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Autophagy Stimulates Apoptosis in HER2-Overexpressing Breast Cancers Treated by Lapatinib

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ABSTRACT

HER2-overexpressing breast cancers often show hyperactivation of the HER2/AKT/mTOR signaling pathway. Lapatinib is an oral dual tyrosine kinase inhibitor (TKI) that targets both EGFR and HER2 to inhibit the proliferation of breast cancer cells. However, it is obscure whether and how lapatinib could induce autophagy in breast cancer cells, an important cell response with drug treatment. In this study, we investigated the apoptosis and the autophagy in the HER2-overexpressing breast cancer cells BT474 and AU565 treated with lapatinib, and further examined their relationship. Lapatinib inhibited the proliferation and the rate of DNA synthesis in HER2-positive cells, as observed by MTT, colony formation and EDU assays. Lapatinib not only induced apoptosis accompanied by an increased expression of cleaved Caspase-3 and cleaved PARP, but it also induced autophagy in vitro, as confirmed by electron microscopy (EM), acridine orange (AO) staining and LC3-II expression. Meanwhile, lapatinib inhibited the phosphorylation of HER2, AKT, mTOR, and p70S6K, whereas that of AMPK was activated. When the cells were pre-incubated with 3-Methyladenine (3-MA), the specific autophagy inhibitor, the growth inhibitory ratio and apoptosis rate were frustrated, whereas colony formation and DNA synthesis ability were encouraged. In addition, 3-MA application could up-regulate Caspase-3 and PARP expression, compared with the treatment with lapatinib alone. The addition of 3-MA could attenuate the inhibitory role on HER2/AKT/mTOR pathway and the active role on AMPK that was raised by lapatinib. Therefore, lapatinib simultaneously induced both apoptosis and autophagy in the BT474 and AU565 cells, and in these settings, autophagy facilitates apoptosis. J. Cell. Biochem. 114: 2643–2653, 2013.

KEY WORDS: BREAST CANCER; LAPATINIB; APOPTOSIS; AUTOPHAGY; mTOR; AMPK

Breast cancer is the leading cause of cancer deaths among females in the United States [Ma and Jemal, 2013]. Approximately 20–30% of breast cancers are characterized by the overexpression of HER2 due to genetic mutation [Slamon et al., 1987]. An excess of the HER2/neu receptor plays an important role in pathological processes such as tumorigenesis and progression in breast cancer. HER2-positive has also been shown to correlate with less responsive to hormonal therapy and chemotherapy. However, treatments that specifically target HER2 are very effective. Trastuzumab, a humanized monoclonal antibody directed against HER2/neu, is one of the most important types of HER2 inhibitors currently in clinical use [Hudis, 2007]. However, the clinical efficacy of

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trastuzumab is limited because a significant number of patients with HER2 over-expressing tumors will be initially or eventually resistant to trastuzumab [Pohlmann et al., 2009].

Lapatinib, designed by the pharmaceutical company Glaxo-Smith-Kline (GSK), is an orally active drug for breast cancer and other solid tumors. It is a dual tyrosine kinase inhibitor (TKI) that interrupts both HER2/neu and epidermal growth factor receptor (EGFR) pathways. It was approved by the U.S. Food and Drug Administration (FDA) in combination therapy for the treatment of patients with advanced or metastatic breast cancers whose tumors over express HER2 [Burris et al., 2005; Medina and Goodin, 2008].

It has been reported that lapatinib inhibits receptor signal processes by binding to the ATP-binding pocket of the EGFR/HER2 protein kinasedomain [Liu and Gray, 2006]. The kinase is the switch for auto-phosphorylation and the subsequent downstream signaling through the phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway and its target protein p70S6K. Misoperation or loss of control in any of the channels mentioned above would result in uncontrolled cell proliferation, enhanced angiogenesis and metastasis, and increased resistance to apoptosis [Zhou and Huang, 2010]. Thus, the HER2/AKT/mTOR pathway is where lapatinib plays a role on the HER2-positive breast cancer cells.

However, a slave to its destiny, lapatinib was subjected to intrinsic and acquired resistance. To date, autophagy is a remarkable mechanism [Chen et al., 2011]. The role of autophagy has created a two-edged sword for tumor cells. Several recent studies have shown that autophagy may be another mechanism of programmed cell death, although some other data demonstrated that autophagy could protect cancer cells against metabolic stress, including drug resistance [Vazquez-Martin et al., 2009; Paillas et al., 2012; Zou et al., 2012]. Because the role of autophagy in cancer remains controversial and depends on the context, it is constructive to learn the role of autophagy in lapatinib-treated cancer cells. To test the hypothesis that autophagy may promote or block lapatinib toxicity, it is critical to identify the function of autophagy in lapatinib-treated cells by blocking autophagy with a specific inhibitor, such as 3-Methyladenine (3-MA), Chloroquine (CQ), and Bafilomycin A1 (BA), to abolish autophagosome formation [Seglen and Gordon, 1982].

Therefore, in this study, we investigated the inhibitory function of lapatinib through the HER2/AKT/mTOR pathway. Because inhibition of PI3K/AKT/mTOR pathway may act as a bridge between apoptosis and autophagy, we further illustrated the relationship between apoptosis and autophagy induced by lapatinib in HER2-positive breast cancer cells.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

The lapatinib (Lap.) was donated by Glaxo-Smith-Kline, the Cell-Light™ EdU DNA Cell Proliferation Kit was from Ribobio, the dimethyl sulfoxide (DMSO), thiazolylblue tetrazolium bromide (MTT), Giemsa stain, acridine orange (AO), 3-Methyladenine (3-MA), Bafilomycin A1 (BA), and Chloroquine diphosphate salt (CQ) were purchased from Sigma–Aldrich (St. Louis, MO). Lapatinib and other compounds were prepared in DMSO as a stock. The DMSO concentration never exceeded 0.1% (v/v), and equal amounts of the solvent were added to the control cells.

The antibodies against p-HER2 (Tyr1221/1222), HER2, p-mTOR (Ser2448), mTOR, p-p70S6K (Thr389), p70S6K, p-AKT (Ser473), AKT, p-AMPKα (Thr172), AMPKα, Beclin-1, PARP, cleaved Caspase-3 (Asp175), Caspase-3, and LC3B are rabbit monoclonal and were purchased from Cell Signaling (Beverly, MA). The antibody against β-actin is mouse monoclonal and was purchased from Boster (Wuhan, China).

CELL CULTURE

The human breast cancer cell lines BT474 and AU565 were obtained from the American Type Tissue Culture Collection. The cells were cultured in DMEM (BT474) or RPMI1640 (AU565) supplemented with 10% fetal bovine serum (FBS) and were maintained in a humidified environment containing 5% CO_2 and air at 37°C.

