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The Regulatory Mechanisms of Autophagy by Choline Kinase in Tamoxifen-Resistant Breast Cancer Cells

by

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A Thesis Submitted to the Department of Biomedical Sciences in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Science in Department of Biomedical Sciences at the Seoul National University Graduate School

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ABSTRACT

The Regulatory Mechanisms of Autophagy by Choline Kinase in Tamoxifen-Resistant Breast Cancer Cells

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Introduction: Breast cancer cells (BCCs) that acquired resistance to therapeutic agents are a critical target in the clinical treatment. Choline kinase-α (Chk-α) and choline-containing metabolites have been considered as an oncogene and cancer metabolic marker to activate PI3K and MAPK signaling through a positive feedback loop in invasive and drug-resistant BCCs. Autophagy, which is controlled by PI3K or MAPK signaling pathway, is a major catabolic pathway to promote cancer cell survival and restrict necrosis. The interrelationship between Chk-α and autophagy are not well understood. Therefore, this study was undertaken to explore intracellular signaling mechanism of autophagy regulated by Chk-α in tamoxifen-resistant BCCs.

Methods: Human breast cancer cell lines, estrogen-receptor (ER)-positive MCF-7

and tamoxifen-resistant MCF-7/TAM were used and shChk-α transduced cells ware established using lentivirus. mRNA transcripts and protein expressions were assessed by quantitative real-time RT-PCR, Western blot analysis and immunostaining. Choline-containing metabolites from cell lysates were analyzed using using 1H-nuclear magnetic resonance (1H-NMR) spectroscopy. The intracellular signaling events were evaluated by Western blot. Cell proliferation activity was evaluated by MTT assay. For cell cycle assay, fluorescence of propidium iodide was measured using flow cytometry. Immunofluorescence staining of activated caspase-3 was performed for the analysis of cell death.

Results: Chk-α expression and phosphocholine levels were increased in tamoxifen-resistant BCCs, MCF-7/TAM compared to control BCCs, MCF-7. Downregulaton of Chk-α using lentivirus containing shRNA showed the significant decrease in Chk-α expression as well as phosphocholine in shChk-α- transduced BCCs. The autophagy-related protein LC3 was compared in shChk-α transduced BCCs and control BCCs without any stimuli. The significant increases in LC3 protein levels and the number of autophagosome-like structures were observed in shChk-α transduced or a inhibitor of Chk-α, CK37-treated BCCs, whereas Beclin-1 levels were not altered. Downregulaton of Chk-α expression and activity by shChk-α transduction and CK37 treatment attenuated the phosphorylation of AKT, ERK1/2, and mTOR in both MCF-7 and MCF-7/TAM. Cell proliferation ability of MCF-7/TAM was decreased as compared with MCF7. The shChk-α transduction of MCF-7 resulted in a decrease in cell proliferation ability and induced caspase-3-

dependent death. In shChk-α transduced MCF-7/TAM, the proliferation ability significantly decreased but activated caspase-3 was not observed.

Conclusions: Our results suggested the downregulation of Chk-α expression and activity in tamoxifen-resistant BCCs, MCF-7/TAM suppressed the PI3K/AKT/mTOR and MAPK signaling pathway which are related with autophagy and cell proliferation, thereby leading to the activation of autophagy and the induction of dormant phenotype.

Keywords: Choline kinase, Metabolomics, Breast cancer, Autophagy, Drug resistance, Signaling pathway, LC3B, PI3K/AKT/mTOR, MAPK

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LIST OF TABLES AND FIGURES

Chapter 1

LIST OF ABBREVIATIONS

PC: Phosphocholine

Chk-α: Choline kinase alpha

NMR: Nuclear magnetic resonance

CPMG: Carr Purcell Meiboom Gill

TSP: Tri-methylsilyl propionate

RT-PCR: Reverse transcription polymerase chain reaction

PI3K: Phosphatidylinositide 3-kinases

MAPK: Mitogen-activated protein kinases

ERK1/2: Extracellular signal-regulated kinases1/2

mTOR: mammalian target of rapamycin

CONTENTS

INTRODUCTION

Breast cancer is one of the highest incidence rates of malignant cancer in women's health worldwide [1], approximately 75% of primary breast cancers are estrogen receptor (ER) positive [2]. Tamoxifen, an antagonist of the estrogen receptor is the most commonly used treatment for patients with ER-positive breast cancer [3]. However, approximately 30% of ER-positive breast cancers do not respond to tamoxifen treatment (de novo resistance) and the majority of tumors that initially respond to tamoxifen treatment develop resistance over time (acquired resistance) [4]. These breast cancer cells (BCCs) that acquired resistance to therapeutic agents can cause recurrent and metastatic breast cancer, therefore the drug-resistance BCCs are an important target in the clinical treatment of breast cancer.

Choline-containing metabolites are emerging as a non-invasive metabolic hallmark to identify malignant transformation and determine response to therapy in cancer *in vitro* and *in vivo* using magnetic resonance spectroscopy [5-10]. Increased phosphocholine (PC) is one of the metabolic characteristic of cancer and is primarily due to increased expression or activity of choline kinase-α (Chk-α), the enzyme that converts choline to the membrane precursor PC and the an increase in the Chk-α expression and activity was observed in several human tumor tissues including breast, prostate, brain and ovarian tumors with respect to normal tissues

[11-15]. Chk-α expression and activity also have been reported to be associated with poor prognosis in lung cancer as well as breast cancer [16]. Overexpression or downregulation of Chk-α altered the invasiveness and drug resistance of diverse cancer cells including breast and lung cancer cells [17,18]. Downregulation of Chk-α with small interfering RNA (siRNA) decreases PC level and proliferation ability but promotes differentiation in malignant BCCs [19]. The change in phosphocholine following estrogen administration may reflect an increase in the rate of choline phosphorylation [20], The higher content of phosphorylcholine has been reported in the tamoxifen-treated BCCs [21]. Therefore, Chk-α has been proposed as a prognostic marker for cancer progression and a molecular target for the development of novel anti-cancer drugs.

Recently, the complex reciprocal interactions between oncogenic signaling and choline metabolism have been suggested. The oncogenic RAS influences choline metabolism, thus, Chk-α activity and PC levels increased in RAS-transformed NIH3T3 cells through MAPK signaling pathway [22]. The block of PI3K/AKT signaling pathway inhibits choline uptake by the regulation of choline transport. PI3K/AKT signaling also is associated with Chk-α activation [23]. The downregulation of Chk-α attenuates simultaneously PI3K/AKT and MAPK signaling, inhibits cell proliferation and markedly decreases the anchorage-independent survival [24]. These studies propose Chk-α and choline metabolites as an oncogene and cancer metabolic marker to activate MAPK and PI3K signaling

through a positive feedback loop.

Recent study has reported the interrelationship between autophagy and lipid metabolism as well as a critical function for autophagy in regulating intracellular lipid stores [25]. Autophagy is a major catabolic pathway for delivery and the clearance of damaged or superfluous proteins to lysosomes or the vacuole, and subsequent degradation by the cell's own lysosomal system [26]. Autophagy which plays a dual role both in cell survival and cell death is often deregulated under pathological conditions, provides a protective function to limit tumor necrosis and inflammation, and to mitigate genome damage in tumor cells in response to metabolic stress [27-29]. Tamoxifen-resistant cells selected from ER-positive BCCs show an increased ability to undergo antiestrogen-induced autophagy without induction of caspase-dependent cell death. It is suggested that autophagy plays a critical role in the development of anti-estrogen resistance in breast cancer cells [30].

