmentation and upregulated Mff expression, similar to the results obtained via supplementation with erlotinib (Fig. 2a–c). However, Mdivi-1 treatment abrogated the promotive effect of erlotinib on mitochondrial fragmentation.

Notably, the fragmented mitochondria could be the result of increased mitochondrial fission and decreased mitochondrial fusion. To verify the alterations of mitochondrial fission/fusion, qPCR was performed to analyze the transcription factors that are related to mitochondrial fission/fusion. In response to erlotinib treatment, the transcription of 6-fission factors such as Drp1 and Mff were significantly upregulated (Fig. 2d–g), indicative of mitochondrial fission activation by erlotinib. In contrast, the transcription and expression of pro-fusion factors, such as Mfn2 and Opal1 were obviously downregulated in response to erlotinib treatment (Fig. 2d–j), suggesting that mitochondrial fusion was repressed by erlotinib. Together, our results confirmed that erlotinib promoted mitochondrial fragmentation in PANC-1 cells.

**Mitochondrial fragmentation induces oxidative stress via mitochondrial ROS (mROS)**

Additional experiments were performed to explore the downstream events of mitochondrial fragmentation. Based on a previous study, mitochondrial fragmentation was associated with cellular oxidative stress via mROS overloading [41]. To confirm this, a mROS probe and flow cytometry were used to quantify mROS levels after erlotinib treatment. As shown in Fig. 3a, b, the level of mROS was significantly elevated in response to erlotinib treatment. To validate whether mitochondrial fragmentation was required for mROS overloading, Mdivi-1 and FCCP were used. FCCP treatment elevated the ROS production in control group, similar to the results obtained...
vis supplementation of erlotinib. However, Mdivi-1 application attenuated erlotinib-mediated mROS overloading (Fig. 3a, b), indicating the necessary role that is played by mitochondrial fragmentation in mROS generation. Excessive mROS production would induce cellular oxidative injury. To confirm this, an ELISA assay was used to observe alterations in the levels of cellular antioxidants.

Compared to the control group, the concentration of Mn-SOD, GSH and GPX were markedly reduced after erlotinib treatment (Fig. 3c–e). In contrast, the level of MDA, an end product of the peroxidation of lipids in the cell membrane, was increased in response to erlotinib treatment (Fig. 3f). Interestingly, blockade of mitochondrial fragmentation via Mdivi-1 could decrease the level...
of antioxidants and suppress the production of MDA (Fig. 3c–f). Excessive oxidative injury can also disrupt cellular energy metabolism. Accordingly, total ATP production was measured using ELISA. Compared to the control group, erlotinib treatment significantly reduced the ATP production in PANC-1 cells (Fig. 3g), and this effect could be reversed by Mdivi-1. Furthermore, we also found that the expression of proteins related to mitochondrial ATP synthesis were notably downregulated in response to erlotinib (Fig. 3h–k); this effect was abrogated by Mdivi-1. Accordingly, our data indicated that mitochondrial fragmentation evoked mitochondrial ROS overloading and oxidative stress in PANC-1 cells.

Mitochondrial fragmentation-mediated mROS promotes HtrA2/Omi liberation

Next, experiments were performed to observe the consequence of mROS-mediated cell oxidative stress. Based on a previous report [42], excessive mROS could cause mitochondrial membrane permeabilization, which facilitates the translocation of mitochondrial proapoptotic factors to the nucleus/cytoplasm [43]. In the present study, an immunofluorescence analysis demonstrated that erlotinib increased the migration of HtrA2/Omi to nucleus when compared to the control group (Fig. 4a, b). Interestingly, this effect of erlotinib could be abolished via Mdivi-1 (Fig. 4a, b). Subsequently, western blotting was performed to quantify HtrA2/Omi liberation. As shown in Fig. 4c–e, compared to the control group, erlotinib treatment increased the levels of cytoplasmic HtrA2/Omi (cyto-HtrA2/Omi) and reduced the expression of mitochondrial HtrA2/Omi (mito-HtrA2/Omi). Similar results were also observed in cytoplasmic cyt c (cyt c) liberation from mitochondria (Fig. 4c–f). However, Mdivi-1 treatment depressed the erlotinib-mediated HtrA2/Omi and cyt c translocation from mitochondria into the cytoplasm. These results indicated that mitochondrial fragmentation accounted for HtrA2/Omi liberation.

