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Original Research

Lapatinib promotes ovarian cancer cell apoptosis through mROS-HtrA2/Omi pathways

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Summary

Mitochondria play a pivotal role in regulating the viability of cancer cells. The role of lapatinib in cancer is poorly understood. This study investigated the downstream response to mitochondrial fragmentation using lapatinib to activate mitochondria. In this study cell viability was measured by MTT and LDH leakage assays. Apoptosis was determined by enzyme-linked immunosorbent assay (ELISA) and Annexin V/PI staining. Mitochondrial fragmentation was measured by qPCR and protein expression was detected by Western blot. Results showed that lapatinib increased ovarian cancer cell apoptosis in SKOV-3 cells via triggering mitochondrial fragmentation. Subsequently, mitochondrial fragmentation increased ROS production and facilitated HtrA2/Omi liberation from the mitochondria into the cytoplasm which inturn activated caspase-dependent cell apoptosis. The present results identified the mROS-HtrA2/Omi axis as a novel signaling pathway that is activated by mitochondrial fragmentation and promotes ovarian cancer cell apoptosis with lapatinib treatment.

Key words: Ovarian carcinoma; Lapatinib; Mitochondria; mROS-HtrA2/Omi.

Introduction

Ovarian cancer is the deadliest gynecologic malignancies in many countries characterized as asymptomatic or nonspecific at the early stage. Majority of patients with ovarian carcinoma are diagnosed with advanced stage disease [1]. The standard treatment strategies, cytoreductive surgery and combination chemotherapies have limited effects on ovarian carcinoma. Tumor relapse and drug-resistance are still a severe problem in clinics [2, 3]. Therefore, there is an urgent need to explore the molecular features of ovarian cancer growth and death in order to control the disease progression and bring effective clinical benefits to patients.

Mitochondria play an important role in regulating the biological behavior of cancer cells through ATP supply, metabolic signal transduction, intracellular calcium homeostasis, and apoptosis [4-7]. As one of the key targets of several chemotherapeutics and radiotherapies, studies have been focusing on mitochondrial homeostasis, especially mitochondrial fission [8]. Excessive mitochondrial fission induces cancer cell injury and subsequent ATP depletion, further impairing cell proliferation leading to apoptosis [9]. These studies have indicated the well-characterized role of mitochondrial fission in regulating cancer cell viability. Unfortunately, the downstream molecular events of mitochondrial fission remain poorly understood.

Activation of mitochondrial fission decreases mitochondrial membrane potential and increases the formation of mitochondrial fragmentation [10]. Mitochondrial fragmentation causes cell death via ROS-induced oxidation. Subsequently, mitochondrial oxidative injury can also activate the HtrA2/Omi-related apoptotic pathway [11]. As a dual tyrosine kinase inhibitor interrupting the HER2/neu and epidermal growth factor receptor pathways, lapatinib is an orally administered drug for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpressing HER2 [12, 13]. However, the role of lapatinib in triggering mitochondrial stress has not been explored in ovarian cancer. In this study, lapatinib was used to treat human ovarian cancer cell line to investigate if it could activate mitochondrial fragmentation affecting the cell viability.

Materials and Methods

The ovarian carcinoma cell line SKOV-3 was purchased and cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin/penicillin at 37 °C in an atmosphere of 5% CO_2. FCCP and mitochondrial division inhibitor 1 were used to activate and inhibit mitochondrial fragmentation, respectively.

After treatment, SKOV-3 cells were seeded in 96-well plates at a density of 4×10^4/100 μl and 10 μl MTT solution was added to each well, and incubated at 37 °C for four hours. 100 μl DMSO was then added in each well to dis-