Autophagic pathways share some signaling molecules including PI3K/AKT and mammalian target of rapamycin (mTOR), which regulates cell growth and protein synthesis in response to nutrient and growth factor availability [31]. Autophagy-related LC3 increased in cells treated with PI3K/AKT inhibitor [32]. The mTOR activation inhibits autophagy, whereas the mTOR inhibitor, rapamycin activates autophagy [33]. The MAPK signaling pathway is involved both in the induction of autophagy and in the maturation of the autophagosome [34].

These studies imply the interrelationship between choline metabolism, autophagy and therapeutic agent-resistance in malignant cancer cells. Especially, the expression and activity of Chk-α might be involved in regulating the autophagy of cancer cells acquired therapeutic agent-resistance. The interrelationship between Chk-α and autophagy in tamoxifen-resistant BCCs is not well understood. In our study, we investigate whether Chk-α expression and choline-containing metabolites are altered in BCCs, MCF-7 (ER-positive cell) and MCF-7/TAM (tamoxifen-resistant MCF-7). Metabolic profiles from cancer cell lysate were analyzed by 1H-nuclear magnetic resonance (1H-NMR) spectroscopy. To explore the interrelationship between Chk-α and autophagy in BCCs, we investigated the alterations of autophagy-related proteins (Beclin-1 and LC3B I/II) and autophagy-controlled signaling pathway (PI3K/AKT/mTOR and MAPK) in MCF-7 and MCF-7/TAM cells which are transduced with lentivirus vector containing Chk-α-shRNA and are treated with Chk-α inhibitor, CK37.

MATERIALS AND METHODS

Cell and cell culture condition

The ER-positive human breast cancer cell line, MCF-7 was obtained from ATCC and tamoxifen-resistant cell, MCF-7/TAM was obtained from professor Sang-kyu Ye (Department of pharmacology, Seoul national university college of medicine). All these cell lines were cultured in Dulbeccos' Modified Eagle's Media (DMEM; Welgene) containing 10% fetal bovine serum (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). MCF-7/TAM cells` medium was further supplemented with 3umol/L tamoxifen (Sigma). All the cells were incubated at 37℃ in a humidified atmosphere of 95% air/5% CO2.

Lentiviral vector infection experiments

The lentiviral derived plasmids that carried the transgene for green fluorescent protein (GFP) were provided from the Thermo Scientific (no. RHS4531). 293T cell were transfected with VSVG, TRP, and Chk-shRNA using lipofectamine 2000 (Invitrogen) for lentivirus packaging and 48 hours later the virus-containing supernatant medium was collected, filtered, and concentrated by ultracentrifugation. The targeted sequences for Chk-α were used 5′-TGAAATTCATTTTCTTTCT-3′

and 5′- TCTTTCTGAGCTTGTTCGG-3′ (NM_001277). In brief, 1x105 breast cancer cells were seeded in a six-well plate and infection the packaging plasmid. After 6 hours remove the medium and replaced culture medium. These cells were incubated for 72 hours and to assess GFP expression by fluorescence-activated cell sorting (FACS) and harvested, respectively.

Cell proliferation, cell cycle and cell death assay

In vitro cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells (1 × 103) were allowed to adhere for 24 hours under high humid environment in 5% CO2 at 37 ℃ in 96 well culture plates. After 24-72 hours, MTT solution (200 µL, diluted in a culture medium to a final concentration of 1 mgmL−1) was added, and the plate was incubated for 2hour. At the end of incubation period, the reaction mixture was carefully taken out and 200 µl of DMSO was added to each well by pippeting up & down several times until the contents were well mixed. The plates were kept on rocker shaker for 10 minutes at room temperature and then read at 540 nm using Multiwell Microplate Reader (Synergy HT, Bio-Tek, USA).

Cell cycle and apoptosis assay were performed to determine whether shChk-α transduction regulates the growth phase and death of BCCs. Cells were trypsinized and centrifuged at 300 × g (1000 rpm) for 5 minutes, then resuspended (1 × 106

cells/ml) and fixed with 70% ice-cold ethanol for 60 minutes, followed by centrifuged, washed and resuspended in PBS contained DNase free RNase. After 60 minutes incubation, propidium iodide (PI, 1mg/ml) was added to the solution to incubate for an additional 15 minutes in the dark. The fluorescence of PI was analyzed using flow cytometer (Becton-Dickinson, San Jose, CA). Cell subpopulations in G0/G1, S and G2/M phases and apoptosis were calculated by gating analysis based on differences in DNA content. At least 20000 cells were analyzed per sample. Cell proliferation characters were indexed by the ratio in S-phase.

RNA isolation, cDNA synthesis, and real time RT-PCR

After cell sorting the cells (MCF-7 and MCF-7/TAM) were seeded in six-well plates in DMEM culture medium and incubated in a humidified CO2 incubator (5% CO2, 37℃). To investigate the expression of choline kinase-α and β, RT-PCR was performed. Total RNA was extracted from cultured cells using the TRIzol (Ambion) extraction method. Briefly, total RNA was extracted with TRIzol and precipitated with isopropyl alcohol, washed in ethanol, and resuspended in RNase-free water. RNA quantity and quality were determined by nano drop spectrophotometer, respectively. cDNA was produced by using SuperScript II reverse transcriptase (Invitrogen). . The following primers were used: choline kinase-α, 5′-CTTGGTGATGAGCCTCGGAA-3′, and 5′-AAGTGACCTCTCTGCGAGAA-3′; choline kinase-β, 5′-

AGTCTCGGTTCCAGTTCTAC-3′, and 5′-CTTCTGCTCGTTGTTCCTCC-3′; β-actin, 5′-CCAACCGCGAGAAGATGACC-3′ and 5′-GGAGTCCATCACGATGCCAG-3′.

Real time RT-PCR products were synthesized using the SYBR Green Realtime PCR Master Mix (ABI) and were analyzed in real-time with the detection system (ABI Prism 7500 Sequence Detection System, PE Applied Biosystems, Foster City, CA, USA). The results were analyzed by the ΔCt method, which reflects the difference in threshold for the target gene relative to that of β-actin in each sample.

Western blot analysis

The cells were lysed in RIPA buffer containing a protease inhibitor cocktail (Sigma), and the proteins were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) for four hours at room temperature and transferred to nitrocellulose membranes for two hours at 4℃. The membranes were blocked with 5% skim milk in Tris-buffered saline and incubated with primary antibodies overnight at 4 ℃, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 30 minutes. The blots were developed using Enhanced Chemiluminescence Reagents (Amersham Biosciences, Piscataway, NJ, USA). The

relative intensity of the bands observed by Western blotting was analyzed using the Image J program. The following antibodies were used in this study: Anti-phospho AKT (Ser473), anti-AKT, anti-phospho ERK1/2, anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-mTOR (Cell Signaling), anti-mTOR (Cell Signaling), LC3B (Cell Signaling), Beclin-1 (Cell Signaling), choline kinase-α (Proteintech Group, Inc., Chicago, IL) and β-actin (Sigma). All antibodies were used according to the manufacturers' instructions. For detection of immune-reactive bands, ECL and ECL Plus were used (General Electric).

1H- NMRS analysis

These cells were harvested, collected as cell pellets containing 3x107 cells per sample, and stored at -80℃ until ready for 1H NMRS analysis. 1H NMRS was performed using methods published previously by Blankenberg *et al* [35]. Freezed cell pellets was thawed with D2O made in PBS, with 1.5mM sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4 (TSP; Cambridge Isotope Laboratories, Inc., Andover, MA) added as internal standard, suspended in a final volume of 900 μL, centrifuged to remove precipitates and then placed immediately on ice until data acquisition.