CELL PROLIFERATION ANALYSIS

Cell survival assay. Cells (2×10^4/200 μl/well) were seeded in 96-well plates. The following day, the lapatinib (2 mM) was diluted in different concentrations (0.25, 0.5, 1, 2, and 4 μM) in the media to incubate the cells. Viable proliferating cells were detected using the MTT assay at different times (12, 24, 36, 48, and 96 h) in five wells each time. MTT was dissolved and sterilized in PBS at 5 mg/ml, and 20 μl was added to each well. The plate was incubated at 37°C for 4 h before the time point when the media was removed. Approximately 150 μl of DMSO was added into each well, and the plate was gently rocked for 10 min to dissolve the dark blue MTT crystals. Cell viability was expressed as optical density (OD), which was detected by use of an Infinite® F500 micro-plate reader (TECAN) at 490 nm. The percentage of cell proliferation was calculated as follows:

$$\text{Cell proliferation ratio}\,\% = \frac{OD_{490\,\text{Lapatinib}} - OD_{490\,\text{blank}}}{OD_{490\,\text{control}} - OD_{490\,\text{blank}}} \times 100\%$$

COLONY-FORMATION ASSAY

To determine long-term effects, cells (1×10^4/2 ml/well) were seeded in six-well plates and were treated with lapatinib at various concentrations (0.0625–2 μM) for 48 h. After being rinsed with fresh medium, the cells were allowed to grow for 20 days to form colonies, which were then stained with Giemsa, and the colonies containing more than 50 cells were counted using Image-Pro Plus.

DNA SYNTHESIS ASSAY

The 100 assay Cell-Light™ EdU DNA Cell Proliferation Kit contains six reagents named component A to component F. Components A, B, C, D, and E are required. Cells (4×10^4/500 μl/well) were seeded in 24-well plates and treated with lapatinib at two concentrations for 48 h. Then, the cells were grown for 4 h in medium containing EDU A (50 μM). The cells were fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4; PBS) for 30 min. Then, the cells were washed with glycine (2 mg/ml) in PBS for 5 min. The cells were treated with 0.5% Triton-X for 10 min before being incubated with the reaction cocktail for 30 min at room temperature and protected from light. Finally, the cell nuclei were labeled with DAPI (4′6-diamidino-2-

phenylindole). (Note: The cells were washed for 3×5 min in PBS before any reagent was added.) The reaction cocktail was used within 15 min of preparation. The reaction cocktail (1,000 μl) contained 938 μl of *dd*H_2O, 50 μl of component B, 10 μl of component C, 3 μl of component D, and 9 mg of component E. The images were visualized and captured by an OLYMPUS IX71 inverted microscope.

CELL APOPTOSIS ANALYSIS

For quantitative analysis of cell apoptosis, 5×10^5 cells with different treatments were harvested, washed with PBS, and stained with Propidium Iodide and Annexin V-FITC (BD Pharmingen™). The presence of Annexin V-FITC-positive cells excluding PI at early time intervals suggested that lapatinib induced cell death by apoptosis. The ratio of apoptosis was analyzed by flow cytometry (BD FACSAria) with CellQuest research software. Apoptosis rate was quantified by measuring the Q4 population on the data acquired by flow cytometry.

AUTOPHAGY ANALYSIS

Electron microscopy. Cells (1×10^7) treated with lapatinib (2 μM), 3-MA (500 μM), or 3-MA (500 μM) and lapatinib (2 μM) were collected and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate for 2 h, then postfixed with 1% OsO_4 for 1.5 h, dehydrated with ethanol, and embedded in epon resin. Ultrathin sections were observed using an electron microscope (JEM-1230). The treatment of 3-MA (500 μM) and lapatinib (2 μM) means 3-MA (500 μM) was added 2 h before lapatinib (2 μM) treatment.

DETECTION OF ACIDIC VESICULAR ORGANELLES (AVOs)

Acidic vesicular organelles (AVOs), as markers of autophagy, were detected by acridine orange staining. Cells (2×10^4/500 μl/well) were seeded in 24-well plates. The following day, the cells were treated with lapatinib at various concentrations. After 4, 8, 12, or 24 h, they were incubated in medium containing 1 μg/ml acridine orange for 40 min. The acridine orange was rinsed with PBS and the samples were observed under an OLYMPUS IX71 inverted microscope. The acidic autophagic vacuoles fluoresced bright orange, while the cytoplasm and nucleus of the stained cells fluoresced bright green. Meanwhile, 20 nM of BA was added 2 h before adding acridine orange to balance out other acidic components.

CONFOCAL MICROSCOPY

The green fluorescent protein (GFP)-fused LC3 was also used to detect autophagy. Cells (2×10^4/500 μl/well) were seeded in 24-well plates preloaded with sterilized glass cover slips. The adherent cells were transfected with the GFP-LC3 expression plasmid (0.8 μg/well) using Lipofectamine 2000 according to manufacturer's instructions. After 24 h, the transfected cells were treated with lapatinib or other agents for another 24 h. It is worth noting that 3-MA or CQ was added 2 h before lapatinib treatment. Then, the cells on the cover slips were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at 4°C. After washing 3×5 min in PBS, DAPI was added for observing nucleus. Next, cover glasses were carefully mounted onto microscope glasses. Finally, the slides were sealed and analyzed using NikonA1 MP^+/A1R MP^+ multiphoton confocal microscopes.

WESTERN BLOTTING ANALYSIS

Cells with different treatment were harvested, washed with ice-cold PBS, centrifuged 2 min at 4°C. The protein was lysed in cell lysis RIPA Buffer (containing 1% protease inhibitor) for 20 min on ice and then subjected to ultrasonic cracking. After centrifugation at 12,000*g* for 20 min at 4°C, the protein concentrations of the supernatant fluid were determined using the BCA protein assay kit (Thermo). The protein samples were separated by 7.5–12% SDS–PAGE and transferred onto nitrocellulose membranes. Tris buffered saline-Tween-20 (TBS-T) containing 5% non-fat milk was used for blocking the membranes. The membranes were incubated with the primary antibodies overnight at 4°C and then with the secondary antibodies conjugated with horseradish peroxidase (HRP). After washing with TBS-T three times, enhanced chemiluminescence reagent was used in accordance with the manufacturer's recommendations, and the resulting membranes were exposed to Kodak X-OMAT processor.

STATISTICS

The statistical significance of the differences among the data were calculated using two-tailed Student's *t*-test. A two-sided *P*-value <0.05 was considered statistically significant. Every experiment was repeated three times. All values are expressed as the mean ± standard error (SE).