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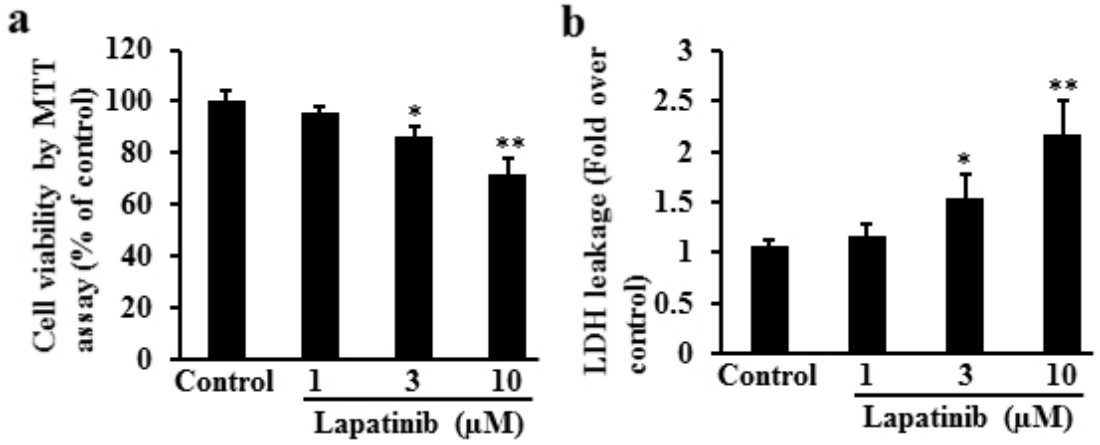

Figure 1. — Lapatinib inhibited SK-OV-3 cell proliferation. SK-OV-3 cells were treated with lapatinib at 1, 3, 10 μM for 48 h. Cell viability was measured by MTT (a) and LDH leakage (b) assays. Data were expressed as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ *vs.* control group.

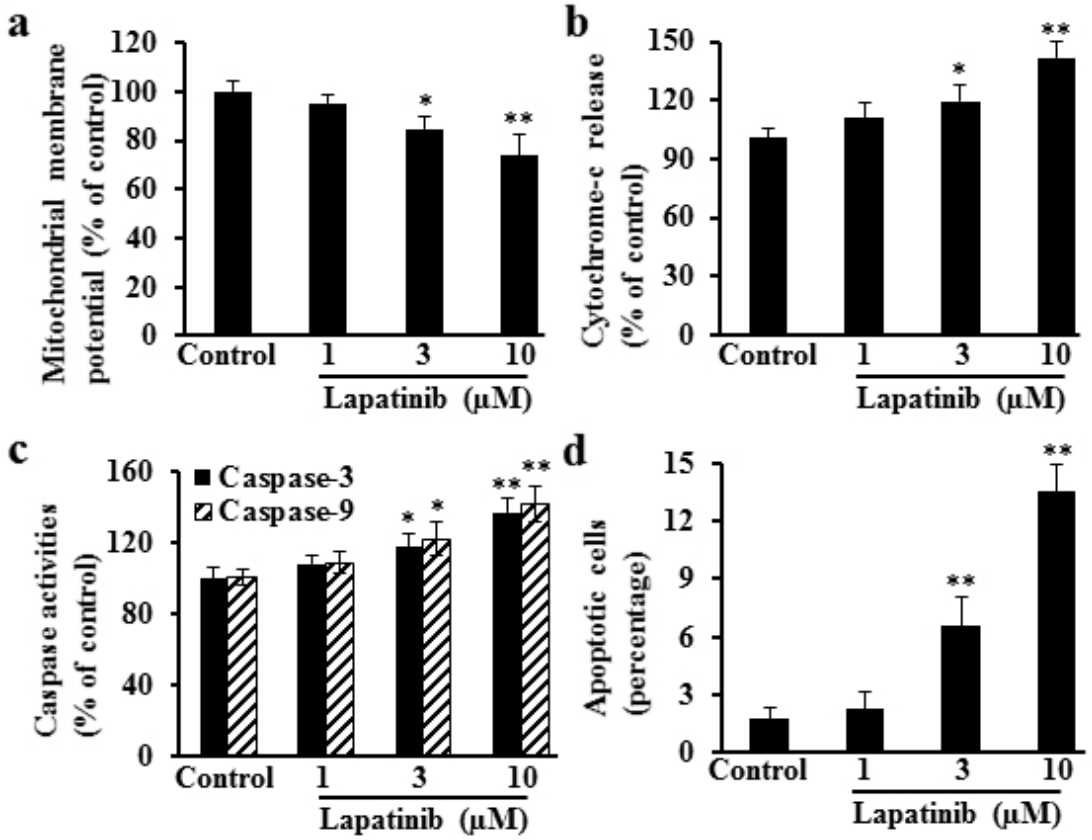

Figure 2. — Lapatinib induced SK-OV-3 cell apoptosis. After treatment, lapatinib caused MMP depolarization (a); increased the release of cytochrome-c (b); increased caspase activities (c); and increased apoptosis detected by Annexin V/PI staining (d). Data were expressed as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ *vs.* control group.

solve the formazan. The absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm.