One-dimensional standard 1H magnetic resonance and CPMG spectra were acquired on a Bruker Avance 600 system (14.1 T) equipped with a 5-mm TXI

croyprobe spectrometers (Bruker BioSpin Corp.). Spectra were acquired at 20℃ ±1℃ with the following sequence parameters; flip angle = 90°, spectral width = 16kHz , relaxation delay = 2s, 32k data points, and 128 scans. Chemical shifts were calibrated relative to the TSP signal at 0 ppm.

Magnetic resonance spectra were processed using MestReNova software (Mestrelab research) and jMRUI software. The time-domain spectra were apodized with an exponential function (1 Hz) and then Fourier-transformed, phased and baseline-corrected. The spectra were referenced to the TSP signal at 0.00 ppm. The assigned resonances were also referenced to the alanine signal at 1.48 ppm in cases where the TSP signal was split due to protein binding. To reduce the complexity of the 1H NMR spectroscopy data for the subsequent multivariate analysis, the spectra were binned by 0.005 ppm intervals and normalized by integration values over the region of 0.99 - 5.59 ppm. The above binning interval was chosen to quantify choline, phosphocholine, and glycerophosphocholine more accurately by allowing each of the signals to span at least two bins. As the aliphatic lipid signals were vastly different from sample to sample, only the regions that are not affected by those signals were used (1.44 - 1.91 ppm and 2.15 - 5.59 ppm). Within those regions, the water region (4.61 - 5.03 ppm) was excluded in the normalization due to its irregular behavior. The binning and normalizations were performed using an in-house Perl program.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde, blocked with 2% bovine serum albumin (Sigma) in PBS, reacted with rabbit anti-Beclin-1, anti-LC3B and caspase-3 primary antibodies, visualized using an anti-rabbit IgG conjugated with Texas red and counterstained with DAPI. These fluorescently stained cells were then observed under a fluorescence microscope as previously described using LAS software (Leica) for image acquisition.

Statistical analysis

Results of the cell growth assay were presented as mean ± SE in three independent experiments and were analyzed using one way ANOVA followed by T-test. Mean differences with $p<0.05$ were considered statistically significant.

RESULTS

To evaluate the Chk-α expression is altered in tamoxifen-resistant BCCs, the Chk-α and β expressions in MCF-7 and MCF-7/TAM cells were analyzed by RT-PCR and Western blot. The expression levels of Chk-α mRNA and protein were increased in MCF-7/TAM as compared with MCF-7 (Fig. 1A and B. *, $p<0.05$.). Choline-containing metabolites in MCF-7 and MCF-7/TAM were analyzed using 1H–NMR spectroscopy. As shown in figure 1C, phosphocholine (PC) were increased in MCF-7/TAM as compared to MCF-7, while glycerophosphocholine (GPC) and choline (Cho) decreased. This result suggests the high level of Chk-α expression might be associated with the acquirement of tamoxifen resistance in MCF-7 cells.

Next, Chk-α downregulated BCCs (MCF-7-shChk-α and MCF-7/TAM-shChk-α) were established using lentivirus containing Chk-α shRNA (Fig. 2A) and GFP (Fig. 2B and C). The expression levels of Chk-α and -β mRNA in shChk-α transduced or control cells were evaluated by RT-PCR and real-time RT-PCR using specific primers against endogenous. As shown in figure 3A and B, Chk-α mRNA was significantly decreased in shChk-α transduced cells as compared to control cells (*, $p<0.05$, shChk-α transduced vs control). However, the level of Chk-β mRNA was not changed in shChk-α transduced cells. Next, the expression levels of

Chk-α protein were analyzed using Western blot. Chk-α protein levels are lower in shChk-α transduced cells compared with the control cells (Fig. 3C and D, *, $p<0.05$, shChk-α transduced vs control).

To analyze the alteration of choline-containing metabolite in shChk-α transduced cells, metabolites profiles was investigated using 1H-NMR spectroscopy. As we expected, 1H-NMR spectra obtained from lysates of shChk-α transduced cells showed that PC levels, the product of choline kinase enzyme activity, were lower than those of control cells (Fig. 4A, and B).

Chk-α expression and choline-containing metabolites are associated with the proliferation and survival of cancer cells. We here investigated the effect of downregulation of Chk-α on proliferation ability of BCCs. MCF-7/TAM is the slowly growing cells as compared to MCF-7. The downregulation of Chk-α resulted in the decrease in proliferation ability of MCF-7/TMA and MCF-7 cells (Fig. 5A and B). MTT assay showed that the proliferation rate of MCF-7 and MCF-7/TAM cells were lower than that of the control. (*, $p<0.05$, *, $p<0.001$, shChk-α transduced vs control). Cell cycle analysis assessed by flow cytometry revealed that the cells population in S phase were significantly decreased by shChk-α transduction in MCF-7/TAM cells but not MCF-7 cells (Fig. 5C and D, shChk-α transduced vs control). To investigate Chk-α downregulation induce the cell death via apoptosis, the activated caspase-3 was evaluated by immunofluorescence staining. Interestingly, activated caspase-3 was observed in

only shChk-α-transduced MCF-7 cells but not shChk-α-transduced MCF-7/TAM cells (Fig. 5E and F).

Many cancer therapeutic agents have been reported to induce autophagy. We observed the autophgosome-like structure in MCF-7/TAM cells (data not shown). Interestingly, shChk-α transduction induced autophagic structure in MCF-7 cells as well as MCF-7/TAM cells (Fig. 6A and B). These results imply that the interrelationship between Chk-α expression or activity and autophagy.

The protein levels of Beclin-1 and LC3B I/II were assessed by Western blot in shChk-α transduced cells and control cells. Western blot analysis showed shChk-α transduction significantly upregulated LC3B II in shChk-α transduced cells as compared with control cells (Fig. 7B and D, *, $p<0.05$, shChk-α transduced vs control), whereas Beclin-1 levels were not altered (Fig. 7A and C, shChk-α transduced vs control).We further examined whether downregulation of Chk-α involved in the induction of autophagy in BCCs. We investigated the cellular distribution of an autophagosome marker, LC3B using immunostaining in controls and shChk-α transduced cells. LC3B was observed as clusters of small, intensely stained granules, which was mainly distributed in the cytoplasm near nuclear of MCF-7/TAM (Fig. 8B). Autophagosome formations visualized using immunostaining with LC3B antibody increased in shChk-α transduced cells as compared with control cells (Fig. 8A and B).

To investigate that an inhibition of Chk-α activity leads to autophagy, after treatment with Chk-α inhibitor, CK37, LC3B expression was assessed by immunostaining and Western blot. Immunostaining results showed that LC3B increased in CK37-teated cells (Fig. 10). This observation is consistent with results observed in shChk-α transduced cells.