At the molecular level, HtrA2/Omi is primarily expressed in the inner membrane of mitochondria. Based on a recent study, the liberation of HtrA2/Omi from mitochondria into the cytoplasm is dependent on cardiolipin oxidation and mPTP opening [15, 44]. First, the oxidation of cardiolipin lowers the affinity of HtrA2/Omi to the mitochondria. Second, the opening of mPTP provides a channel for HtrA2/Omi leakage [45]. Given the role of mitochondrial fragmentation in cellular oxidative stress via mROS overproduction, we examined whether mROS was required for the mitochondrial fragmentation-mediated HtrA2/Omi liberation via modulating cardiolipin oxidation and mPTP opening. To support our hypothesis, cardiolipin oxidation was determined via staining with NAO, which is a cardiolipin probe. Under physiological conditions, NAO could interact with cardiolipin to display a green fluorescence. In response to cardiolipin oxidation, NAO cannot bind to oxidized cardiolipin, and thus the green fluorescence is reduced. As shown in Fig. 4g, h, the fluorescence of cardiolipin was significantly downregulated in response to erlotinib, and this effect was reversed by Mdivi-1. To verify whether mROS was responsible for cardiolipin oxidation, mitoQ was used to neutralize the mitochondrial fragmentation-produced mROS. Interestingly, mitoQ treatment also reversed the green fluorescence intensity of cardiolipin (Fig. 4g, h), similar to the results obtained via supplementation with Mdivi-1. These results verified the role played by mROS in cardiolipin oxidation. In addition, we also found that the mPTP opening rate was significantly increased in response to erlotinib (Fig. 4i), and this effect was inhibited by Mdivi-1 or mitoQ (Fig. 4i). Together, our data demonstrated that the mitochondrial fragmentation-mediated mROS regulated HtrA2/Omi liberation via inducing cardiolipin oxidation and mPTP opening.

Released HtrA2/Omi induces caspase-9-dependent apoptosis

After it is released into the cytoplasm, HtrA2/Omi can interact with and activate mitochondrial apoptosis in a manner that is dependent on caspase-9 activity [11]. Notably, an early feature of caspase-9–related apoptosis is the reduction of mitochondrial potential. In the present study, a JC-1 kit was used to stain for the mitochondrial potential. The results indicated that erlotinib treatment significantly reduced the mitochondrial potential (Fig. 5a, b), and this effect was inhibited by Mdivi-1. To confirm
whether HtrA2/Omi accounted for the mitochondrial potential collapse, two independent siRNAs were used. After knockdown of HtrA2/Omi, the mitochondrial potential was analyzed again. Compared to the erlotinib-treated group, the loss of HtrA2/Omi stabilized the mitochondrial potential (Fig. 5a, b), an effect that was similar
to the results obtained via treatment with Mdivi-1. Furthermore, the last characteristic of caspase-9-related apoptosis is the activation of caspase-9, an effect that is accompanied by an increase in proapoptotic proteins. In the present study, the protein activity (Fig. 5c) and expression (Fig. 5d, e) of caspase-9 were both upregulated in answer to erlotinib stress and these effects could be repressed by Mdivi-1 or HtrA2/Omi siRNA. As a consequence of caspase-9 activation, the levels of proapoptotic factors such as Bad and Bax were significantly increased in response to erlotinib treatment, and this effect was negated by Mdivi-1 treatment or HtrA2/Omi siRNA transfection (Fig. 5f–j). By comparison, the expression of antiapoptotic proteins, including Bcl-2 and survivin, were obviously downregulated by erlotinib (Fig. 5f–j) and were reversed to near-normal levels with Mdivi-1 treatment or HtrA2/Omi knockdown. Together, our results indicated that mitochondrial fragmentation activated caspase-9-dependent apoptosis via HtrA2/Omi.

Mitochondrial fragmentation also modulated PANC-1 cell proliferation via mROS-HtrA2/Omi pathways

To this end, we asked whether mitochondrial fragmentation was involved in PANC-1 cell proliferation via the mROS-HtrA2/Omi pathways. First, the EdU assay was conducted to observe cellular proliferation. As shown in Fig. 6a, b, compared to the control group, erlotinib treatment significantly reduced the ratio of EdU-positive cells; this effect was repressed by Mdivi-1 (Fig. 6a, b). In addition, the neutralization of mROS via mitoQ and knockdown of HtrA2/Omi via siRNA transfection also reversed the number of EdU-positive cells after erlotinib treatment (Fig. 6a, b). These results indicated that mitochondrial fragmentation affects the cell proliferation in PANC-1 cell via the mROS-HtrA2/Omi axis. Further, the cell proliferation is primarily regulated by CDK4 and Cyclin D1. Cyclin E and Cyclin D1 interact with each other and generate cyclin-dependent kinase (Cdk)4/6-cyclin D and/or Cdk2-cyclin E complexes, which accelerate transition from the G0/G1 to S stage, according to the previous study [46]. We have provided the references for this. With the help of a western blotting assay, we found that the expression of CDK4 and Cyclin D1 were both reduced in response to erlotinib treatment, and this effect was negated by Mdivi-1 (Fig. 6c–e). Interestingly, the neutralization of mROS via mitoQ and knockdown of HtrA2/Omi via siRNA transfection also reversed the levels of CDK4 and Cyclin D1. Together, our results confirmed that PANC-1 cell proliferation was regulated by erlotinib via mitochondrial fragmentation in a manner that was dependent on the mROS-HtrA2/Omi pathways.