After treatment, the culture medium was collected and centrifuged at 3000 rpm for 10 minutes. The supernatant was used to measure the LDH activity according to the manufacturer's instructions on a microplate reader at 490 nm.

Mitochondrial membrane potential (MMP) was measured with the fluorescent probe JC-1. After treatment, SKOV-3 cells were incubated with 2 μM JC-1 at 37 °C for

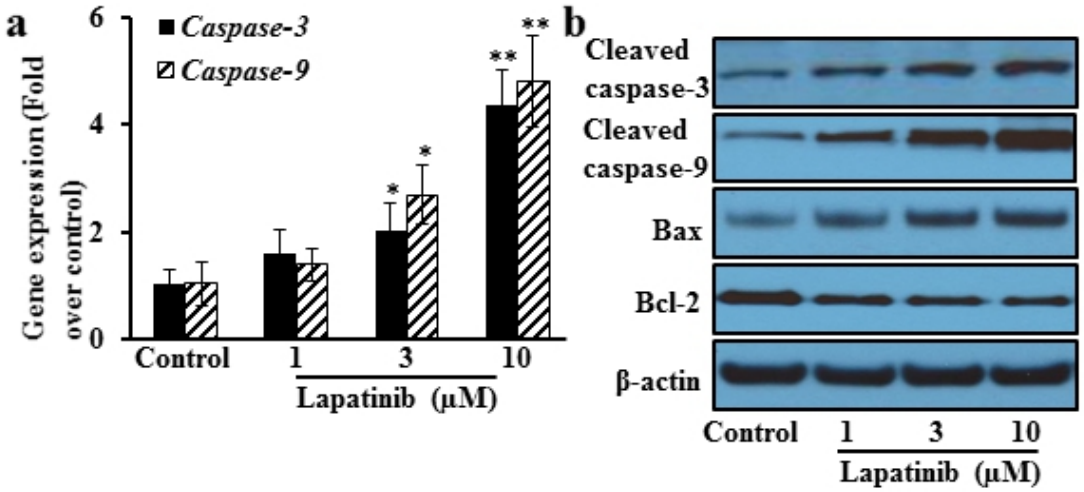

Figure 3. — Lapatinib changed apoptosis-related gene and protein expression in SK-OV-3 cells. After treatment, lapatinib increased caspase gene expression (a); increased pro-apoptotic protein expression and decreased anti-apoptotic protein expression (b). Data were expressed as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ *vs*. control group.

15 minutes in the dark. The fluorescent dye JC-1 labels mitochondria with a low membrane potential green and a high membrane potential red. The fluorescence was assessed with excitation at 490 nm and with emission at 590/530 nm on a fluorescence microplate reader.

SKOV-3 cells were fractionated after treatment. The cytochrome-c was measured by the assay kit according to the manufacturer's instructions. The optical density was measured on an ELISA plate reader with a wavelength of 490 nm.

After treatment, SK-OV-3 cells were lyzed and analyzed for caspase-3 (Ac-DEVD-Amc, 390/475 nm) and caspase-9 (Ac-LEDH-Afc, 400/505 nm) activities using the fluorescent assay kit, respectively, according to the manufacturer's instruction. After treatment, 2×10^6 SKOV-3 cells were harvested and washed with pre-chilled PBS, and then re-suspended in 500 μl binding buffer. Five microliters of Annexin V-FITC and PI were added and then samples were incubated for 10 minutes in the dark at room temperature. Analysis was performed by FACScan flow cytometer. Data were analyzed by FlowJo V10.

Mitochondrial ROS and Mff levels were measured by flow cytometry. After treatment, cells were incubated with MitoSOX red mitochondrial superoxide indicator in the dark at 37 °C for 30 minutes. Cells were washed twice and analyzed with a FACScan flow cytometer. Data were analyzed by FlowJo V10.