The MAPK and PI3K/AKT/mTOR signaling pathways are frequently dysregulated in cancer and these two pathways cooperate to promote the survival and proliferation of cancer cells [30]. Here we analyzed the AKT and ERK phosphorylation in shChk-α transduced cells using Western blot analysis. shChk-α transduction resulted in the significant decrease in the phosphorylation of ERK1 and ERK2 at the activation Thr202/Tyr204 and Thr185/Tyr187, respectively (Fig. 11A and B, *, $p<0.05$, shChk-α transduced vs control) but the difference in phosphorylation level of AKT at Ser 473 was not observed between shChk-α transduced and control. These results suggest that downregulation of Chk-α regulates the MAPK and PI3K/AKT signaling pathways directly or indirectly. We examined the mTOR phosphorylation in shChk-α transduced cells and control. A significant decrease in the phosphorylation of mTOR at Ser 2448 shChk-α transduced cells was observed (Fig. 11C, *, $p<0.05$, shChk-α transduced vs control). These data show that downregulation of Chk-α interrupts PI3K/AKT, mTOR and MAPK signaling pathway simultaneously, leading to autophagy in MCF-7 and MCF-7/TAM cells. Overall, our results suggested the downregulation of Chk-α

expression and activity in MCF-7/TAM cells decreased PI3K/AKT, mTOR and MAPK signaling pathway, which is associated with the autophagy-induced dormancy (Fig. 12).

DISCUSSION

In this study, we investigated the relationship of therapeutic agent-resistance, choline metabolilites and autophagy in ER-positive human BCCs (MCF-7) and tamoxifen-resistant BCCs (MCF-7/TAM) and further explored the signaling pathway involved in autophagy in Chk-α downregulated BCCs. Here we have shown that the Chk-α expression and PC were significantly increased in MCF-7/TAM as compared to MCF-7 and selective downregulation of Chk-α by using lentivirus containing shRNA led to dramatically decrease PC level and attenuated the phosphorylation of ERK, AKT and mTOR signal molecules and induced LC3BI/II expression, thereby leading to autophagy (Fig. 12).

The implication of Chk-α in the regulation of normal cell growth as well as and cancer has been extensively demonstrated [13,15,36-38]. The production of PC by an increase of the Chk-α expression and activity has been described as an essential process in cell growth induced by growth factors and the treatment with specific drugs to target Chk-α results in a blockage of the DNA synthesis [37]. Chk-α is overexpressed in diverse cancer cells including breast, lung, colorectal, bladder, prostate and ovarian tumor [12-16,39-41]. High concentrations of PC constitute a common characteristic in cancer cell lines derived from human tumors [42]. NMR-based metabolomics studies revealed the abnormal phospholipid metabolism in cancer and increased levels of PC associated to cell

malignancy [43,44]. Chk-α and PC has been proposed as a useful marker for diagnosing cancer progress and for monitoring response to drug therapy [45-47].

On this study, our result showed the high expression of Chk-α and elevated level of choline-metabolites, PC in tamoxifen-resistant BCCs, MCF-7/TAM. Recently, it has been shown that overexpression of Chk-α increases invasiveness and drug resistance to 5-fluorouracil (5-FU) of human breast cancer cells. This resistance induced by Chk-α was consistent with an increased expression of thymidylate synthase and higher efflux that facilitates cellular detoxification [18]. The treatment with PI3K and mTOR inhibitor, PI-103, resulted in a concentration- and time-dependent decrease in PC and Chk-α levels in human prostate and colon carcinoma cell lines [48]. Chk-α and its metabolites have been described as a key mediator of the AKT pathway [24,49].

Autophagy may be dysregulated in several disorders, including metabolic diseases, neurodegenerative disorders, infectious diseases and cancer. Recently autophagy is becoming an attractive target for anti-cancer therapies. Autophagy assessed by the accumulation of autophagosomes is frequently upregulated in cancer cells following treatment with tamoxifen or imatinib [50,51]. Our study showed that the expression of LC3 leading to the induction of autophagosome formation as a marker for autophagy was increased in tamoxifen-resistant and shChk-α transduced BCCs. Activation of the PI3K/AKT and mTOR signaling pathway promotes necrotic cell death via suppression of autophagy [52]. Deregulation of the PI3K/AKT and mTOR pathway has been shown to be involved

in breast cancer doxorubicin- and tamoxifen-resistance through the regulation of multidrug resistance proteins [53-55]. The MEK/ERK pathway influences chemotherapeutic drug resistance as ectopic activation to doxorubicin and paclitaxel in BCCs [56,57].

Our results strongly support a link between Chk-α and the PI3K/AKT, mTOR and MAPK signaling pathway. Because Chk-α protein levels are decreased that Chk-α could be regulated at downstream of mTOR [58]. In the present study, we have investigated possible mechanisms underlying NMR-detectable metabolic changes with the focus on the decrease in PC levels. The aim was to establish the link between PI3K signaling and phospholipid metabolism and, in turn, to help optimize the use of NMR-detectable changes as potential biomarkers for inhibitors of this pathway. Inhibitors that act at the different key components in the PI3K/AKT, mTOR and MAPK network could be used to further clarify the association between NMR-detectable metabolic changes and the these signaling pathway [59]. As a central signal integrator, mTOR receives signals arising from nutrients and mTOR inhibition triggers autophagy [60].

In conclusion, the elevated levels of Chk-α and PC might be involved in the tamoxifen-resistance in ER-positive BCCs. A decrease in Chk-α and PC levels led to the suppression of PI3K/AKT, mTOR and MAPK signaling pathway, which is associated with the induction of autophagy in BCCs, especially tamoxifen-resistant BCCs, MCF-7/TAM. Monitoring the changes in Chk-α and PC levels by NMR

may provide noninvasive biomarkers of dysregulation of autophagy through PI3K/AKT, mTOR and MAPK pathway in therapeutic agent-resistant breast cancer. The identification of these signaling cascades has led to the preclinical and clinical development of specific targeting drugs for the treatment of tamoxifen- or the other drug-resistant breast cancer. Moreover, understanding the signaling pathways involved in the regulation of autophagy as well as the autophagy process itself represents new directions in the development of anticancer therapies. The combination therapies of autophagy modulating agents and specific targeting drugs for different subtypes of breast cancers, especially drug-resistant cancer are currently being considered.

Figure 1: Increased choline kinase-α expression and phosphocholine in Tamoxifen-resistant BCCs. A and B: The Chk-α and β expression in MCF-7 and MCF-7/TAM cells were analyzed by RT-PCR and real-time RT-PCR. *p<0.05. C: Choline-containing metabolites in MCF-7 and MCF-7/TAM were analyzed using 1H–NMR spectroscopy.

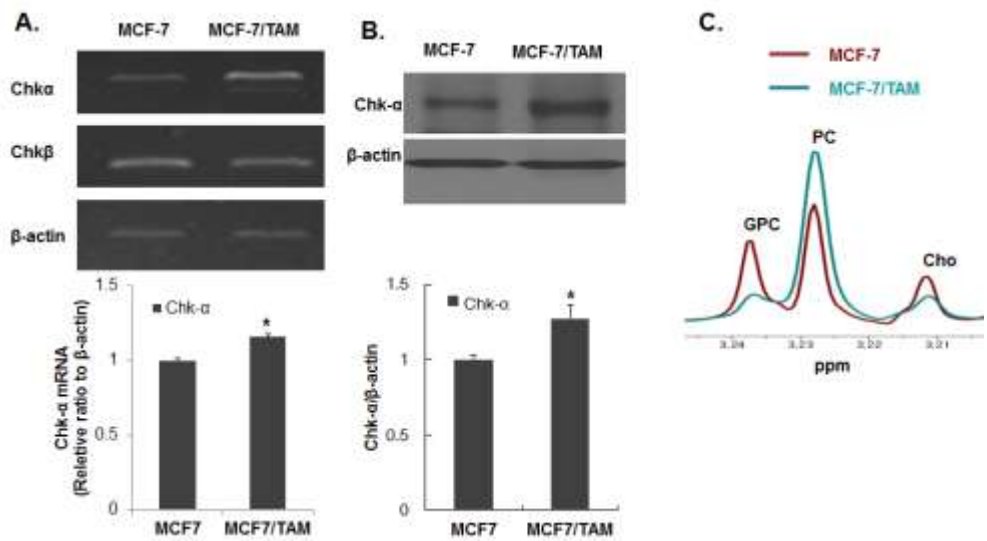


Figure 2 : Establishment of choline kinase-α downregulated BCCs using lentivirus carrying shChk-α. A: Chk-α downregulated BCCs were established using lentivirus containing Chk-α shRNA. B and C: MCF-7-shChk-α, and MCF-7/TAM-shChk-α were established using lentivirus containing Chk-α shRNA and GFP.