Discussion

According to the previous findings, mitochondrial fission has been acknowledged as a potential target to reduce the proliferation, migration and survival of PANC-1 pancreatic cancer cells [10]. Excessive mitochondrial fission promotes mitochondrial fragmentation [15]. Fragmented mitochondria induce damage to mitochondrial structure and function, eventually interrupting the cellular ATP supply and activating the apoptosis response [47, 48]. However, the detailed molecular mechanism by which mitochondrial fragmentation triggers mitochondrial damage and cellular apoptosis remains unclear. Our study provides an answer to this question. We used different doses of ERL to screen its proapoptotic effect in two types of cancer cell lines. Then, we used the minimal lethal dose of ERL to investigate its apoptotic mechanism, with a focus on mitochondrial damage. We observed the minimal lethal dose of ERL has an ability to induce the mitochondrial fragmentation and this finding may explain one of the mechanisms by which ERL mediated cancer cell apoptosis. Notably, whether higher dose of ERL could activate other signaling pathway to induce cell apoptosis requires further investigation. Our data illustrated that erlotinib treatment promoted mitochondrial fragmentation that occurred via increased mitochondrial fission and decreased mitochondrial fusion. Subsequently, excessive mitochondrial fragmentation triggered mROS overloading, leading to cellular oxidative stress and disordered energy metabolism. In addition, mROS overproduction was closely associated with cardiolipin oxidation and mPTP opening, favoring HtrA2/Omi liberation from mitochondria into the cytoplasm. As a consequence of HtrA2/Omi leakage, reduction of the mitochondrial potential and caspase-9 activation were
**Figure 3: JC-1 staining for mitochondrial potential**

(a) Cont, ERL+ Cont, ERL+ si-HtrA2/Omi, ERL+ si2-HtrA2/Omi, ERL+ Mdivi-1. Scale bar: 50 μm.

**Figure 4: Caspase-9 activity**

(b) Ratio of red to green fluorescence intensity for different treatments: ERL+ Cont, ERL+ si-HtrA2/Omi, ERL+ si2-HtrA2/Omi, ERL+ Mdivi-1.

(c) Caspase-9 activity (folds) for different treatments: ERL+ Cont, ERL+ si-HtrA2/Omi, ERL+ si2-HtrA2/Omi, ERL+ Mdivi-1.

**Figure 5: Bax and Bad expression**

(d) Cont, ERL+ Cont, ERL+ si-HtrA2/Omi, ERL+ si2-HtrA2/Omi, ERL+ Mdivi-1.

**Figure 6: Bcl2 and Survivin expression**

(e) Bax expression for different treatments: Ctrl, ERL+si-cont, ERL+si-HtrA2/Omi, ERL+si2-HtrA2/Omi, ERL+Mdivi-1.

(f) Bad expression for different treatments: Ctrl, ERL+si-cont, ERL+si-HtrA2/Omi, ERL+si2-HtrA2/Omi, ERL+Mdivi-1.

**Figure 7: Bcl2 and Survivin expression**

(g) Bcl2 expression for different treatments: Ctrl, ERL+si-cont, ERL+si-HtrA2/Omi, ERL+si2-HtrA2/Omi, ERL+Mdivi-1.

(h) Survivin expression for different treatments: Ctrl, ERL+si-cont, ERL+si-HtrA2/Omi, ERL+si2-HtrA2/Omi, ERL+Mdivi-1.