RESULTS

LAPATINIB CAN INHIBIT THE GROWTH OF BREAST CANCER CELLS

Lapatinib caused the death of breast cancer cells in a time- and dose-dependent manner. Lapatinib exerted the potent inhibitory effects on both BT474 and AU565 cell growth short-term (Fig. 1A) and long-term (Fig. 1B). When restricting the dose from 1 to 4 μM, the inhibitory effect was significantly time-dependent. By 48 h after 2 μM treatment, the cell death rate reached almost 50%. Therefore, a 48 h incubation with lapatinib seemed to be sufficient for the half induction of cell death. When the cells were treated with lapatinib at various concentrations (from 0.25 to 4 μM) for 96 h, there seemed to be little difference in the anti-proliferative activity (Fig. 1A). Lapatinib can also prevent DNA synthesis in breast cancer cells (Fig. 1C), just 500 nM of lapatinib for 48 h could has an appreciable impact on reducing the number of nuclei stained red, which represented the active nuclei at peak synthesis.

LAPATINIB INDUCED APOPTOSIS OF BREAST CANCER CELLS

The viability at 48 or 96 h of BT474 and AU565 cells exposed to 250 nM or 2 μM lapatinib were analyzed, respectively, by flow cytometry (Fig. 2A). According to the results, lapatinib could promote BT474 and AU565 apoptosis both time- and dose-dependent. To evaluate the intrinsic pathway of apoptosis, the ratios between Caspases-3/cleaved Caspases-3 and PARP/cleaved PARP were detected by Western blot analyses (Fig. 2B). The cleaved PARP was clearly detected in both BT474 and AU565 cells treated with 1 μM (24 h) or 2 μM (8 h) lapatinib. The cleaved Caspases-3 was increased obviously in the BT474 cells treated with 4 μM (24 h) or 2 μM (72 h) lapatinib and in the AU565 cells treated with 4 μM (24 h) or 2 μM (48 h) lapatinib.

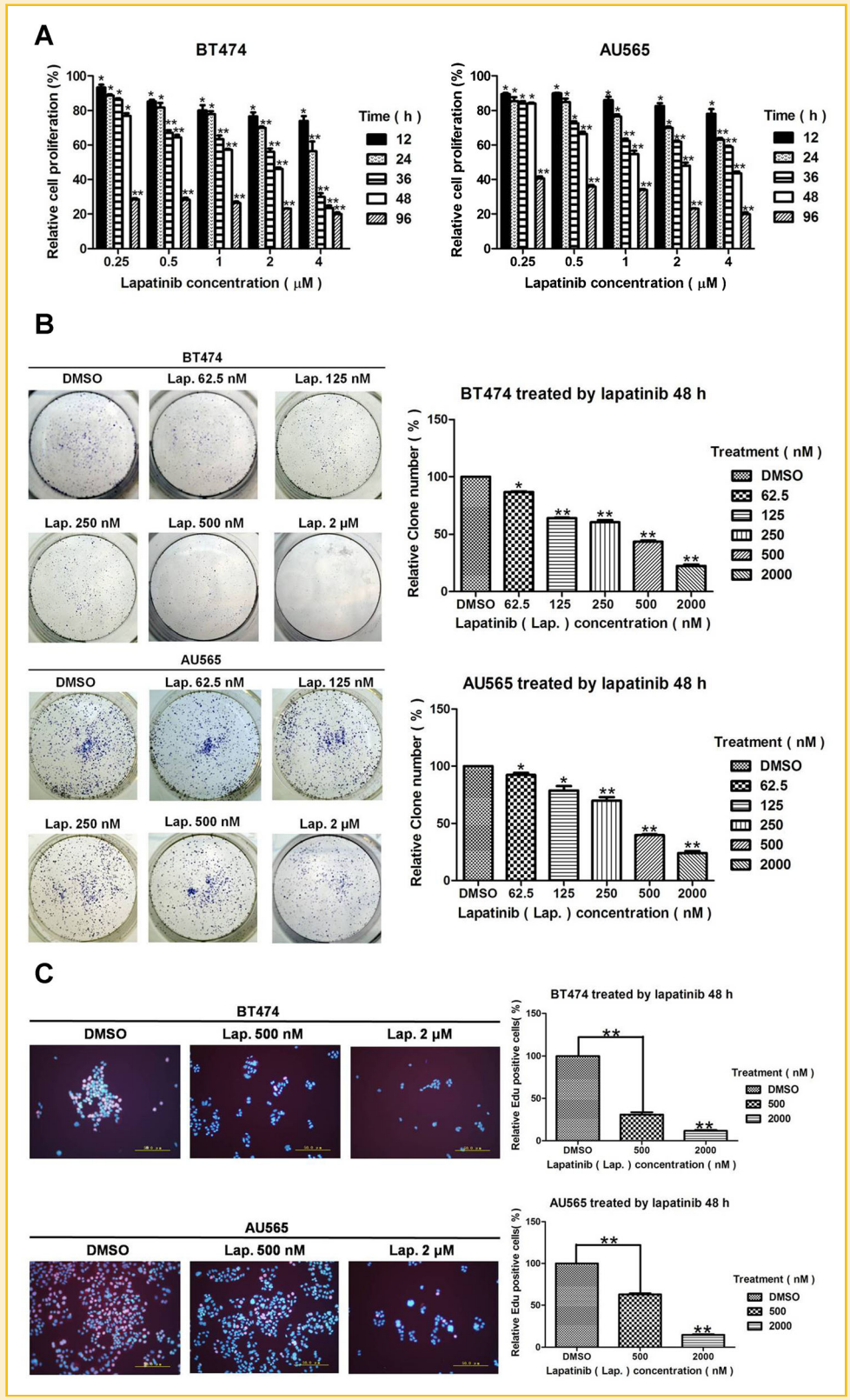

Fig. 1. Lapatinib can inhibit breast cancer cell proliferation. A: Growth curves. BT474 and AU565 cells were treated with DMSO or various concentrations of lapatinib (0.25–4 μM). Cells were harvested; OD values were measured at 12, 24, 36, 48, and 96 h. B: Colony formation rate. The BT474 and AU565 cells were treated with DMSO or various concentrations of lapatinib (0.0625–2 μM) for 48 h. Colonies were observed after 20 days and counted using Image-Pro Plus. C: DNA synthesis ability. DNA cell proliferation was detected using the Cell-Light™ EdU Kit and visualized in an inverted fluorescence microscopy. The nuclei with active DNA synthesis ability were stained red. The blue nuclei represent the total nuclei. $^{*}P < 0.05$, $^{**}P < 0.01$ for lapatinib treatment versus control vehicle. The data are expressed as the average of three independent experiments.