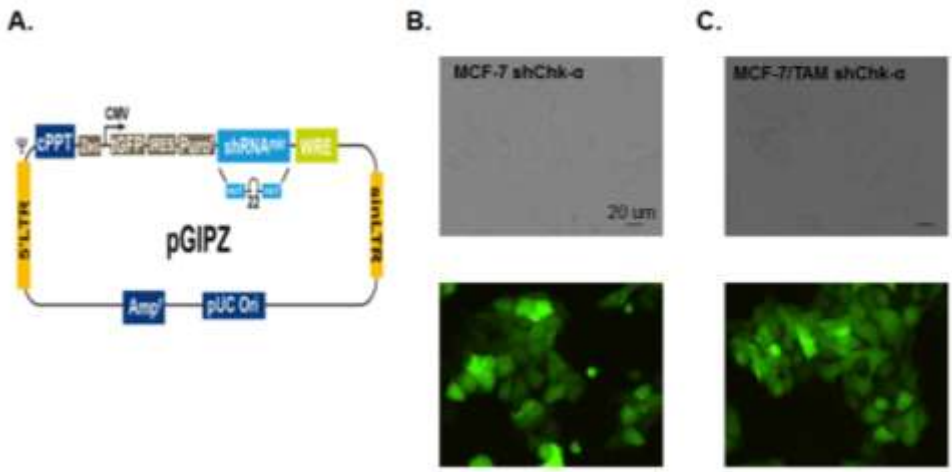


Figure 3: Downregulation of choline kinase-α in shChk-α transduced BCCs.

A and B: The expression levels of Chk-α and -β mRNA in shChk-α transduced or control cells were evaluated by RT-PCR and real-time RT-PCR. C and D: The expression levels of Chk-α protein were analyzed using Western blot. *p<0.05.

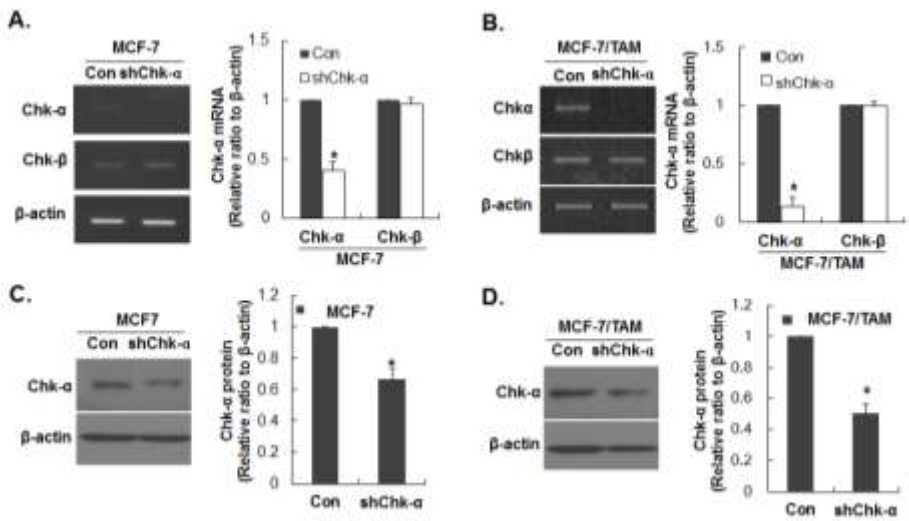


Figure 4: Remarkable decrease of phosphocholine in shChk-α transduced BCCs using 1H-NMR spectroscopy. A and B: 1H-NMR spectra obtained from lysates of shChk-α transduced cells showed that PC levels. *p<0.05.

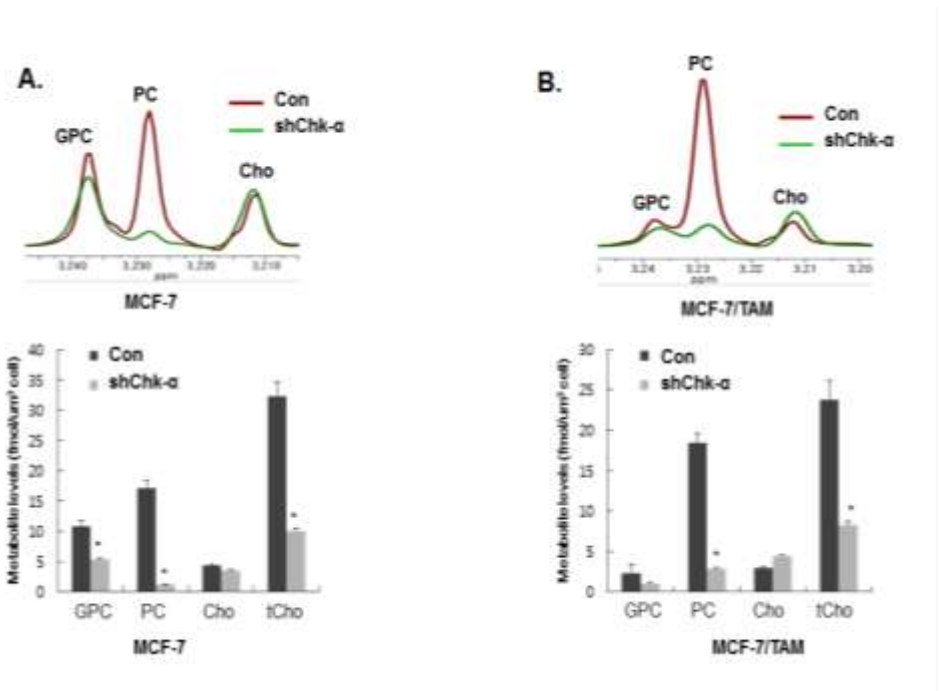


Figure 5: Decreased proliferation ability of shChk-α transduced BCCs.

A and B: MTT assay showed that the proliferation rate of shChk-α transduced MCF-7 and MCF-7/TAM cells were lower than that of the control. *$p<0.05$. **$p<0.001$. C and D: The analysis of the stages of cell division by flow cytometry with propidium iodide staining. The cell cycle analysis revealed that the cell populations in S phase were significantly decreased and cell population in G0/G1 were increased in shChk-α transduced MCF-7/TAM cells as compared with control. *$p<0.05$. The cell populations in S phase between shChk-α transduced MCF-7 cells and their control was no different. E and F: Immunofluorescence staining of activated caspase-3. Immunostaining result showed that the activated caspase-3 was detected in shChk-α transduced MCF-7 cells.