RNA was extracted with TRIzol reagent and cDNA was generated by adding 0.5 μg of the total RNA to SuperScript master mix and performing reverse transcription. Quantitative PCR was conducted using SYBR Green Supermix with comparative C_t value method to quantify the expression in different samples. The mRNA levels were normalized to that of a house-keeping gene β-actin. Protein was extracted and quantified with the BCA protein assay kit. Equal amounts of protein (50 μg) were subjected to electrophoresis on the 4-12% (v/v) SDS-polyacrylamide gel. Protein was then electroblotted from gel to a PVDF membrane. The membrane was blocked with 5% non-fat milk at room temperature for one hour, and incubated with indicated primary antibodies overnight, followed by incubating with the secondary antibody for one hour. The signals were detected by enhanced chemiluminescence kit (ECL).

Data were expressed as mean ± SD of triplicate samples and analyzed by one-way analysis of variance followed by Dunnett's t test with SPSS 19.0 software. $p < 0.05$ was considered significant difference.

Results

MTT and LDH leakage assays were used to determine the anti-proliferative activities of lapatinib on SKOV-3 cells. Comparing to the control group, lapatinib treatment reduced the viability of SKOV-3 cells in a dose-dependent manner (Figure 1).

Apoptosis-related markers were identified after lapatinib treatment. Compared to control, lapatinib treatment decreased MMP (Figure 2a), increased release of cytochrome-c (Figure 2b) as well as activities of caspase-3/9 (Figure 2c), and induced apoptosis in SKOV-3 cells (Figure 2d). Expression of pro-apoptotic genes and proteins increased, but anti-apoptotic proteins expression decreased after lapatinib treatment (Figure 3).

Lapatinib treatment significantly increased the fluorescence intensity of Mff, an activator of mitochondrial fragmentation, similar to the results of positive control FCCP, an agonist of mitochondrial fragmentation (Figure 4a). To further confirm the promotive effect of lapatinib on mitochondrial fragmentation, Mdivi-1, an antagonist of mitochondrial fragmentation, was used. Mdivi-1 treatment abrogated the promotive effect of lapatinib on mitochondrial