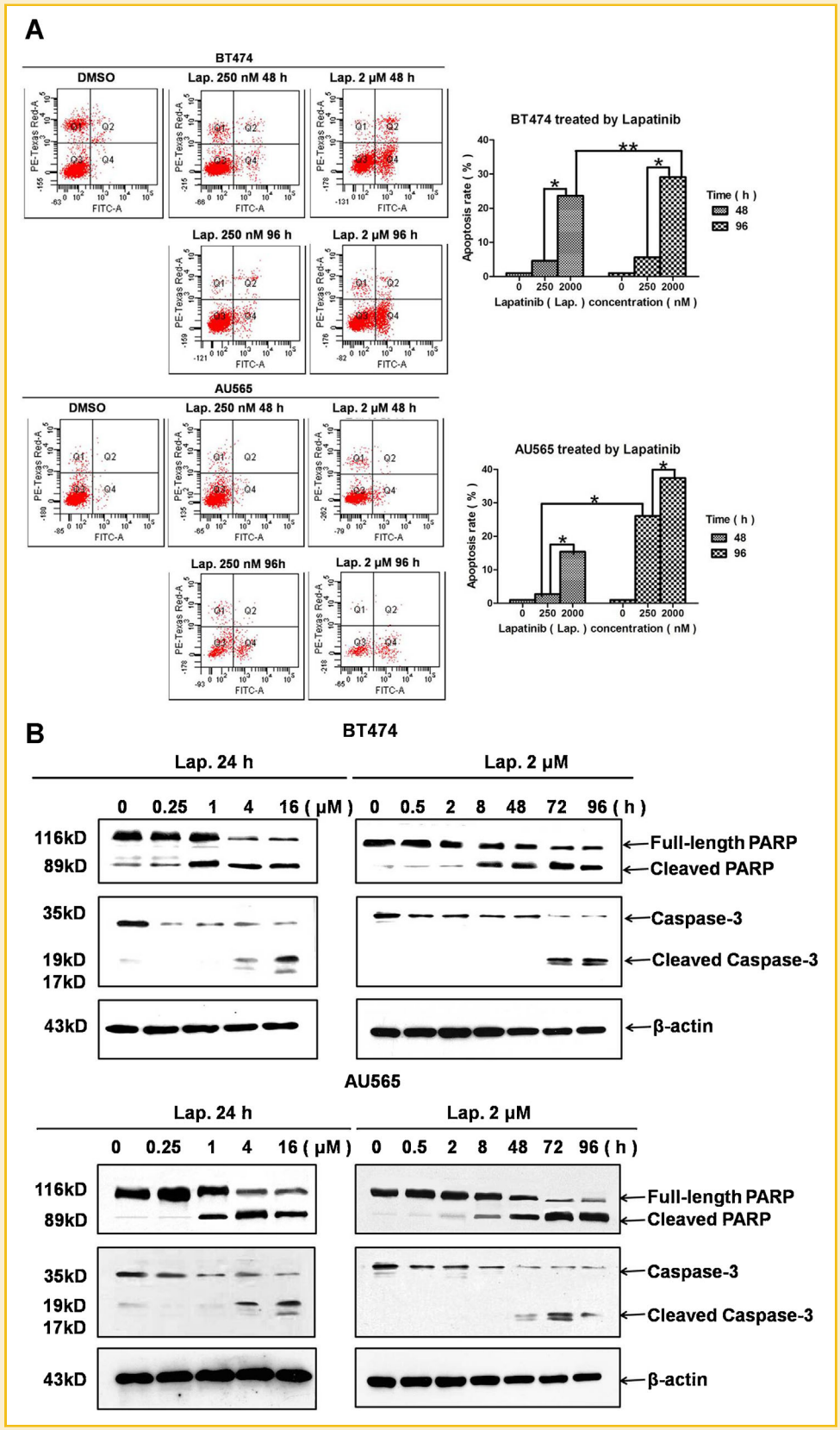

Fig. 2. Apoptosis of breast cancer cells following treatment of lapatinib at different time periods and doses. A: Apoptotic rate. Apoptotic rate was detected by gating on breast cancer cells with flow cytometry and detecting the percent of Annexin V staining as percentage of total breast cancer cells. $^{*}P < 0.05$, $^{**}P < 0.01$ for lapatinib treatment versus control vehicle. B: Western blots were performed to observe the cleaved Caspase-3 (Asp175), Caspase-3, cleaved PARP and full-length PARP protein expression. The more cleaved Caspase-3 and cleaved PARP expression represents for the higher level of apoptosis.

LAPATINIB SUPPRESSES TUMOR GROWTH THROUGH THE HER2/AKT/MTOR PATHWAY

Both BT474 and AU565 cells are HER-2 over-expressing breast cancer cells. Lapatinib is a dual HER2- and epidermal growth factor receptor (EGFR)-specific tyrosine kinase inhibitor. Exposure of BT474 and AU565 human breast cancer cells to lapatinib resulted in a dose- and time-dependent reduction of phosphorylation of HER-2, while the total HER2 showed insignificant variations in both cell lines (Fig. 3A, B), unless HER2 degradation when exposing to lapatinib with higher dose (16 μM) or longer time (72 h). Activation of HER2/neu leads to breast cancer cell proliferation, presumably by inducing the activation of the AKT/mTOR pathway. To examine whether lapatinib can inhibit the activation of AKT and mTOR, BT474 cells and AU565 cells were treated with various concentrations of lapatinib for 24 h. The results indicated that the amount of phospho-AKT and phospho-mTOR decreased significantly following treatment (Fig. 3A,B), yet the levels of AKT and mTOR were hardly affected. However, the expression of both phospho-p70S6K and p70S6K were reduced (Fig. 3A,B). In addition, AMPK has recently been shown to phosphorylate raptor, a scaffold in the mTORC1 complex. We next examined whether the inhibitory effects of lapatinib on mTOR signaling were concurrent with AMPK activation. Lapatinib increased the phosphorylation of AMPKα at Thr172 with time- and dose-dependent manner (Fig. 3A,B), which reflected AMPK activity in the BT474 and AU565 cell lines.

LAPATINIB LEADS TO AUTOPHAGY OF BREAST CANCER CELLS

Autophagy is featured by the formations of the autophagosome and autolysosome, which was confirmed by electron microscopy (EM), acridine orange (AO) staining, Western blotting, and confocal microscopy. Transmission electron microscopy revealed the presence of a number of the large vacuoles, double-membraned autophagosomes (indicated by arrows) in the lapatinib-treated cells (Fig. 4A) compared to the untreated controls and the 3-MA pretreated samples. At a higher magnification, the peripheral cytoplasm revealed vesicles containing phagocytosed organelles, free ribosomes, and endoplasmic reticulum.

To verify whether the autophagic pathway that lapatinib induced was time-dependent, acridine orange staining of the live cells was employed to visualize the acidic autophagy lysosome in the control and lapatinib-treated cells at different times (4, 8, 12, and 24 h) (Fig. 4B). Here, lapatinib treatment markedly increased the amount of autophagy lysosome in the cells (right) and the concentrated dye in the vesicles fluoresced bright red, whereas the cytoplasm and the nucleus showed dominant green in the lapatinib-treated cells, especially at 24 h. In contrast, the majority of the untreated cells exhibited mainly green fluorescence with minimal red fluorescence.