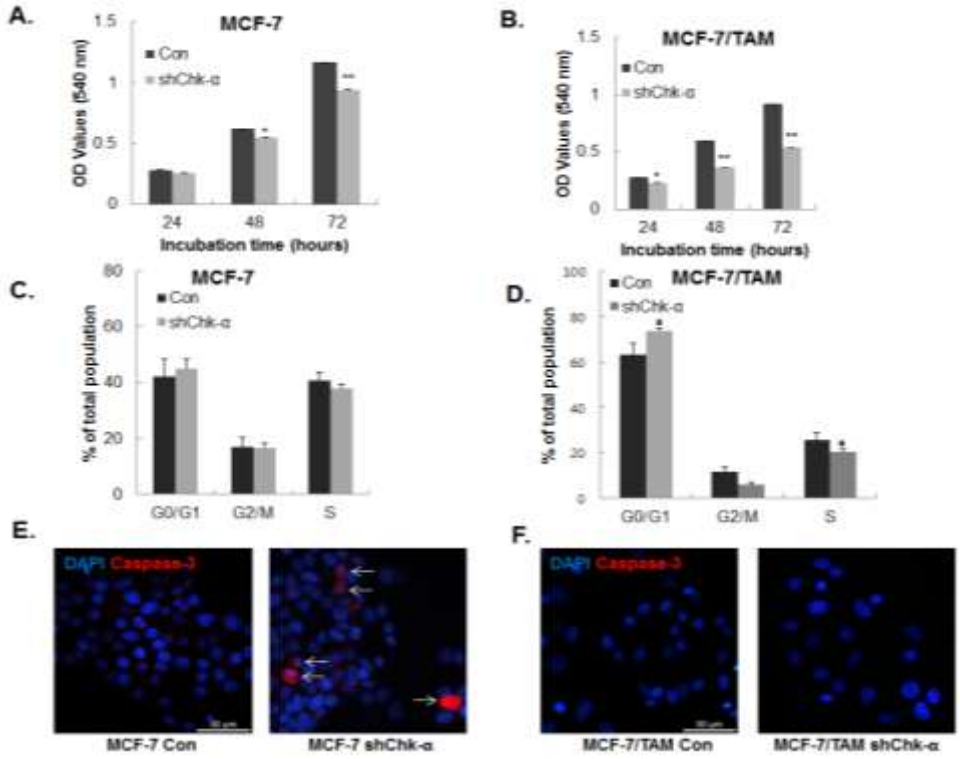

A.
MCF-7
Con
shChk-α
OD Values (540 nm)
1.5
1
0.5
0
24
48
72
Incubation time (hours)
B.
MCF-7/TAM
Con
shChk-α
OD Values (540 nm)
1.5
1
0.5
0
24
48
72
Incubation time (hours)
C.
MCF-7
Con
shChk-α
% of total population
80
60
40
20
0
G0/G1
G2/M
S
D.
MCF-7/TAM
Con
shChk-α
% of total population
100
80
60
40
20
0
G0/G1
G2/M
S
E.
DAPI Caspase-3
MCF-7 Con
MCF-7 shChk-α
F.
DAPI Caspase-3
MCF-7/TAM Con
MCF-7/TAM shChk-α

Figure 6: Autophagosome-like structure in shChk-α transduced BCCs. shChk-α transduction induced autophagic structure in MCF-7 cells as well as MCF-7/TAM cells.

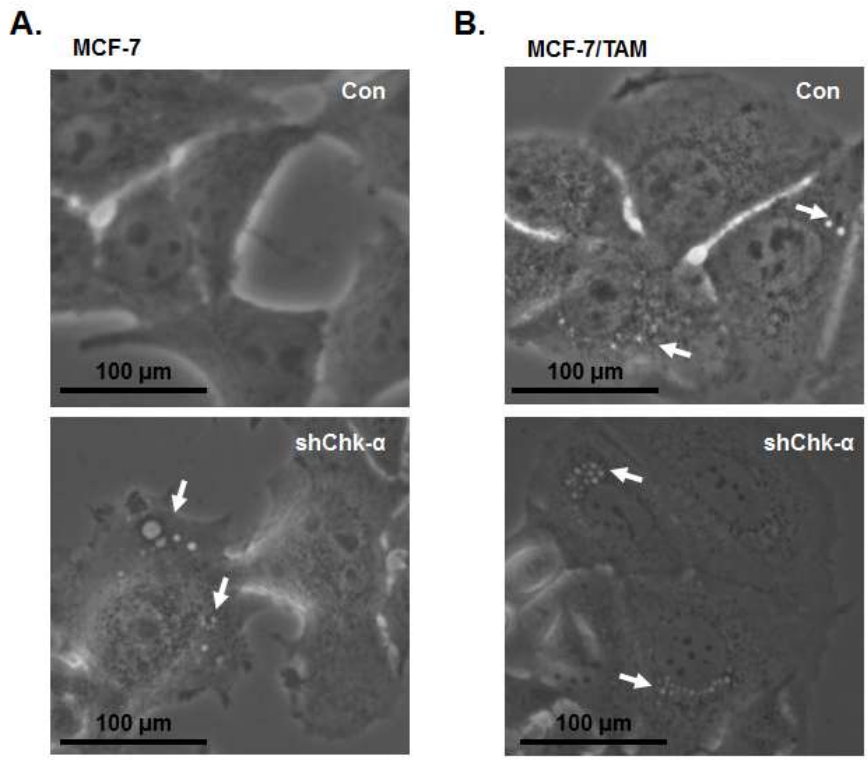


Figure 7: Significant increase of LC3B, autophagy-related protein in shChk-α transduced BCCs. The protein levels of Beclin-1 and LC3B I/II were assessed by Western blot in shChk-α transduced cells and control cells. LC3B II expression was significantly increased in shChk-α transduced cells compared to control cells *p<0.05. shChk-α transduced vs control

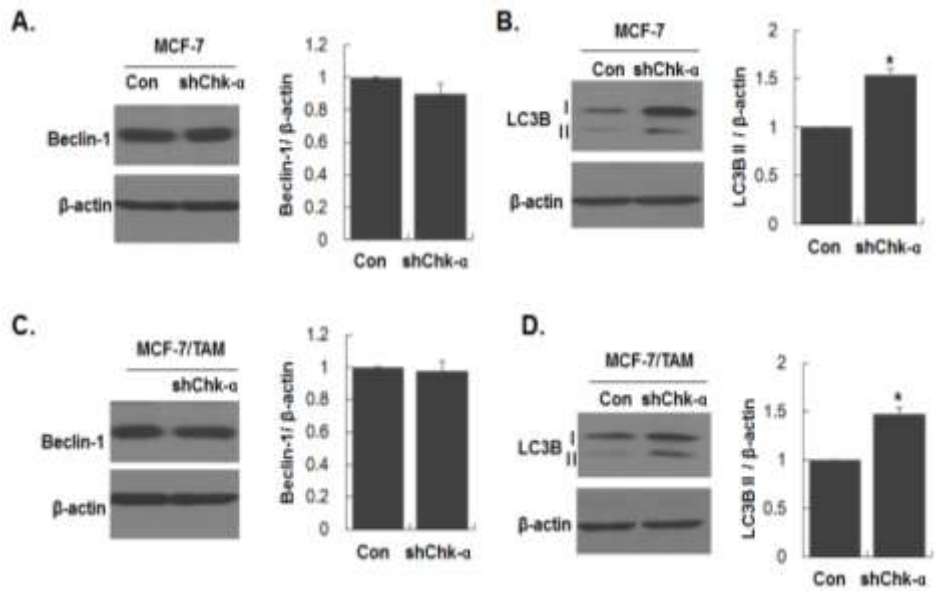


Figure 8: Distribution of the autophagosomal marker, LC3B in shChk-α transduced BCCs. The cellular distribution of an autophagosome marker, LC3B evaluated by immunostining was detected in in MCF-7/TAM cells and shChk-α transduced cells.