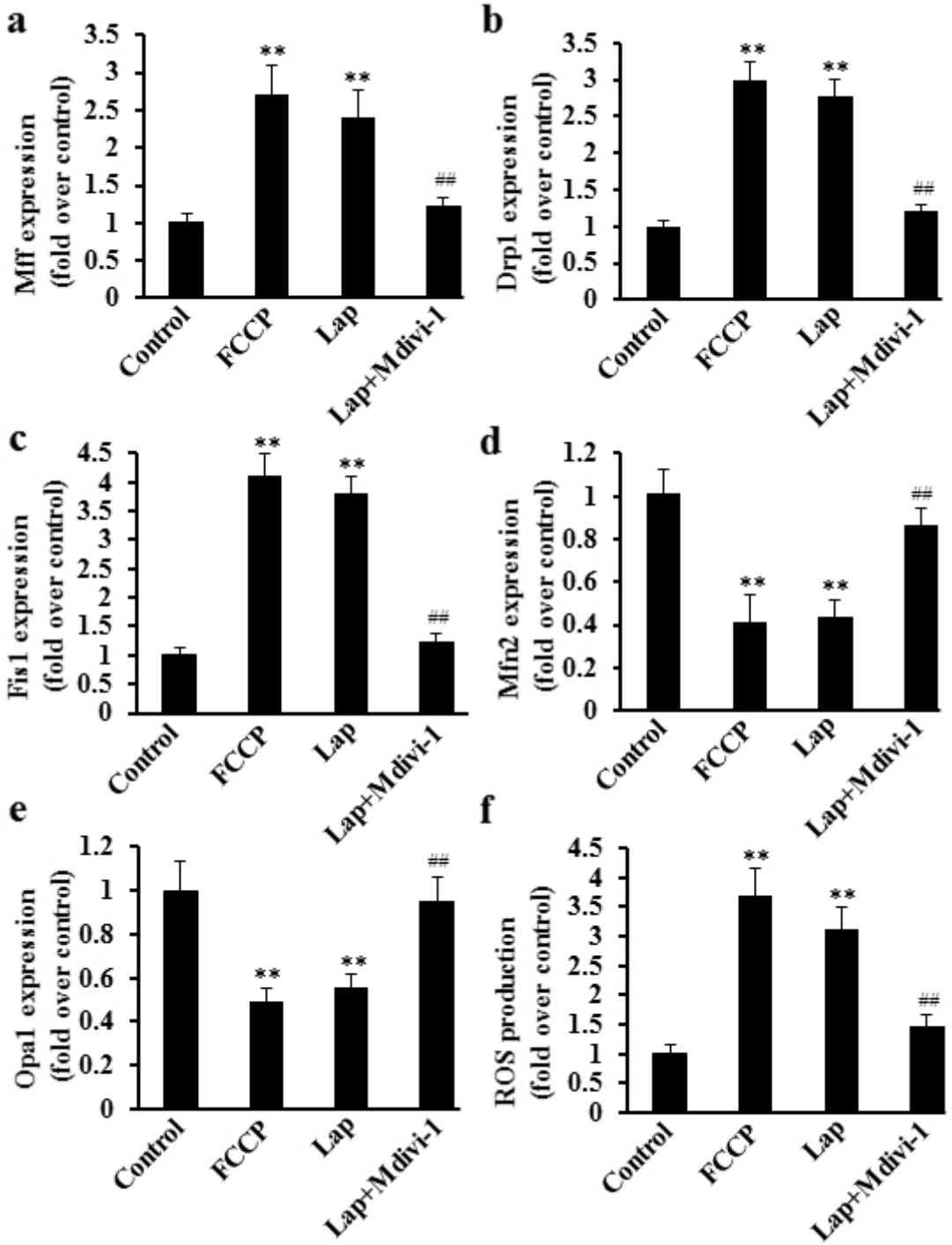

Figure 4. — Lapatinib (Lap) activated mitochondrial fragmentation in SK-OV-3 cells. The relative Mff fluorescence intensity was evaluated after lapatinib treatment (a); The alterations of mitochondrial fission/fusion-related factors were measured using qPCR (b-e); mROS in SK-OV-3 cells were measured after lapatinib treatment (f). Data were expressed as mean ± SD (n = 3). $^{**}p < 0.01$ *vs.* control group; $^{\#\#}p < 0.01$ *vs*. lapatinib alone group.

fragmentation. The mitochondrial fragmentation could be the result of increased mitochondrial fission and decreased mitochondrial fusion. Therefore, qPCR was performed to analyze the transcription factors related to mitochondrial fission/fusion. After lapatinib treatment, pro-fission factors Drp1 and Fis1 were significantly up-regulated. In contrast, pro-fusion factors Mfn2 and Opa1 were clearly down-regulated (Figures 4b-e).

Mitochondrial fragmentation was associated with cellular oxidative stress. In this study, mitochondrial ROS

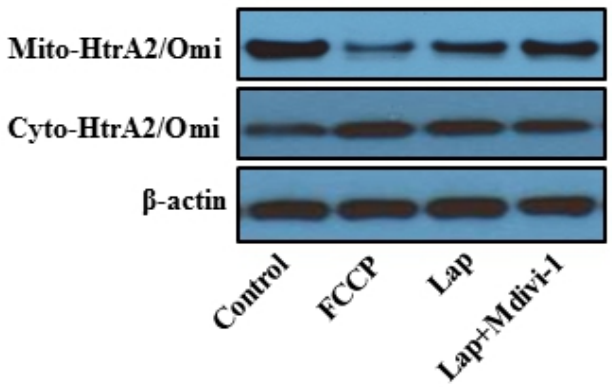

Figure 5. — Lapatinib (Lap) induced HtrA2/Omi liberation. Cytoplasmic HtrA2/Omi (Cyto-HrA2/Omi) and mitochondrial HtrA2/Omi (Mito-HtrA2/Omi) protein expression was determined by western blot.

(mROS) levels were quantified after lapatinib treatment. Results showed mROS level significantly increased after lapatinib treatment. To validate whether mitochondrial fragmentation was involved in mROS overproduction, Mdivi-1 and FCCP were used. Mdivi-1 application attenuated lapatinib-mediated mROS overproduction (Figure 4f), indicating the necessary role of mitochondrial fragmentation in mROS generation.