Another useful tool for the study of autophagy is a green fluorescent protein (GFP)-conjugated form of LC3. This structure can fuse with an acidic endosome coming from the autophagosome, and the proteins in the lumen of this compartment are then degraded. Induction of autophagy by lapatinib was demonstrated by monitoring BT474 and AU565 cells transfected with a GFP-LC3 expressing vector. Confocal microscopy revealed that the diffuse GFP fluorescence in control BT474- and AU565-GFP-LC3 cells changed to bright green vacuolar fluorescence, often overlapping with the DAPI blue staining following exposure to lapatinib (Fig. 4C). The puncta produced by lapatinib were similar to those of serum starvation but were more obvious than those of 3-MA, DMSO and 3-MA pretreatment, and lapatinib treatment. The increase of puncta could also been detected in CQ pretreatment and lapatinib compared with that of CQ treatment only. The conversion of the microtubule-associated protein 1 light chain 3-I (LC3-I) to the phosphatidylethanolamine-conjugated form LC3 (LC3-II) was another indicator of autophagy. According the Western blot assay, it was clearly seen the increase of LC3-II in groups with treatment of 3-MA and lapatinib, lapatinib, CQ and lapatinib versus without lapatinib (Fig. 4D). We further examined the expression level of Beclin-1 and LC3-II (two of the key autophagy-related genes) by Western blotting (Fig. 5A,B). Following the assay, the amount of Beclin-1 changed little with variations after exposure to lapatinib. The conversion of LC3-I to LC3-II induced by lapatinib was in a dose- and time-dependent manner. We observed that the proportion of LC3-II after treatment with lapatinib peaked at 16 μM (24 h) in both the BT474 and AU565 cells. Reducing the dose to 2 μM caused it to peak at 72 h in both the BT474 and AU565 cells.

AUTOPHAGY MAY PROMOTE APOPTOSIS INDUCED BY LAPATINIB

Interesting functional links had been revealed between apoptosis and autophagy. To investigate whether lapatinib-induced autophagy could cause cell apoptosis, the classical autophagy inhibitor 3-MA, which inhibits the sequestration of the autophagy process, was enrolled. We tested both the colony formation and DNA synthesis abilities in the BT474 and AU565 cells after exposure to lapatinib (2 μM) 48 h with 2 h of pre-incubation of 3-MA. There seemed to be no difference between cells incubated with 3-MA alone compared with the untreated cells (control), but the group with 3-MA pretreatment showed increased cell proliferation ability compared to the cells treated with lapatinib only. Using flow cytometry, we further investigated whether 3-MA could protect cells from the apoptosis induced by lapatinib (Fig. 7A); the results were in accordance with those obtained from the plat colony assays (Fig. 6A) and EDU tests (Fig. 6B). Finally we observed the expression of proteins related to apoptosis and autophagy by Western blot analysis. The expression of cleaved PARP and cleaved Caspase-3 increased less in the cells pre-treated with 3-MA than in the cells treated with lapatinib alone (Fig. 7B). Following the observations of the effects of lapatinib on the HER2/AKT/mTOR pathway, which we verified above, we examined the role of 3-MA on this pathway. Clearly, 3-MA was able to weaken the inhibitory role of lapatinib on HER2, mTOR, AKT, and p70S6K phosphorylation, besides it lessened the activation of lapatinib on p-AMPKα (Thr172) (Fig. 7C).

DISCUSSION

Research on autophagy and its effects on cell metabolism and physiology have increased dramatically during the past decades. The regulation and contribution to cell metabolism of autophagic process have been characterized in much detail [Maria Cuervo, 2004; Mizushima et al., 2008]. Functional autophagy has been shown to be critical formal embryonic development, malfunction of autophagy