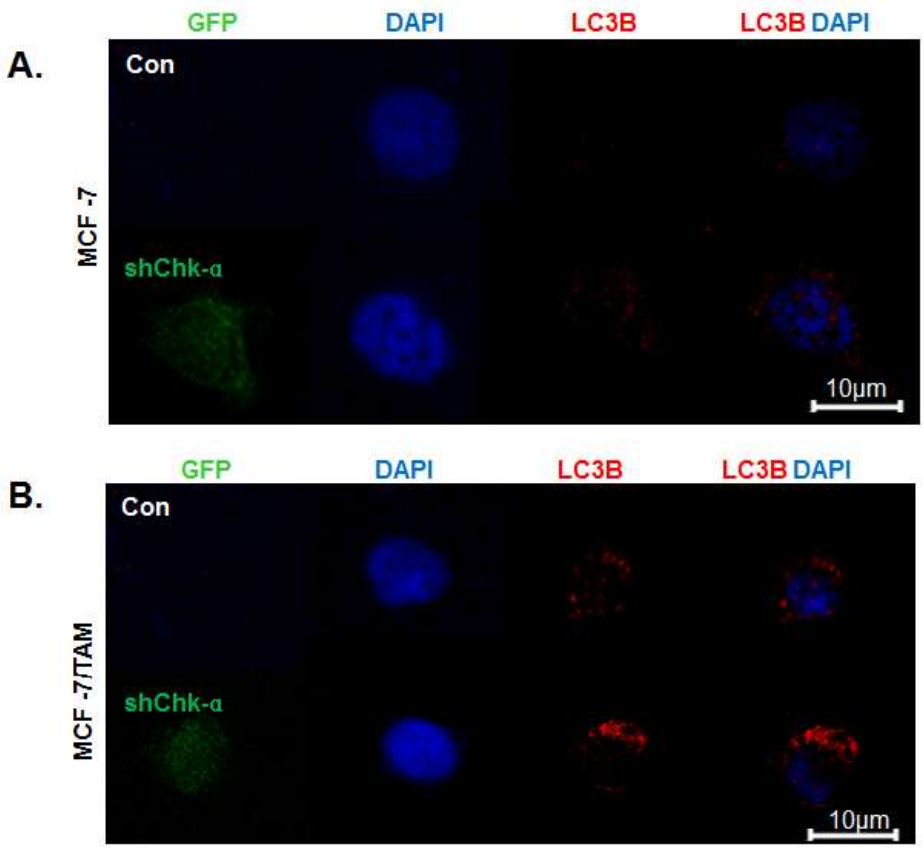


Figure 9: Significant increase of LC3B, autophagy-related protein in choline kinase inhibitor, CK37-treated BCCs. LC3B expression was assessed by Western blot. LC3B II expression was significantly increased in CK37-treated cells compared to control cells *p<0.05. shChk-α transduced vs control

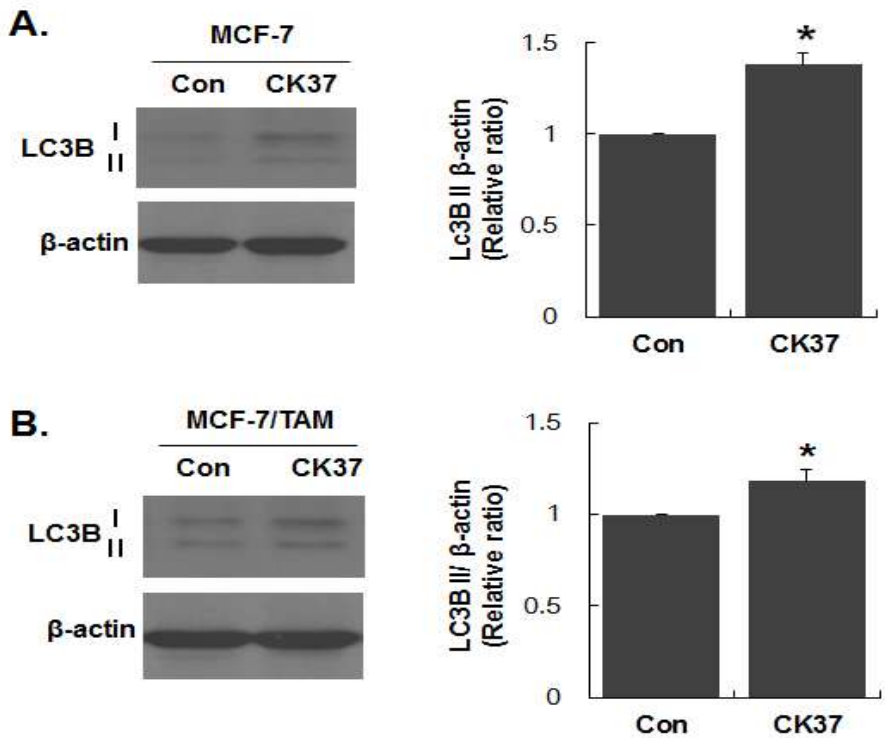


Figure 10: Distribution of the autophagosomal marker, LC3B in choline kinase inhibitor, CK37-treated BCCs. The cellular distribution of an autophagosome marker, LC3B was evaluated by immunostaining in untreated or CK37-treated cells. LC3B clearly was detected in in MCF-7/TAM cells and CK37-treated cells.

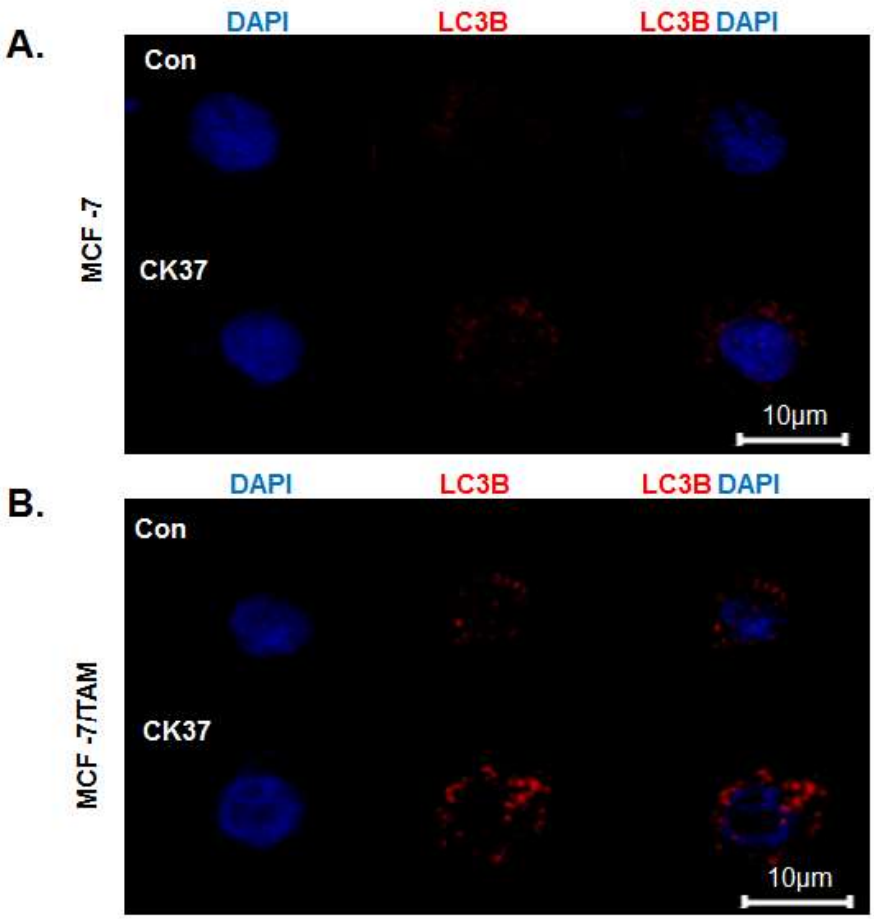


Figure 11: Attenuated phosphorylation of AKT, ERK and mTOR in shChk-α transduced BCCs. A-F: AKT and ERK and mTOR phosphorylation in shChk-α transduced cells were analyzed using Western blot. *p<0.05.