Overproduction of mROS increased permeability of mitochondrial membrane, facilitating the translocation of mitochondrial pro-apoptotic factors to nucleus/cytoplasm. In this study, Western blot was performed to quantify HtrA2/Omi liberation. As shown in Figure 5, lapatinib treatment increased levels of cytoplasmic HtrA2/Omi, but decreased the expression of mitochondrial HtrA2/Omi. Mdivi-1 addition repressed the lapatinib-mediated HtrA2/Omi translocation from mitochondria into cytoplasm.

Discussion

As the most severe gynecological cancer in women, about 75% of the affected patients with epithelial ovarian cancer are being diagnosed with advanced-stage of the disease. Although the response rate to chemotherapies is good, overall survival is still limited due to the high relapse rate of recurrent resistance [14-16]. Therefore, there is an immense need to search for new therapeutics against ovarian cancer.

Mitochondrial fission has been considered as a potential target to inhibit the proliferation, migration, and survival of cancer cells. Mitochondrial fragmentation causes damage to mitochondrial structure and function, eventually interrupting the cellular ATP supply and activating apoptosis [17, 18]. In this study, lapatinib was used to activate mitochondrial fragmentation and further downstream events were observed based to this treatment. Our results showed that lapatinib treatment increased mitochondrial fission, but decreased mitochondrial fusion. Subsequently, excessive mitochondrial fragmentation triggered mROS overload, leading to cellular oxidative stress. Moreover, mROS overproduction induced HtrA2/Omi liberation from mitochondria into the cytoplasm, resulting in up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic factors. This study demonstrates for the first time lapatinib-activated mitochondrial fragmentation via HtrA2/Omi pathway.

The mitochondrial network undergoes moderate fission and fusion to meet the requirements of cellular metabolism under different physiological conditions [19, 20]. However, uncontrolled mitochondrial fission generates massive amounts of fragmentation and disrupts mitochondrial homeostasis. This acts as an apoptotic trigger and activates mitochondrial fragmentation limiting tumor cell proliferation and inducing apoptosis [10]. Consistent with these findings, the present study demonstrates mitochondrial fragmentation initiated ovarian cancer SKOV-3 cell death. Strategies to promote mitochondrial fragmentation would be of importance in the anti-cancer drug design.

Mitochondrial fragmentation modulated cell viability through ROS-mediated cell oxidative injury and the HtrA2/Omi liberation-induced caspase activation [11, 21]. Mitochondrial fragmentation generated excessive amounts of ROS, and ROS overproduction augmented the liberation of HtrA2/Omi from mitochondria into cytoplasm, where Htra2/Omi decreased the mitochondrial membrane potential and induced caspase activation. HtrA2/Omi is primarily expressed in the inner membrane of mitochondria. Liberation of HtrA2/Omi from mitochondria into the cytoplasm is dependent on cardiolipin oxidation and mPTP opening [22, 23]. Firstly, oxidation of cardiolipin lowers the affinity of HtrA2/Omi to the mitochondria. Secondly, the opening of mPTP provides a channel for leakage of HtrA2/Omi [24, 25]. In this study, lapatinib was used to activate mitochondrial fragmentation and our results showed that lapatinib-mediated SKOV-3 cell apoptosis could be inhibited by Mdivi-1, an antagonist of mitochondrial fragmentation. To the best of the author's knowledge, this is the first study on the effects of lapatinib during mitochondrial stress. This study indicated that the anti-cancer activities of lapatinib against ovarian cancer depends on the activation of mitochondrial fragmentation by up-regulating mitochondrial fission and down-regulating mitochondrial fusion via the Mros-HtrA2/Omi pathways. Further human study is required to validate the tumor-suppressive effects of mitochondrial fragmentation in response to lapatinib treatment.

Author Contributions

XLY conceived and designed the research. YQY, HMZ and WMC performed experiments and statistical analysis. YQY and XYC drafted the manuscript.

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Conflict of interest

All authors read and approved the final manuscript. The authors declare no conflict of interest.

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