**Supplementary Figure:**

(i) Western blot analysis for Bax, Bad, Survivin, Bcl2, and GAPDH.
noted, and these alterations were accompanied by an upregulation of proapoptotic proteins and a downregulation of antiapoptotic factors. Overall, we demonstrated for the first time that erlotinib-activated mitochondrial fragmentation mediated PANC-1 apoptosis via the mROS-HtrA2/Omi pathways. This finding fills the knowledge gap regarding how mitochondrial fragmentation induces mitochondrial damage and triggers the apoptotic pathway.
Mitochondrial fission and fusion are a part of mitochondrial dynamics. Under physiological conditions, the mitochondrial network undergoes moderate fission and fusion to fill the requirements for cellular metabolism [49, 50]. Mild levels of mitochondrial fission help the mitochondria in generating daughter mitochondria, whereas moderate levels of mitochondrial fusion provides the energy for communication between the mitochondrial network [51, 52]. Interestingly, uncontrolled mitochondrial fission generates massive amounts of fragmented mitochondria and disrupts mitochondrial homeostasis. Previous studies have identified mitochondrial fragmentation, which is produced by mitochondrial fission, as the apoptotic trigger in various disease models. For instance, in fatty liver disease, mitochondrial fragmentation promotes the apoptosis of hepatocytes and the progression of liver fibrosis by decreasing mitophagy [53]. In neurodegenerative illness such as Alzheimer’s disease, excessive mitochondrial fragmentation disturbs mitochondrial energy metabolism and causes neuronal oxidative injury [54]. In addition, in rectal cancer, activated mitochondrial fragmentation limits tumor proliferation and augments cancer apoptosis [11]. In accordance with these findings, our data also illustrated the necessary role played by mitochondrial fragmentation in initiating pancreatic cancer PANC-1 cell death. Thus, mitochondrial fragmentation would be considered as a tumor-suppressor and strategies to promote mitochondrial fragmentation are of significant importance in the design of anti-cancer drugs.

Although the proapoptotic effect of mitochondrial fragmentation has been well-documented, the detailed mechanisms by which mitochondrial fragmentation induces mitochondrial damage and activates cellular apoptosis are incompletely understood. In the present study, we found that mitochondrial fragmentation modulated mitochondrial homeostasis and cell viability through two mechanisms. One mechanism was driven by the promotion of mROS-mediated cell oxidative injury, and the other involved the HtrA2/Omi liberation-induced caspase-9 activation. First, mitochondrial fragmentation generated superfluous amounts of mROS, and the excess mROS induced cardiolipin oxidation and mPTP opening [55]. Subsequently, oxidized cardiolipin and increased mPTP opening worked together to augment the release of HtrA2/Omi from mitochondria into the cytoplasm, where HtrA2/Omi reduced the mitochondrial potential and induced caspase-9 activation. This information was also consistent with previous studies. In cardiac ischemia–reperfusion injury, excessive mitochondrial fragmentation-induced mitochondrial DNA damage evokes mROS overproduction and cardiolipin oxidation [14, 15]. Additionally, in oral cancer, mitochondrial fragmentation-related cardiolipin oxidation and mPTP opening eventually contribute to caspase-involved cellular apoptosis [56].

In the present study, we used erlotinib to activate mitochondrial fragmentation and found that erlotinib-induced PANC-1 cellular apoptosis could be inhibited by Mdivi-1, which is an antagonist of mitochondrial fragmentation. To the best of our knowledge, this is the first study to investigate the role of erlotinib in mitochondrial stress. Although erlotinib has been tested in several human clinical studies [3, 57], its pharmacological mechanism has not been adequately explored. Our study proposed that the anti-cancer property of erlotinib relied on the activation of mitochondrial fragmentation by upregulating mitochondrial fission and downregulating mitochondrial fusion. Notably, the dose selection of ERL was according to a previous study [26] and this selection may be also relied on the types of cancer cell lines. In clinical practice, different doses of ERL have been used according to the tumor staging and pathologic grading. Further insight is required to figure out the appropriate concentration of ERL on different types of pancreatic cancer. Besides, there are several limitations in the present study. Although we used two pancreatic cancer cell lines to screen the role of erlotinib, an animal study is necessary to further support our finding. In addition, human evidence is also required to validate the tumor-suppressive effects of mitochondrial fragmentation in response to erlotinib treatment.

Conclusion
Collectively, our results reported that mitochondrial fragmentation, which was activated by erlotinib, regulated the viability of the PANC-1 pancreatic cancer cell line via the mROS-HtrA2/Omi pathways. This conclusion provides a potential target to modify pancreatic cancer viability via augmenting mitochondrial fragmentation and activating the mROS-HtrA2/Omi pathways.

Additional file

**Additional file 1: Figure S1.** The proapoptotic effect of erlotinib on PANC1 cells using Annexin V/PI staining. Early apoptosis (Annexin V+/PI− cells) and late apoptosis (Annexin V+/PI+ cells) were counted. *p* < 0.05 vs. control group.

**Authors’ contributions**
JW, JC, and LW were involved in the conception and design, performance of experiments, data analysis and interpretation, and manuscript writing. KPW, XPH, YLZ, HK and SZ were involved in data analysis and interpretation. All authors read and approved the final manuscript.
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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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