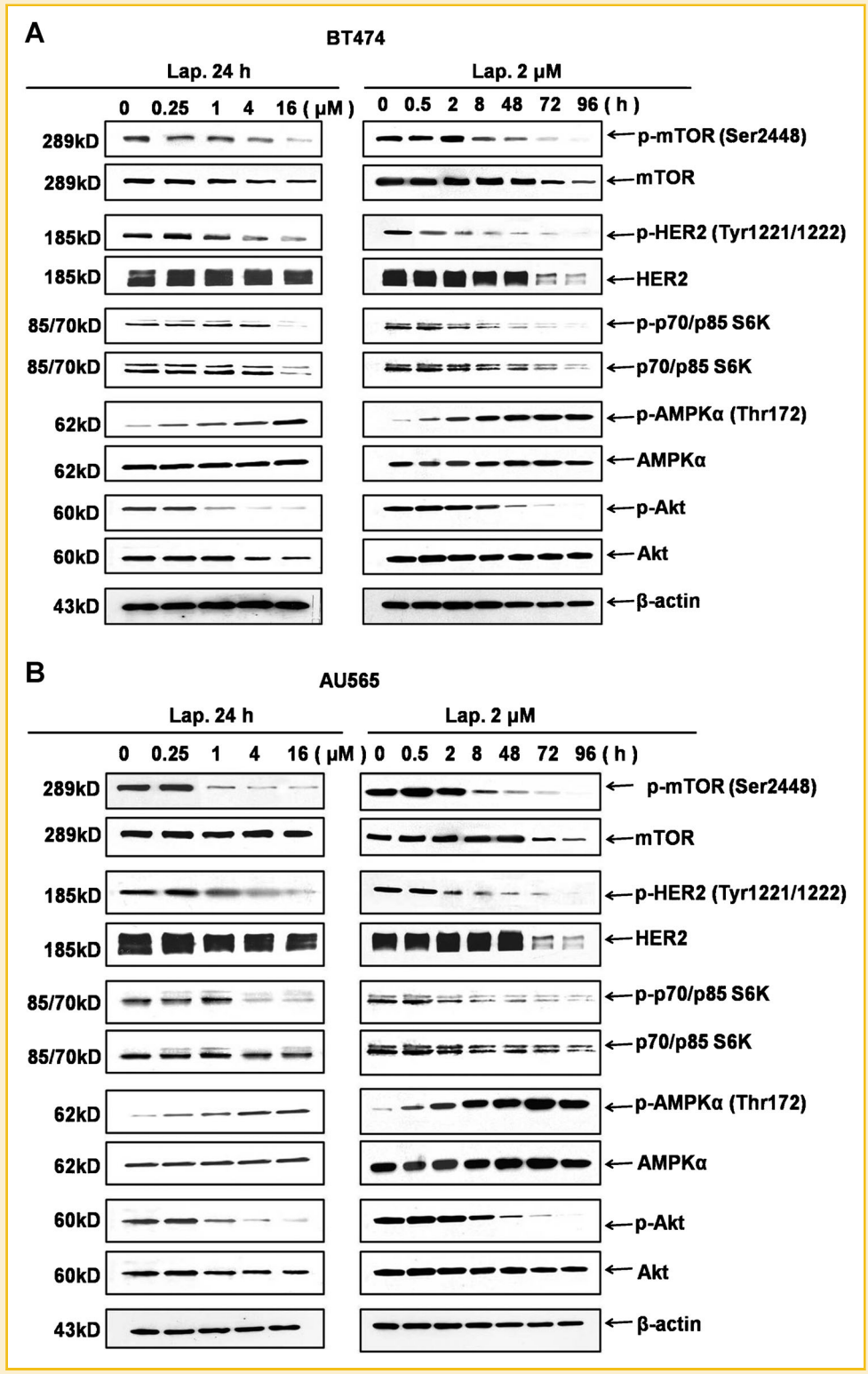

Fig. 3. Dose-dependent and time-dependent activity of lapatinib on HER-2, m-TOR, AKT, p70S6, and AMPKα expression and phosphorylation in BT474 (A) and AU565 (B) cells. Both cell lines were treated with increasing doses of lapatinib (0.25–16 μM) for 24 h or with 2 μM lapatinib for increasing durations (0.5–96 h) and compared to cells treated with DMSO only.

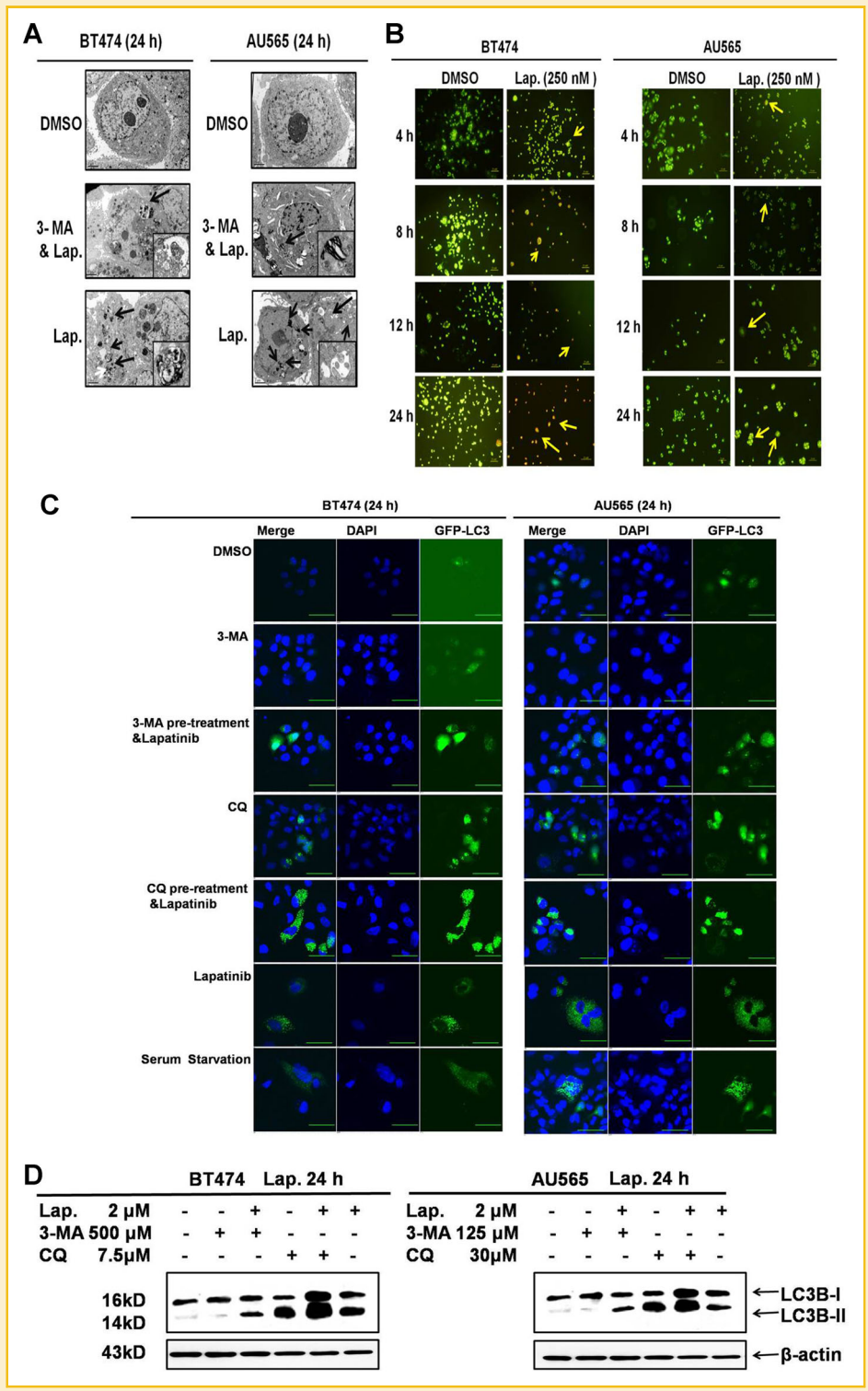

Fig. 4. Lapatinib induced the autophagy of HER2 positive breast cancer cells. A: Electron microscopy pictures were taken of BT474 and AU565 cells treated for 24 h with DMSO (control), 500 μM 3-MA pretreatment and 2 μM lapatinib, or 2 μM lapatinib alone. Numerous autophagic vacuoles (black arrows) and details with an enlarged scale (block diagrams) were observed in the lapatinib-treated cells compared to the DMSO-treated cells. B: After treatment with 2 μM lapatinib for 4, 8, 12, and 24 h, BT474 and AU565 cells were stained with AO, as described in the materials and methods section, and detected by an inverted microscope. Acid autophagy lysosome was stained orange and apparent red fluorescence could be observed (yellow arrows). The BT474 and AU565 cells treated with DMSO served as the negative control. C: The BT474 and AU565 cells expressing GFP-LC3 were treated with DMSO, 3-MA, 3-MA pretreatment and lapatinib, Chloroquine (CQ), CQ pretreatment and lapatinib, lapatinib (Lap.) or incubation without serum for 24 h, then fixed and mounted with DAPI-containing mounting medium and observed under a confocal microscopy. The increase in punctate green fluorescence is an indicative of autophagy, comparable to the autophagy induced by serum starvation. D: The LC3 expression in cells with treatment of 3-MA pretreatment and lapatinib, CQ pretreatment and lapatinib or lapatinib alone shown apparently transformation from LC3-I to LC3-II compared with lapatinib untreated. Note: 3-MA and lapatinib (3-MA pretreatment and lapatinib) or CQ and lapatinib (CQ pretreatment and lapatinib) mean BT474 and AU565 cells were pre-incubated with 3-MA or CQ for 2 h before incubation with lapatinib.