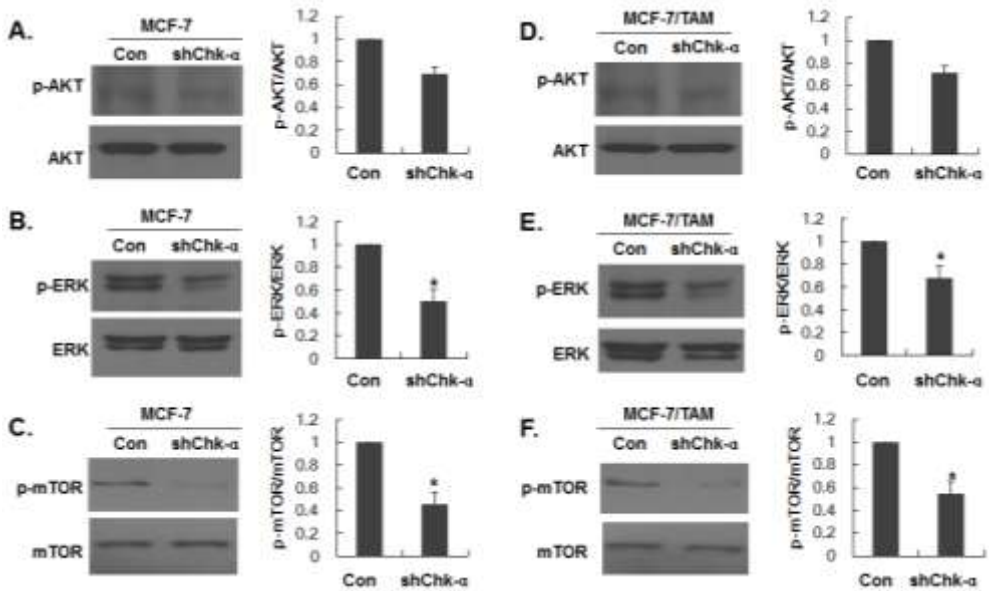
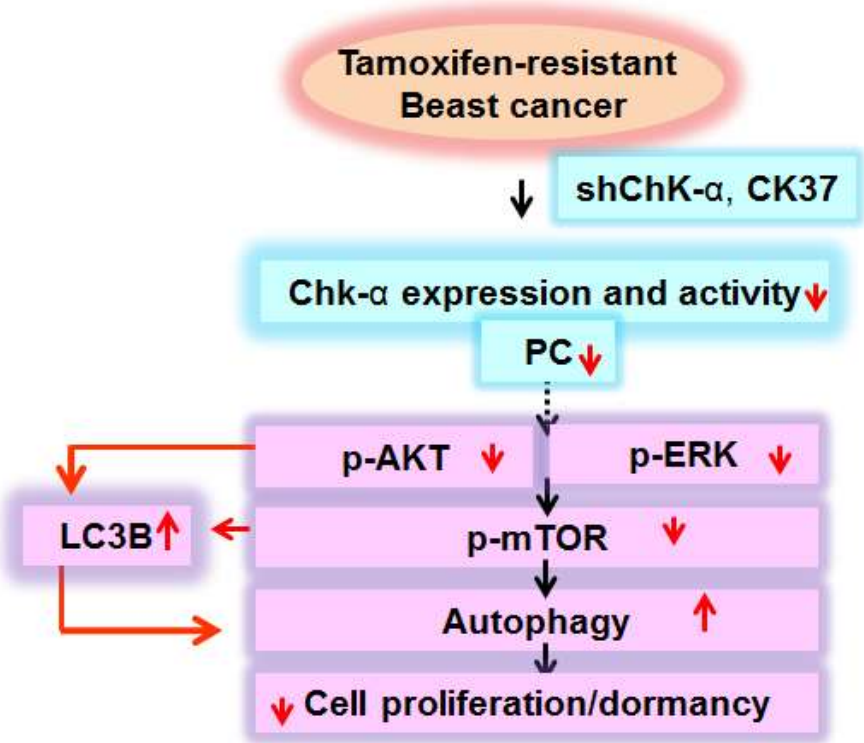


Figure 12. Schematic diagram of the regulatory mechanisms of autophagy by choline kinase in tamoxifen-resistant breast cancer cells.



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국문 초록

서론: 약제저항성을 획득한 유방암세포의 표적 치료는 유방암치료에 가장 중요한 과제이다. 유방암세포에서 choline kinase-α (Chk-α) 유전자 발현과 포스포코올린 대사체 증가는 악성도가 높은 유방암에서 관찰되고 있으며 자가포식은 암세포 치료제내성을 제공하는 기전으로 여겨지고 있다. 본 연구에서는 약제저항성을 획득한 유방암세포에서 Chk-α 발현에 따른 자가포식 조절기전을 규명하고자 한다.

실험방법 및 결과: 사람유방암 세포주, MCF-7과 tamoxifen에 저항성을 획득한 유방암 세포, MCF7/TAM를 사용하였다. 각각의 유방암세포에 shRNA를 포함하는 렌티바이러스를 이용하여 Chk-α의 발현을 감소시키고 CK37을 처리하여 Chk-α 활성을 억제시켰다. 실시간 정량 RT-PCR, Western blot, 면역염색을 통해 Chk-α의 발현을 평가하였으며 1H 핵자기공명분광학을 이용하여 콜린 대사체의 변화를 대조군 세포와 비교 분석하였다. MCF7/TAM 세포는 MCF-7세포에 비해 Chk-α의 발현과 포스포코올린 대사체가 증가되었으며 다수의 자가포식소체가 형성하고 자가포식 관련 단백질인, LC3B 발현이 증가되었다. shChk-α에 의한 Chk-α의 발현 감소는 포스포코올린 대사체의 감소와 세포증식능력의 감소를 유도하였다. shChk-α에 의한 Chk-α의 발현 감소와 CK37 처리에 의한 Chk-α 활성 억제제로

자가포식 표지 단백질인 LC3B 발현 증가를 유도하였고, AKT, ERK1/2와 mTOR의 인산화가 감소 되였다. MCF-7 세포는 shChk-α에 의해서 세포 증식능력이 감소 될 뿐만 아니라 caspase-3 의존적인 세포 사멸이 일어났으나 MCF7/TAM 세포에서는 shChk-α에 의해서 증식능력이 현저히 감소하였으나 caspase-3에 의한 세포사멸이 관찰되지 않았다.

결론: Tamoxifen 약제저항성을 획득한 유방암 세포인 MCF-7/TAM 세포는 Chk-α의 발현과 효소활성을 감소시키면 PI3K/AKT/mTOR 및 MAPK 신호전달 경로를 저해하여 자가포식을 유도하고 암세포의 증식을 억제하여 휴면 상태로 될 수 있을 것으로 사료된다.

주요어: 콜린키나제, 대사체학, 유방암, 자가포식, 치료제 저항성, 신호전달기전, LC3B, PI3K/AKT/mTOR, MAPK

학 번: 2010-30843