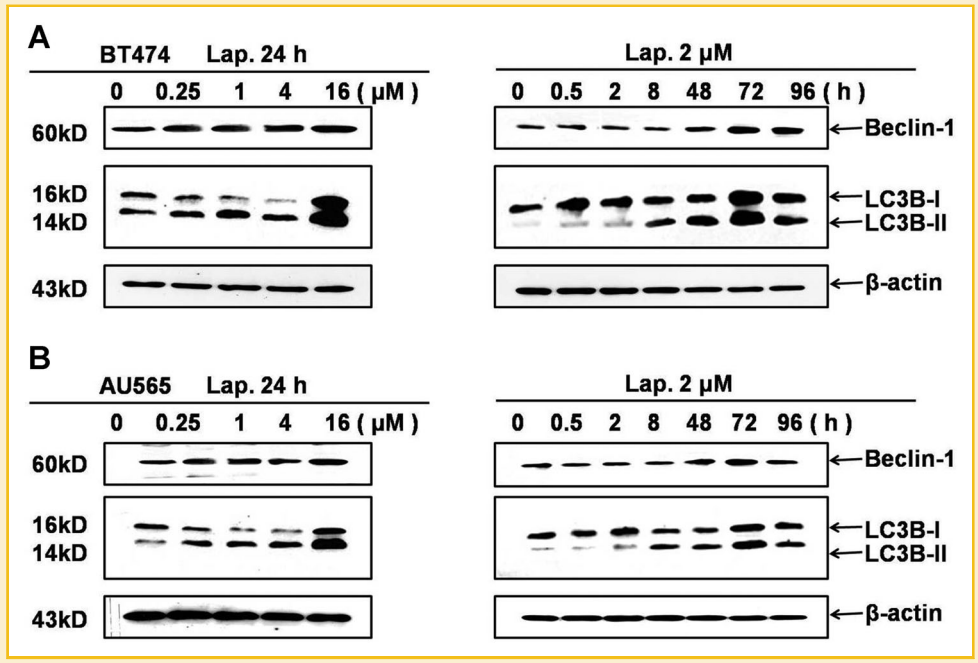

Fig. 5. Lapatinib induced the autophagy of breast cancer cells in a dose-dependent and time-dependent manner. After exposure to lapatinib with various doses (0.25–16 μM) for 24 h or 2 μM for different amounts of time (0.5–96 h), the BT474 (A) and AU565 (B) cells were harvested and analyzed by Western blot analysis using antibodies against Beclin-1 and LC3B. The conversion of LC3-I to LC3-II induced by lapatinib was in a dose- and time-dependent manner.

contributes to a variety of diseases, including cancer, neurodegeneration, cardiovascular disorders, and microbe infection. Most often, autophagy may function primarily as a cyto-protective mechanism through its contribution to maintain nutrient and energy homeostasis during starvation conditions and removal of defective proteins, damaged or ganelles and invasive pathogens. However, activation of autophagy can also be harmful, for example, autophagy might allow cancer become resistant to chemotherapy or excessive autophagy can cause undesirable cell death. Although this is true for a majority of toxic insults associated with the activation of autophagy, there are ample examples of anticarcinogen-induced autophagic cell death [Yousefi et al., 2006; Cui et al., 2007]. In this context, crosstalk between autophagy and other modes of cell death is of particular interest, especially the links between autophagy and apoptosis.

Several studies pointed out that autophagy may be indispensable for apoptosis by preceding and further turning on apoptosis. Cui et al. showed that autophagy induction was essential to the oridonin-induced human breast cancer MCF-7 cells undergoing apoptosis, indicating that execution of apoptosis is preceded by and even depends on the occurrence of autophagy. In these settings, autophagy inhibitors, like 3-MA, delay apoptosis while conversely, broad-range caspase inhibitors fail to inhibit autophagy. Here, our results from colony formation, cell proliferation assay, flow cytometry analysis, and Western blot showed that employing 3-MA, the specific inhibitor of autophagy pathway, decreased the apoptotic level induced by lapatinib, indicating that autophagy facilitated the cell apoptosis, which was consistent with the previous reports [Crighton et al., 2006; Green and Chipuk, 2006; Yousefi et al., 2006].

Moreover, activation/inhibition of autophagy can influence drug response, which was consistent with our findings. Martin and his colleagues demonstrated that obatoclax potentiated the cytotoxicity of lapatinib in human colon and breast cancer cells by inducing autophagic cell death [Martin et al., 2008, 2009]. However, whenever treated the cells with Chloroquine (CQ) and Bafilomycin A1 (BA) to inhibit the autophagy, we obtained the opposite results to 3-MA blocked BT474 and AU565 cells (data not shown). Also, BA or CQ-inhibited autophagy promote apoptosis in BT474 cells but little function in AU565. And these facts allowed us to suppose that the phenomenon that autophagy preceded apoptosis probably is highly celltype- and compartment-specific. Further studies on the relationship between autophagy and HER2 positive cell features remained to be explored.

In other cellular settings, autophagy may rather antagonize or delay apoptosis [Abedin et al., 2006; Li, 2009]. The well-described examples are the cases that an autophagic survival response has been attributed to MCF-7 cells following nutrient starvation or exposure to tamoxifen [Schoenlein et al., 2009] and that paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells [Ajabnoor et al., 2012]. In fact, we have established the stable resistant cells to lapatinib in BT474 and AU565 cell lines. Notably, the autophagosome formation increased a lot in the lapatinib resistant cells. Combined the literature and our preliminary data, we believed that autophagy might be regarded as opposite survival mechanism of the parental and resistant breast cancer cells to lapatinib.

Plenty of molecules can affect both autophagy and apoptosis, suggesting that they may act as molecular switches of these cellular

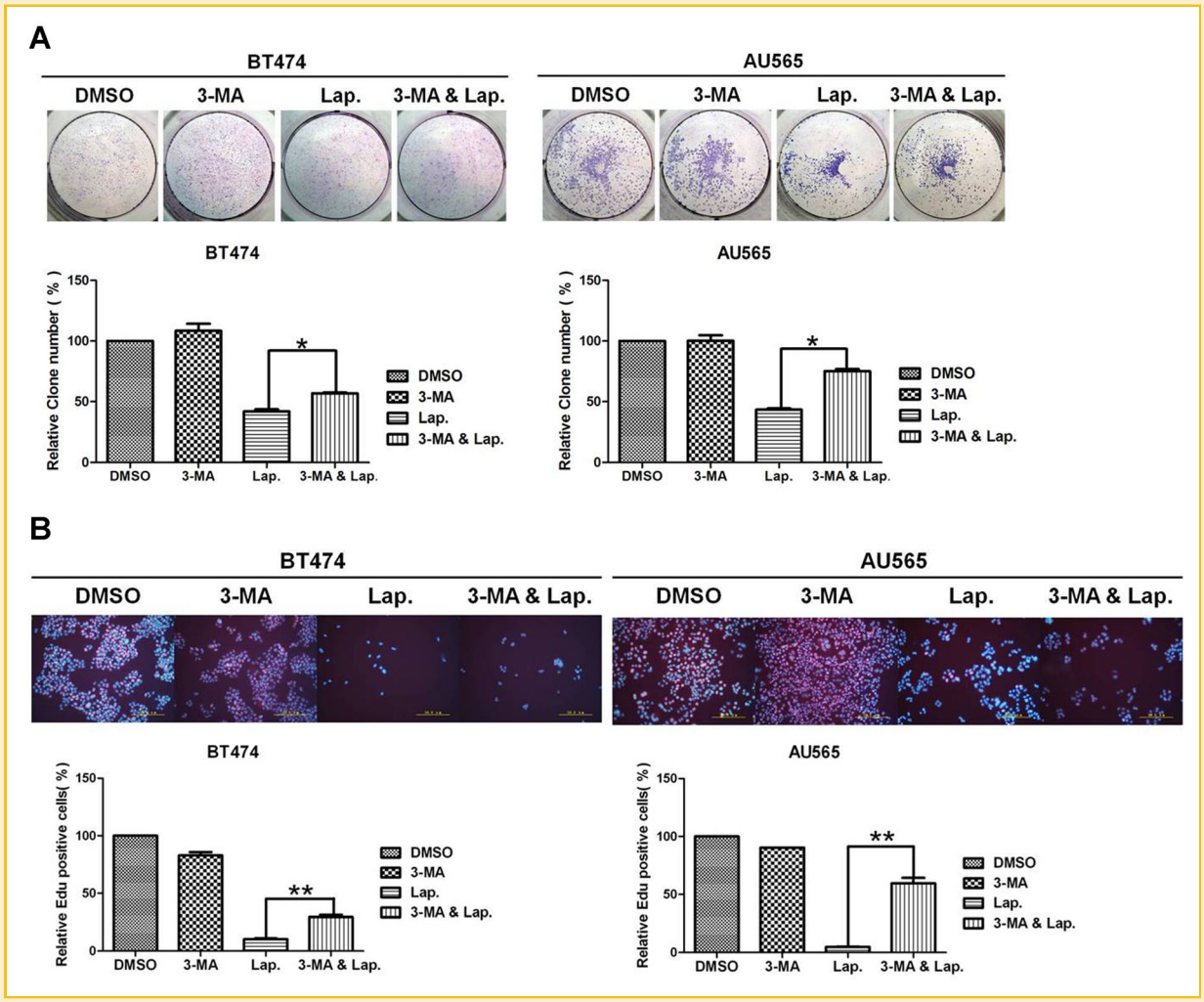

Fig. 6. The autophagy inhibitor 3-MA may weaken the anti-proliferative activity of lapatinib. The 3-MA was used for inhibiting autophagy in the BT474 and AU565 cells and investigating the relationship between autophagy and anti-proliferation. The cells were treated with DMSO, 3-MA, lapatinib, or 3-MA pretreatment and lapatinib. Various indicators were examined. A: Clone formation ability. B: DNA synthesis ability.

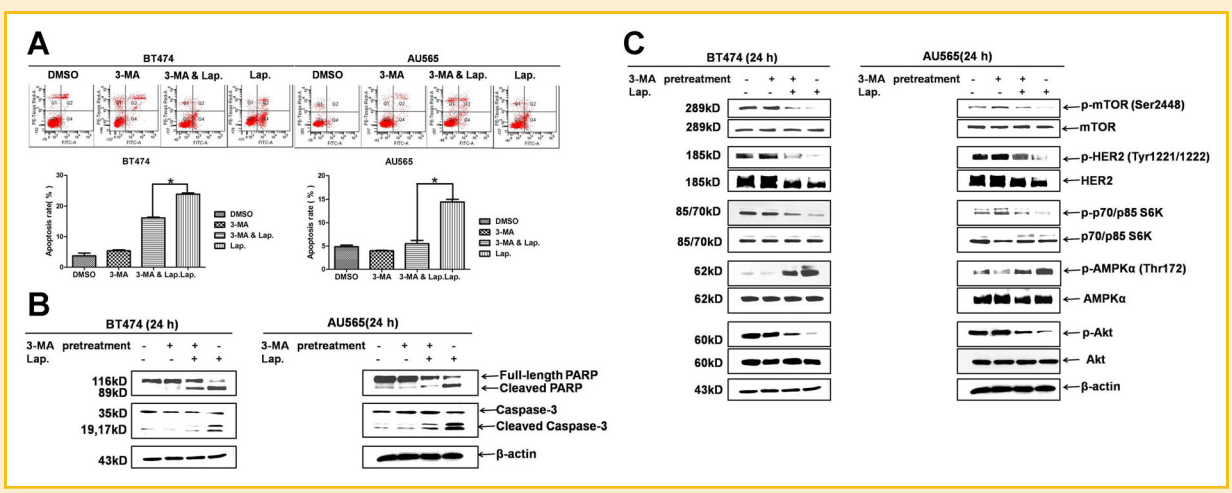

Fig. 7. Autophagy promote apoptosis in BT474 and AU565 cells. When BT474 and AU565 cells were pre-incubated with 3-MA for 2 h, indicators related with apoptosis were detected versus lapatinib treatment alone. A: Apoptosis rate by flow cytometry assay. B: Expression of PARP and Caspase-3 and their cleaved forms. C: The expression of key molecules in HER2/Akt/mTOR and AMPK pathway were detected with Western blot.

processes in response to cell damage signaling. The PI3K/AKT/mTOR and AMPK pathways can influence cell survival through both apoptosis and autophagy and have been pointed as an important target for cancer therapy [Morgensztern and McLeod, 2005; Georgakis and Younes, 2006; Cidado and Park, 2012]. From all the evidences, we drew a conclusion that there were crosstalks between autophagy and apoptosis in lapatinib-treated BT474 and AU565 cells. According to our findings, HER2/Akt/mTOR and AMP-kinase activities were profoundly affected by lapatinib, p-HER2, p-Akt, p-mTOR, and p-70S6 activities were inhibited while p-AMP-kinase activity was activated. However, when autophagy was blocked by 3-MA, both inhibition of p-HER2, p-Akt, p-mTOR, p-70S6, and activation of p-AMP-kinase were reversed, suggesting that lapatinib-induced autophagy exerted its synergic effect on apoptosis through HER2/Akt/mTOR and AMPK pathway.

Consequently, autophagy plays a key role in the pro-apoptotic effect of lapatinib in both BT474 and AU565 cells lines. Moreover, inhibition of HER2/Akt/mTOR and activation of AMP-kinase mediates the effect of lapatinib on the inhibition of cell proliferation and induction of apoptosis in an autophagy-dependent manner.

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